PLANT FOODS AND DIETARY SUPPLEMENTS: BUILDING SOLID FOUNDATIONS FOR CLINICAL TRIALS

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PLANT FOODS AND DIETARY SUPPLEMENTS: BUILDING SOLID FOUNDATIONS FOR CLINICAL TRIALS

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EDITORIAL
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Editorial: Plant Foods and Dietary Supplements: Building Solid Foundations for Clinical Trials

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Editorial on the Research Topic

Plant Foods and Dietary Supplements: Building Solid Foundations for Clinical Trials

Clinical trials are the generally accepted gold standard for querying the safety and efficacy of interventions, but they are time- and cost-intensive. Given their high price, it is critical that each clinical trial advance our understanding to the greatest degree feasible. While it is to be expected that many clinical trials will not reject the null hypothesis given the many differences between preclinical models and humans, as well as between ethnobotanical or even epidemiological and clinical contexts, too often when a clinical trial does not reject the null hypothesis myriad post-trial concerns emerge that it would have been better to resolve pre-trial [e.g., whether a different version of the intervention (dose, formulation, timing, etc.) might have been effective for a slightly different outcome or population] and leave the outcome open to different interpretations. Thus, despite the 2,269 papers on curcumin (or turmeric or curcuminoids) published (in English) in the year ending on November 24, 2021 and the 355 "curcumin" clinical trials listed in clinicaltrials.gov (same search terms, all years), the web site of the US National Institutes of Health's National Center for Complementary and Integrative Health says (https://www.nccih.nih.gov/health/turmeric): *Much research has been done on substances from turmeric, but their health effects remain uncertain.* Similarly large volumes of published research on many other natural products (NP) also shed little light.

With the goals of increasing the yield of clear, evidence-based public health guidance from preclinical, epidemiological and clinical research by increasing the application of good practices to the foundational research as well as to its translation to clinical trials, and of addressing more of those myriad concerns before rather than after the clinical trial, this special edition expands on a 2019 review (1). The papers collected here delve into ways to increase the replicability and clinical relevance and thus also the public health-relevant yields from NP research across the spectrum from chemical characterization to clinical trials.

Increased clarity on which components within a chemically complex NP participate in modulating specific biological outcomes should increase the information gained from clinical trials of these products. A related question is "how much of each key chemical reaches the *in vivo* site of action?" Aspects of this include product stability and replicability, bioavailability and metabolism. Optimization of methods for biochemical characterization and standardization of chemically complex NP are described in this topic by Abraham and Kellogg, Coskun et al., Floyd et al., and Lyu et al..

Interactions among the constituents of chemically complex products may contribute to their biological activities, as highlighted by Seigler et al. Replicability thus requires that we ask "what other, as yet unidentified chemicals may contribute to biological activities of this material?".

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Abraham and Kellogg, Coskun et al., and Funk and Schneider describe the utility of untargeted chemical characterization in detecting such constituents. The foregoing highlight the challenges, which Coskun et al. and Wright et al. note are particularly critical for NP used in clinical or translational research; they describe applications of orthogonal methods (leveraging different scientific principles) to increase replicability.

Biologically active product constituents must reach their targets at sufficient concentrations for activity. Lyu et al. describe the importance and development of methods for testing the disintegration and dissolution (D&D) of capsules to be used in clinical trials, while Floyd et al. stress the importance of assessing D&D in biorelevant media representing both fed and fasted conditions; this may avoid the need to develop a novel dosage form for clinical applications.

Floyd et al., Weaver and Hodges, and Wright et al. note the challenge and importance of evaluating absorption and pharmacokinetics for chemically complex products. Heterogeneity—genetic, epigenetic, dietary, etc, among humans, as well as between species—may alter product metabolic rates and the formation of biologically active metabolites (2). Chilton et al. demonstrate the application of several approaches to detect human genetic variants which critically modify metabolic flux and the health effects of food (or other NP), with implications for optimizing clinical trial inclusionary criteria and interpretations.

Floyd et al. describe the challenges of designing pre-clinical studies appropriately for translation to human studies. They highlight the importance of selecting the optimal animal models and dosing regimens for the outcomes of interest and for relevance to the population(s) of interest, and note the importance of considering potential sex differences. Weaver and Hodges note that while stringent inclusionary criteria may decrease the sample size required to provide a reasonable likelihood of avoiding a false positive or false negative result, greater inclusivity may allow greater generalizability. Floyd et al. add that other factors such as circadian rhythm and diet should be documented if not controlled, since they may strongly modulate pharmacokinetics.

Moving further toward translation, issues of safety, optimal dosing regimen, tolerability, ability to mask the intervention in controlled trials, optimal trial population(s) and outcome(s), and regulatory compliance are all critical. Clinical researchers must consider the possibility of "floor effects" for ingredients present in the diet (3), and of "drop in" for supplements available over the counter.

Weaver and Hodges adapted general best practices guidelines for human nutrition randomized controlled trials (4) specifically to plant-based interventions. Both Funk and Schneider and Weaver and Hodges note that while clinical trials are the gold standard for testing efficacy and providing evidence of causality, they should be undertaken only where they address a novel question of substantial public health significance.

Funk and Schneider note that clinical trials based on traditional uses may be less likely to succeed where the effect of interest is more likely to respond to a placebo, and that translation of ethnobotanical research may be complicated by cultural differences in symptomatology as well as in other behaviors or even pharmacogenetics.

Wright et al. provide a description of the development of a clinical trial-ready botanical product which meets requirements for toxic contaminants, is comparable in chemistry and dosing to the preclinical research and traditional products, minimizes participant burden (critical for recruitment, retention and compliance), and provides a good match to the placebo control.

Weaver and Hodges note the importance of compliance with regulatory requirements, including those for data integrity and participant privacy and safety. Transparency must be ensured through pre-study registration of the trial protocol including the statistical analysis plan. A CONSORT [Consolidated Statement on Reporting (Clinical) Trials] checklist provides guidance for reporting herbal interventions (5). Equally essential for transparency, these authors describe the importance of thorough reporting and FAIR (Findable, Accessible, Interoperable, Replicable) data for the advancement of knowledge, as well as for compliance with requirements of funding agencies.

Full adoption of the best practices described by authors in this topic will both increase the value of knowledge gained from translational research using chemically complex NP and increase the utility of NP clinical trial results for improving our understanding of NP effects on human health. Adoption of these practices will provide a more solid foundation for building the evidence base for the use of NP for health.

AUTHOR CONTRIBUTIONS

BS and MJ wrote the editorial. SM and CW contributed to the editorial. All authors contributed to planning and editing the topic collection.

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Conflict of Interest: CW is a scientific advisor for Produce for Better Health.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Dissolution Study on Grape Polyphenol Hard Gelatin Capsule Dietary Supplements

Weiting Lyu[1,2], Thamer Omar[3], Harna Patel[1], David Rodriguez[4], Mario G. Ferruzzi[5], Giulio M. Pasinetti[6,7], James W. Murrough[7,8], Fernando J. Muzzio[3], James E. Simon[1,2] and Qingli Wu[1,2]**

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Methods for a dissolution study by ultra-high performance liquid chromatography/triple quadrupole mass spectrometry (UHPLC-QqQ/MS) analysis of grape polyphenol dietary supplements, namely, grape seed extract (GSE) and resveratrol (RSV) capsules, were developed following the guidance of United States Pharmacopeia (USP) <2040>. Two dissolution media, 0.1 N hydrochloric acid (pH 1.2) and 0.05 M acetate buffer (pH 4.6), were evaluated with dissolution apparatus (USP 1), 100 rpm rotation speed, and 900 ml dissolution medium volume. Dissolution profiling was performed over 120 min. Major phenolic compounds of gallic acid, catechin, epicatechin, and procyanidin B2 were quantitated to obtain the dissolution profile of GSE capsules, and trans-RSV was used for RSV capsules. Results indicated that the released trans-RSV for RSV capsules in both of the dissolution media meets the USP standards, and that for the GSE capsules, all the four marker compounds passed the dissolution test in the HCl medium but did not reach a 75% release within 60 min in the acetate buffer. These promising results suggest that the general USP dissolution protocols are adequate for the successful release of RSV capsules in HCl medium and acetate buffer and GSE capsules (in HCl medium), but may be inadequate for GSE capsules in acetate buffer. These results showed that under a low pH of 1.2 (simulated stomach environment), bioactive compounds were released on time from the GSE capsules and met the USP guidelines; however, under a higher pH of 4.6 (simulated duodenum environment), the same biomarkers failed, suggesting the need to further improve the dissolution of GSE over a wider range of pH environments to enhance bioavailability and efficacy.

Keywords: grape seed extract, resveratrol, UHPLC-QQQ/MS, polyphenol, bioavailability

INTRODUCTION

Grape (*Vitis vinifera*) is native to southern Europe and Western Asia and is grown all over the world today. It has gained a high level of interest from the public health sector because of its numerous active components (1). Grapes are rich in polyphenols, 60–70% of which are found in grape seeds. These active constituents are linked with a broad spectrum of pharmacological and therapeutic benefits, namely, antioxidant, cardioprotective, hepatoprotective, anticarcinogenic, antidiabetic, antimicrobial, and antiviral (2–9). The major phenolic compounds in grape that contribute to the described health benefits are gallic acid, (+)-catechins, (−)-epicatechin, (−)-epicatechin-3-O-gallate, procyanidin dimers (B1–B5), procyanidin C1, and procyanidin B5-3′-gallate (1).

For active ingredients, the United States Pharmacopeia (USP) has established performance standards to detect their release from dosage forms in capsules and tablets that may occur as a result of formulation design or manufacturing processes in order to ensure safety and bioavailability (10, 11). While several quality control tests of dietary supplements (DSs) in the United States have been described in the USP, mandatory testing has not yet been implemented, and the USP does not have a guideline for resveratrol or grape seed extract, although there is one for grape seeds oligomeric proanthocyanidins. The vast majority of marketed DSs do not have USP-approved testing protocols. Importantly, the release of bioactives can be impacted by the manufactured product (e.g., nature of material and composition of the capsule, as well as the insert materials used in the manufacturing such as capsule, cellulose and/or gelatin, commonly used materials, all meeting FDA requirements but not all exhibiting uniform dissolution and breakdown properties) (12). Although the USP general chapter <20 40> describes a set of standardized test protocols tailored for specific combinations of dosage form and ingredient content category and dissolution protocols for vitamin/mineral DSs, it does not specify a dissolution protocol for testing grape-based DSs (10).

As part of the Consortium for Advancing Research on Botanical and Other Natural Products (https://ods.od.nih.gov/Research/Dietary_Supplement_Research_Centers.aspx) Program, grape-derived DSs and the major polyphenols in these products were investigated in support of an NIH-funded U19 clinical trial to ensure that bioactive compounds would be released at specific concentrations over time, thus bridging the link between chemistry, product formulation, and delivery with acceptable predictable release times to achieve a more robust quality control. In this brief research report, methods for dissolution study with UHPLC-QqQ/MS analysis on grape polyphenol DSs, namely, grape seed extract (GSE) capsules and resveratrol (RSV) capsules, were developed following the guidance of the USP <2040>.

MATERIALS AND METHODS

Reagents and Materials

Chemical Reagents

Standard compounds, namely, trans-RSV, (+)-catechin, (-)-epicatechin, and gallic acid were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, United States). HPLC-grade water, acetonitrile (ACN), and formic acid (FA) were obtained from Thermo Fisher Scientific Co. (Fair Lawn, NJ, United States). ACS-grade hydrochloric acid, glacial acetic acid, and sodium acetate were also purchased from Thermo Fisher Scientific Co. (Fair Lawn, NJ, United States).

Drug Material Sourcing and Manufacturing

Two kinds of grape-based dietary supplements were investigated. MegaNatural® Grape seed polyphenol extract (GSE) was purchased from Polyphenolics Company (Madera, CA, United States), which was produced from grapes that were grown in California, United States, certified by Halal (IFANCA). The final GSE product was processed by hot water extraction at a ratio of 30–50:1 (dry seed: extract). Synthetic trans-resveratrol (RSV) was purchased from BannerBio Nutraceuticals, Inc. (Nanshan District, Shenzhen, China).

Original GSE and RSV materials were delivered to Eagle Nutritionals (Carlstadt, NJ, United States) to prepare the capsules. Briefly, for GSE capsules, 450 mg GSE powder and 50 mg silica were filled into #0 purple/white hard gelatin capsules. For RSV capsules, 450 mg RSV powder was encapsulated using #0 green capsules.

TABLE 1 | Dynamic multiple reaction monitoring (dMRM) parameters of biomarkers in grape seed extract (GSE) and resveratrol (RSV) capsules.

	Compound	Retention time (min)	MS/MS transition (dMRM)		Fragementor voltage (V)	Collision energy (V)
			Precursor ion (*m/z*)	Product ion (*m/z*) (quantifier/qualifier)		
GSE capsule	gallic acid	1.28	169	125.0/79.1	86	12/24
	catechin	2.05	289.1	245.2/123.1	120	12/36
	procyanidin B2	2.21	577.1	289.0/407.0	145	25/25
	epicatechin	2.34	289.1	245.2/203.1	134	12/20
RSV capsule	*trans*-resveratrol	1.67	227.1	185.0/143.0	115	17/29

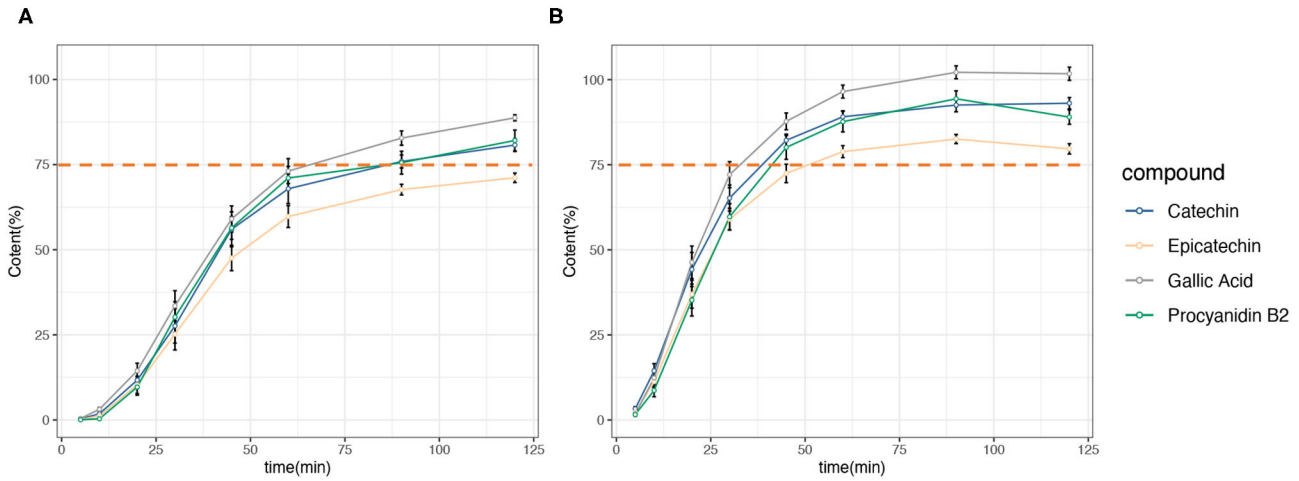

FIGURE 1 | Dissolution profile of grape seed extract (GSE) capsules showing the four major components: catechin, epicatechin, gallic acid, and procyanidin B2. **(A)** 0.05 M acetate buffer (pH 4.6). **(B)** 0.1 N hydrochloric acid (pH 1.2).

Equipment

Analysis of Dissolution Samples

The instrument used for chemical analysis was an Agilent 1,290 Infinity II UHPLC (Agilent Technologies, Palo Alto, CA, United States) coupled with a 6,470 (Agilent Technologies, Santa Clara, CA, United States) triple quadrupole mass spectrometer with electrospray ionization (ESI) source. Agilent MassHunter Optimizer (version B.07.00) was used for standard compound-related parameter optimization, and MassHunter Workstation software Data Acquisition (version B.08.00) and Quantitative Analysis (version B.07.01) were used for data processing. The column used for compound separation was a Kinetex^{TM} (Phenomenex Inc., CA, United States) C18 column; the particle size was 2.6 μm, and the size was 100*2.1 mm.

Dissolution Apparatus

All the dissolution testing reported in this study was performed in a 708-DS, eight-spindle, eight-vessel USP dissolution apparatus type I (basket), with automated online UV-Vis measurement (Agilent Technologies, Palo Alto, CA, United States). This apparatus consists of the following: a vessel, which is made of a glass material; a motor; a metallic drive shaft; and a cylindric basket. The vessel is cylindrical with a hemispherical bottom and has a 1-L capacity. The device used has eight vessels and is partially immersed in a water bath. The water bath keeps the temperature inside the vessel at 37 +/−0.5°C during the test. To prevent evaporation, plastic covers on the top of each vessel are used. A rotating shaft is placed in a position that ensures its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly. The basket is connected at the bottom of the rotating shaft. The shaft and basket are made of stainless steel. The basket is positioned so that the distance between the inside bottom of the vessel and the bottom of the basket is kept at 25 mm +/−2 mm during the test (10, 11).

Dissolution Testing

The GSE and RSV capsules were tested for dissolution based on recommendations by the FDA and USP 39 general chapters <2040> and <711> (10, 11). The dissolution apparatus was USP type I (basket method). The capsules were immersed and agitated in 900 ml of an appropriate medium (0.1 N HCl medium, pH 1.2, or pH 4.6 acetate buffer) at 100 rpm rotation speed, and the temperature was 37 ± 0.5°C. The 0.1-N HCl medium was selected to model the pH of the stomach (1.2) while 0.05 M acetate was used to mimic the pH of the duodenum (4.6). Because trans-RSV is light-sensitive, the instrument was protected from light using tin foil. Twelve samples were tested per case. Basket method was preferred in this study, because it can prevent a capsule from floating. During the test, 1 ml of each solution was withdrawn from the dissolution vessel at 5, 10, 20, 30, 45, 60, 90, and 120 min using a syringe equipped with a cannula. The cannula was then removed from the syringe, and a 0.45-mm polytetrafluoroethylene (PTFE) filter was used to filter each sample.

LC-MS Method

Preparation of Standard Solution

Gallic acid, catechin, procyanidin B2, and epicatechin were chosen as markers, because they are important bioactive polyphenols in GSE (1), and based on our previous study, the amount of these four compounds are dominant in GSE. To prepare the reference solution of marker compounds in the GSE capsules (gallic acid, catechin, procyanidin B2, and epicatechin), each standard was accurately weighed and diluted serially using 70% methanol with 0.1% FA to make a 5,000 to ∼0.1 ng/ml reference solution. For the RSV capsules, trans-RVS was accurately weighed and diluted serially using 70% methanol with 0.1% FA to make a 5,000 to ∼0.1 ng/ml reference solution.

Calculation of Marker Compounds in GSE Capsules

To calculate the percentage of marker compounds in each dissolution sample, each of the marker compounds in GSE capsules dissolved in the two-dissolution media (0.1 N HCl medium and pH 4.6 acetate buffer) was quantified. Briefly, one GSE capsule was extracted by sonication for 30 min using each of the dissolution media. The extracted solvent was diluted ten times and then analyzed using the LC-MS method mentioned in Section GSE Capsule Dissolution Samples (LC-MS Method). Three replicates were made in parallel for quality control purposes.

GSE Capsule Dissolution Samples (LC-MS Method)

For LC parameters, mobile phase A was 0.1% formic acid (FA) in water, and mobile phase B was 0.1% FA in ACN. The gradient was 2 to 10% B in 0.4 min, and raised to 30% B from 0.4 to 3 min, then raised to 60% B in 0.5 min and dropped to 2% B in 0.1 min, with a flow rate of 0.4 ml/min. The column was equilibrated with 2% B for 0.4 min between injections. The column was thermostatted at 30°C, and the autosampler was set to 4°C. The injection volume was 1 μl.

For MS parameters, nitrogen was used as the nebulizing and drying gas. The nebulizer was set to 35 psi and the drying gas to 300°C with a flow rate of 12 L/min. The sheath gas was set to 250°C with a flow rate of 12 L/min. The nitrogen used for MS electrospray ionization was generated from a Parker Balston NitroFlow60NA nitrogen generator.

Each GSE dissolution sample was diluted 10 times using 70% methanol acidified with 0.1% FA and centrifugated at 12,000 rpm for 10 min. The supernatant was directly injected into UHPLC under dynamic multiple reaction monitoring (dMRM) mode. The dMRM parameters are listed in **Table 1**.

RSV Capsule Dissolution Samples (LC-MS Method)

For LC-MS parameters, mobile phase A was 0.1% FA in water, and mobile phase B was 0.1% FA in can with a flow rate of 0.4 ml/min. The gradient was 25 to 60% B in 3 min, and the column was equilibrated with 25% B for 0.5 min between injections. The injection volume was 1 μl. The column was thermostatted at 30°C, and the autosampler was set to 4°C. Nitrogen was used as the nebulizing and drying gas. The nebulizer was set to 35 psi and the drying gas to 300°C with a flow rate of 12 L/min, while the sheath gas was set to 250°C with a flow rate of 12 L/min. The nitrogen used for MS electrospray ionization was generated from a Parker Balston NitroFlow60NA nitrogen generator.

All the RSV dissolution samples were diluted using a solution of 70% methanol acidified using 0.1% formic acid (1:100). The prepared sample was centrifugated at 12,000 rpm for 10 min. The supernatant was directly injected into UHPLC. The scan mode was dMRM. The parameters are presented in **Table 1**. Brown Eppendorf tubes were used to protect the RSV dissolution samples from light.

RESULTS

Calculation of GSE Marker Compounds Dissolved

For all the tested GSE capsules, the percentage of four marker compounds (gallic acid, catechin, epicatechin, and procyanidin B2) released from GSE capsules was calculated based on analytically measured amounts. The dissolution profile is presented in **Figure 1**, and the detailed data are shown in **Supplementary Material**. In general, the amount of four marker compounds in both the dissolution media increased rapidly. For the 0.1N HCl acid medium, the GSE capsules released 96.49, 89.09, 87.65, and 78.84% of gallic acid, catechin, procyanidin B2, and epicatechinin, respectively, within 60 min, meeting the general chapter USP disintegration and dissolution standards. However, for the acetate buffer with a pH of 4.6, the GSE capsules released 73.09, 67.9, 71.06, and 59.75% of gallic acid, catechin, procyanidin B2, and epicatechin, respectively, within 60 min, failing to meet the USP guidelines of 75% release within 60 min.

Calculation of RSV Marker Compounds Dissolved

The percentage of trans-RSV released from the RSV capsules was also calculated based on the LC-MS data. The dissolution profile is illustrated in **Figure 2**, and detailed data are shown in **Supplementary Material**. For this case, the amount of trans-RSV in both dissolution media increased rapidly, and the release was >75% within 60 min, meeting the general chapter USP disintegration and dissolution standards.

DISCUSSION

In this study, a novel dissolution test and LC/MS analysis were developed to evaluate the performance of grape polyphenol dietary supplements, namely, GSE and RSV capsules.

For the GSE capsules, the marker compounds released rapidly in both media and under conditions found in the stomach (at pH 1.2) met the USP dissolution guidelines. However, the same GSE capsules failed to meet that USP guideline of a 75% release within 60 min in the acidic medium with a pH of 4.6.The reasons for dissolution test failure can be broadly classified into two categories: dissolution procedure and capsule quality (12). It is possible that these marker compounds may be extracted less efficiently with the dissolution media than with sonicated organic solvent mixtures. Also, in this study, dissolution method type I was used where the capsules were placed inside the basket with a 40-mesh size. The openings of the 40-mesh size may be small to allow the release of all dissolved products. Any coacervate, which could result during the disintegration and dissolution of GSE capsules in 0.1 N hydrochloric acid (pH 1.2), cannot pass into the bulk dissolution medium, as shown in **Figure 3**. If capsule residues could not completely pass through the basket into the dissolution medium, the percentage of dissolved GSE components would be lower than expected and would finally lead to incomplete release (<100%). It is worth noting that using a spiral capsule sinker with large opening (10-mesh size) could solve this problem. Moreover, a previous study has reported

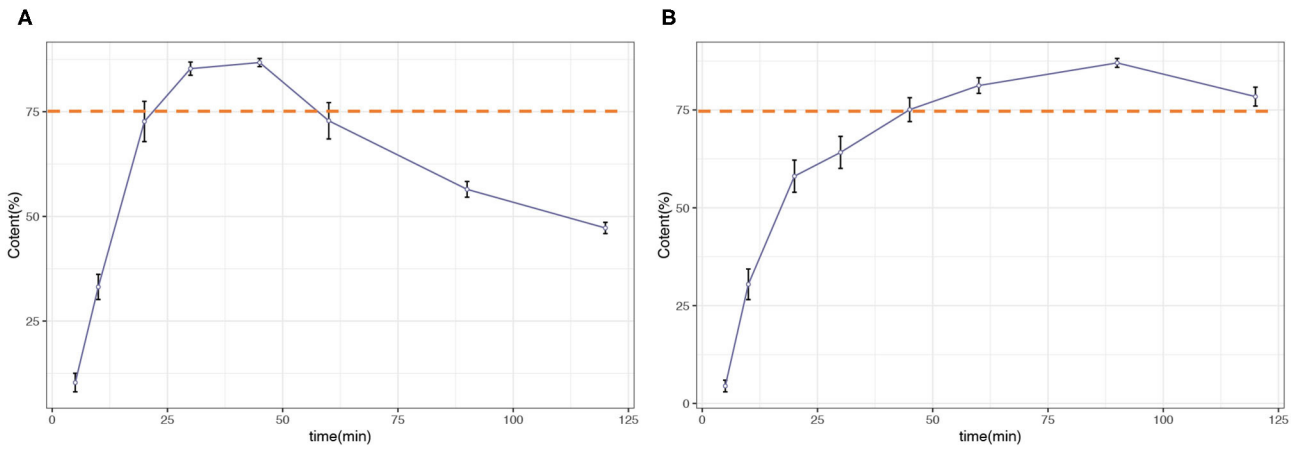

FIGURE 2 | Dissolution profile of resveratrol (RSV) capsules. **(A)** 0.1 N hydrochloric acid (pH 1.2). **(B)** 0.05 M acetate buffer (pH 4.6).

FIGURE 3 | GSE capsule residues within the dissolution basket after 2 h dissolution in 0.1 N hydrochloric acid (pH 1.2) (as indicated by arrowheads).

that some products were sensitive to chosen test conditions, namely, beaker size and the equipment used in dissolution study (13). Because of the limitation of our laboratory and costs, we did not optimize these parameters, and these aspects might be further improved. The results also indicate that the bioactive compounds may be not released properly from GSE capsules in the 0.05 M acetate buffer (pH 4.6), while these compounds were successfully released from GSE capsules in 0.1 N hydrochloric acid (pH 1.2).

For the RSV capsules, trans-RSV released was more than 75% within 60 min in both the dissolution media. However, for the 0.1-N HCl acid medium, the amount of trans-RSV dropped after 45 min. Due to the stability and solubility of trans-RSV being highly influenced by pH and temperature, trans-RSV might be degraded during the experiment. Hence the concentration obtained by UHPLC-QqQ was much lower than the amount that was released. Photochemical and photocatalytic degradation of trans-RSV is another possible reason for the

reduction, owing to cis-isomerization, which occurs when the *trans*-isomer is exposed to sunlight, or artificial or natural UV radiation at a wavelength of 254 or 366 nm (14–18). In this study, even though the dissolution apparatus was covered with tin foil to protect RSV from light, and brown Eppendorf tubes were used when preparing the RSV capsule samples, light-sensitive trans-RSV may still be exposed to visible light during the experiment. Hence, it is worthwhile to note that special attention must be paid to trans-RSV dissolution testing. These results demonstrate the need to improve the dissolution apparatus for these light-sensitive compounds. Our findings suggest that product formulation needs to be considered in all such studies that examine the bioavailability of a botanical product in animal or human trials and in as rigorous a manner as botanical authentication and chemical profiling of the actual product. That is, the material of the capsule itself and the excipients used and blended into the actual botanicals need to be subjected to such dissolution tests to ensure that the correct concentrations needed in animal and human studies are delivered.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

WL, TO, FM, JS, and QW designed the study. WL, TO, and HP conducted the experiments, performed data analysis, and drafted the manuscript. FM, JS, and QW supervised the overarching project, and all the co-authors, namely, DR, MF, GP, and JM, discussed the original concept and overall objectives with DR and MF providing key insights into botanical ingredients and formulation. GP and JM provided criteria under which the botanicals were needed for applications in the planned clinical trials. All authors contributed to the article, reviewed and strengthened the final version of the manuscript, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.780260/full#supplementary-material

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REVIEW
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Chemometric-Guided Approaches for Profiling and Authenticating Botanical Materials

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Botanical supplements with broad traditional and medicinal uses represent an area of growing importance for American health management; 25% of U.S. adults use dietary supplements daily and collectively spent over $9. 5 billion in 2019 in herbal and botanical supplements alone. To understand how natural products benefit human health and determine potential safety concerns, careful *in vitro*, *in vivo*, and clinical studies are required. However, botanicals are innately complex systems, with complicated compositions that defy many standard analytical approaches and fluctuate based upon a plethora of factors, including genetics, growth conditions, and harvesting/processing procedures. Robust studies rely upon accurate identification of the plant material, and botanicals' increasing economic and health importance demand reproducible sourcing, as well as assessment of contamination or adulteration. These quality control needs for botanical products remain a significant problem plaguing researchers in academia as well as the supplement industry, thus posing a risk to consumers and possibly rendering clinical data irreproducible and/or irrelevant. Chemometric approaches that analyze the small molecule composition of materials provide a reliable and high-throughput avenue for botanical authentication. This review emphasizes the need for consistent material and provides insight into the roles of various modern chemometric analyses in evaluating and authenticating botanicals, focusing on advanced methodologies, including targeted and untargeted metabolite analysis, as well as the role of multivariate statistical modeling and machine learning in phytochemical characterization. Furthermore, we will discuss how chemometric approaches can be integrated with orthogonal techniques to provide a more robust approach to authentication, and provide directions for future research.

Keywords: metabolomics, adulteration, multi-omics, dietary supplements, biochemometrics, chemometrics, botanicals, authentication

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INTRODUCTION

Botanical medicines and dietary supplements represent a growing facet of personal health and medical care for Americans; the 2017 survey from the Council for Responsible Nutrition found that botanicals make up ca. 39% of total dietary supplement usage for adults in the United States (1), and US sales of herbal supplements totaled $9.6 billion in 2019, an annual increase of 8.6% (2). The use of botanical medicines and dietary supplements has come to include patients receiving

disease therapy, such as cancer (3) and chronic obstructive pulmonary disease (COPD) (4). The increase in economic and biomedical relevance of botanicals have led to a rise in research interest surrounding their potential health benefits, including support from the National Institutes of Health (5). The US National Library of Medicine's clinical trial tracker (clinicaltrials.gov) had >140 active clinical trials involving "herbal" or "botanical" preparations listed (accessed July 30, 2021) (6). However, the veracity of biomedical research, whether it is *in vitro* studies or clinical trials, is predicated on the authenticity and purity of the botanical(s) being studied. Botanical products are inherently complex chemical mixtures that can vary depending on abiotic and biotic factors during growth and post-harvest processing. Complicating this is the fact that products can be obtained from multiple producers and growers, potentially with multiple sources of raw material and processing techniques. Thus, to ensure the authenticity, efficacy, and safety of botanical dietary supplements, complex multi-faceted methods are required. This review focuses on chemometric and orthogonal methods for profiling, analyzing, and comparing botanical systems. We first provide opportunities and limitations of traditional botanical product authentication, followed by an overview of alternative chemometric approaches, then delve into a plethora of multivariate statistical approaches for botanical evaluation and present a workflow for how researchers can rationally select an analytical model based on data types and goals.

OPPORTUNITIES AND LIMITATIONS OF TRADITIONAL APPROACHES

Morphology

Plant morphology is the traditional approach to botanical product authentication, based on leaf shape and size and arrangement, color, life cycle changes, and other phenotypic factors. The combination of modern resources for plant identification and expansive collections of medicinal plant herbarium vouchers allows for fairly accurate morphological characterization (7, 8). Although trained specialists provide the most accurate identifications, guidebooks and phone applications provide a simple, inexpensive avenue for authentication. Increased accuracy results from micromorphology which allows species-specific evaluations of pollen shape, pore size, and other microscopic traits (9, 10). Recently, machine learning and image processing software have led to high-thruput identification of medicinal plants based on predefined characteristics and extensive training datasets (11, 12).

Despite its strengths, morphology-based identification is limited and often impractical, especially for rare plants. Similar environments and evolution pathways can result in unrelated plants with strong morphological resemblances but differing medicinal properties. Furthermore, important morphological information is lost when plants are dried or powdered, such as leaf shape and texture. Morphology also varies between plant parts, and recorded information for identifying plants based on below ground parts rarely exists. While certain root characteristics are useful, such as stone cells, auxiliary root angle, and rhizome length, the literature for species level identification is lacking and often contradictory between labs (13–16). Taxonomic identification is further complicated by vernacular names, which vary based on culture, location, language, and subspecies (17, 18).

Genetics

Genetic approaches, namely DNA barcoding and genome sequencing, are powerful tools for herbal product authentication. DNA can be extracted from fresh or dried tissue and is often effective with post-processed material (19). Primer-based methods are the most straightforward approach to DNA based identification: predefined primers for single genes (ITS2), a combination of genes (*matK* and *rbcL*), or chloroplast genomes (18, 20–23) amply specific fragments know to vary between species and have potential to differentiate morphologically and genetically similar species (24). Extensive sequence libraries exist which simplifies species identification; rare and understudied species are not thoroughly represented though (24). As sequencing becomes increasingly advanced and affordable, the applicability of genetic marker-based identification of a broad range of botanicals will increase.

DNA barcoding, including random amplification of polymorphic DNA (RAPD) (25) and inter-simple sequence repeats (ISSR) (26), provides a robust evaluation of genome diversity through examination of the presence/absence of more than 20 random fragments of polymorphic DNA at a time. Primer-based approaches amplify random segments of DNA to compare polymorphic variations among species. Although DNA barcoding is reliable, it is time consuming and requires meticulous method optimization for each application. Further, there is low resolution at the species or sub-species level (27). Recent advances in metabarcoding, which combines next-generation sequencing with bioinformatics, has greatly improved the ability to detect adulteration and supplementation in herbal products (28–30). Notably, the EU and other governing bodies suggest metabarcoding to evaluate the identity and safety of botanical products (31). For example, Seethapathy et al. used metabarcoding to determine that over 24% of Ayurvedic herbal products tested do not contain the botanical as labeled (28). However, metabarcoding is expensive and requires a reference DNA library and pre-defined genetic markers. So, for rare species or those without sequenced genomes, metabarcoding is ineffective as a quality control approach (32).

While genetic approaches have proven useful for botanical product quality control, there are limitations. Plant tissue is damaged and degraded during processing procedures, hindering extractions of high-quality DNA (33). Since genetic approaches do not provide quantitative data, there is limited ability to determine relative abundances of different species within a product. Thus, DNA barcoding does not allow trace contamination, as from shared equipment, to be discerned from intentional, large-scale adulteration of products. A final limitation is the inability to evaluate medicinal properties through barcoding based approaches. The medicinal value of a product is largely based on its chemical constituents. Without

detailed chemical analysis, the presence and relative abundance of specific medicinal compounds is unknown. So, while genetics may be able to detect adulteration, it cannot determine a products actual medicinal value. Thus, chemical evaluation serves to both authenticate botanicals and provide information of a product's bioactive potential.

TARGETED ANALYSIS OF BIOMARKERS

A simple and common approach to herbal product quality control is the use of small-molecule based targeted analysis. This approach uses individual and small groups of compounds specific to the botanical in question. Using a targeted analysis allows quick verification the product contains the plants as advertised. This section outlines the targeted analysis workflow, with examples and explanations of the pros and cons of targeted approaches.

Single Biomarker Approach

The first step in using small molecule chemistry to serve as biomarkers of quality and authenticity is to identify a targeted metabolite or small set of metabolites specific to the botanical in question. Since many commercially available botanical medicines and dietary supplements are fairly well characterized in scientific literature, the identification of predominant metabolites (also known as 'marker compounds') is fairly straightforward. These targeted compounds are analyzed by a chemical methodology and compared against reference standards and literature values; common analytical techniques include charged aerosol detection (CAD), ultraviolet-visible (UV/VIS) spectrophotometry, and mass spectrometry (MS), often with chromatographic separation beforehand (liquid chromatography, "LC", or gas chromatography, "GC" being the two primary forms). Nuclear magnetic resonance (NMR) is an analytical technique that has become more quantitative recently (qNMR) to facilitate comparisons between complex botanical samples (34–36).

However, axiomatic to using a defined marker compound is the knowledge of the chemistry of the system at hand and the commercial availability (or the ability to isolate and conclusively identify) of the target marker compounds. While many botanicals on the market have well-developed chemical libraries and/or have monographs detailing their chemical composition [including the German Commission E (37), US Pharmacopeia (38), and Tyler's Herbs of Choice (39)], not every botanical, nor every potential dietary supplement, is as thoroughly studied, and gaps in the literature of even well-known botanicals still exist today. The choice of marker compound also should, but doesn't necessarily, have relevance to the putative biological activity of the botanical medicine or dietary supplement. Finally, standards must be available to construct calibration curves; if they are not commercially available, researchers face the daunting task of isolating and elucidating the structure prior use as a marker compound (40).

Furthermore, tying authenticity to a single compound overlooks the broader chemical landscape present in the botanical product, and can leave products susceptible to potential adulteration. Single-point analyses can be confounded by spiking with specific compounds or mixtures that might bypass quality control procedures. One example is the discovery by Chandra et al. of adulteration in ginkgo (*Gingko biloba*) extracts spiked with either single isolated flavonoids or flavonoid-rich mixtures (41). As the broad category "flavone glycosides" was chosen by the gingko market as an authenticity marker, it was prone to spiking by flavones (e.g., quercetin, kaempferol, and isorhamnetin) to meet the quality criteria. In fact, three out of eight products analyzed in the study that were labeled to contain ginkgo extracts actually resembled those of commercial extracts from Japanese sophora (*Styphnolobium japonicum*) (41). In other cases, botanical dietary supplements have been doped with dyes or other synthetic mixtures to deceive single molecule quality control methods (42). Supplements with alleged weight loss properties were spiked with alkaloid derivatives, ephedra stimulants, or androgenic steroids (43, 44). Spiking and adulteration can also be used to bypass negative controls searching for known contaminants/adulterants; 1,3-dimethylamylamine (1,3-DMAA) is one case study. The United States Food and Drug Administration (FDA) had banned 1,3-DMAA in 2016 and pulled all products containing the stimulant from shelves because of an increased incidence of ER visits correlated with this stimulant, as well as failure to meet regulatory conditions (45). However, investigations by Cohen et al. revealed 1,3-DMAA analogs present in multiple weight loss supplements (five out of six tested), illustrating how adulteration can be used to sidestep regulatory authorities with potentially toxic constituents (45).

Molecular "Fingerprints"

Beyond single molecules for targeted biomarker detection, researchers can collect information on a range of molecules or a "chemical fingerprint" that exemplifies a more robust and nuanced representation of the botanical's metabolite profile. Using multiple components blunts the potential for metabolite spiking (as seen with single marker compound approaches) and can provide more selective and sensitive analysis for distinguishing authentic material. Lv et al. (2016) developed an HPLC-based fingerprint to differentiate species and geographical origins of *Rhizoma coptidis* using six distinct alkaloids (46), while eight organic acids were used to distinguish between *Castanea* spp. Buds (47), and Parveen et al. validated an UHPLC-UV-MS method incorporating 10 standard compounds to distinguish closely related *Tinospora* species (48). Even AOAC's official method for some botanicals incorporates multiple compounds; their method 2015.007 for investigating Ashwagandha (*Withania somnifera*) employs 10 withanolide glycosides and aglycones (49). However, multi-molecular chemical "fingerprints" are more time- and labor-intensive approaches, as they require the quantitation of multiple compounds with different linear ranges and limits of detection and quantitation (LOD and LOQ, respectively). This also does not circumvent the issue with single biomarker approaches needing reliable, commercially available standards in order to determine the overall fingerprint and quantitation for the analysis.

METABOLOMICS

The 'metabolome' is generally defined as the complete set of small molecules produced by an organism or biological sample at any given point in time. Metabolomics, therefore, is the unbiased, holistic measurement of the metabolome (though practically speaking there is no single analytical approach capable of measuring all small molecules in one experiment), and the relative areas or heights of signals within the metabolome can be employed as a basis for comparison between two or more samples. As such, metabolomics provides a powerful tool for understanding the complete chemical makeup of an herbal product, which can be used for efficient and accurate quality control and authentication. Metabolomics characterizes the chemical relationships that underlie variations based upon genotype, origin (50), climate (51), or other biotic or abiotic interactions (52–54). While a variety of analytical inputs can be used to generate metabolome data – including Fourier-Transformed infrared spectroscopy (FT-IR), charged aerosol detection (CAD), ultraviolet-visible (UV/VIS) spectrophotometry, mass spectrometry (MS), and Nuclear Magnetic Resonance (NMR) spectroscopy – the two primary analytical approaches employed for the majority of metabolomics studies are liquid chromatography coupled to mass spectrometry (LC-MS) and NMR spectroscopy. These two provide incredible sensitivity and selectivity in profiling a large fraction of the metabolome of a sample, while also offering detailed structural information crucial for metabolite annotation for the authentication of botanical dietary supplements and medicines (55, 56). The advances of metabolomics techniques is not the focus of this review, the incredible innovation and progress that has been achieved in metabolomics experiments have been discussed elsewhere (57–59).

As relative comparisons are being made across a large dataset (often hundreds to thousands of peaks in a single metabolome data matrix), the chemical identification of the peaks is not necessary at the outset of the experiment and analysis. Thus, untargeted metabolomics studies can compare complex samples with no a priori knowledge of their constituents (60) and do not require the acquisition of analytical standards to complete comparative analyses, a distinct advantage over the targeted or fingerprinting approaches described above.

CHEMOMETRIC APPROACHES FOR PATTERN RECOGNITION AND SIMILARITY DETERMINATION

While a valuable tool for authentication of herbal products, the innate complexity of metabolomic datasets can be daunting when developing novel quality control approaches. One of the major challenges facing metabolomic (or other molecular fingerprinting approaches) is not the collection of the data, but instead the processing, analysis, and interpretation of the expansive datasets that are often generated. In metabolomics, the data matrices often have more columns (independent variables, such as *m/z*-retention time pairs or NMR signal buckets) than rows (samples) and are known as "landscape" matrices. "Chemometrics" refers to the application of statistical methods to discover relevant analysis and maximize the information obtained from the chemical datasets (61). For the authentication of botanical materials, chemometric pattern recognition approaches are the most prevalent. There are a variety of multivariate mathematical–statistical methods for prediction and pattern recognition (**Figure 1**), which have disparate criteria for successful application to complex chemical datasets.

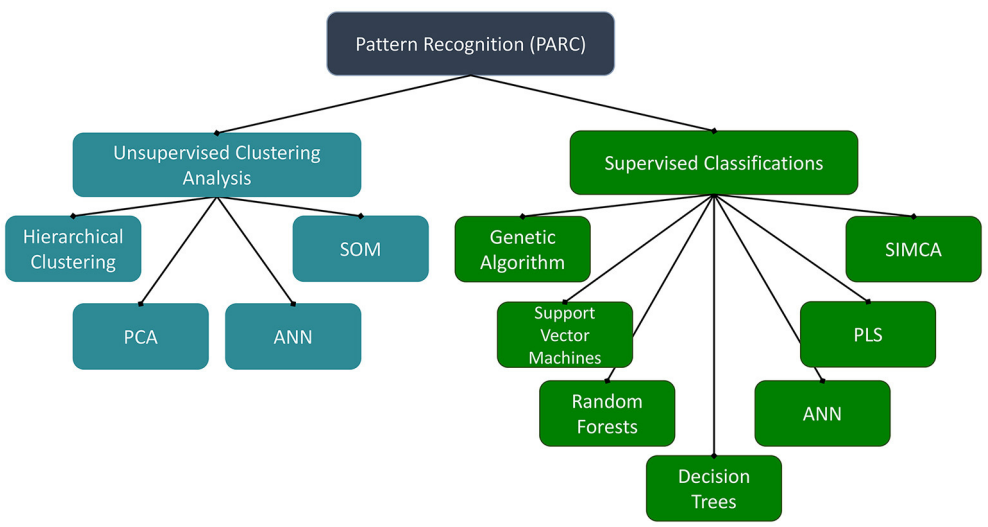

FIGURE 1 | Pattern recognition methods. ANN, artificial neural networks; PCA, principal component analysis; PLS, partial least squares; SIMCA, soft independent modeling of class analogy; SOM, self-organizing maps.

Data Preparation for Chemometric Analysis

In any statistical analysis, the robustness of the predictions and inference is limited by the quality of the data that is input into the model. For chemometric analysis, there are a number of aspects of the dataset that will contribute to the overall quality and reliability of the resulting model. One aspect of note is the reproducibility of analytical data. Variations in extraction protocol, sample handling as well as the mass spectrometer detection itself (mass analyzer, detector, and even the chromatography components) preclude facile comparisons between labs. This can potentially lead to differing raw spectral data, as well as variations in results obtained (62).

Raw spectral data, from any analytical source (LC-MS, GC-MS, NMR, FT-IR, etc.) must be processed in order for the statistics to be effective. For some spectral data (e.g., ^{1}H-NMR and FT-IR), the data is traditionally sliced into "bins" that are then used as individual features in the dataset (63, 64). Mass spectrometry data is obtained as discreet features (unique *m/z*-retention time pairs), yet still requires multi-step "preprocessing" to identify peaks and align the data. There are numerous methods and workflows to preprocess spectral data, and have been examined and reviewed exhaustively elsewhere (65–71). While most open access preprocessing software yields similar performance in detection of actual peaks ("true" features) from the data [as examined by Li et al. (68)], the abundance of parameters needed to fine tune in order to develop a robust final dataset can be challenging for researchers. The subsequent scaling, centering, and normalization of the dataset can also play a factor in the resulting statistical analysis (72, 73). Thus, careful treatment of the raw data during preprocessing is critical to downstream chemometric analyses in order to obtain reproducible and reliable interpretations of the data. The potential for variations in the processing of the data is a persuasive argument in favor of the trend in metabolomics to encourage open science by depositing the spectral data, as well as metadata associated with the preprocessing parameters used, in accordance with the FAIR (Findable, Accessible, Interoperable, and Reusable) data principles (74).

Unsupervised Approaches

Unsupervised methods are the (relatively) simplest ways of classifying large chemical datasets, designed to analyze data that can only be arranged in one matrix. These methods are "unsupervised" in the sense that no data classifications are known before the analysis; instead data structures are revealed through these pattern recognition methods. Researches should be aware of the differences between hard and soft classification techniques.

Principal Component Analysis (PCA)

Principal component analysis (PCA) is an unsupervised approach which projects multivariate data (with k features/variables) onto a smaller dimensional space ($<k$-1). As such, PCA is often referred to as a projection or dimension reduction method. The metabolite profile is reduced to uncorrelated principal components (PCs) which represent the total variation present in the metabolome. The first principal component accounts for the maximum percentage of the overall variance, the second principal component (orthogonal to the first) accounts for the second largest amount of variance, and so on until all the variation in the data is accounted for or the number of principal components reaches the limit (i.e., the number of features-1) (75). The principal components are plotted in a pair-wise fashion (typically the first two, which explain the most variation) on a 2-dimensional plane – known as a "scores" plot – that demonstrates the spatial relationship between different samples. Points which cluster together have similar correlations in the PC variations, which translates to similarities in their overall chemical profile. Likewise, dissimilar samples are located further from one another in the two-dimensional graph. A second corresponding graph associated with PCA is the loadings plot, in which the features (variables) are arranged in a two-dimensional plot using the same PCs as the scores plot. The spatial representation of the loadings mirrors that of the scores, thus enabling the determination of which features are more prevalent in certain clusters of samples. Zhang et al. (76) developed an approach to authenticate juices from different berry fruits using untargeted metabolomics. Using PCA generated from LC-QTOF-MS spectra, they were able to discriminate between blueberry, cranberry, apple, and grape juice (**Figure 2**). The corresponding loadings plot yielded 18 characteristic markers that were able to categorize the juices (76). Additionally, Farag et al. differentiated ten cinnamon accessions from the main cinnamon species using ^{1}H-NMR metabolomics combined with unsupervised chemometric approaches (77). The scores plot (**Figure 3**) distinguished between *Cinnamomum cassia* and *C. verum*, with PC1 and PC2 comprising 77% of the variability in the model. The loadings plot suggested nine key metabolites which could be used to differentiate between cinnamon accessions, including cinnamaldehyde and eugenol; the exclusive presence of eugenol in *C. verum* samples suggested its potential as an authentication marker (77). Thus, PCA represents a robust and potent chemometric tool in the evaluation of different samples and their authenticity/purity.

However, while PCA can demonstrate clusters of samples based upon their chemical profile, it is not able to provide quantitative metrics around the degree of similarity between samples, nor ranking how similar samples are to one another. Furthermore, PCA relies on a subsection of the overall principal component model to visually represent similarities and differences between the samples; this is often an *ad hoc* choice of PCA components which can mask outliers or shift the overall spatial relationship between samples, leading to the possibility of specious results and subsequent conclusions. Integration of multiple PCs into a single quantitative comparison may circumvent this. Termed the composite score, it has potential to facilitate comparisons between multiple samples using the entirety (or at minimum a significant subset) of the principal component model to quantify similarity between samples (78). This approach was used recently by Wallace et al. to differentiate *Hydrastis canadensis* supplements from potential adulterants (79).

Suggestions for future use: PCA is a powerful unsupervised clustering tool with accessible computational resources to

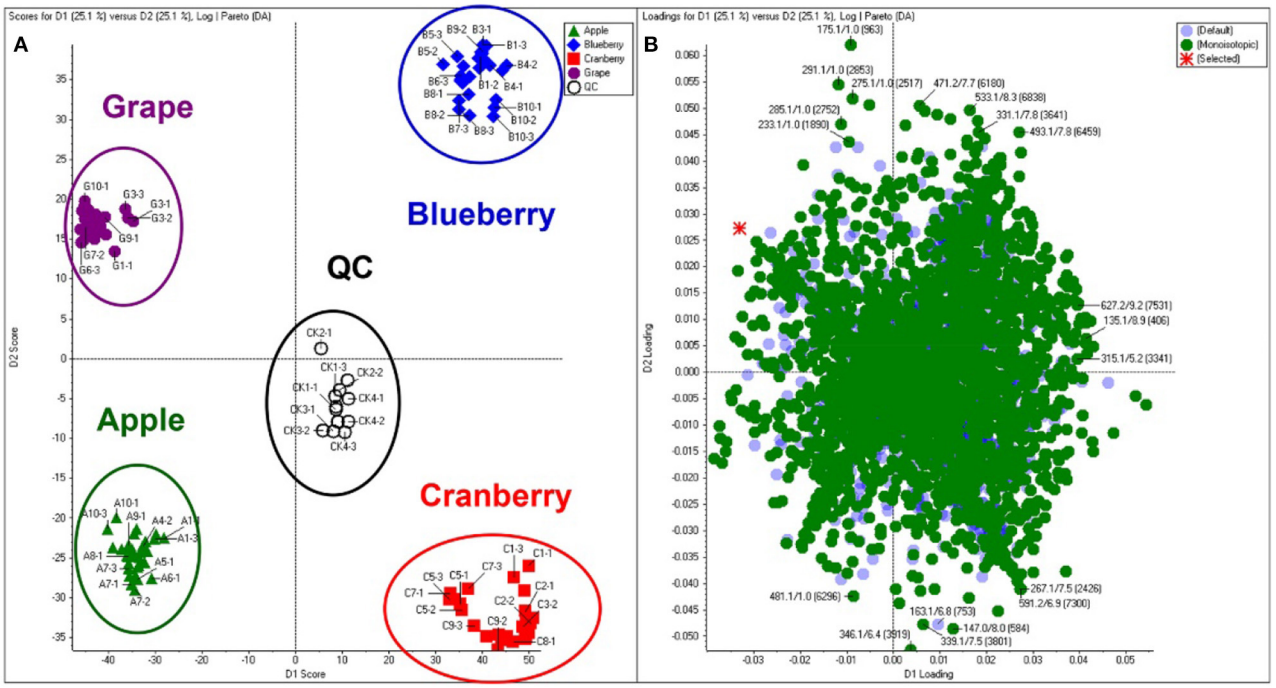

FIGURE 2 | Principal component analysis (PCA) scores **(A)** and loadings **(B)** plot demonstrating differentiation between fruit juices based upon untargeted metabolomic analysis. Reproduced with permission from Zhang et al. (76). Copyright 2018, American Chemical Society.

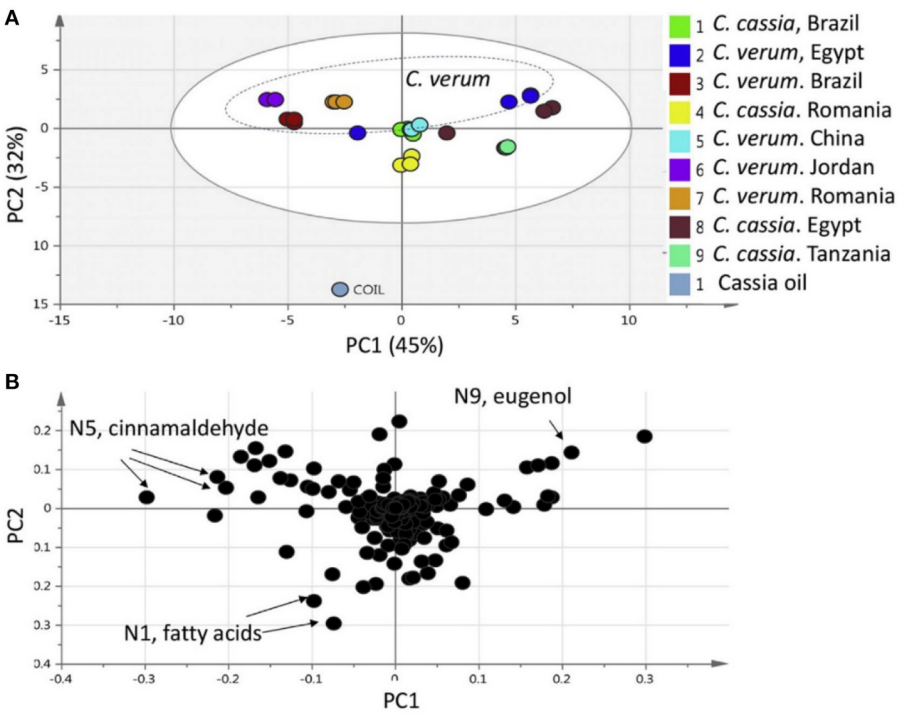

FIGURE 3 | Principal component analysis (PCA) from Cinnamomum verum and C. cassia from different geographical origins, and representative commercial oil, using ^{1}H-NMR ($n = 3$) metabolomics. The scores plot **(A)** demonstrates clusters at distinct spatial points in the PC1-PC2 scores plot, and loadings plot **(B)** highlights major contributing molecules to the separation of the samples. Reproduced with permission from Farag et al. (77). Copyright 2018, Elsevier Ltd.

simplify analysis, making it an ideal first step in any chemometric analysis. PCA can be used prior to any supervised approach to confirm expected clustering among samples, and that apparent distinctions result from true variations in sample metabolomes, not as a result of overfitting to predefined categories. Alone, PCA can be used to determine if adulterated and pure samples differ while simultaneously identifying biomarkers likely responsible for any variation. Thus, PCA has potential for a quick and easy approach to botanical authentication based on metabolite profiles. Possible sample clustering that may be identified using PCA is species proximity, cultivation procedures, or origin of plant growth. For any authentication study requiring more detailed information of how samples are related or identification of unknown with a single model, other approaches should be performed concurrently with PCA.

Hierarchical Cluster Analysis (HCA)

Hierarchical clustering analysis (HCA) uses distances between sample groupings (clusters) to organize samples into taxonomies; objects with the highest similarity cluster together, and generated clusters are treated as a new, independent feature which are clustered with the next most similar variable. Similarity is calculated as distance between variables through a variety of algorithms, including Euclidian, Mahalanobis, or city block (Manhattan); similarly, there are various linkage rules for amalgamating the cluster analysis, such as minimum or maximum similarity between variables, group average (average similarity between every possible pair of data points), or Ward's Method (sum of the squared distance between each pair of data points). Proximity matrixes are used to compare the calculated similarity of all groups. The shorter the distance, the more similar the variable, and thus more likely to be related. However, since the similarity (distance) and linkage can be calculated using different combinations of rules, the results of cluster analysis are difficult to compare between studies. In the case of sample authentication, each botanical sample is treated as a variable and clusters are formed based on similarity in peak heights (or other metabolite features) so that the most chemically similar samples group together. HCA has been used to distinguish *Cirrhosae bulbus* from common adulterants using UPLC-ELSDA fingerprinting (80). Zhou et al. demonstrated the use of HCA in discriminating between two bitter melon (*Momordica charantia*) chemotypes with different medicinal properties (81). While PCA was able to distinguish the two chemotypes, HCA allowed a deeper insight into how each variety differed within the groups (81), and the combination of PCA and HCA predicted biomarkers for easy chemotype distinction of unknown samples. NMR chemical fingerprinting of Sarsaparilla species (*Decalepis hamiltonii*, *Hemidesmus indicus*, *Pteridium aquilinum*, and *Smilax* spp.) revealed four clear clusters, which were further confirmed by patterns in the NMR spectra (**Figure 4**) (82). In addition to detection of herbal adulteration, HCA provides opportunity to detect contamination with pharmaceutical drugs; Cebi et al. used HCA to classify coffee and tea blends adulterated with sibutramine, an illegal weight-loss drug (83).

Suggestions for future use: HCA models share similar possible directions as PCAs, with additional information of how samples are related chemically within generated clusters. A potential study may evaluate differences in chemotypic and genotypic based hierarchical clustering for authentication. It is possible genetic approaches may discover adulteration by non-target species but miss contamination with synthetic compounds; chemotypic approaches may simultaneously provide species distinction and chemical authentication. Similarly, comparisons of HCAs generated via multiple analytical techniques (H-NNR vs. LC-MS) may provide a deeper understanding of sample relationships though inclusion of additional compounds.

Self-Organizing Maps

Artificial neural networks (ANN) is a collective term for several machine learning methods. The most common unsupervised ANN approach is self-organizing maps (SOM). Section 5.2.3.4 provides an overview of supervised ANN in natural product authentication.

Self-organizing maps (SOMs), sometimes referred to as Kohonen maps or Kohonen networks, is a neural network-based algorithm that reduces the input dimensionality to represent sample patterns; SOM forms a 2-dimensional map where similar samples are mapped closer together. The benefit of this approach is that SOMs account for non-linear information in the data, and each variable's importance to the model can be derived from the weights associated with each map "point" (84). Torrecilla et al. (85) employed SOM to analyze extra virgin olive oils and detect adulteration via the addition of other oils. Using random and non-random noise to simulate adulteration, the SOM was constructed which yielded a misclassification rate $<1.3\%$ (68). Using previous research, Menezes et al. generated a library of terpenes present in three tribes of Annonaceae species (521 molecules) for use in training a SOM (86). The model was able to classify unknown samples into the three predefined tribes with 80% average accuracy (86). Similar approaches have been demonstrated using diterpenes to classify Lamiaceae spp. (87) and flavonoids to classify Asteraceae spp. (88).

Suggestions for future use: Menezes et al. provide an SOM method very applicable to natural product authentication (86). Using previous metabolomics data to classify botanical samples, despite variations in analytical and collection techniques, provides an opportunity to create authentication models without extensive benchwork. This approach should be applied to commercial supplements with well-defined chemistry to develop predictive models for existing products.

Supervised Approaches

Supervised statistical methods require the data matrix have both independent and dependent variables, the latter of which can be nominal (categorical) or numerical in nature. Nominal dependent data are ideal for clustering data into pre-defined classes, such as "pure" and "adulterated," whereas numerical data can allow for the ranking, quantifying, and comparing variables against each another. Many machine learning approaches are supervised models based on training datasets. Simply, a set of samples with known dependent variables are used to train, generate, and validate a model, which subsequently predicts the classification of additional, unknown samples (or the remainder

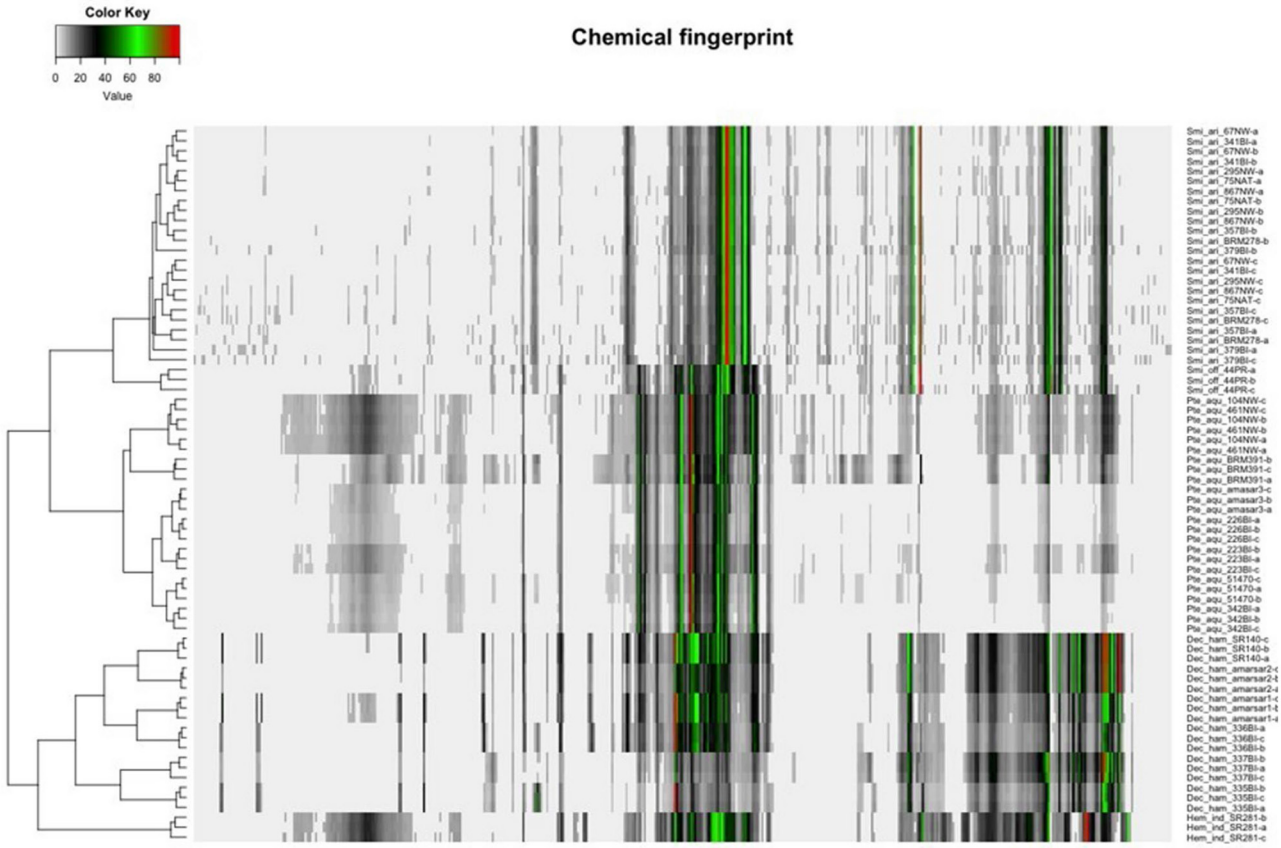

FIGURE 4 | Unsupervised clustering analysis of four species of Sarsaarilla using cheometric modeling of ^{1}H NMR data. Peak patterns are also provided to illustrate differences between four major species. Reproduced under a Creative Commons Attribution 4.0 license from Kesanakurti et al. (82).

of the data). However, as the numbers of samples in a metabolomics data set are generally fewer than the number of variables, supervised techniques are prone to overfitting the data (89); even so far as to be able to fit a model to completely random data (90). Therefore, model validation is critical before any interpretation of the model is reliable, and often quality criteria of the model are reported such as R^2 (a measure of the fit of the model) and Q^2 (the ability of the model to predict unknown samples) (91).

Partial Least Squares (PLS)

PLS is a dimension reduction tool similar to PCA. PLS condenses complex data to simpler latent variables which explain shared features between correlated samples, but with a dependent variable to supervise the construction of the model. The goals of PLS are akin to linear regression: classification of dependent variables and understanding the independent variables (metabolite features) that are predictors of this classification. A PLS model plots the latent components among the independent variables that best explain variations in dependent variables, and samples are projected onto the model space. The resulting scores plot allows simple visualization of sample clustering based on the reduced variables; the loading plot provides information about specific variables which contribute the most covariance to the model. The two primary types of PLS analyses- PLS-R and PLS-DA are defined by the nature of the dependent variable.

PLS-R

Partial least squares-regressions model variations among the independent variables to explain a numerical dependent variable. While PLS-R is uncommon for quality control of botanical products, it has been employed with biomarker identification, biochemometrics, and detection of adulteration (92). For example, PLS-R was employed to differentiate between *Hydrastis canadensis* (goldenseal) and four common adulterants using FT-NIR data (64). Following preprocessing and filtering the spectral data, PLS-R modeling successfully clustered pure goldenseal from non-target species, as well as differentiated between various goldenseal parts (roots and shoots) (64). In this case, the plot consisted of latent variables which reduced the spectral data as guided by a gradient in contamination as the dependent variable. This study also highlights the importance of preprocessing and filtering data; unprocessed data was unable to distinguish species using PLS-R (80). Partial least squares is also one of the primary predictive chemometric approaches: when there are correlations

are drawn between the dependent data set (often bioactivity or other quantitative data) and the independent chemical data from which the model is derived. This approach, known as biochemometrics when using bioactivity data, is explained more fully below.

Suggestions for future use: Some future applications of PLS-R in herbal product authentication could include evaluating products with known and specified variations in ingredients-teas with varying percentages of *Ilex paraguariensis* (yerba mate) and ashwagandha root for example. PLS-R may also be useful for discovering biomarkers to quickly differentiate between bioactive and inactive products though detailed bio-chemical analysis of commercial supplements and subsequently screening additional products for identified markers. This may bypass some typical issues with single marker analysis, as described in section Single Biomarker Approach, by using commercially available products for biomarker discovery as opposed to predetermined pure plants.

PLS-DA

Partial least squares-discriminate analysis (PLS-DA) models the data similar to PLS-R, but with the caveat that the dependent variable be a binary descriptor (e.g., "class1" vs. "class2", "authentic" vs. "adulterated", etc.), which are coded as −1 and 1, or 0 and 1. The resulting scores plot is typically able to discriminate between the two groups, as it is guided by the classification of the samples. PLS-DA is one of the most common chemometric tools applied to chemical data for authentication and discrimination among botanical products. The study by Ismail et al. demonstrates this approach by differentiating between different grades of gaharu (agarwood, *Aquilaria malaccensis*). Using ^{1}H-NMR metabolomics, a PLS-DA model was able to differentiate between "high grade" and lower grades of gaharu (**Figure 5**), and the resulting loadings plot also highlighted aquilarone derivatives that discriminated the different quality classes (93). Windarsih et al. also employed PLS-DA analysis to differentiate between authentic *Cucuma xanthorrhiza* ("Java ginger") and samples adulterated with *C. aeruginosa*. PLS-DA yielded a robust model (R^2 and Q^2 of 0.993 and 0.986, respectively) which separated authentic from adulterated samples (94).

One of the limitations of PLS-DA is that the categorization is restricted to a binary class designation. If there are more than two main categories, the discriminant analysis requires pair-wise comparison, complicating the analysis and potentially limiting the conclusions which can be drawn. This is exemplified by Barbosa et al.'s study to differentiate and authenticate paprika grown in three different areas (La Vera and Murcia in Spain and the Czech Republic) (95). The PLS-DA classification plots were done as iterations of one region vs. the other two, to comprehensively demonstrate that the three regions were distinct from one another (a classification rate of 100%) (**Figure 6**).

Suggestions for future use: PLS-DA has excellent potential in herbal product quality control since binary categorical classes can encompass multiple facets of plant differentiation. These applications range from classifying samples based on geographic origin, plant parts, species or subspecies, or adulteration status. Although PLS-DA requires pre-determined classifications of data, the loading plots can guide discovery of biomarkers for quick screening of unclassified samples. An interesting study would model one chemical dataset for multiple classifications of the same samples to evaluate how clustering and model validation (R^2 and Q^2) change to determine the most reliable classifications for authentication.

Soft Independent Modeling of Class Analogies (SIMCA)

SIMCA is a supervised expansion of PCA: samples are grouped into predefined classes and PCA is performed on each class, so that each group is projected onto a separate PC space. To detect adulteration, there are only two classes: authentic or adulterated, so one-class PCA's for authentic or adulterated samples can be generated (79). A new, unknown sample's classification is predicted by projecting it to the PC space and calculating the Q statistic (or Q residual), quantifying the similarity of the unknown PCA to the training set's PCA (79). The Q-statistic predicts if the new sample belongs in the authentic, adulterated, both, or neither class. Thus, SIMCA distinguishes similarities among samples and unknowns rather than defining the differences between groups (96). Wallace et al. intentionally adulterated *Hydrastis canadenis* with varying concentrations of *Copits chinesis* and used untargeted metabolomics with SIMCA analysis to differentiate between pure and tainted samples. Using one-class modeling, the Q statistic of "unknown" adulterated products was calculated and found to fall above the 95% confidence interval for pure samples, successfully identifying even the lowest percentage of contamination (5%) and providing a higher resolution of differentiation than PCA alone (**Figure 7**) (79).

Suggestions for future use: SIMCA is a powerful classification tool with digestible graphic outputs. SIMCA can be used for classification problems where the output for each sample is already known, such as adulterated vs. pure. It is a straightforward tool for analysis of binary classifications but becomes more complicated as more categories are added. Thus, it should be reserved for problems focused on identifying contaminated samples when deep machine learning modeling is not necessary. A suggested approach to botanical quality control is to perform unsupervised PCA to identify and confirm a binary clustering of samples followed by SIMCA to predict the classification of unknown products.

Machine Learning Models

While easily interpretable, models such as SIMCA and PLS are inherently linear algorithms, capable of modeling only linear latent covariance. As biological data are often non-linear, it is probable that the related chemical data also has a non-linear latent structure. Thus, non-linear machine learning methods can be uniquely suited to examine relationships from metabolomic or other chemical data.

Decision Trees

Decision trees are a machine learning approach that use hierarchical decisions to determine sample classification based

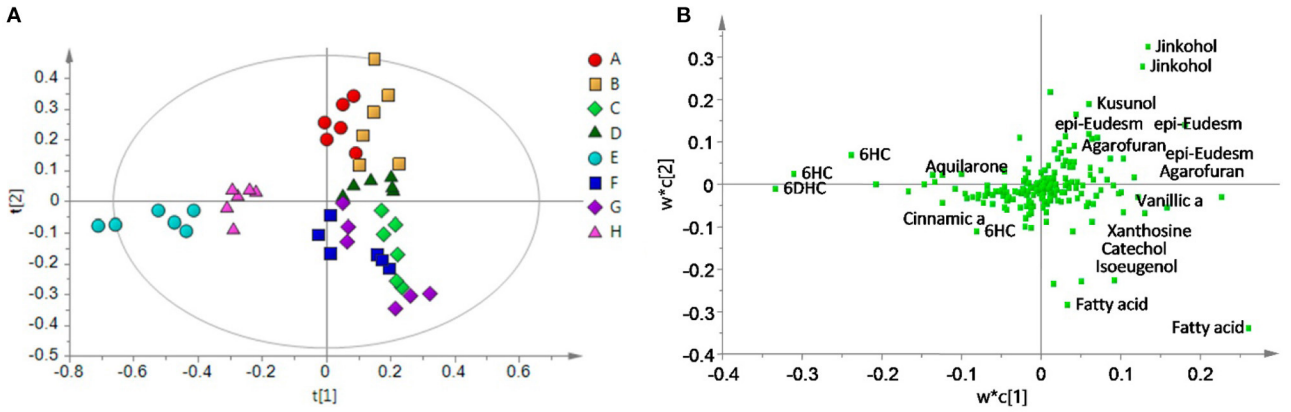

FIGURE 5 | PLS-DA analysis of gaharu (Aquilaria malaccensis) woods by ^{1}H-NMR untargeted analysis. The PLS-DA scores plot **(A)** effectively discriminated between lower grade products ("E" and "H") and higher grades. And the corresponding loadings plot **(B)** demonstrated that the lower quality products contained higher levels of aquilarone derivates. Reproduced under a Creative Commons CC BY 4.0 license from Ismail et al. (93).

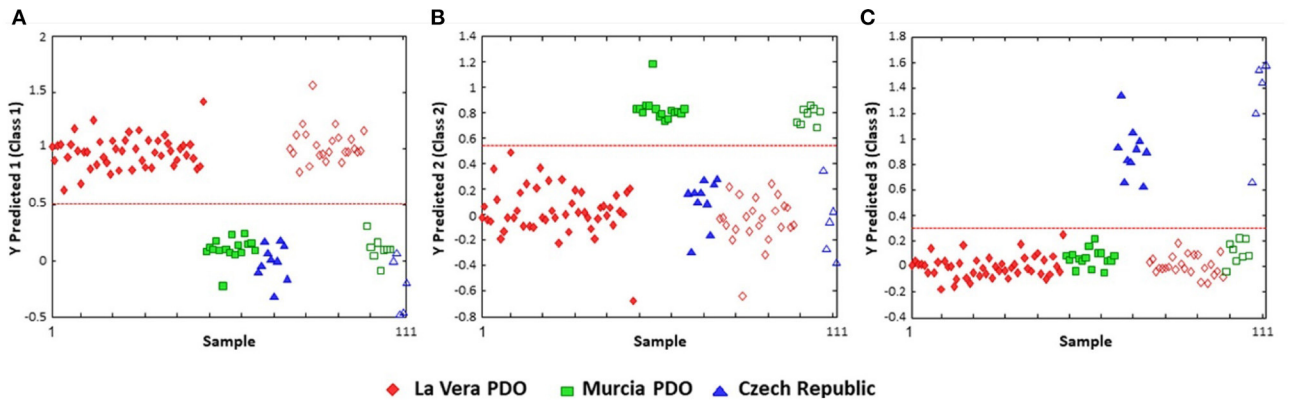

FIGURE 6 | PLS-DA discrimination plots according to the geographical region of production of paprika. **(A)** La Vera PDO vs. other regions; **(B)** Murcia vs. the other two; **(C)** Czech Republic vs. other classifications. The dashed red line indicates the classification boundary between the two designations. Open symbols represent the training data; the solid symbols are the test data. Reproduced with permission from Barbosa et al. (95). Copyright 2020, American Chemical Society.

on training data. Trees are displayed upside down, with the bulk data at the top being split based on features that best distinguish the data at each step. These distinctions are typically based on the presence or ratios of specific metabolites that separate one classifier from another. The result is a tree split into branches at decision nodes that end with leaves, or the classification groups. Decision trees are commonly referred to as classification and regression trees (CARTs) to encompass both distinct variables (classification) and numerical or continuous variables (regression). In the case of botanical product quality control, samples are classified based on species, purity, or other relevant factors. Classification trees were used to classify different cultivars of avocados based on HPLC-CAD metabolomics (97). Training data that comprised of spectra from 32 avocado samples of three varieties generated a tree which guided classification of unknown avocado oil samples into cultivar classes or no class based on specific, model generated rules (**Figure 8**) (97). A strength of decision trees is the ability to classify an unknown sample into a "no class" group to avoid overfitting or forcing a sample into a classification group. A creative application of decision trees is to predict the need of specific safety tests and evaluations for botanical products, as demonstrated by Little et al. the group used an *in silico* decision tree model to analyze the need for safety assessments of botanical products based on UHPLC with UV, CAD, and HRMS metabolomics, structure identification, consumer exposure, and existing safety evaluations (98). The developed tree used chemical data and previous records to determine if any tests are necessary for consumer safety depending on the presence of certain metabolites in the sample and database information of safety data (98). This study highlights the versatility of decision trees in quality control – they can not only identify botanical adulteration, but they can also ensure safe practices while developing botanical supplements.

Suggestions for future use: Decision trees have the appeal of a visually appealing output for a complex machine

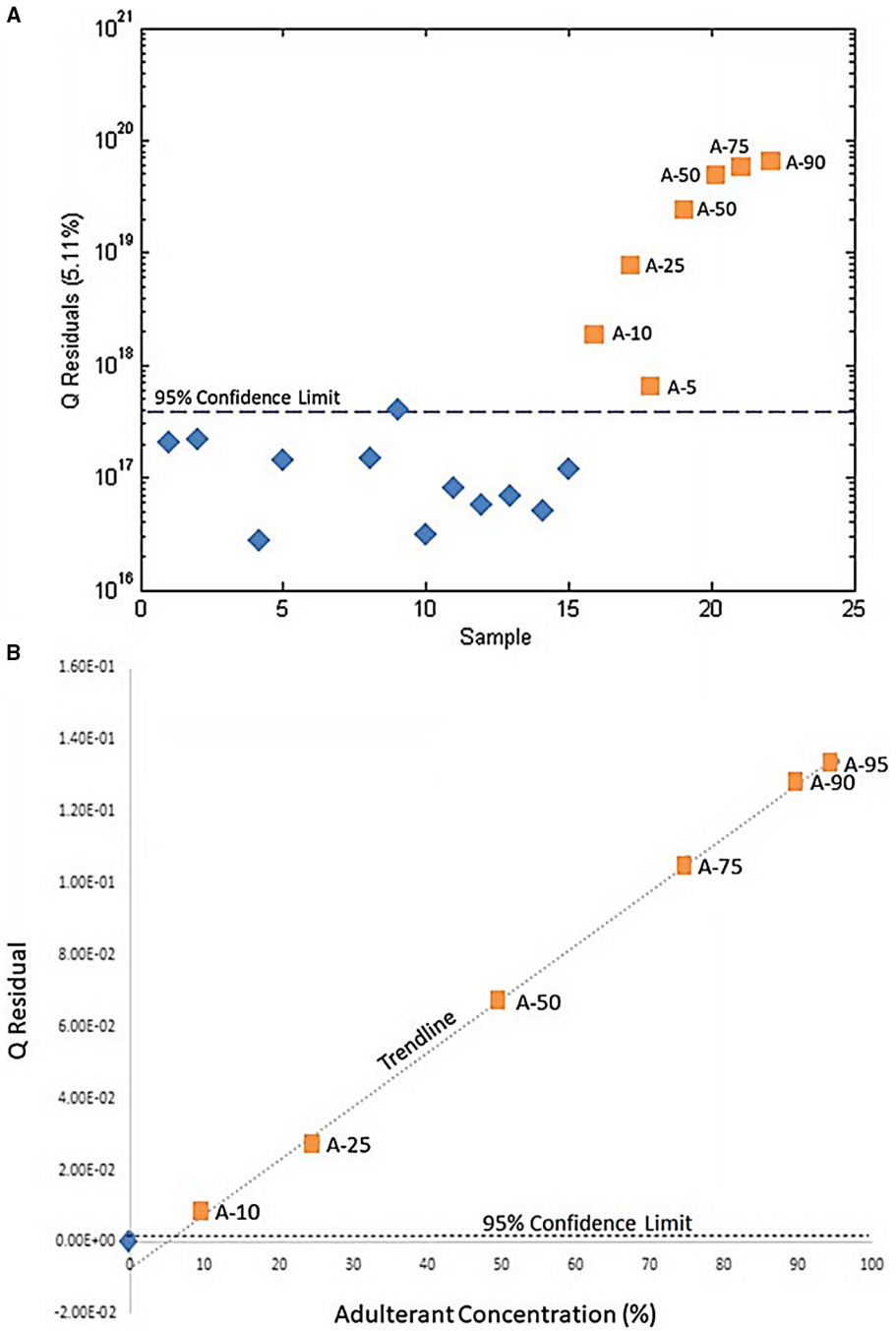

FIGURE 7 | Use of SIMCA to determine adulteration of H. canadenis by C. chinesis. **(A)** SIMCA demonstrating that pure H. candenis samples (blue diamonds) are below the 95% confidence interval and adulterated samples (orange squares) are above the 95% confidence interval. **(B)** The Q-residual of each adulterated sample. The blue diamond represents the mean Q-residual for the unadulterated H. canadenis samples. Reproduced with permission from Wallace et al. (79). Copyright 2020, Springer Nature.

learning model. They hold promise for discerning unknown product identification, detecting adulteration of products with known contaminants, and discovering biomarkers for various classifications. Once the decision tree model is built with training data, it is relatively straightforward to feed an unknown's chemical data through the model to predict its classification. This is an exciting possibility for quality control, especially when the most common adulterants are known to base relevant decision trees around. It should be noted that the decision algorithms at each node are based on separating the data available from the

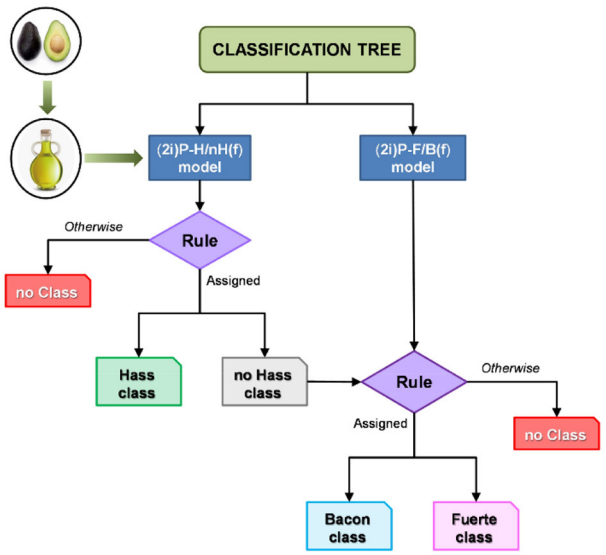

FIGURE 8 | Use of classification trees to identify unknown avocado oil samples as a specific cultivar or as no class based on HPLC-CAD metabolite profiles of a training set. Reproduced with permission from Martin-Torres et al. (97). Copyright 2019, John Wiley and Sons.

previous split, but the split progressions may not actually be the most reliable representations of divisions in the data. Random forests (described below) increase the accuracy of node splits but lose clear visual representation of the model.

Random Forests

Developed in 2001 by Breiman (99), random forest (RF) methods build an ensemble of decision trees, each of which is trained using the dependent variable(s). Each tree produces an outcome, and the aggregate outcome from all the trees (aka the forest) is reported as the outcome of the model. Random forest holds several advantages over other methods. The multiple decision trees produce more accurate classifications compared to a single decision tree algorithm, and it is less prone to overfitting than other supervised approaches (100). Additionally, one other advantage of random forest is that it can be used for both classification and regression problems. Deklerck et al. used random forests to classify heartwood samples of *Pericopsis elata*, a protected timber species. Using Direct Analysis in Real Time ionization coupled to time-of-flight mass spectrometry (DART-TofMS) on wood slivers, the random forest model using cross-validation was able to correctly predict *P. elata* samples (101). To analyze *Zanthoxylum* seed oils, Houet al. built a random forest classification model that differentiated between the two main species (*Z. bungeanum* and *Z. armatum*) with 100% accuracy from cross-validation. Even simplifying the model to only the most important chemical features, the cross-validated model still maintained 100% accuracy (102). Random forests also have the ability to be a predictive machine learning tool, and provide correlative predictions between dependent variables and the associated independent chemical dataset (103).

Suggestions for future use: Random forests can be used for the same purposes as decision trees where increased reliability of decisions at each node is necessary. This includes instances of fewer chemical data or a smaller number of training samples. Since random forests combine multiple decision trees, the computational input is much higher, so model building and training takes longer. Thus, random forests are not the best option when expecting a quick turn-around. However, there is potential application in developing random forest models for detection of adulteration of complex botanical products and mixtures. The extent of random forests in detecting contamination and purity of extremely complex samples in a high-throughput manor should be explored.

Support Vector Machine

Support vector machines (SVMs) are another supervised machine learning technique that can be employed for regression or classification analyses. The objective of the SVM algorithm is to find a plane in a *k*-dimensional space (*k* representing the number of features) that distinctly classifies the data points into groupings so that it has the maximum margin (i.e., maximum distance) between data points of both classes. Similar to other supervised machine learning or multivariate approaches of chemometrics data (where the number of features outstrips the number of samples), SVM can be prone to overfitting, so training on a smaller subset of samples, followed by cross-validation, is key to generating robust classification or predictive models.

Martín-Torres et al. (97) used SVMs to differentiate between the geographical origins as well as the botanical variety of avocados. Samples from five different countries (on three continents) representing seven avocado varieties were analyzed using normal phase HPLC-UV/VIS, after which the data was interpreted using two different multivariate approaches. The authors found that PLS-DA and HCA were unable to resolve the differences in geographical origin or between main groupings of variety. However, a three-input class SVM classification model (3iC) correlated the three different continents of origin (Africa, Americas, and Europe), as well as between the three dominant varieties ("Bacon", "Fuerte", and "Topa-Topa"); the latter having 100% correct assignments and precision and sensitivity of 1.00 (104). SVMs were also used to classify *Paris polyphylla* via fusion of Fourier-transformed infrared spectroscopy (FTIR) and UV-VIS spectroscopy data (105). Pan et al. used untargeted LC-MS metabolomics to profile five different *Uncaria* species in order to authenticate the source of Uncariae Rammulus Cum Uncis (Gou-Teng). A SVM model correctly categorized both training and test samples, and was used to classify 20 commercial Gou-Teng (GT) samples (106). The model predicted 16 of the samples were *Uncaria rhynchophylla*, while four did not match any of the *Uncaria* species. These four samples exhibited LC-MS chromatograms that were substantially different from the others, and thus it was believed that these were other *Uncaria* species or mixtures of *Uncaria* species. This represented a significant advantage over other (un)supervised techniques for discrimination purposes. And using data fusion techniques of mid-infrared (MIR) (transmission and reflection mode) and

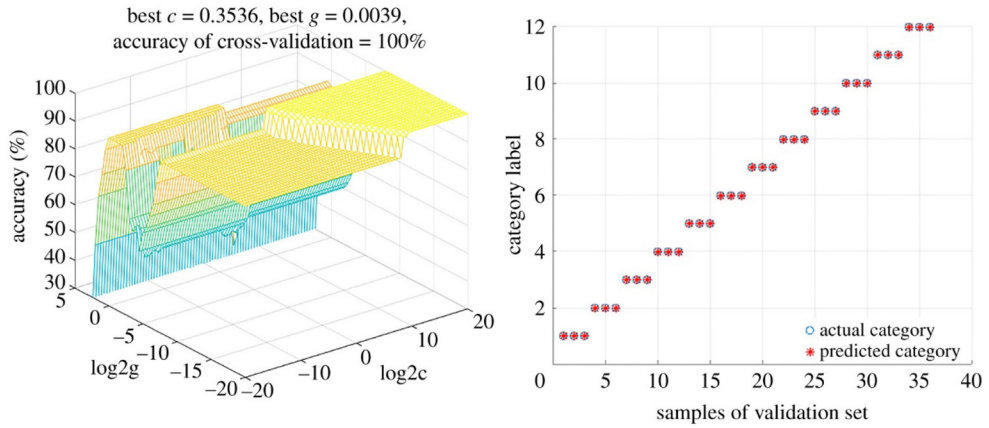

FIGURE 9 | Support Vector Machine (SVM) model for differentiating between Dendrobrium species. Using a low-level fusion strategy of MIR and NIR spectral data from 12 Dendrobrium sp. Reproduced under a Creative Commons Attribution License 4.0 from Wang et al. (107).

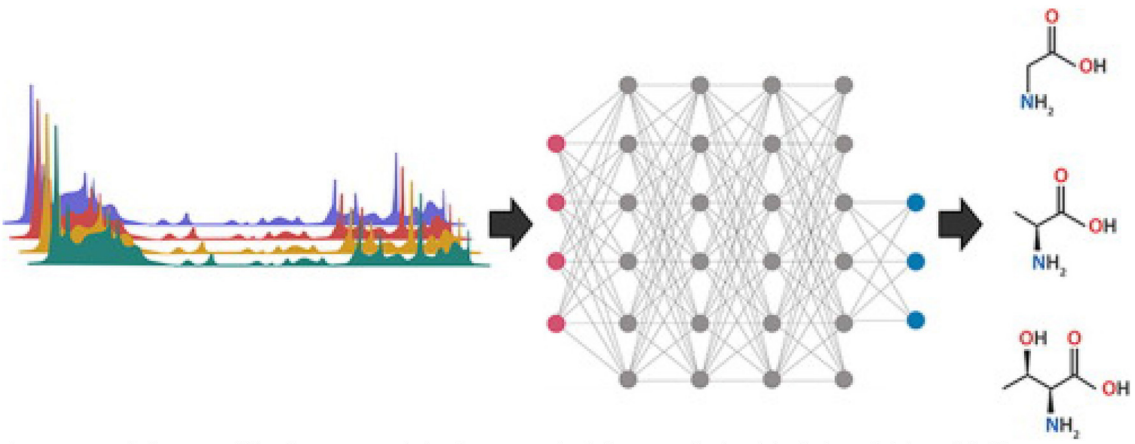

FIGURE 10 | Metabolomics workflow for ANN. Metabolite features are loaded as inputs (red circle), which are fed through hidden neuron networks (gray circles), and categorized into output categories (blue circles). Each connection (gray line) has a weight, and each neuron has a bias, which are used for the activation functions. Reproduced with permission from Pomyen et al. (112) Copyright 2020, Elsevier Ltd.

near-infrared (NIR) spectra followed by SVM analysis facilitated the discrimination of 12 different *Dendrobrium* species (107). SVM provided perfect discrimination (100% accuracy rates) for both calibration and validation sets (**Figure 9**).

Suggestions for future use: SVMs have practical applications for both classification of test data and prediction of unknown samples origin, species, or cultivar. SVMs may prove useful for distinction of genetically and chemically similar plants that cannot be differentiated by other clustering models, either supervised or unsupervised. For example, many sub-species of herbs have overlapping genotypes due to crossbreeding and PCA analysis can fail to separate the most closely related cultivars using chemical data (108); SVMs may provide a deeper level of distinction. This application can be applied to authentication of products commonly adulterated with very similar species that lack the promised medicinal output. Since SVM models can automatically handle missing data, SVMs can be used for metabolomes with variable metabolite profiles and lower resolution analytical techniques.

Genetic Algorithms

Genetic algorithms (GA) are based on the processes of evolution, including natural selection, reproduction, and mutations. These processes take place over multiple generations of increasingly accurate and simple solutions to a complex problem (109). In the case of botanical authentication and quality control, the problem may be product identification, detection of adulteration, or biomarker discovery. The solutions are the subset of metabolites and their ratios that best classify samples based on predefined classes or distinctions. As a brief example, consider Gil et al.'s study which used a GA to identify the region of rose wine origin (110). At generation 0, every combination of the 79 polyphenols present in the samples as detected by UPLC-MS were evaluated for their ability to distinguish between origin

region. Solutions with the highest fitness, or its distinguishing power as determined by linear discriminant analysis, were selected for reproduction. During reproduction, two solutions were mixed in a cross-over like process to create a new generation of unique solutions with higher fitness than the previous. The selection and reproduction processes were repeated for five generations, and each of the final combinations of polyphenols was tested for its accuracy in cross-validation tests. Those with the highest accuracy were further evaluated for their ability to discriminate wine origin regions in an unknown validation sample set. The GA model was able to discover a set of 4 polyphenols that had 86.7% accuracy (110). GA also provides the opportunity for simultaneous sample and variable selection for improved speed and accuracy for unsupervised clustering of samples and biomarker discovery (111). This bi-clustering approach opens the door for high-throughput metabolomics authentication of botanical materials.

Suggestions for future use: Potential for GA in botanical product quality control ranges from geographic identification to generation of a subset of biomarkers for subsequent analysis. The speed of GA modeling is ideal for situations requiring fast turn-around, so it is practical for developing authentication schemes for new products or products with increasing rates of adulteration. It should be noted, however, that GAs can be difficult to interpret, since the steps the model takes to combine data and reach a solution are not defined for the user and models fed the same data often reach different solutions. Thus, users should only use GA when the intermediate steps are not necessary for model validation.

Artificial Neural Networks (ANN) With Known Outputs

ANN are the backbone of deep learning machine learning models. Mimicking the human brain and neurons allows computers to recognize complex patterns in sets of training data and predict the classification of a new dataset using the resulting model. There are three main sections of an ANN: an input layer, an output layer, and hidden layers in between (**Figure 10**) (112). Each metabolite from the complete set of samples is treated as an individual input and connections are generated randomly through multiple hidden layers to generate an output response. Hidden layers are comprised of "neurons" that connect metabolites with a random numeric weight and have a randomly assigned bias (**Figure 10**) (112). Together the weights and bias generate an activation function to determine if a neuron will be activated for use in the next hidden layer. This process of forward progression is repeated until the model predicts an output (such as adulterated or pure). Typically, the first prediction is incorrect since the weights and bias are random, so the model uses backwards progression using the prediction error to modulate weights and biases throughout the hidden layers. Through multiple rounds of forward and backward progression with a variety of inputs belonging to each output category, the model can predict the output of new data by processing the new inputs through the meticulously developed hidden layers (112, 113). The history of ANN in metabolomics, as well as an in-depth explanation of different ANN models for spectral data is reviewed by Mendez et al. (113).

Binetti et al. used ANN with merceological, NIR, and H-NMR data to classify olive oil cultivars (114). Using H-NMR spectral data, ANNs were able to classify unknown samples with >99% accuracy, despite variable environmental, harvesting, and processing conditions (114). Additionally, ANN modeling of headspace solid-phase microextraction (HS-SPME) coupled with GC-TOF-MS of 374 honey samples over two years provided 94.5% accuracy in prediction of honey origin when data from both collection years was combined (115). These studies are promising for herbal product classification - botanical material analysis is typically complicated by temporal, environmental, and procedural variations. In addition to classification and identification, ANN modeling has potential to predict the chemical and medicinal properties of supplements without extensive bioassays and robust chemical profiling (116). Using species classification and extraction procedures as inputs, Tusek et al. used an ANN to predict chemical features, including total phenolic content and extraction yield, and antioxidant potential of nine medicinal plants (116).

Suggestions for future use: ANNs hold immense potential for herbal product authentication. Since training data covers a range of environmental, temporal, and procedural variables, the predictive nature of the resulting model has very high accuracy. This is critical for commercial products that have limited information about harvest and processing procedures. An interesting study would determine if a single ANN model built on samples with a range of preparations (powdered, dried, capsules) and environmental factors can successfully classify and authenticate various types of new products. Additionally, prediction of medicinal properties using ANN should be expanded to allow confirmation of desired effects from commercial products quickly and accurately. This will take authentication a step further from identifying product constituents and increase efficacy of botanicals on the market. Users should take caution when using ANN to not over interpret their results. While ANNs are powerful classification tools for large data sets, they do not provide information on the chemical distinctions on which the model is built. Thus, the model does not allow interpretation about specific chemicals responsible for classification of samples.

Precautions for Using Classification Models

Each model describe above has benefits to the natural product community, and there are examples highlighting their usefulness in the literature. However, each model also has pitfalls. It is crucial for researchers to understand the dangers of overinterpreting their outputs. One such downfall is overfitting data, or forcing data points into a category due to the lack of a "unknown" output option within the model. Almost all of the models described in this review are prone to overfitting, but some models, like decision trees and random forests, reduce this possibility by including an unknown option or compiling the output of multiple models into the output. It is important to validate each model by withholding a sample's data as a validation set with a

known output, as well as reporting the Q^2 and R^2, as described in section Unsupervised Approaches.

An additional warning is that not every model is applicable in each situation. Despite a model seeming to fit a research goal, it is possible the type, quantity, or quality of data is not applicable to a given algorithm. Multivariate statistics and metabolomics projects require careful planning prior to data collection to ensure desired models can be used. For suggestion of models to use in different situations, see section Conclusions and Future Directions.

COMBINING ORTHOGONAL DATASETS

While modeling chemical data through chemometric approaches can leverage the immense information contained therein to investigated nuanced differences between samples, being able to differentiate samples based on their geographic origin, taxonomic relationship, or adulteration level, the chemical composition represents only one facet of potential data to be analyzed. Incorporating additional data sources, whether it is from orthogonal chemical analyses, bioactivity/toxicity data, or genetic data, has the potential to develop discriminatory models that are even more robust in authenticating botanical products. Often, combinational approaches can increase the efficacy and reliability of natural product quality control, and should be implemented when feasible.

Multiple Chemical Analyses Inputs

There is no single chemical analysis able to profile every metabolite present in a complex sample; each approach has some detractors. Ultra violet-visible spectroscopy (UV-VIS) requires a chromophore that can absorb energy within these wavelengths of light (often 180–800 nm); mass spectrometry (MS) can only monitor structures that are ionizable; nuclear magnetic resonance (NMR) is not as sensitive in detecting low-abundance compounds (55, 117). Therefore, combining different chemical investigations of a metabolome can better represent the chemical diversity present in a sample, and consequently allow for more precise modeling and differentiation between samples. These 'data fusion' approaches have been used with different botanical products to evaluate their authenticity and detect adulteration. Spiteri et al. combined 1H-NMR with LC-MS to discriminate between commercial honey. The PCA was constructed considering each technique separately, and then combining NMR and LC-MS together. The authors found that the discriminating potential increased through data fusion, allowing better separation of the four different floral origins with no misclassification observed (118). NMR and LC-MS were also combined to detect adulteration of a commercial botanical dietary supplement which had resulted in the hypotensive collapse of several consumers. The product was purported to contain the species *Crataegus oxyacantha*, *Olea europea*, *Capsella bursa-pastoris,* and *Fumaria officinalis*. However, the analysis revealed the presence of indole alkaloids belonging to the genus *Rauwolfia*, such as ajmaline, reserpine and yohimbine. Subsequent quantitative analysis determined reserpine was present in pharmacologically-relevant doses (119).

Chemometric analyses using multiple analytical inputs have also been used to elevate and extract more information from more common and less expensive analyses, such as infrared analysis (IR) and ultraviolet-visible spectroscopy [UV-VIS, often abbreviated as LC methods (HPLC or UPLC) or diode array detectors (DAD)], to provide robust data and allow clear discriminate model formation. Combining three different types of detectors: diode-array detection, evaporative light scattering detection and mass spectrometry, Deconinck et al. constructed fingerprints for three common herbal products—*Rhamnus purshiana*, *Passiflora incarnata* L. and *Crataegus monogyna*. Using unsupervised projection chemometric analyses, the researchers were able to detect the presence of these plants in three different herbal matrices as well as in commercial preparations containing multiple botanicals (120). Wu et al. reported that fusing data obtained from polyphyllin content, FTIR spectra, and UPLC chromatograms yielded correct discrimination of Paridis Rhizome samples according to botanical and geographical origins by PLS-DA modeling. The authors reported that SVM and RF provided similar results (105). Two-step fingerprints, built upon mid infrared spectroscopy (MIR) and HPLC chromatograms, were analyzed by k-nearest neighbors and SIMCA to screen for five regulated plants used in commercial dietary supplements (121). And Zhou et al. fused two different infrared technologies – Fourier transform mid-infrared (FT-MIR) and near infrared (NIR) – to detect the origin of 210 *Panax notoginseng* samples from five cities in Yunnan Province, China. Random forest was used to establish classification models, which resulted in a classification accuracy of 95.6% (122). Data fusion of orthogonal analytical approaches has the potential to cover complementary facets of chemical space, and the subsequent modeling can be seen to be more powerful in its ability to discriminate between botanical samples as a means of authentication and adulterant detection.

Biochemometrics

The ability to profile large swaths of a metabolome without iterative methods of separation and purification means metabolomics approaches have an advantage for screening for bioactive metabolites (92, 123, 124). Integrating metabolomic fingerprinting with biological activity data allows for supervised methods to statistically model correlations between variations in biological response with differences in chemical composition across samples. These methods, collectively known as "biochemometrics", have become a driver for bioactive molecule discovery. Several statistical methods have been utilized for this purpose, including hierarchical clustering analysis (125), partial least squares (92, 126, 127), and partial least squares-discriminate analysis (128, 129). Of these, PLS and PLS-DA have emerged as the foremost multivariate approaches for biochemometric analysis. These approaches utilize different variable metrics to ascribe correlation (and thus importance) to the chemical signals with the variable importance in projection (VIP) plot, the S-plot, and the selectivity ratio being among the leading metrics (92, 130–132). Biochemometrics holds great promise for botanical examination and authentication, as it could leverage

relevant biological activity to determine a targeted fingerprinting method which has relevance to the biological function of the plant (as opposed to *ad hoc* choices of metabolites). Kim et al. used a biochemometric method to evaluate 17 different species of grape myrtle (*Lagerstroemia* spp.) based on their ability to increase glucose uptake *in vitro*. From the PLS-DA model (using the glucose update as the dependent variable), the *Lagerstroemia* sp. were grouped into two clusters, and from the S-plot the authors identified three main metabolites (myricetin-3-O-β-D-rhamnoside, quercetin 3-O-β-D-rhamnoside, and corosolic acid) that predicted glucose uptake activity and could be used as a discriminatory model for identifying bioactive species from the genus (**Figure 11**) (133). The integration of biological activity as an orthogonal dataset, and as a continuous numeric dependent variable in the dataset, allows for supervised chemometric methods to provide greater interpretation of the discriminatory model creation and identification of bioactive components. This can lead to the development of fingerprinting or authentication tools that correlate with the relevant biological effects of the botanical in question.

Multi-Omics Integration for Botanical Control

While metabolomic approaches provide ample opportunity for accurate, robust, and time-efficient authentication of complex botanical products, combining chemical data with other -omics approaches may yield the most effective solutions. Most commonly, metabolomics modeling is combined with genomics data. As mentioned in section Genetics, DNA barcoding and metabarcoding can lose accuracy at the species and subspecies level. Similarly, clustering of metabolites can lose resolution of chemically similar plants. Combining DNA barcoding using *rpoC1* and LC-MS metabolomic fingerprinting allowed species-level distinction between nine Phyllanthus species (134). Integration of genetics and metabolomics is easily the most common approach to botanical product identification, as outlined in **Table 1**. There are instances when metabolomics and genetics in combination cannot differentiate between species, so additional analytic approaches are employed, such as electronic noise (141), microscopy (142, 143), high-resolution melting analysis (144), Raman spectroscopy (145), or multiple metabolomic approaches (146). **Figure 12** demonstrates that integrating multifaceted -omics approaches can be achieved in a single study to increase the power of distinction and authentication of herbal products (141).

Although genetics is most integrated with genetics, there is potential to expand to lipidomics, proteomics, and transcriptomics. Lipidomics, the study of the complete set of lipids in an organism, is analytically similar to metabolomics; different extraction and analytical instrument methods target lipids. The same research lab could seamlessly transition from metabolite to lipid analysis since the instrumentation is often the same. On its own, lipidomics has been useful for detection of adulteration of white rice – RF and SVMs were used to discriminate pure and adulterated samples using LysoPCs and lysoPEs as novel lipid biomarkers (147). Using the same UPLC-MS instrument, Anagbogu et al. combined lipid and metabolite analysis to identify 30 genotypes of coffee; joining the two approaches increased species level resolution (148). Proteomics also uses similar instruments as metabolomics, but it has more variable methods that may complicate inter-lab experimentation. Peptide analysis allowed differentiation between mountain-cultivated ginseng and cultivated ginseng with 52 variable peptides between the groups (149), and MALDI TOF-TOF/MS yielded five proteins with potential to authenticate *Ophiocordyceps sinenis*, a traditional fungal medicine (150). Given the limited successful studies utilizing integrated -omics approaches for botanical product authentication and evidence

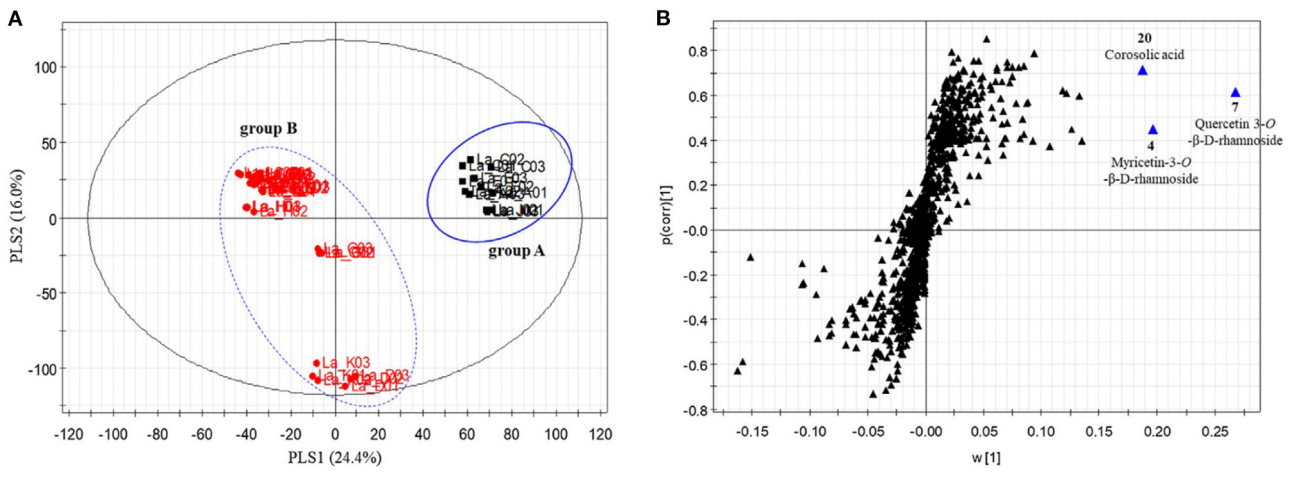

FIGURE 11 | Partial least squares-discriminate analysis of Lagerstroemia samples. Scores plot **(A)** from the PLS-DA model accounted for 40.4% of model variability, and demonstrated two distinct clusters of samples. The S-plots **(B)** revealed two flavonol glycosides (myricetin-3-O-β-D-rhamnoside and quercetin 3-O-β-D-rhamnoside) and corosolic acid as potential discriminatory biomarkers with activity in stimulating glucose uptake. Reproduced with permission from Kim et al. (133). Copyright 2020, Elsevier Ltd.

TABLE 1 | Orthogonal approaches to integrate genomics and metabolomics data analysis of botanicals.

Botanicals	Product type	Genetic approach	Metabolomic approach	Modeling	Author	Year (Ref.)
Qin jiao	Dried root powder	ITS2 barcoding	Q-TOF-MS H-NMR	ANOVA PCA OPLS-DA	Li et al.	2020 (135)
***Hypericum* taxa**	Essential oil Dried leaf	ITS2 barcoding ITS1 barcoding	GC-MS LC-HRMS LC-DAD-MS HPLC-DAD	PCA Biplots Mantel test	Zeliou et al.	2020 (136)
Salvia* subg *Perovskia	fresh leaf and root	trnH-psbA barcoding ITS2 barcoding	UHPLC-QTOF-MS	PCA	Bielecka et al.	2021 (137)
***Hypericum* spp.**	Cultured leaf	ITS1 barcoding ITS2 barcoding Chromosome number genome size	HPLC-DAD	PCA HCA	Brunakova et al.	2021 (138)
Sarsaparilla	Dried root	rbcL barcoding matK barcoding genome skimming DNA probe	H-NMR	HCA	Kesanakurti et al.	2020 (82)
***Glycyrrhiza* spp.**	Dried root powder Dried root stick Dried root capsule	rbcL barcoding matK barcoding ITS barcoding trnH-psbA barcoding	H-NMR UHPLC-UV	PCA SIMCA CDA	Simmler et al.	2015 (139)
***Echinacea* spp.**		Genome skimming metabaroding matK barcoding rbcL barcoding	HPLC-UV		Handy et al.	2021 (140)

that each approach has potential to identify adulteration, there is a gap in the botanical products community developing methods and statistical approaches for combined datasets. This is not a trivial undertaking; often the data sets generated for genomics, proteomics, and metabolomics experiments are very different, and their integration can be a challenge. The wide variety of expertise required to generate high quality data is also a factor in the wider implementation of a multi-omics approach to botanical authentication; these disparate techniques have different methodological proficiencies and even reagents and laboratory setups, necessitating broad proficiency in a single lab or a reliable collaboration between different laboratory groups. For data integration, the R tool mixOmics (including PCA, PLS, and PLS-DA tools) may prove useful for combined biomarker discovery and species identification (**Figure 13**) (151).

WHEN TO USE WHAT

This review highlights the number of chemometric techniques that can be applied to datasets in order to help authenticate botanical materials or detect adulteration. However, the diversity of approaches that are possible can be daunting to researchers unfamiliar with chemometric analysis and multivariate analysis/machine learning. While there is a bit of trial and error in selecting a chemometric approach, there are some points to consider in determining which technique to employ in analyzing a dataset. The decisions and chemometric options available to a researcher analyzing data are summarized in the following workflow (**Figure 14**).

First, is there response data collected with the chemical information? This could take the form of classification identifiers (e.g., "pure" vs. "adulterated"), control or QC datasets, taxonomic identification, quantitative data (e.g., temperature, geographic coordinates, elevation), or bioactivity data (inhibitory studies, cell studies, *in vivo* experiments, toxicological data, etc.). For datasets which do not contain any dependent information (only chemical input from FTIR, UV-VIS, MS, NMR, etc.), unsupervised analyses are recommended to understand the shape and relationships between samples without any guiding variables or observations. For a hierarchical analysis, where the similarity relationship between samples is ranked by distance, hierarchical cluster analysis (HCA) is the foremost choice. For examining

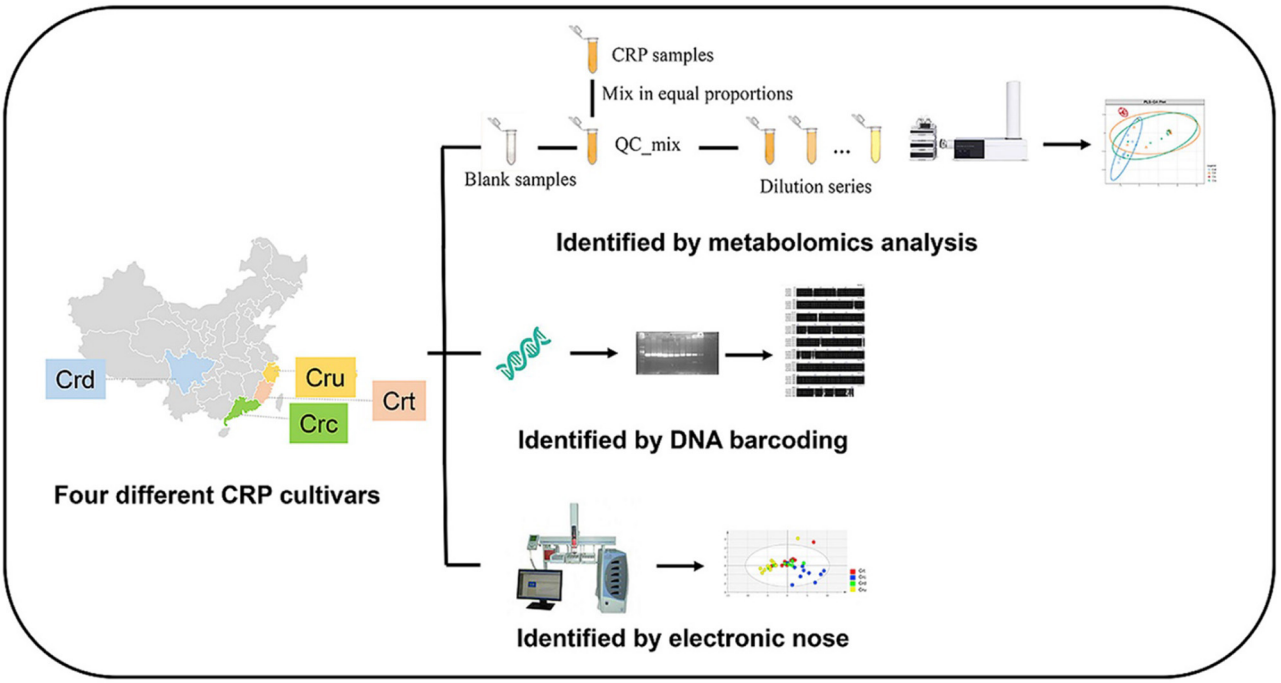

FIGURE 12 | Integration of metabolomics, DNA barcoding, and electronic noise increases accuracy of Citri Reticulatae Pericarpium cultivar distinction compared to each method alone. Reproduced with permission from Li et al. (135) Copyright 2020, Springer Nature.

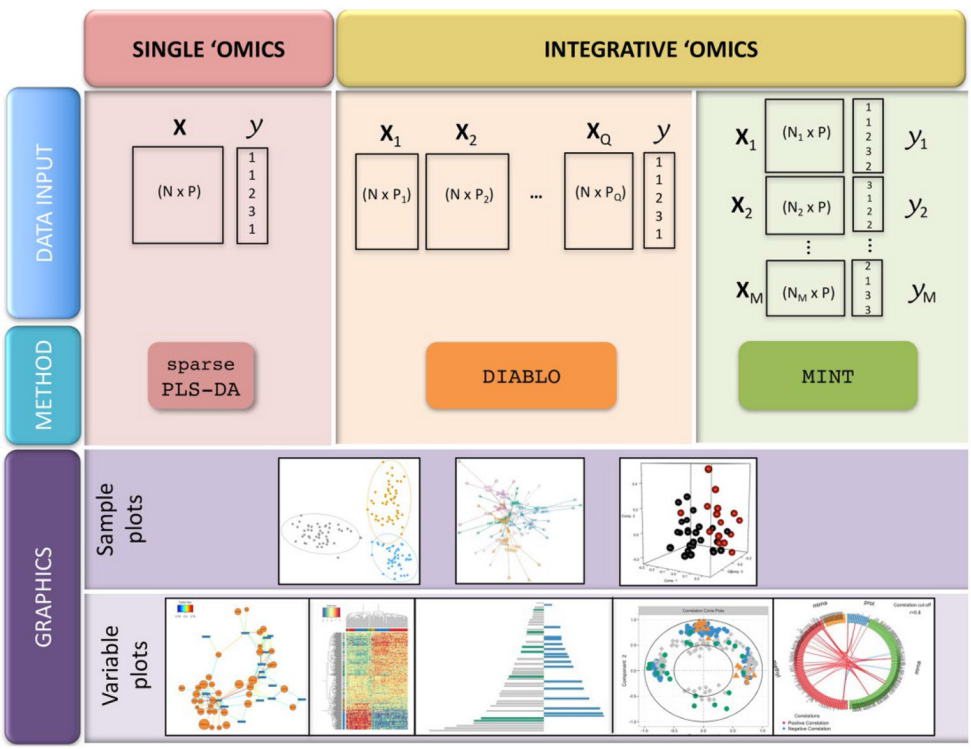

FIGURE 13 | Integration of multiple-omics datasets and potential outputs using the mixOmics R package. Reproduced under a Creative Commons CC BY 4.0 license from Rohart et al. (151).

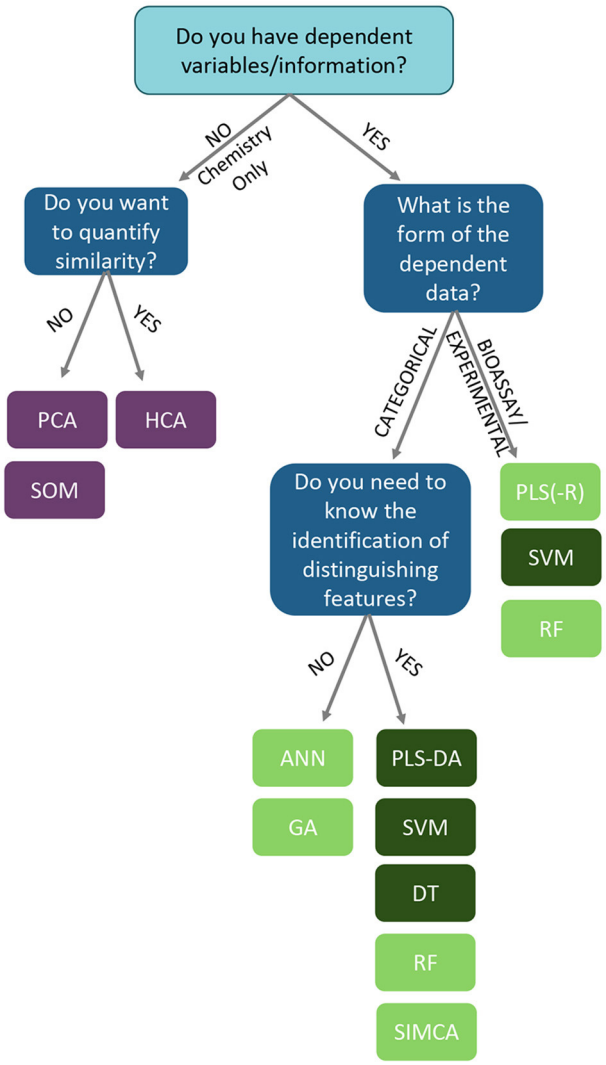

FIGURE 14 | A decision tree to establish which analysis is more appropriate to analyzing complex chemical data. Based upon the presence of response data, and how the overall analysis needs to be structured/interpreted. ANN, artificial neural networks; DT, decision trees; GA, genomic algorithms; HCA, hierarchical clustering analysis; PCA, principal component analysis; PLS, partial least squares; PLS-DA, partial least squares-discriminate analysis; RF, random forests; SIMCA, soft independent modeling of class analogies; SOM, self-organized maps; SVM, support vector machines. Light green boxes represent "soft" classification techniques. Dark green boxes represent "hard" classification techniques.

similarities between samples without a hierarchy, principal component analysis (PCA), self-organizing maps (SOMs), and k-means clustering are viable options.

For experimental sets which contain dependent variables, chemometric options include numerous supervised analyses, which require response or dependent variables to train models. Generally, an unsupervised approach (PCA) should be applied to the metabolomics data set to ensure clustering occurs without predefined categories before delving into supervised analysis. Within the supervised approaches, the chemometric options vary depending on whether the response data are categorical or numerical in nature. Categorical dependent data, such as class assignments (e.g., "authentic" and "unknown") enable supervised analysis to generate models that maximizes differences between the two classes. When choosing a classification methodology, one can consider whether the particular chemometric approach is "soft" or "hard." These designations relate to a method's rigidness in assigning an unknown to a particular class. A "soft" classification rule estimates the probability associated with each class and subsequently provides a class prediction based on the largest estimated probability. In comparison, "hard" classification delivers a final class prediction without probabilistic reasoning behind the classification. Of the reviewed approaches, SIMCA, PLS, random forest (RF), genomic algorithms (GA), and artificial neural networks (ANN) are generally considered "soft" computational approaches (**Figure 14**, light green boxes) (152), while other techniques, such as PLS-DA, decision trees (DT), and support vector machines (SVM) (153) are "hard" methodologies (**Figure 14**, dark green boxes).

At this point, the last decision is the degree of interpretability the model will have for the researcher. A highly interpretable algorithm means that one can easily understand how any individual predictor (variable) is associated with the response, so it's easier to relate the final classification structure back to specific variables contributing to model responses. Techniques like partial least squares-discriminant analysis (PLS-DA), support vector machines (SVM), decision trees (DT), soft independent modeling of class analogies (SIMCA), and random forests (RF) are able to provide interpretable models. If interpretation is not essential (a "black box" approach), and only the final classification of the data is important, models like artificial neural networks (ANN) or genetic algorithms (GA)are prime options.

Numeric dependent variables are frequently obtained from biological activity experiments, and thus enable the use of prediction algorithms to correlate the dependent variable with variations in the chemical information. For the biochemometric analysis of these orthogonal datasets, partial least squares approaches (PLS, PLS-R) are most common in teasing out these relationships (92). However, newer machine learning approaches, such as SVM and RF, have the ability to provide predictive capabilities and understand relationships with input variables. As an example, Deng et al. employed random forests to provide geographical classification of green teas (which outperformed several other chemometric techniques), but also were able to correlate the geography with several isotopic indicators (103).

As with data-collection, where multiple orthogonal techniques facilitate a greater coverage of the overall chemical composition of the samples, multiple data analysis techniques are often utilized to gain a more comprehensive perspective of the data structure and relationship between samples. It is common to begin with unsupervised approaches (e.g., PCA) to glean a preliminary understanding of how samples are relating to one another, then followed up with supervised or machine learning methods to further classify the samples and obtain information about potential biomarkers or bioactive constituents. Zhang et al., in authenticating berry juices, first used PCA to identify clusters of juices by origin, then followed with PLS-DA to

determine relevant biomarkers (76). PCA and HCA were employed to reveal well-differentiated clusters for black peppers, then followed by supervised PLS-DA for a prediction model for additional unknown samples (154). Thus, merging chemometric methods, when possible, offers researchers a potentially more rigorous analysis of their botanical data, which is essential to draw relevant and robust conclusions about authentication and adulteration questions.

CONCLUSIONS AND FUTURE DIRECTIONS

As the demand for botanical medicines and dietary supplements grows, in terms of relevance to human health as well as economic importance, ensuring reliable determination of starting materials for research, safety, and production considerations remains a challenge. Plant-based formulations pose a particularly unique hurdle due to their inherent chemical complexity as well as their variability. Non-targeted chemical fingerprinting techniques, including metabolomics, hold immense potential for describing the chemical composition of botanicals. However, organizing that highly complex information and deducing relevant conclusions from it can represent a major obstacle for researchers. This review has sought to address this hurdle by presenting examples of major chemometric techniques that can be employed to distill complex chemical data into models for authentication and classification of unknown samples. The adaptation of statistical models to wrangle large, complex datasets represents a significant advancement in modeling botanical chemical data. While the chemometric analysis methods profiled in this review are the most common, and some of the most powerful, approaches in use for botanical authentication, it is by no means an exhaustive list. Other variations of unsupervised and supervised techniques have been reported, and there is considerable research being undertaken to advance the capabilities of these statistical and machine learning approaches. And the combination of complementary methods (e.g., biological data and metabolomics, chemical profiling and genomics, or multi-omics techniques) has the potential to provide even more efficient and robust tools to advance authentication and discovery efforts.

AUTHOR CONTRIBUTIONS

JK and EA conceived, wrote and reviewed the manuscript, and secured funding. All authors contributed to the article and approved the submitted version.

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REVIEW
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Do Certain Flavonoid IMPS Have a Vital Function?

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Flavonoids are a vast group of metabolites that are essential for vascular plant physiology and, thus, occur ubiquitously in plant-based/-derived foods. The solitary designation of thousands of known flavonoids hides the fact that their metabolomes are structurally highly diverse, consist of disparate subgroups, yet undergo a certain degree of metabolic interconversion. Unsurprisingly, flavonoids have been an important theme in nutrition research. Already in the 1930s, it was discovered that the ability of synthetic Vitamin C to treat scurvy was inferior to that of plant extracts containing Vitamin C. Subsequent experimental evidence led to the proposal of Vitamin P (permeability) as an essential phytochemical nutrient. However, attempts to isolate and characterize Vitamin P gave confusing and sometimes irreproducible results, which today can be interpreted as rooted in the unrecognized (residual) complexity of the intervention materials. Over the years, primarily flavonoids (and some coumarins) were known as having Vitamin P-like activity. More recently, in a NAPRALERT meta-analysis, essentially all of these Vitamin P candidates were identified as IMPs (Invalid/Improbable/Interfering Metabolic Panaceas). While the historic inability to define a single compound and specific mode of action led to general skepticism about the Vitamin P proposition for "bioflavonoids," the more logical conclusion is that several abundant and metabolically labile plant constituents fill this essential role in human nutrition at the interface of vitamins, cofactors, and micronutrients. Reviewing 100+ years of the multilingual Vitamin P and C literature provides the rationales for this conclusion and new perspectives for future research.

Keywords: flavonoids, Vitamin P, Vitamin C, invalid metabolic panaceas (IMPS), vitamins, micronutrients, cofactors

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ORIGIN OF THIS STUDY

The original motivation for this study came from the recent proof of the existence of invalid/improbable/interfering metabolic panaceas (IMPS) (1) as over-studied natural products that have an implausible plethora of reported biological activities, making them panaceas—in theory. At the same time IMPS are very commonly found in plant-derived food products, implying that IMPS play potential roles in human nutrition. Following the premise that unrecognized life-essential biological functions of molecules may potentially blur their experimentally observable *in vivo* and *in vitro* effects, we sought to unravel the link between flavonoids and Vitamin P (VitP).

The commonly used synonym, "bioflavonoids," already hints at the potentially blurred scientific standing of VitP. In contrast, flavonoids are well-defined phytochemicals that are nearly ubiquitous in plants and likely represent the most well-studied class of plant constituents. Interestingly, yet not surprisingly, flavonoids comprise the by far largest group in the top-38 known IMPS (1).

MOTIVATION OF THIS STUDY

Building on more than a century of primary literature published in English, French, and German, the present study compiles existing evidence and presents an intriguing new view for the connection between the existence of Vitamin P (VitP) as a known, but difficult to identify, cofactor of vital body functions in humans, and flavonoid dietary plant constituents that have been designated as IMPS. The study develops a set of rationales for the ability of flavonoids and some phenylpropanoids, structurally and biogenetically closely related plant phenols, to possess biological functions that are essential for healthy human life. These functions involve peripheral collagenous, vascular tissue, and include the permeability of vascular capillaries and cerebral tissue. Accordingly, flavonoids and related compounds must impact cardiovascular and general human health significantly. Despite them being phenols, chemically, this study also brings about new rationales why their categorization as "polyphenols" has mostly confusing biochemical and biological implications that make this term problematic.

The surprisingly elusive nature and lack of an assigned single chemical entity of these, otherwise essential, bioactive molecules that constitute VitP may well be rationalized by the dietary omnipresence and metabolic interconversion of the flavonoid species, as they have been experimentally associated with both VitP and Vitamin C (VitC) bioactivities. This directly leads into the metabolomic complexity of plant phenols: Thousands of flavonoids and a somewhat smaller number of coumarins exist with the basic ring structure required for VitP activity. However, the activity and quantity of various flavonoids varies tremendously. The most commonly sold, rutin, has very little activity, whereas epicatechin is reported to have much greater activity.

OVERCOMING FACILE STRUCTURE-BIOACTIVITY PARADIGMS

The observation that P is the last letter in the historic "vitamin alphabet" (**Table 1**) which has not been linked to a triplet of a strictly deficient diet, biochemical processes, and well-defined chemistry indicates why the scientific community has hesitated to assign VitP the role of a vitamin, cofactor, or micronutrient. To achieve a new overall perspective, this study took a two-fold approach by (A) collating existing knowledge via a comprehensive review of 100+ years of relevant literature; and (B) evaluating the findings with respect to contemporary knowledge in phytochemistry, biochemistry, and biology.

This strategy also allowed for the application of recent insights from the authors' long-term research on botanical dietary supplements to the dietary role of flavonoids. Our extended body of prior work (see go.uic.edu/botanicalcenter) for an overview and list of 260+ relevant publications. This indicates the importance of several factors contributing to bioactive principles from plants: metabolomic diversity; biosynthetic and metabolic relationships; static and dynamic residual complexity of chemical composition; complexity of biochemical pathways; the multi-layer nature of mechanisms of action; polypharmacology and "synergistic" properties. Collectively, the proven interplay of these factors challenges paradigms that seek to connect, in the most facile manner, single chemical species with well-defined biochemical processes and/or clear-cut biological outcomes.

Accordingly, this study prioritized collecting evidence for the role of VitP as an essential nutritional factor over the question of whether VitP "truly" is a vitamin. The present work followed four hypotheses and addresses three key questions:

(H1) Similar to therapeutic botanical interventions, nutrition acts through multiple, often chemically closely related components, which need to be considered jointly when trying to understand biological effects.
(H2) As the abundance (concentration) and biological significance of plant constituents are uncorrelated, important contributing compounds can be (are frequently?) overlooked when they are minor, difficult to analyze, and/or when the preceding discovery of high-abundance components discourages further or a more in-depth search.
(H3) Common terminology obstructs the quintessential structural differences of congeneric compounds (e.g., flavanonol vs. flavones vs. flavonols vs. chalcones) by using facile common denominators to group chemical entities ("phenol" and "polyphenol") into inhomogeneous cohorts.
(H4) Combined with the equally common assignment of broad bioactivity terms ("antioxidant"), oversimplifying terminology precludes the ability to establish specific correlations between chemical species and essential biological functions.
(Q1) What is the relationship between flavonoids, VitP, and VitC?
(Q2) Is VitP a single chemical entity or a "complex" of related compounds?
(Q3) What is the role of metabolic, including microbial, transformation in the action of VitP?

In line with these hypotheses and questions, the overarching objective of the study was to compile the available evidence to investigate whether VitP exists, which essential biological role it may fulfill, and which natural compounds can be reasonably associated with these activities. The ultimate goal was to inspire and provide direction for future experimental studies.

Abbreviations: IMPS, invalid/improbable/interfering metabolic panaceas; PAC, proanthocyanidin; OPAC, oligomeric proanthocyanidin; VitC, Vitamin C; VitP, Vitamin P.

TABLE 1 | The alphabet of vitamins, their chemical description, deficiency conditions or diseases, and basic discovery facts.

Vitamin	Chemistry	Deficiency	Brief discovery facts
A	Retinol	Visual impairment (among others)	Magendie 1816; Hopkins 1912 (Nobel Prize 1929) from milk, McColumn and Davis and Mendel and Osborne 1913; chemical structure by Karrer 1913
B_1	Thiamine	Beriberi	Kanehiro 1884 germ theory rejection, Williams 1934 structure elucidation; mainly from rice bran
B_2	Riboflavin	Stomatitis (among others)	Kuhn, György, and Wagner 1933–1939 from egg white and whey
B_3	Niacin	Pellagra	Chemical discovery by Weidel 1873; Elvehjem 1937 extraction from liver
B_5	Pantothenate	Impaired energy production	Essential yeast growth factor by Williams 1933, structure by Williams 1940; Lipmann coenzyme A discover 1946 (Nobel Prize 1953)
B_6	Pyridoxine	Metabolic disorders	Discovery György 1934, isolation Lepkovsky 1938
B_7	Biotin	Hair, nail, skin disorders	Bateman 1916, Boas and Parsons 1927, Kögl and Tönnis 1936, among others; consolidated structure by 1940
B_9	Folate	Anemia	Anemia reversal with yeast by Wills 1941; isolation from spinach leaves [Lat. *sing.* folium] by Mitchel, Snell, William 1941
B_{12}	Cobalamins	Anemia	Recognition of pernicious anemia 1847 to 1887, liver concentrate treatment by Whipple, Murphy, and Menot (Nobel Prize 1934), structure by Todd 1955 (Nobel Prize 1957) and via X-ray by Hodgkin (Nobel Prize 1964)
C	Ascorbate	Scurvy	Citrus fruit effects known empirically for long; György, Svirbely, and King late 1920s to mid-1930s, see main text
D	Calciferol	Bone deficiencies	Discovery from cod liver oil by McCollum and David 1914; connection with steroids by Windaus (Nobel Prize 1928); isolation and elucidation by Bourdillon, Rosenheim, King, Callow, and Windaus until mid-1930s
E	Tocopherol/-trienol	Neurological deficiencies	Recognized 1922 and isolated from wheat germ 1936 by McLean Evans; structure by Fernholz 1938
F	Essential fatty acids	General health deficiencies	Discovery and recognition as fats rather than vitamins 1923–1930
G	Now B_2		
H	Now B_7		György 1933–1939 (H [German] for Hair (Haar) and Skin (Haut))
I	Not assigned		
J	Choline (or B_2)	General health deficiencies	Choline isolation by Stricker 1849, elucidated by Baeyer 1957, vitamin J effect proven by Best 1932
K	Menaquinone (K_2), phylloquinone (K_1), menadione (K_2)	Hemorrhages	Recognized by Dam 1929, structure by Doisy 1932 (Nobel Prize 1939)
L	Anthranilic acid	General health deficiencies	Discovery from indigo Fritzsche 1841, structure by Friedländer 1910
M	Now B_9		Named M after research done in monkeys
N	Alpha-lipoic acid	General health deficiencies	Discovery Snell 1937, elucidation by Reed and Eli Lilly scientists 1950s
O	Carnitine	General health deficiencies	Discovery 1905, structure until 1927, function until 1965, biochemistry Fraenkel since 1950s
P	"Bioflavonoids"	Capillary fragility	Recognition parallel to Vitamin C; see main text
Q-Z	Not assigned		

FLAVONOIDS AS IMPS AND FOOD INGREDIENTS

Recently, a systematic meta-analysis of world literature on bioactive natural products, encoded in NAPRALERT (2), led to the discovery that certain plant-based metabolites (often considered as "secondary metabolites") have received massive, but essentially non-productive, attention in the literature. Termed as invalid metabolic panaceas (IMPS), these compounds show bioactivity in virtually all known biological endpoints, frequently due to bioassay "interference," but despite major research efforts they fail to succeed in their development as drugs ("improbable leads") or other effective intervention agents such as dietary supplements. IMPS lack the essential characteristics of highly specific leads such as well-defined structure-activity relationships, stability, and other desirable properties. Collectively, IMPS can undermine natural products and nutritional discovery research (1).

However, these findings do not preclude important biological functions for these compounds. In fact, highly abundant molecules may play crucial roles in biological systems—even if their roles are "passive" (i.e., not involving a definable active site, receptor, or analogous target) and not drug-like, but rather function as essential components. While located at the "extreme end" of such a concept, water could still serve as an example. Focusing on phenolic compounds that belong to the group of flavonoids (**Supplementary Material 1**), the possible existence of un(der)recognized vital biological functions is illustrated in studies of VitP, which was first co-discovered with VitC in the 1930s by Szent-Györgyi.

The close connection between VitP and flavonoids becomes immediately evident when doing literature database searches for "Vitamin P." Performed with both PubMed and Scopus, they yield in the range of ∼800 articles. A manual inspection of all titles and abstracts reveals that almost all these articles published since the mid-1950s use the term "Vitamin P" as a synonym for rutin (vast majority of articles), hesperetin, quercetin, or unidentified flavonoid mixtures ("flavonoids"). The bulk of publications on VitP, however, has been published between 1937 and 1964. Based on these and other insights from database searches that included the refinement of search terms, the present study did not rely as much on database searches, but performed extensive manual back- and forward-tracing of citations in order to generate an as much as possible unbiased and comprehensive collection of reports of experimental outcomes that are directly related to the character and existence of VitP. Tracing evidence back particularly to the earlier VitP literature, including *in vivo* experiments, also provided a means of assessing whether the (over)simplification of "flavonoids" as "Vitamin P" and the idea that rutin could be used as synonymous with VitP were justified, and how these assumptions may have impacted the understanding of VitP in the more recent literature. The authors acknowledge that the manual tracking of literature involves a certain degree of human bias and is limited by the effort that can reasonably be spent. However, the results of database searches led to many, but by far not all, articles cited in the present study. This includes reports that are key to the understanding of VitP, but do not appear in typical database searches, likely due to limitations in their keywords and how the information of these earlier works has been extracted for database purposes.

In order to understand the relationship of flavonoid IMPS and VitP, it is necessary to place the discovery of vitamins and, in particular, VitC into perspective.

FLAVONOIDS VS. POLYPHENOLS VS. ANTIOXIDANTS

As workers in different fields have very differing concepts of what constitutes a "polyphenol," this term has caused much confusion. This has recently been highlighted by a consortium of scientists (3). Because the confounding effect of using "polyphenol" is difficult to avoid even within a well-defined context, the present work avoids the term altogether as it does not add any useful meaning, but actually would introduce more confusion, especially in a broader context. However, one potential explanation and resolution is that the term "polyphenol" was initially intended and used only for poly*meric* (not poly*hydroxylated*) aromatic ("phenolic") constituents, and that its wider adoption beyond these clear definitions led to a dangerous deviation from its original meaning.

The present focus on flavonoids as a chemical substance class is justified by the available experimental evidence for the existence of VitP. It is important to realize that many flavonoids are not polyphenols as they are not polymeric and only consist of a single flavonoid moiety. Proanthocyanidin researchers typically propose to limit use of "polyphenols" to these polymeric phenylpropanoids and hydrolyzable tannins. In the context of most current uses, the term "polyphenol" is meaningless, should be avoided, and be replaced with the specific type of compound involved in a particular study. This would also bode well on future discussions of DRIs (see below).

Another source of confusion at the interface of chemical structure and bioactivity arises from the fact that virtually all phenols are antioxidants, as they possess unsubstituted (free) phenolic hydroxyl. This suggests that there should not be anything special about flavonoids as antioxidants as an explanation for VitP activity. Interestingly, VitC also is a (powerful) antioxidant, albeit by a very different chemical mechanism. However, with regard to terminology, it is important to emphasize that a present study is neither about "polyphenols" nor about their alleged "antioxidant" effects.

Notably, Health Canada has recently concluded that claims or statements or claims about Oxygen Radical Absorbing Capacity (ORAC) are unacceptable on foods as relationships between ORAC scores and human health effects have not been established. Underlining their strict requirements on antioxidant claims, the authority stated that a specific antioxidant function can be valid when linked to a well-substantiated physiological effect in healthy subjects, as determined by controlled human clinical trials (https://tinyurl.com/48w79wzy).

BRIEF HISTORY OF VITAMINS

Early in the twentieth century, the research of several investigators indicated that a family of organic substances found in foods were essential for human life. One of these early investigators, Funk, recognized that small amounts of these substances were essential, and that their absence was responsible for many common diseases such as beriberi, pellagra, and scurvy (4–6). Funk termed these substances vitamins, or "vital amines," based on the original discovery of thiamin, an amine found to be involved in beriberi. In 1920, Drummond (7) proposed that the term be shortened to "vitamin" as non-amine essential compounds were discovered. He further proposed that thiamine and riboflavin be called Vitamins A and B, respectively, to contrast them from Funk's anti-scurvy factor, which he called Vitamin C (8). Eventually, a series of vitamins was

discovered and labeled alphabetically in the order of discovery (**Table 1**).

This alphabetic listing is primarily historic, because not all vitamins have equally well-defined deficiency diseases, levels of biological activity, and assignments to a single chemical species. For example, the bioactivities of vitamins A and D involve multiple carotenoid and steroidal species, respectively, ranging from nutritionally necessary to actual effector molecules. Furthermore, the B series exemplifies that the vitamin originally designated under one category may turn out to be multiple, in this case even chemically unrelated groups of compounds. Collectively, this means that the historic vitamin designation should be reinterpreted with today's knowledge of complex biochemical systems rather than with the expectation that it is a single compound with a clearly defined biological endpoint.

Although modern definitions differ somewhat, an essential organic chemical compound (or set of related compounds) is called a vitamin when it cannot be synthesized by the organism at all nor in sufficient quantities and, therefore, must be obtained through diet. Thus, the term vitamin is conditional upon the circumstances and particular organism. For example, VitC (ascorbic acid) is a vitamin for humans and guinea pigs, but not for rats and most other animals (9). The well-known Vitamins A, C, D, E, K, and Vitamins of the B complex were among those subsequently discovered in the early part of the twentieth century and later accepted as vitamins (10). In contrast, the proposed VitP was almost forgotten. Putting VitP into proper perspective requires a review of VitC discovery and basic physiology, as follows.

VITAMIN C AND SCURVY

By the mid-1700s, scurvy debilitated and killed those whose diet was largely based on meat and starch and devoid of fresh vegetables and fruits. Although the problem was widespread, this was especially difficult for the British navy, as numerous British sailors were afflicted with this disease (10, 11). Attempting to ameliorate this situation, a Scottish physician, James Lind, had observed the curative and preventive powers of citrus fruits and wrote an essay (1757) recommending mandatory consumption of citrus fruits and lemon juice by sailors in the British Navy, eventually leading to their colloquial designation as "limeys." However, it would take a century for scientists to understand why citrus fruits were so effective against scurvy (10).

Commonly recognized symptoms of scurvy are loss of weight; swollen, soft, spongy, or ulcerated gums; loose carious teeth; hemorrhages; necrosis of the bones; swollen joints; edema; hardening of the skin and often perifollicular or petechial hemorrhages, sometimes bloody conjunctiva and occasionally anemia (12). In the absence of VitC, the development and maintenance of intercellular substances degrades. This involves the collagen of all fibrous tissues and of all non-epithelial cement substances, such as intracellular material of the capillary wall, cartilage, dentin, and bone matrices (12). It has been the prevailing view that scurvy and the deficiency states of VitC are marked by unduly fragile capillaries, despite a considerable body of evidence that there is no direct association of capillary strength and VitC in humans nor in guinea pigs [see (13) and citations therein].

In 1907, two Norwegians, Holst and Fröhlich, reported the existence of a substance that, based on observed biological effects, had the ability to cure the symptoms of scurvy (14). Furthermore, they were able to demonstrate that the absence of this substance produced the symptoms of scurvy in guinea pigs, which are unable to synthesize the substance endogenously. This work was largely ignored because, at that time it was generally accepted that only lipids, proteins, and carbohydrates were needed for growth and development of animals.

In approximately 1924, the Hungarian scientist Szent-Györgyi began to study animal, vegetable, and synthetic oxidizing systems (15). By 1928, he had isolated and accumulated about 30 grams of a strongly reducing substance, which he called hexuronic acid, from adrenal tissue, citrus species, and cabbage. Further, Szent-Györgyi also provided a sample of hexuronic acid to Haworth, an eminent chemist who, in turn, passed the sample to colleagues who determined the structure (16). Interestingly, in his early studies, Szent-Györgyi did not carry out bioassays to establish that his substance was the antiscorbutic compound, but provided a sample to another investigator, Zilva, who, in 1932, declared that Szent-Györgyi's hexuronic acid was not VitC (17–20).

Perhaps slightly after Szent-Györgyi had begun his studies on hexuronic acid in 1928, King at Pittsburgh began complementary studies. King's contribution involved the isolation of VitC from lemon juice in 1931–1932 and study of its antiscorbutic activity in guinea pigs (21, 22). The research groups of King and Szent-Györgyi connected when a Hungarian-American, Svirbely, who worked with King at the University of Pittsburgh until 1931, returned to Hungary and worked with Szent-Györgyi. By early 1932, Svirbely had established that hexuronic acid was the antiscorbutic factor identical to VitC (16, 23–25). Within 2 weeks of each other in the spring of 1932, first Waugh and King (26), King and Waugh (27), and then Svirbely and Szent-Györgyi (16) published articles declaring that VitC and hexuronic acid were the same compound, thus VitC was subsequently named ascorbic acid (28, 29). Later work by Szent-Györgyi and his collaborators, with the diet of Sherman et al. (30) for induction of scurvy in guinea pigs, was the first to demonstrate VitC avitaminosis (16, 24) and finally explained its link with the treatment and prevention of scurvy (11).

Szent-Györgyi was awarded a Nobel Prize in Medicine in 1937 for his work with regard to VitC. However, controversy remains over whether both Szent-Györgyi and King deserved equal credit for the discovery of VitC. Szent-Györgyi's further accomplishments included the discovery of the role of adenosine triphosphate and actin-myosin, elucidation of many phases of the Krebs cycle, and studies on the influence of free radicals in tumor formation (31, 32). Haworth received the 1937 Nobel Prize in Chemistry for his investigations on the chemistry of carbohydrates and VitC.

VITAMIN P EMERGED FROM VITAMIN C RESEARCH

Many biochemical processes involve VitC and its oxidized counterpart, dehydroascorbate, as cofactors: hydroxylase metabolism; (nor)adrenaline biosynthesis in the adrenal cortex; formation of dopamine and noradrenaline (syn. norepinephrine) in adrenergic synapses; and metabolism of aromatic amino acids (**Supplementary Material 5**). The most characterized function of VitC involves collagen biosynthesis, where it takes part in the post-translational hydroxylation of prolyl and lysyl residues in unfolded procollagen chains, which is essential for folding collagen into triple helices prior to collagen secretion by fibroblasts. Hydroxyproline residues contribute to the stiffness of the collagen triple helix, and hydroxylysine residues hydrogen bond with carbohydrates and form intramolecular cross-links that give structural integrity to the collagen mass. The under-hydroxylation of procollagen, which is degraded, appears to be a major factor in the pathophysiology of scurvy. VitC deficient subjects usually show reduced urinary excretion of hydroxyproline (33).

From the collagen pathophysiology perspective, the VitC story is quite satisfying. An avitaminosis condition could be treated by a single biologically active compound, designated as VitC, and chemically identified as ascorbic acid. Structure activity relationships have been established (**Supplementary Material 2**) and specific biological targets identified. However, the VitP story emerges from the VitC narrative. Although Szent-Györgyi was awarded the Nobel Prize for the discovery of VitC, according to some, he was never fully satisfied, because his experiments with VitC showed something was "missing" (22, 29). This is where VitP enters the picture.

Early VitC studies were performed with natural product extracts, which contained VitC along with other compounds (impurities) that accounted for the residual complexity, i.e., minor chemical species in nature-derived agents that impact biological test systems and bioactivity profiles of the assay material (34). VitC became the focus of these studies as it was the major component of the extracts. Moreover, the role of putative impurities was obscured by the apparent success of VitC to reverse most, but not all, biological effects of a scorbutic diet in guinea pigs and humans.

EARLY EVIDENCE FOR VITAMIN P AS A FLAVONOID

Although the structure of VitC was still unknown in the 1920s, several investigators at the time provided evidence that VitC interacted with a second factor of unknown composition (35–38). Based on these reports and his own experiments, with isolated and characterized VitC, Szent-Györgyi also believed that there was an additional chemical factor that played a significant role in curing scurvy (22, 29). He felt that this additional substance was of similar importance and related activity with VitC. In the dietary absence of both VitC and this unknown entity, the symptoms of scurvy prevailed and concealed symptoms of the deficiency of the second substance (39). Along with other investigators, he found that in certain pathological conditions characterized by an increased permeability or fragility of the capillary wall, highly purified or synthetic VitC was ineffective for reducing the permeability, whereas the condition was readily cured by administration of extracts of Hungarian red pepper (called "Vitapric") or lemon juice. These extracts were effective in cases of decreased resistance of the capillary wall toward whole blood (vascular type of hemorrhagic purpura), as well as in cases where the capillary wall showed increased permeability toward plasma protein only, such as observed in various septic conditions. As little as 40 mg of this active fraction given daily intravenously to a human restored normal capillary resistance in 2 weeks. Spontaneous bleeding ceased, the capillary walls lost their fragility toward pressure differences, and no more plasma protein left the vascular system on increased venous pressure (39).

In a second publication, Szent-Györgyi et al. demonstrated that when guinea pigs were fed a diet that induced scurvy, those that also received 1 mg daily of the crystalline flavonoid fraction of lemon juice (called citrin), survived much longer than those that did not. Those animals not receiving citrin died in 28 days, whereas those with citrin lived 44 days on average. Both groups showed typical symptoms of scurvy, but the group that did not receive citrin had major increases in hemorrhages of several types in comparison to group that received citrin (40, 41). The authors argued that these results indicated that experimental scurvy, as it is commonly known, is a deficiency disease caused by the combined lack of VitC and other components of lemon extract. Accordingly, they proposed the name "Vitamin P" (VitP) for the substance responsible for the action on (vascular) permeability. The decision to call this material a "vitamin" later proved to be a source of controversy (31). Experimental difficulties in preparing a strictly VitP deficient diet and, thereby, establishing a hard biochemical link to a deficiency syndrome are one reason why the scientific community has found it difficult to embrace VitP as a vitamin. The intertwinement with VitC further obscures the potential link between deficiency and disease. However, as discussed below, evidence for VitP as an essential nutritional factor should take precedence over the formal question of its designation as a "true" vitamin vs., e.g., being a micronutrient.

Zacho investigated the impact of citrin (see **Supplementary Material 3** for a comprehensive description) in capillary resistance in guinea-pigs ($n = 36$) fed with various diets using the sucking-cup method adopted from the human clinical use to the animal model (42). His findings confirmed that citrin was effective in restoring normal capillary resistance from scorbutogenic dietary conditions. Unfortunately, several workers, notably Zilva (19, 43), were not able to duplicate the effects of citrin and its interactions with VitC as claimed by Moll (44), Lotze (45), and Parrot and Sevestre (46). Even Szent-Györgyi was not always able to duplicate his own results (47, 48). However, in other cases, the initial observations of activity of citrin and other flavonoid fractions could be repeated (13). Although the difficulty to repeat the original work may have been for a variety of reasons, one major problem was that the preparations used by Szent-Györgyi et al. were not consistent

in composition (46, 47, 49–51). From today's perspective, these reported reproducibility issues could be interpreted as a form of (chemical) residual complexity. Researchers attempted to isolate the active component of citrin. Upon fractionation, the active fraction from lemon juice was found to consist primarily of flavonoid glycosides. The experimental description of the production of citrin, the phytochemical methods available at the time, and the incomplete compound purification (without crystallization to constant m.p.) most likely yielded materials with variable chemical composition.

Despite the fact that Armentano et al. (52) found that use of lemon juice and preparations of paprika rich in VitC produced favorable results in certain patients with bleeding diseases, namely vascular purpura and protein permeability of the capillaries, the same results were not observed with purified VitC. From these studies, it appeared that purified and synthetic VitC were less effective than unpurified VitC from natural sources for treating thrombocytopenic purpura. These observations reinforced the notion that bioactive minor compounds, attributed to residual complexity, were present in the VitC-rich extracts. Lavollay demonstrated that injection of pure, synthetic VitC caused elevation of capillary resistance in non-scorbutic guinea pigs (2 mg per 400 g animal), whereas injection of the flavanone, epicatechin (1 μg), was 10,000–20,000 times more effective than VitC. Others also have not detected any increase in capillary resistance following administration of VitC alone to apparently healthy persons on inadequate diets or to persons with scurvy (45, 49, 52, 53). Interestingly, a very recent meta-analysis of 17 qualifying clinical trials has associated the flavonoid quercetin with lowered systolic blood pressure as a clinical endpoint, in the absence of lipid and glucose metabolic effects (40).

Even today, residual complexity plagues programs aimed at natural product discovery. The ambiguities in the reported interaction of flavonoid preparations such as citrin and VitC foreshadowed the ongoing difficulties that have been experienced with performing biological assays on nature-derived materials. The intricacies of purity and the chemical integrity of assay materials has recently been recognized as a persistent problem in biological assays (54).

EXPANDING THE REPERTOIRE OF VITAMIN P CANDIDATES

With the VitP hypothesis in hand, a search was undertaken to identify a chemical principle that enhanced the activity of VitC for treating the cause and symptoms of scurvy. A major problem in the search for a specific compound that could be identified as VitP proved to be that a series of structurally diverse flavonoids from other sources also produced similar results. For example, the glucosides of the flavonoid aglycones, hesperetin, and eriodictyol, were both active. In comparison, the flavonoid fractions of *Citrus* extracts contained heterosides of quercetin. In some cases, comparative activities were measured, for example, citrus fruit concentrates were reported to have 20 times the activity of hesperidin (49, 51). In his publications, Szent-Györgyi concluded that VitP should be a flavanone with exceptional properties owing to its activity vis-à-vis oxidative agents (47). However, Javillier and Lavollay noted that the activity was not associated with specific flavonoid structures, but also with many flavonoids including flavonols, flavanones, and their glycosides, as well as catechins and their oligomeric proanthocyanidins [**Figure 1**; (50)].

Catechins from cutch (*Senegalia catechu* [syn. *Acacia catechu*], catechu) proved to be a source of VitP-like activity. Although Lavollay et al. found the original crystalline (+)-catechin from cutch to be inactive (55, 56), the mother liquor was quite active. Notably, (–)-epicatechin, from the mother liquors was 500–1,000 times more active than the flavonoid fraction from citrus extracts (55–57). This example of "dynamic" residual complexity (54) plausibly had resulted from an epimerization event that occurred during recrystallization of a compound isolated by bioassay guided fractionation. Based on LC/MS and LC/MS/MS analyses, it is now known that the major monomeric catechins of cutch are (+)-catechin, (–)-epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin-3-*O*-gallate (58). Extracts of gambier, *Uncaria gambir* (Rubiaceae), a source of the catechin oligomers called proanthocyanidins, were also found to be active. Gambier extracts contain primarily (+)-catechin and only small amounts of (–)-epicatechin (59).

In subsequent studies, the French investigators found that coumarin and the coumarin derivatives esculin and esculetin also were quite active (60). A flavonoid dihydrochalcone, phloridzin, even showed activity in microgram quantities (50). Coumarins are widespread in nature, however, phloridzin is best known as an apple skin component and is only occasionally found in other plants.

In 1948, Masquelier founded a company that prepared an extract of the bark from French maritime pine, *Pinus pinaster* Aiton subsp. *atlantica* Villar, called Pycnogenol®, that had VitC-like properties [**Supplementary Material 4**; (61)]. Chemical identification studies showed that this extract is primarily composed of proanthocyanidins that are biopolymers of (+)-catechin and epicatechin with two or more flavonoid subunits. The extract also contained monomeric (+)-catechin and epicatechin.

Lavollay and Sevestre demonstrated that Bordeaux wine, from which the ethanol was removed, possessed Vitamin P activity when injected into guinea pigs and humans (62). Masquelier et al. later discovered the presence of oligomeric proanthocyanidins in red wines (63, 64). About 1970, it was discovered that grape seeds were also rich in oligomeric proanthocyanidins. Early research on the effects of grape seed extracts on the permeability of vascular capillaries was designed to unravel the underlying cellular and molecular mechanisms of action of oligomeric proanthocyanidins and other flavonoids (65).

During the 1970's, Masquelier began producing a grape seed extract for medicinal uses as an alternative to the pine-derived Pycnogenol®. Grape seeds contain a similar, but not identical complement of the oligomeric proanthocyanidins (OPCs; syn OPACs) to pine bark. Masquelier's product, called "MASQUELIER's® Original OPCs," was extracted from the seeds of *Vitis vinifera* L. This product contained catechin, epicatechin,

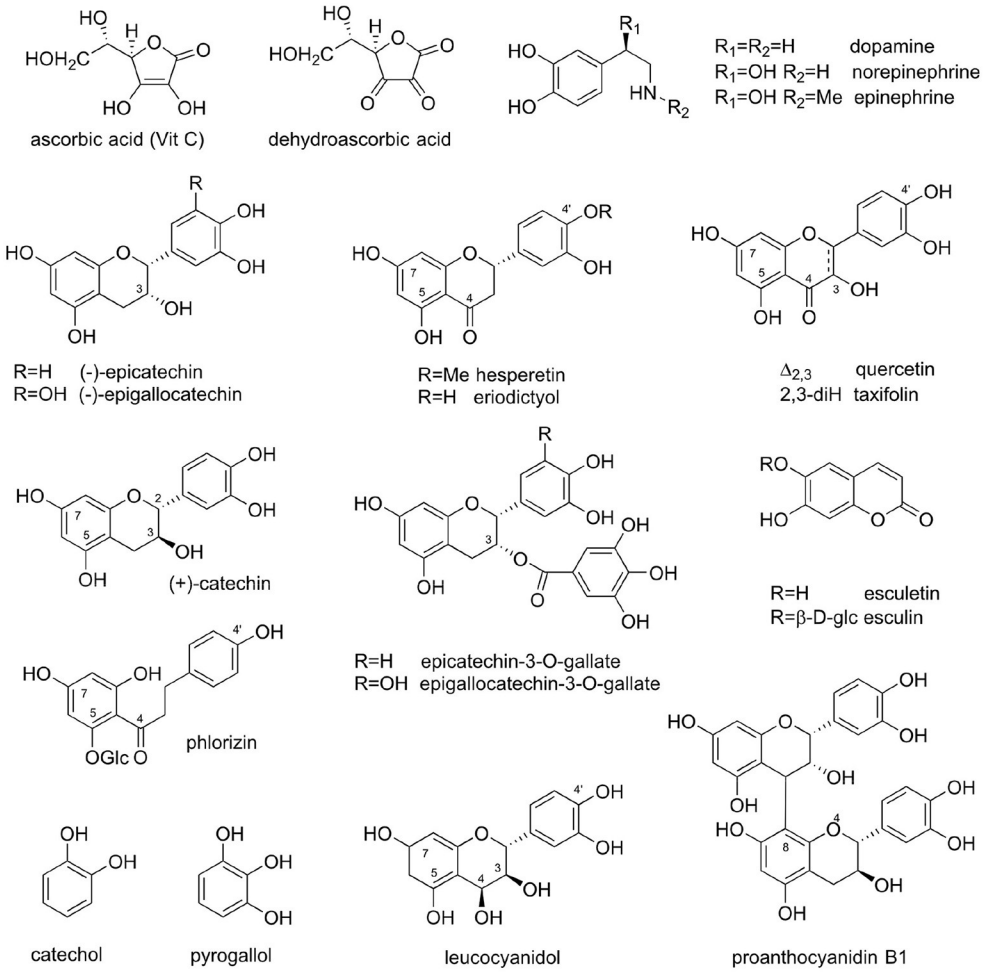

FIGURE 1 | Natural products featured in the Vitamin P story: Vitamin C (ascorbic acid); dehydroascorbate; dopamine, noradrenaline (norepinephrine), and adrenaline (epinephrine); hesperetin and eriodictyol; (–)-epicatechin and (–)-epigallocatechin; quercetin and taxifolin; (+)-catechin; (–)-epicatechin-3-*O*-gallate and (–)-epigallocatechin-3-*O*-gallate (EGCG); esculetin and esculin; phlorizin; catechol; pyrogallol; leucocyanidol; and proanthocyanidin B1 [epicatechin-(4β → 8)-catechin].

and OP(A)Cs (dimers to pentamers) (66). Detailed analyses have established that the product is standardized to contain about 85% (w/w) flavonoids, of which 50–60% (w/w) are monomeric and dimeric catechins. The same product was evidently devoid of OP(A)Cs (65, 67).

In vitro studies indicate that Pycnogenol® constituents demonstrate a high percentage of binding to collagen fibers, promote synthesis of collagen and elastin, and inhibit their proteolytic degradation (68). Masquelier's OP(A)Cs have been demonstrated to bind collagen and elastin in blood vessel walls, promote collagen synthesis and polymerization, and inhibit degradation of collagen and elastin in a study conducted in guinea pigs (69). The OP(A)Cs of grape seed show strong collagen protection. By binding to collagen, they also may offer protection of elastin and collagen in vascular tissues from degradation by elastase and collagenase, respectively (65).

Flavonoids such as (-)-epicatechin and dimeric procyanidins in human diets can be absorbed and reach the bloodstream and other organs (67, 70, 71). OP(A)Cs consisting of larger numbers of monomeric units are poorly absorbed and mostly pass through the digestive system unaltered. In this process, a portion of certain OP(A)Cs bind to membranes of the gastrointestinal tract. These substances have a key role in stabilizing membranes by preventing their disruption via chemical and biological agents, and regulating membrane-associated events. In many instances, OP(A)Cs appear not to bind directly to degradative enzymes, but to bind to matrix macromolecules and prevent their degradation by various factors such as temperature, oxidative stress, inflammation, and proteinases (67, 70–74). The original lemon and paprika extracts studied by Szent-Györgyi and others are rich in VitC and a variety of flavanones, but essentially lack catechin and proanthocyanidins (75–77).

Fibrillar collagen is a strong and viscoelastic biomaterial arranged into highly organized hierarchical structures. Type 1 collagen is the most abundant of all collagen types and is defined as an interwoven coiled trimer, containing repeated sequences of proline and hydroxyproline (78). The interaction of OP(A)Cs and collagen is believed to be stabilized by hydrogen bonding between the protein amide carbonyl and hydrophobic bonds. The relatively great stability of OP(A)C-protein complexes suggests structural specificity. Indeed, two new trimeric and tetrameric A-type OP(A)Cs capable of strengthening the micromolecular backbone of teeth via intermolecular and intermicrofibrillar cross-linking have been reported (79). The applications in dentistry include disease prevention (caries) and partial tooth repair.

In conclusion, while astute experimental observations concerning the residual complexity of early VitC preparations from natural sources led to the VitP hypothesis, the residual complexity of VitP formulations (also prepared from natural extracts) led to considerable confusion in identifying a "lead compound" in the search for Vitamin P. It has not been possible to attribute VitP activity to a specific food, preparation, or compound. This problem, coupled with the difficulty in attributing a deficiency disease that could clearly be linked to Szent-Györgyi's lemon extracts or to VitP might have led scientists to abandon VitP as a useful hypothesis. Nonetheless, despite the difficulties in understanding the relation of Vitamins C and P, two things have been established: (i) the existence of a distinct biological effect of VitP, i.e., the influence of a series of naturally occurring flavonoids and coumarins (**Figure 1** and **Supplementary Material 1**) on vascular permeability; and (ii) the influence of these natural products on the anti-scurvy VitC effect (45, 49, 65).

EVIDENCE FOR VITAMIN P AVITAMINOSIS

One of the early objections to consideration of VitP as a true vitamin lay in the difficulty of establishing disease symptoms that were related to the identified series of compounds. This shortcoming was addressed by later studies of Casley-Smith et al. who worked mostly with rats, which have the ability to synthesize VitC endogenously. When fed a diet lacking flavonoids, the rats exhibited definite structural alterations in blood capillaries and tissues. A diet of this type, for the time period employed, produced considerable increases in capillary fragility. These fine structural alterations were quite different from those reported for VitC avitaminosis and implied a different deficiency (80, 81). Other work demonstrated that lack of flavonoids gives rise to cerebral edema due to the increased permeability of the blood-brain barrier (82). In skin, a flavonoid-deficient diet greatly increased capillary fragility, which was reversed by the addition of flavonoids to the diet (80).

Medicinal preparations involving a semisynthetic flavonoid compound, *O*-(β-hydroxyethyl)-rutoside, were developed about 1960 (83). The corresponding preparation, Venoruton®, has been widely used in Germany and Switzerland for the treatment of edema and other vascular disorders. Oral administration of Venoruton® increased conjunctival capillary resistance in rabbits. This preparation has few negative side effects and, importantly, is soluble in water, whereas rutin and other flavonoids with similar activity have limited water solubility.

Studies with guinea pigs and humans implied that natural flavonoids and coumarins acted as vitamins in those animals, and that VitC and VitP deficiency states are quite distinct. The structural effects of VitC deficiency in both guinea pigs and humans have been studied by a number of workers who noted a significant reduction in the amount of collagen, with swelling of the fibroblasts and endoplasmic reticulum. The basement membranes of the blood capillaries (and associated lymphatics) were often very tenuous and disrupted, although they sometimes appeared thicker (80). In contrast, in VitP avitaminosis in rats, the basic lesion consisted of the opening of some blood capillary endothelial intercellular junctions. Unlike in VitC avitaminosis, the endothelial cells were intact, without a pale, grossly swollen cytoplasm (80). These effects were largely prevented by feeding coumarins as well as flavonoids such as troxerutin (80, 83, 84).

The studies by Casley-Smith implied that the natural flavonoids and coumarins were vitamins. VitC and VitP deficiency states are sufficiently distinct to justify the assignment of VitP as the underlying factor. It seems probable that some of the changes in VitC deficiency observed in many studies were due to a concomitant VitP deficiency. Whereas both conditions had many open endothelial junctions and somewhat altered basement membranes, all the gross distortions of the endothelial cells observed in VitP avitaminosis were considerably lessened by flavonoids and coumarins.

EARLY IDEAS ON THE MECHANISM OF ACTION OF VITAMIN P

Probably because adrenal glands were an original source of VitC, and due to the fact that adrenaline was a major factor in capillary blood flow, much attention was focused on adrenalin as a link to VitP activity (49, 62, 85). *In vitro* experiments showed that adrenaline is readily oxidized by catechol oxidase, the cytochrome system, amine oxidases, and peroxidases (86). Javillier and Lavollay supposed that VitP slowed down oxidation and, thereby, the resulting inactivation of adrenaline (50, 87–89); adrenaline can also be inactivated by non-oxidative enzymes, such as catechol-*O*-methyltransferase. The ability of many different types of flavonoids and coumarins to affect the persistence of adrenaline was evaluated in a subsequent extensive study (85).

It is likely adrenaline is involved in the action of VitP: it binds to α-1 receptors that are involved with vasoconstriction, smooth muscle contraction of the bladder neck, and glycogenesis. Adrenaline also binds to α-2 receptors, which are involved with vein constriction, central attenuation of the sympathetic nervous system, inhibition of insulin release, and relaxation of the intestine. Other interactions include β-1 receptors that provide positive inotropic and positive chronotropic signals to the heart, lipolysis, and renin release, as well as β-2 receptors, providing bronchodilation, vasodilation, gluconeogenesis,

relaxation of the uterus, and relaxation of the intestine. The ubiquitin receptor (vascular dopamine) DA1 is involved with vasodilation, whereas the DA2 receptor prevents presynaptic noradrenaline release [http://www.urology-textbook.com/adrenal-glands-catecholamins.html; (90)]. The plasma half-life of adrenaline is ∼20 s. Inactivation depends mainly on monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT) availability.

Early experiments with flavonoids examined their role in the metabolism of adrenaline. Injection of quercitroside inhibited decomposition of adrenaline in dogs and cats. In addition, quercitroside extended the physiological action of adrenaline in cats (46). In guinea pigs that were scorbutic, a number of other substances were shown to inhibit oxidation of adrenaline *in vitro*, however, most of them, e.g., catechol and pyrogallol, were not substances that would normally be found in the diet or in animals (56, 57).

Alternatively, other investigators concluded that the activity of VitP consisted of inhibiting the oxidation of VitC. In 1947, Masquelier isolated a flavan-3,4-diol, leucocyanidin (syn. leucocyanidol), from the seed coats of peanuts (91–93). He found that extracts containing flavan-3,4-diols could protect VitC from oxidation *in vitro*. He also demonstrated that rutin, esculin, and proanthocyanidin B1 all inhibited *in vitro* oxidation of VitC mediated by Cu^{2+} ions and ascorbic acid oxidase. In subsequent work, Masquelier referred to "leucocyanidol" as "OPCs" (oligomeric proanthocyanidins) although in his 1951 papers, he refers to the tested substance as a monomer (91–93). Later, Bate-Smith and Ribéreau-Gayon confirmed that the original "leucocyanidol" isolated was indeed a flavan-3,4-diol (94). Overall, Masquelier examined the health benefits of probable mixtures of these compounds and based on his subsequent studies, developed a vasculo-protective medicine in 1950. However, the (somewhat surprising) limited availability of peanut skins necessitated examination of other plants sources of active antioxidant substances (29).

At first sight, the role of VitP as an "antioxidant" in biological systems might appear an attractive explanation for many aspects of the biological role of flavonoids, because it can easily be rationalized why so many diverse flavonoids and related phenylpropanoids display VitP activity. However, "antioxidant" bioactivity does not adequately explain the apparent link between VitC and VitP activity, especially as VitC could, presumably, act as its own antioxidant. Flavonoid compounds such as flavan-3-ols, flavan-3,4-diols, and oligomeric proanthocyanidins (including procyanidin B1) have radical scavenging activity and, therefore, have been proposed to serve as "antioxidants." It is now known that vascular function is strongly influenced by oxidative stress and that diminishing oxidative stress also reduces inflammatory stress (65). However, flavonoids and related compounds, as well as extracts containing these compounds, may be involved in a multitude of biological functions and may have multiple (pleiotropic) effects that during evolution have not been selected for a strong effect on a single well-defined target (65, 67), but rather the opposite.

PLAUSIBILITY OF FLAVONOIDS AS BIOACTIVE COMPOUNDS

The NAPRALERT Database (2) was consulted to investigate the plausibility of flavonoids as bioactive compounds. Plant natural products from 421 families, 3,714 genera, and 16,011 species are represented in NAPRALERT. A much smaller number of plants have been examined for biological activity. A NAPRALERT search revealed that ∼50,000 biological experiments have been published on flavonoids. About half of these are based on commercially available materials. The other half included flavonoids from about 1,700 species of living organisms, primarily plants. Almost 7,000 compound names associated with these experiments are represented. The qualitative activity, i.e., overall active to inactive activity, reported for these compounds is 2:1 (3:1 *in vivo*) which means that 66-75% of flavonoids have been reported as being bioactive in their respective assays.

Flavonoids also have been reported to have bioactivity in a very broad variety of bioassays. They undoubtedly interact with many different systems in mammals. These pleiotropic interactions may be detrimental or beneficial. Note that flavonoids and related phenolics often interfere with bioassays and, therefore, are described as pan-assay interference compounds (PAINS) (95). PAINS interfere with *in vitro* assays through various mechanisms including fluorescence, redox, or through generalized binding to enzymes. As a result, flavonoids represent 10 of the 22 natural products that have been identified as most likely being natural product invalid metabolic panaceas (IMPS) (1).

ARE VITAMIN C AND VITAMIN P MUTUAL CO-FACTORS?

One early experimental approach to explore this question used guinea pigs, which like humans, lack the ability to synthesize VitC. They were divided into five groups. A control group was given VitC, whereas other groups received no or sub-optimal levels of VitC, but were given various levels of OP(A)Cs. Even though deprived of VitC, those that received an appropriate amount of OP(A)Cs survived as long as those that got adequate amounts of VitC. Lotze concluded that VitC and OP(A)Cs have a marked "synergy" because flavonoid co-administration of OP(A)Cs and VitC made it possible to decrease the dosage of VitC (22, 45, 53, 96). Pleiotropic effects (multiple biological effects from the same phytoconstituent) may also explain bioactivity that cannot be rationalized by classical reductionist models (67).

DOES METABOLIC TRANSFORMATION CONTRIBUTE TO FLAVONOID BIOACTIVITY?

When considering bioactivity research that is dominated by live animal assays, it is necessary to consider the contribution of the dynamic complexity of metabolites to the observed activity. The stability and metabolism of catechins and other flavonoids

under digestive conditions is relatively poorly understood. However, several common food additives including VitC, milk, and citrus juice enhance the stability of epicatechin, epigallocatechin, epigallocatechin gallate, and epicatechin gallate, when incorporated into tea beverages. From *in vitro* digestion studies, without additives, <10% of epigallocatechin gallate and epicatechin gallate were recovered. Addition of VitC increased recovery to 54 and 74%, respectively, while epicatechin recovery was 82%. Of all substances tested, juice preparations promoted stability best. Epicatechin recovery was then between 86 and 95%. Of the juices tested, lemon juice was most effective. Catechins are most stable in aqueous solutions at about pH 4 (97, 98).

VitC and VitP may play a joint role in the biological activity of VitC (99, 100). VitC reacts with *p*-hydroxybenzyl alcohol to produce two epimeric forms 2-*C*-(*para*-hydroxybenzyl)-3-keto-hexulosonic acid (99). These authors also indicated that VitC can react with leucoanthocyanidins (flavan-3,4-diols) and proanthocyanidins (such as proanthocyanidin B1) to produce adducts. Both epi(gallo)catechin and epi(gallo)catechin-3-*O*-gallate are very similar with regard to structure and reactivity (100).

Metabol(om)ic relationships including xenobiotic conversions, which exist between the flavonoid structures and structurally distinct derivatives and/or reactive species may be the actual carriers of the VitP effect. Recently evolving studies provide compelling evidence for gut microbiota and microbiome playing a key role in intestinal flavonoid metabolism. Recent reviews and studies [see (101–103) and references therein] have already summarized the impact of such processes on flavonoid bioavailability, the deglycosylation and formation of metabolites that unfold the actual effects at the site of action, the inter-individual differences of health outcomes, and connections with cardiovascular health.

In addition to the property of flavonoids to form a network of readily interconverting congeners that primarily differ in the degree of (un)saturation and oxidation of the pyran/C-ring, flavonoids yield a common set of very small molecule degradation products when exposed to gut microbes. Among these microbial metabolites are hydroxybenzoic acids (HBAs), including salicylates, and other small phenolic acids (104). Considering the relatively high abundance of many flavonoids, HBAs can be produced in physiologically relevant amounts. Existing evidence regarding the catabolism of the flavan-3-ol subgroup has been compiled and discussed in recent reviews (105, 106).

CAN WE SHED LIGHT ON HISTORICAL REPORTS?

Unambiguous data show that the activity of Vitamins C and P are related. In a number of studies, guinea pigs on a scorbutogenic diet developed symptoms of scurvy and the animals also developed reduced capillary resistance. This latter effect was reversed by addition of citrin, hesperidin, or other flavonoids to the diet. Interpretation of data from different methods for determination of capillary resistance and lack of experimental details contributed to variation in the results of many of the studies reviewed by Scarborough and Bacharach (49).

Other problems result from the composition of scorbutic diets used to study the role of VitC and VitP in guinea pigs and rats. Many early studies, including those of both King and Szent-Györgyi, were based on a scorbutic diet that consisted mainly of freshly ground oats (*Avena sativa*), dried milk powder, salt, and butter fat. Notably, oats contain (–)-epicatechin, a compound with pronounced VitP-like activity (107). The presence or absence of (–)-epicatechin and related flavanols in the diets employed, opens questions about the interpretation of the results of many of these early studies, and/or the role of (–)-epicatechin in VitP activity.

Nonetheless, we concur that VitP activity is real. At the same time, the strict definition of a vitamin as a single chemical entity (SCE), the absence of which generates a disease (avitaminosis), does not apply because of the complex composition and interrelated nature of the compounds that can serve as VitP. In this respect, VitP resembles a complex similar to Vitamin B. While the definition of Vitamins P1, P2, P3, etc. is premature, they differ from the B-series by being structurally related.

Much of the vast reported evidence suffers from being chemically inconclusive, due to a lack of rigor in the chemical characterization of the intervention materials. While this, in part, reflects the progress in chemical and structural analysis made since performing the biological assays that characterized the vitamins, other potential explanations for the gap in our current understanding of VitP may lie in the following shortcomings: (a) a general trend toward bioassays that are driven by reductionist hypotheses (single agent, single target) vs. consideration of pleiotropic activity/targets (multiple biological effects originating from the same phytoconstituent); (b) the general lack of consideration of metabolic activation, in particular the conversion of flavonoids with poor PK properties into metabolites that can be absorbed; (c) a tendency to ignore all forms of residual complexity, particularly the impurity of the natural products used in bioassays (static RC), as well as stability/conversion during bioassay and/or chemical processing (e.g., heating during recrystallization, triggering epimerization and other chemical reactions).

IS VITAMIN P A SINGLE CHEMICAL ENTITY? IS IT A VITAMIN?

Considering the evidence available to date, VitP does not exist as a single chemical entity, but consists of a group of analogous, and often congeneric, compounds. The search for VitP will unlikely lead to a single isolated compound. This challenges existing paradigms built on more simplistic ligand/target models and indicates that VitP function involves higher complexity on both sides of biological action, i.e., the chemistry of the agents and the biochemistry of the biological targets and networks. As mentioned above, a number of flavonoids and flavonoid glycosides have been considered to be VitP. However, these compounds mostly have limited activity in comparison to certain

coumarins (e.g. esculetin; **Figure 1**), as well as the flavonoids phloridzin and epicatechin. Epicatechin and oligomeric catechins (such as procyanidin B1) are widely distributed among plants and are found in many food plants.

Whether the catechins and/or OP(A)Cs constitute a vitamin (complex), related or unrelated to VitC, is, in part, a question of definitions. Notably, it has not been demonstrated unambiguously that VitC *alone* is adequate for resolving the symptoms of scurvy (49). In the opinion of Masquelier, flavan-3-ol oligomers (i.e., catechol oligomers or oligomeric proanthocyanidins [OP(A)Cs]) are the only flavonoids that have a justified claim to VitP activity (22). Furthermore, there is significant evidence that coumarin derivatives, which share a biogenetic and ADMET relationship with flavonoids, have VitP activity and also interact in a powerful manner with VitC (45, 53). Notably, while representing constituents present in certain dietary plants, coumarins themselves are excluded from becoming dietary supplements due to their approved drug status.

Although VitP has not been widely accepted as a vitamin by the scientific community, numerous commercial dietary supplements are currently sold as "VitP," sometimes using synonymous terms such as "bioflavonoids." The variety of chemical structures associated with commercial products sold with the label "VitP" is as diverse as all the structures shown in this article (**Figure 1**), with the exception of the coumarins, and always has a focus on flavonoids.

As detailed above, the designation of VitP as a vitamin has historical roots and stems from the era of vitamin discovery (ca. 1918–1948; **Table 1**), when nutrients present at relatively low concentrations in certain foods were recognized as causing deficiency symptoms or diseases and as being essential, and were labeled in a more or less organized alphabetical order of discovery. Compared to the well-established vitamins, VitP is chemically elusive yet biologically relatively well-documented. Reflecting the overall complexity of describing it chemically and biologically, its status as a vitamin remains hypothetical. Its "metabolic network chemistry" and interdependence on other "co-factors," particularly its established relationship with Vitamin C, may eventually move its label from vitamin to the broader micronutrient category. However, in the view of the authors, the elusive and partially hypothetical nature as well as its to-be-resolved classification tends to hide the importance of VitP and certain "bioflavonoids" as an essential factor of human health.

WHAT ABOUT DIETARY REFERENCE INTAKES OF FLAVONOIDS?

Based on all available/reviewed data, it is currently not feasible to define Dietary Reference Intakes (DRI) for VitP. Review of recent references does not clarify how the DRI of flavonoid substances can be accomplished. Several recent reports (108–111) outline how DRIs can be approached for certain compounds. Accordingly, DRIs are potentially feasible for the carotenoid lutein (not to be confused with the flavonoid, luteolin), but it is impossible to carry out the required steps for flavonoids. Although a single compound may ultimately be shown to provide most VitP activity, a number of congeners may be involved in its interaction with VitC. Collectively, much additional information will be required before determination of a VitP DRI is possible.

POTENTIAL DIRECTIONS FOR ESSENTIAL FLAVONOID RESEARCH

Flavonoids are apparently closely related, yet still form a rather heterogeneous group of metabolically interchangeable essential nutrients with poorly understood biological profiles. The historic and ubiquitous nature of flavonoids in the human diet suggests that they may have become essential dietary nutrients at some point in the evolution of the human species. In fact, it is difficult to devise a diet that is completely deficient in these compounds as flavonoids cover a wide range of chromatographic polarity. Moreover, the highly generic nature of flavonoids as bioactive compounds suggests that no single compound emerged as playing a decisive role. This seems to indicate that a more generalized activity might be at work, which is strikingly well-aligned with the *Screening Hypothesis* by Firn and Jones (112) and Jones and Firn (113).

At the same time, this does not rule out the possibility of specific biological targets, which remain to be determined. In fact, there may be multiple specific biological targets that are impacted by various chemical members of the VitP family (65, 67). The relevant literature associates VitP activity with 12 flavonoids (**Figure 1** and **Table 2**), which belong to various subclasses (flavanones, flavan-3-ols, flavans, flavanonols, chalcones, flavan-3,4-diols, and proanthocyanidins), each of which has a large number of closely related and relatively widely occurring congeners. Thus, the total number of structural and metabolically related flavonoids that contribute to the overall VitP activity could be substantial. At the same time, some members of this "flavonoid network" likely have more pronounced VitP bioactivities than others. In the understanding of the authors, based on the evidence summarized above, the flavan-3-ols including the proanthocyanidins could potentially be such an important subclass.

From a more general bioactivity perspective, the difficulty in discerning a specific biological VitP activity for a specific compound is a direct result of the behavior of many flavonoids in bioactivity assays that makes them "Invalid Metabolic Panaceas" (IMPs). Nevertheless, this apparent dilemma can also be understood as an outline of opportunities for future experiments that seek to connect a network of multiple phytochemicals with the multitude of biological effects that collectively constitute the VitP activity. The promiscuity and questionable validity of the biological effects assigned to highly prominent flavonoid IMPs has been recognized by Ingólfsson et al. (114) and is also reflected in the human protein-protein interactions that have recently been presented

TABLE 2 | Description of the compounds shown in **Figure 1** with an emphasis on their role in the vitamin P story.

Compound	Class	Origin	Known or proposed interactions
Ascorbic acid	γ-lactone	Not biosynthesized in humans, but prevalent in many plant and animal species	VitC and VitP work together
Dehydro-ascorbic acid	γ-lactone	Not biosynthesized in humans, but prevalent in many plant and animal species	
Dopamine	Catechol Phenylethylamine	Mammalian neurotransmitter	VitP slows down oxidation of dopamine *in vivo*
Norepinephrine	Catechol Phenylethylamine	Mammalian neurotransmitter	VitP slows down oxidation of norepinephrine *in vivo*
Epinephrine	Catechol Phenylethylamine	Mammalian neurotransmitter	VitP slows down oxidation of epinephrine *in vivo*
Epigallocatechin	Flavan-3-ol Pyrogallol	Plant-derived natural product	Likely present in plant extracts that show VitP activity
Epicatechin	Flavan-3-ol Catechol	Plant-derived natural product	Present in plant extracts that show VitP activity. Shows VitP activity as purified compound
Hesperetin	Flavanone	Plant-derived natural product	Glucosides present in plant extracts that show VitP activity
Eriodictyol	Flavanone Catechol	Plant-derived natural product	Glucosides present in plant extracts that show VitP activity
Quercetin	Flavonol Catechol	Plant-derived natural product	Glucosides present in plant extracts that show VitP activity. Studied as a purified compound for it biological benefits
Taxifolin	Flavonol Catechol	Plant-derived natural product	Glucosides likely present in plant extracts that show VitP activity
Phlorizin	Dihydrochalcone	Plant-derived natural product	Likely present in plant extracts that show VitP activity
Catechin	Flavan-3-ol Catechol	Plant-derived natural product	Present in plant extracts that show VitP activity
Epicatechin-3-O-gallate	Flavan-3-ol Catechol Pyrogallol	Plant-derived natural product	Present in plant extracts that show VitP activity
Epigallocatechin-3-O-gallate	Flavan-3-ol Pyrogallol	Plant-derived natural product	Present in plant extracts that show VitP activity
Esculetin	Coumarin Catechol	Plant-derived natural product	Present in plant extracts that show VitP activity. Shows VitP activity as purified compound
Esculin	Coumarin	Plant-derived natural product	Present in plant extracts that show VitP activity. Shows VitP activity as purified compound
Catechol	Catechol	Degradation product of plant-derived natural products	Shows VitP activity as purified compound
Pyrogallol	Pyrogallol	Degradation product of plant-derived natural products	Shows VitP activity as purified compound
Leucocyanidol	Flavan-3,4-diol Catechol	Plant-derived natural product	Present in plant extracts that show VitP activity
Procyanidin B1	Flavan-3-ol Catechol	Plant-derived natural product	Present in plant extracts that show VitP activity

by do Valle et al. (115): studying associations between 65 IMPs and closely related compounds vs. 299 diseases, yielded 1,525 known and 17,910 unknown associations within the human interactome consisting of 17,651 proteins and 351,393 interactions. While the study recognized the known poor PK properties of these compounds, the cited contemporary approaches that use nanoparticles are not suitable to explain for enhancement of bioavailability in the context of food and vitamins/micronutrients contained therein.

In addition to seeking answers to the above pivotal questions, more general insights were gained from the study with regard to the overarching goals (see Motivation of This Study). Reinterpretation of previously-documented outcomes can inspire new directions for flavonoid/VitP/VitC research as summarized by the following points.

- Newly developed research hypotheses should take into account that multiple chemically related, yet distinct constituents are required to unfold an essential biological role.
- Low-abundance ("micro") components are more challenging to work with, but fully valid as putative agents; they provide important opportunities for discovery, even in the presence of other known bioactive factors that might overshadow such "micronutrients."
- Specific and distinctive chemical and biological terminology is essential, whereas blanket terminology (e.g., "antioxidant polyphenols") tends to oversimplify and prevent progress.

By highlighting previously unrecognized connections between documented outcomes, naming known culprits, and outlining recent advances as well as remaining challenges equally, this

study hopes to inspire future interdisciplinary research that ideally can clarify the nature of Vitamin P and advance it from its resilient last place in the vitamin alphabet.

AUTHOR'S NOTE

In 1960, my [DS] personal interest in this topic originated in the seminar class of Dr. Charles Schwartz of Southwestern Oklahoma State University, Weatherford, OK, in which I wrote an assigned report on vitamins. Obviously, that report and the attendant presentation were much too broad for a single 50-min seminar but provided an early introduction to the VitP literature.

AUTHOR CONTRIBUTIONS

DS and GP wrote the manuscript. DS, JF, JG, and GP conceived and organized the structure and wrote the first draft of the manuscript. JB, AB-R, and JM contributed to the critical revision of the paper. All authors approved the final manuscript for the publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.762753/full#supplementary-material

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REVIEW
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Perspective on Improving the Relevance, Rigor, and Reproducibility of Botanical Clinical Trials: Lessons Learned From Turmeric Trials

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Plant-derived compounds, without doubt, can have significant medicinal effects since many notable drugs in use today, such as morphine or taxol, were first isolated from botanical sources. When an isolated and purified phytochemical is developed as a pharmaceutical, the uniformity and appropriate use of the product are well defined. Less clear are the benefits and best use of plant-based dietary supplements or other formulations since these products, unlike traditional drugs, are chemically complex and variable in composition, even if derived from a single plant source. This perspective will summarize key points–including the premise of ethnobotanical and preclinical evidence, pharmacokinetics, metabolism, and safety–inherent and unique to the study of botanical dietary supplements to be considered when planning or evaluating botanical clinical trials. Market forces and regulatory frameworks also affect clinical trial design since in the United States, for example, botanical dietary supplements cannot be marketed for disease treatment and submission of information on safety or efficacy is not required. Specific challenges are thus readily apparent both for consumers comparing available products for purchase, as well as for commercially sponsored vs. independent researchers planning clinical trials to evaluate medicinal effects of botanicals. Turmeric dietary supplements, a top selling botanical in the United States and focus of over 400 clinical trials to date, will be used throughout to illustrate both the promise and pitfalls associated with the clinical evaluation of botanicals.

Keywords: botanical, clinical trial, curcumin, turmeric, curcuminoids, dietary supplement

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INTRODUCTION

Research of plant-derived products (e.g., extracts and/or dried plant parts) stands at the complex intersection of science, consumerism, industry, and federal regulation connecting stakeholders with differing and only partly overlapping interests and expectations. Nowhere is this more apparent than when examining the design and results of published botanical clinical trials and their therapeutic impact (1). In contrast to FDA-approved drugs, the regulatory environment for botanical dietary supplements in the United States (US), which only allows their sale under the explicit provision that the products not be marketed for the treatment or prevention of any specific disease, does not provide a strong commercial incentive for financing appropriately

powered and designed (e.g., dose finding or equivalency) clinical studies (2, 3). The often-lacking defined chemical composition of botanical products, as well as their non-uniformity, adds a layer of complexity for scientists, clinicians, and consumers alike when attempting to understand the medical implications of published trials (4). Thus, consumers often become the final arbiters of information derived from trials of readily available botanicals, and may use a product with a chemical composition distinct from that studied to treat a medical condition for which definitive efficacy and safety data are also lacking (5).

While approximately one third of the earth's plants have been used traditionally as medicines, often in combination, <10% of traditional medicinal plants have been the focus of scientific research (6). Despite this absence of scientific evaluation, a majority of populations in developing nations continue to rely on traditional remedies for disease treatment, while in developed nations, such as the United States, over the counter botanical sales continue to expand (7). How can we best marshal limited commercial and government resources to improve the quality and significance of information derived from botanical clinical trials to better understand the benefits and limitations of plant-derived products? Using turmeric as a test case, given its rich history of ethnobotanical use (8), the impressive number (>400) of modern clinical studies conducted to understand its best use (9, 10), and its current rank as one of the top selling botanicals in the United States (4, 11), we will summarize key points to be considered when designing or evaluating results from botanical clinical trials.

SCIENTIFIC PREMISE SUPPORTING CLINICAL EVALUATION OF A BOTANICAL

Ethnobotanical Evidence

In contrast to pharmaceutical development, which usually begins with a specific biological target and works backwards to find a silver bullet, clinical evaluation of botanicals often has its nidus in ethnobotanical evidence of therapeutic effects of a particular plant, mechanism unknown (12). Indeed, the majority of plant-derived compounds developed into pharmaceuticals were identified following ethnobotanical leads (6). For some plants, centuries of use by specific populations, often supported by written texts, provides a compelling source of information for disease-specific treatments despite an absence of modern studies to confirm effects. Turmeric is one such plant, having been used as an anti-inflammatory in Ayurvedic medicine for thousands of years, up until the present (8). Using modern scientific methods, turmeric clinical trials have offered evidence in support of this traditional anti-inflammatory use (9, 13). While likely not common in antiquity, obesity-associated diseases, like insulin resistance or non-alcoholic fatty liver disease, for which inflammation is a key driver, have been the most studied conditions in turmeric clinical trials, representing almost one third of citations and yielding strong evidence of efficacy (13). Anti-inflammatory effects of turmeric are also strongly supported by studies related to musculoskeletal diseases, the second most commonly studied condition, half of which have focused on osteoarthritis, with the majority of studies reporting clinical improvements (13).

Reliance on ethnobotanical evidence can have limitations, however. For clinically silent disease processes, such as age-related bone loss, ethnobotanical footprints do not exist. In these cases, mechanistic pre-clinical studies in the same or mechanistically similar conditions can sometimes provide direction. For example, in the course of conducting pre-clinical turmeric studies documenting remarkable *in vivo* anti-arthritic efficacy, our laboratory identified direct and indirect inhibitory effects of turmeric on the formation of bone resorbing osteoclasts (14), key mediators of bone loss across all disease states (14–16). Subsequent pre-clinical studies by our laboratory verified anti-resorptive effects of turmeric in a model of menopausal bone loss, a clinically silent disorder, that were subsequently confirmed clinically (16, 17). For other biological processes, such as menopause, symptomatology can be culturally dependent (18), and pharmacogenetic differences between populations can also impact botanical responses (19), a caveat that should be kept in mind when designing–and perhaps most importantly–when interpreting clinical trial results. Similarly, for clinical endpoints more responsive to placebo effects, ethnobotanical evidence may also be less reliable. Menopause again provides a possible example (20), as evidenced by the NIH-funded HALT trial testing black cohosh effects on menopausal vasomotor symptoms where a clinically significant 30% reduction in symptoms was documented in black cohosh—and in placebo—trial arms (21). While placebo responsiveness was not necessarily the reason that this trial did not identify an effect (e.g., criticism of the product used and limited power of the study due to inclusion of multiple arms have also been cited as possible explanations), this caveat must again be considered when designing botanical trials, particularly when estimating effect size to appropriately power the clinical trial.

Pre-clinical Evidence

Even when ethnobotanical evidence of a medicinal effect is strong, botanical clinical trials are vastly improved when mechanistic data are available from appropriately designed pre-clinical studies, particularly those performed *in vivo* (22). In addition to strengthening scientific premise, mechanistic information can also identify biomarkers for inclusion as endpoints, thus improving assessment of pharmacodynamic efficacy and pharmacokinetic sufficiency. For example, pre-clinical data documenting specific, avid binding of turmeric-derived curcumin to amyloid plaques in brains of Alzheimer's Disease (AD) mice has been leveraged, taking advantage of curcumin's natural fluorescence, to image these plaques non-invasively in the retinas of AD mice (23). Subsequently, curcumin has been used successfully to image retinal plaques in aging patients suffering from cognitive decline (24), suggesting a diagnostic tool for a disease where few currently exist. Since curcumin is also reported to reduce amyloid plaques in AD mice (25), an endpoint now accepted, albeit controversially, by the FDA as a measure of AD pharmaceutical clinical efficacy (26), this pre-clinical discovery suggests that curcumin-visualized changes

in retinal plaques could serve as a biomarker for clinical trials assessing the efficacy of drugs–including curcumin–in slowing AD progression.

Similarly, *in vivo* and *in vitro* pre-clinical studies from our own laboratories have demonstrated *in vivo* inhibition of NF-κB activation by curcumin (14), an effect likely attributable to adduct formation between oxidative curcumin metabolites and IκB kinase β (IKKß), the activating kinase upstream of NF-κB (14, 27–29). Furthermore, our laboratories have demonstrated that *in vivo* inhibition of NF-κB activation in a pre-clinical arthritis model is associated with decreased NF-κB-induced cytokines or NF-κB-mediated tissue destructive processes (i.e., formation of bone-resorbing osteoclasts) known to be closely linked with adverse clinical outcome (**Figure 1**) (14–16). Consistent with these pre-clinical findings, in clinical trials assessing curcumin effects on diseases, such as arthritis, where NF-κB activation is known to contribute to pathology, inhibitory effects of curcumin on NF-κB activation and NF-κB downstream pathways have also been reported, with these biomarkers lending credence and mechanistic support to beneficial clinical outcomes (17, 30–33). Indeed, given the central role of NF-κB in mediating inflammation and the significant contribution of inflammation to many disease processes (34), it is perhaps not surprising that benefits of turmeric have been reported in clinical trials across disease types, consistent with its traditional use as an anti-inflammatory (9).

CHOICE OF BOTANICAL PRODUCT FOR STUDY

Botanical Product Composition

One fundamental feature of botanicals not always appreciated by medical researchers is their chemical complexity and variability, even for products derived from the same plant (35). Most plant-derived medicinal compounds are so called secondary metabolites lacking a function within the plant itself, phytoestrogens being one excellent example (36). Secondary metabolites are directed outward (e.g., polyphenolic curcuminoids in turmeric rhizomes), often as a defensive mechanism, protecting the plant from herbivores, insects, or pathogens; thus, their biosynthesis is context-specific and highly regulated but also variable (36, 37). However, even for well-studied plants like turmeric, where curcuminoids have been identified as a primary bioactive principle and are used for extract standardization (4), so called entourage effects are possible (38), with bioactivity resulting from additive and synergistic effects of component parts. In the case of turmeric, ground rhizome—containing polyphenols (3% curcuminoids by weight), terpene-rich essential oils and polysaccharides—is used both in cooking and for preparation of traditional medical formulations (39), whereas the content of most US turmeric dietary supplements is limited to curcuminoids only (98% curcuminoids by weight) (4).

Given reports of enhanced curcuminoid bioavailability when combined with turmeric's essential oils (40), as well as pre-clinical evidence from our laboratories of enhanced or differential *in vivo* bioactivity of polyphenols derived from turmeric (curcuminoids), or from the botanically-related plant ginger (gingerols), when combined with essential oils and/or polysaccharides, it is readily apparent that botanical extracts, even when standardized to an active principle (e.g., curcuminoids or gingerols) may have differential effects (8, 14, 41–46). For example, in pre-clinical arthritis studies testing turmeric rhizome extracts normalized for curcuminoid or essential oil content (**Figure 2**), (14, 41, 42, 46) our laboratories have demonstrated anti-arthritic effects for each type of secondary metabolites, as well as additional effects of polar rhizome constituents. However, when testing clinically-relevant, oral doses of purified curcuminoids vs. essential oils, purified curcuminoids were more potent with greater effects (41, 42, 47). In addition, it

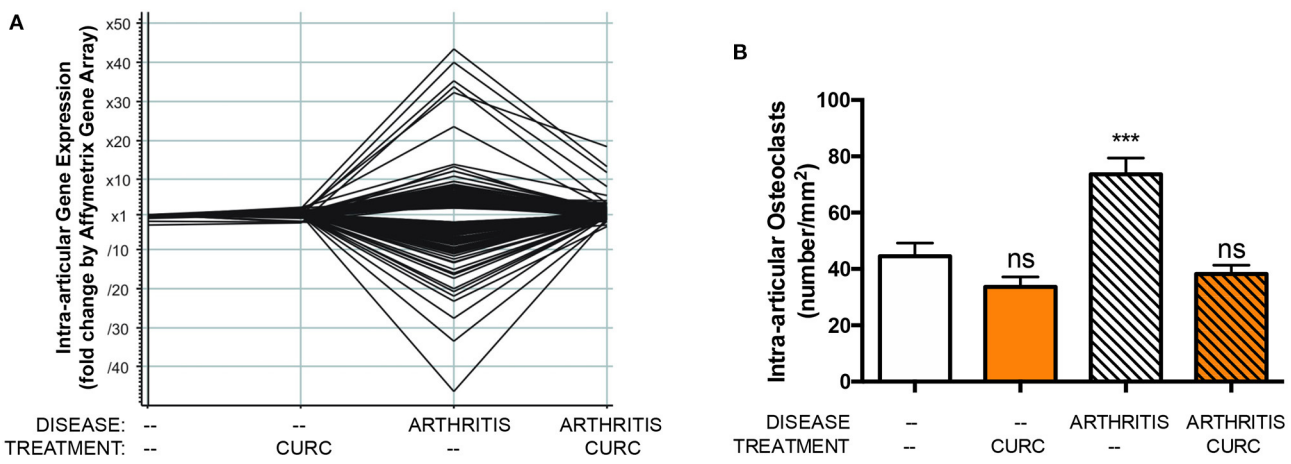

FIGURE 1 | In a pre-clinical arthritis model, consistent with curcuminoid blockade of intraarticular NF-κB activation (not shown), curcuminoid (CURC) treatment significantly altered **(A)** gene expression in arthritic joints, including suppression of over 40 NF-κB regulated genes, and **(B)** inhibited bone-resorbing osteoclast formation in arthritic joints, which is also NF-κB mediated. ns, not significant or ***$p < 0.001$ vs. control (13).

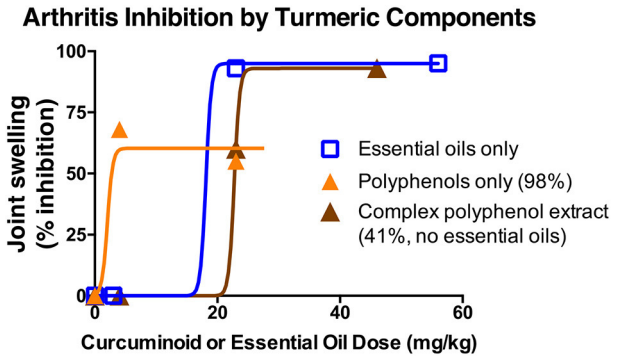

FIGURE 2 | Differential anti-arthritic effects of components extracted from turmeric rhizome in a pre-clinical arthritis model. The anti-arthritic effects of turmeric extracts of different chemical composition were assessed using an ip dosing strategy given reports of altered oral curcuminoid bioavailability when combined with essential oils. Each of turmeric's secondary metabolites [curcuminoids (orange triangles) and essential oils (blue squares)] had significant anti-arthritic effects when administered separately. Interestingly, differential effects were noted for a chemically complex curcuminoid extract (brown triangles) devoid of essential oils but containing polar compounds, such as polysaccharides. Anti-arthritic curcuminoid efficacy was confirmed with oral dosing [50% inhibition; human equivalent dose (HED) of 1 g/d], while protection from oral essential oils was much reduced (20%; HED of 5 g/d) (13).

was notable that *in vivo* anti-inflammatory effects of these same extracts differed for joint vs. hepatic inflammation in the same animals, and also did not necessarily correlate with *in vitro* screening assays (14, 42). Thus, while high throughput screening methods to identify target-specific bioactivity of complex extracts are being developed (48), the utility of pre-clinical data evaluating *in vivo* efficacy of normalized botanical constituents administered alone or in combination can be particularly helpful in choosing a product for clinical study. In addition, entourage effects may also alter active principle bioactivity. The availability of head-to-head pharmacokinetic studies for botanical products normalized to an active principle can serve as a gold standard in this regard. For example, turmeric essential oils, while perhaps of limited anti-inflammatory efficacy in clinically relevant doses, have been variably reported to enhance curcuminoid bioavailability in human pharmacokinetic studies (40, 47, 49). This raises interesting questions not only about botanical product choice for clinical testing, but also regarding assessment of ethnobotanical evidence. If true, the western reductionist approach of using purified curcuminoids rather than complex extracts may not only require higher dosing, but also suggest a corollary question; can intake of lower curcuminoid doses via dietary or traditional medicinal use of essential oil- and curcuminoid-containing turmeric preparations yield biological effects? While this question remains unanswered, it is intriguing that a recent pharmacokinetic study by Mahale et al. (39), examining a turmeric rhizome dose in food analogous to estimated daily dietary intake in India, documented serum curcuminoid levels similar to those reported for therapeutic doses of purified curcuminoid dietary supplement formulated by other means to enhance bioavailability (49–51).

Standardization of the entirety of a plant extract can be difficult, however, because the exact chemical composition can also be dependent not only on the plant and plant part used, but also on growing conditions and method of preparation, including possible fractionation and/or solvents used for extraction, which can differ between products and manufacturers (4, 44, 46). For example, residual levels of 7 different carcinogenic class 1 or toxic class 2 solvents, while below USP limits, were documented by our laboratories in the majority of turmeric dietary supplements tested, suggesting differential modes of preparation, as well as the potential for safety concerns (4). Even when bioactive content is well documented, other aspects of product formulation can confound comparisons of bioactivity in clinical trials and must be considered in clinical trial design. For example, our laboratories have documented that more than half of commercial turmeric dietary supplement sold in the US are enhanced bioavailability formulations and/or include additional botanicals (4). Country-specific regulatory environments can add another layer of complexity to product standardization for botanical clinical trials (3). Reports of botanical product mislabeling in terms of both plant species and chemical content, deliberate adulteration with drugs, or contamination are not uncommon (52). Even in well studied proprietary botanical products, formulations can change over time, possibly altering bioavailability and bioactivity of the standardized active principle. For all of these reasons, besides careful consideration and justification of botanical composition to be tested in a clinical trial, it is absolutely critical that the chemical composition of the specific product and lot(s) used are documented *independently* by the clinical trial researchers *and* reported as an integral part of the clinical trial results—even when commercial products are used—so that research can be replicated and reasons for possible differences between studies can be more rigorously assessed (53). For assay of some active principles, such as curcuminoids (54), standardized methods have been described. In all cases, methodology used to determine product content should be included when reporting clinical trial results.

Botanical Product Dosing

As exemplified by the turmeric clinical trial literature (9), even for diseases where botanical clinical efficacy is reported across a majority of clinical trials (e.g., curcuminoid treatment of diseases attributable to obesity-associated inflammation or joint-inflammation) (13), definitive conclusions as to efficacy (e.g., from metanalyses) or informed clinical use by consumers are often limited since botanical clinical trials often test only a single dose, with the added complication of disparate products being tested across trials for a given clinical condition. In the case of turmeric dietary supplements, for example, because many are formulated as enhanced bioavailability products (4), curcuminoid dosing is difficult to compare across trials even if product curcuminoid content is reported (9). Thus, neither consumers nor biomedical researchers can easily extrapolate information from a given study to support the rational clinical use or clinical evaluation of a different product, unless detailed information on product composition, dosing and pharmacokinetics are all included in clinical trial design and reported and discussed when publishing results. For example,

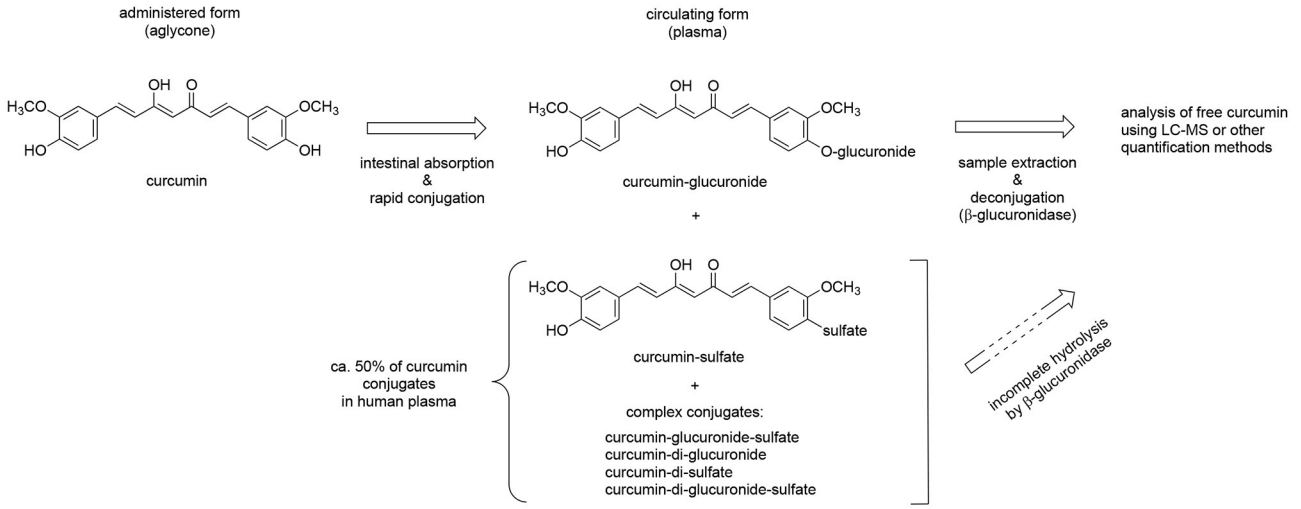

FIGURE 3 | Metabolic conjugation of curcumin and deconjugation for quantitative analysis. Curcumin is consumed in its free (aglycone) form and undergoes rapid phase II conjugation following intestinal absorption. The glucuronide conjugate accounts for about half of the circulating conjugates while sulfate and other, more complex conjugates account for the rest. Free curcumin is low or undetectable in plasma samples. For quantification plasma samples are often deconjugated using β-glucuronidase but this achieves only incomplete hydrolysis of sulfate and complex conjugates; more complete hydrolysis of all conjugates can be achieved using sulfatase. Direct analysis of conjugates is preferred but hampered by the large number of conjugates, lack of standards, and technical challenges. Reduction of the aliphatic double bonds, a significant route of metabolism *in vivo*, and other metabolic events are not illustrated.

while osteoarthritis (OA) is one of the most commonly studied diseases in turmeric clinical trials ($n = 35$ unique citations), yielding generally positive effects in studies that are primarily double-blinded, placebo-controlled, and randomized (77%), the OA clinical trials evaluated approximately 20 different, primarily proprietary, curcuminoid-enriched products without any head to head comparisons or inclusion of pharmacokinetic endpoints; rarely included multiple dosing arms; and frequently omitted information regarding curcuminoid content of the study drug and/or rationalization of the single dosing choice (i.e., anticipated bioequivalency of proprietary enhanced bioavailability products, based on prior pharmacokinetic analyses) (13). Thus, both clinical translation and validation of trial results can be improved when dosing information is clearly stated, well justified, and preferably supported by pharmacokinetic data. Both pre-clinical (scaled for human equivalent dosing [HED]) or clinical pharmacokinetic and pharmacodynamic data can be used to optimize clinical trial dosing. For example, a least effective dose of 4 mg/kg daily curcuminoids blocked joint swelling in a rat arthritis model in our laboratory, yielding no greater effect at a higher dose, with a similar inhibitory effect documented with an oral HED of 1 g/d (**Figure 2**). As even oncologic drugs are sometimes insufficiently studied to determine least effective clinical dose (55), these types of pre-clinical data can help direct botanical clinical trial design.

Pharmacokinetic Analyses

Inclusion of pharmacokinetic endpoints in clinical trial design can help to overcome limitations attributable to the testing of disparate products across trials, facilitating comparisons. This is most particularly true for botanicals, such as curcuminoids, where use of enhanced bioavailability products is common and careful head to head pharmacokinetic comparisons of formulations containing identical amounts of the bioactive are required to determine actual bioequivalency (50). Even when bioequivalency or bioavailability has been reported previously for a product—and most definitely in cases where it has not—as a minimum standard, rudimentary assessments of bioavailability (e.g., assessment of Cmax, the maximum plasma concentration) should be included in clinical trial design. For example, while different approaches have been used to enhance curcuminoid bioavailability, targeting absorption or secondary metabolism (40, 49–51, 56), a rare head to head comparison of different proprietary enhanced bioavailability curcuminoid products in healthy adults did not support prior published pharmacokinetic reports in all cases (49, 56). This demonstrates the importance of documenting Cmax or other pharmacokinetic parameters in clinical trials, particularly when testing botanical products in disease-specific populations.

The design of pharmacokinetic endpoints in botanical clinical trials also can present unique challenges since the *in vivo* metabolic fate of plant-derived compounds can complicate analyses. For example, we and others have demonstrated that curcumin and many other plant-derived polyphenols primarily circulate as glucuronide or sulfate conjugates (57–60), with ingested aglycones being near undetectable (**Figure 3**). Indeed, in the case of curcuminoids, these conjugates can persist in the circulation for over 24 h (e.g., 10% of administered curcuminoids, independent of dose) (61), due in part to enterohepatic recirculation (**Figure 4A**) (60–65). For this reason, and because glucuronide conjugates are difficult to analyze (66), serum samples for curcuminoids and other botanicals

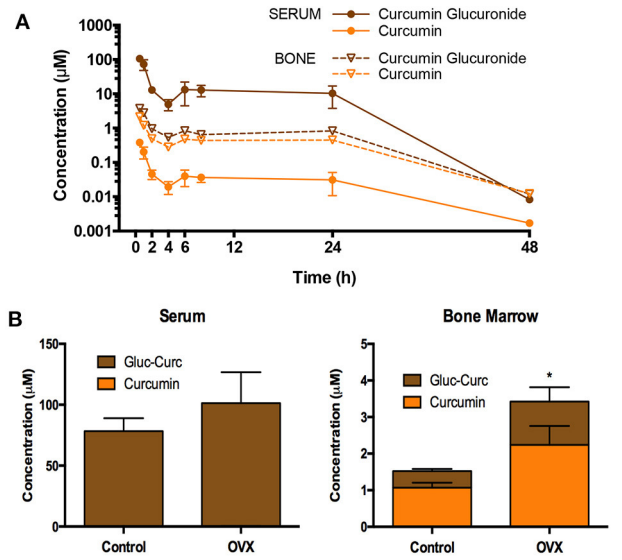

FIGURE 4 | Pharmacokinetics of oral curcuminoids in mice. **(A)** Circulating curcumin-glucuronide (CG) levels predominate and are sustained for up to 24 h in mice following a single oral curcumin dose (HED 2.5 g), with sustained, albeit lower, levels also documented in bone, where ß-glucuronidase-dependent GC hydrolysis to form aglycone curcumin occurs, resulting in higher aglycone concentrations than those perfusing bone. **(B)** Following curcumin ingestion in mature ovariectomized (OVX) mice modeling menopausal bone loss (vs. controls), the capacity of bone to deconjugate curcumin glucuronide distributing to this site persists and is higher in OVX mice. Interestingly, curcumin concentrations in mouse bones are also highest in trabecular bone compartments where menopausal bone loss is most pronounced. Figures are reproduced with permission from John Wiley and Sons (62).

are often pre-treated with deconjugating enzymes to liberate the aglycone prior to pharmacokinetic analyses (51, 58). This practice, however, is not always well documented or characterized, nor are the clinical implications of low *in vivo*, bioactive aglycones always considered (60, 67). For example, data from our laboratories indicate that the common practice of glucuronidase hydrolysis can underestimate curcumin exposure due to incomplete hydrolysis of significant quantities of sulfated or higher order conjugates and suggest the use of sulfatase instead of glucuronidase since the former enzyme achieves a more complete hydrolysis of conjugates (58). Some have questioned the clinical relevance of such measures as conjugates typically lack bioactivity (68). Others postulate that the prolonged circulation of these conjugates provides a ready source of material (e.g., polyphenols) that can be deconjugated locally, and most particularly at sites of inflammation due to the presence of glucuronidase-rich hematopoietic cells, to form the bioactive aglycone (60, 69). Evidence for this later postulate has come from recent studies in our own laboratories. Following oral curcumin administration to mice, bone has the capacity to deconjugate the majority of circulating curcumin glucuronides distributing this site (**Figure 4B**), which has high levels of glucuronidase due to resident hematopoietic marrow cells (60, 62, 67). This deconjugation process is glucuronidase-dependent and can yield local aglycone curcumin concentrations sufficient to inhibit NF-κB-mediated formation of bone-resorbing osteoclasts (60, 62, 67).

In vivo Botanical Metabolism

A further complication in assessing botanical exposure is the possibility that botanicals, besides forming phase II conjugates, may undergo further *in vivo* metabolism to create additional bioactive moieties (28, 70, 71). This has been extensively described for flavonoids (72–74), and curcumin is also susceptible to reductive as well as enzymatic and non-enzymatic oxidative metabolism (**Figure 5**) (66, 75). Again, in the case of curcumin, our laboratories have demonstrated an important role for oxidative metabolites of curcumin (70, 76) in altering protein function via the formation of specific adducts (27, 77–80). While evidence for protein adduction of curcumin *in vivo* is yet lacking, in cell-based assays multiple proteins appear to be targeted by reactive oxidative metabolites of curcumin (28, 67, 77–83), consistent with curcumin's reported pleiotropic effects. Protein adduction appears specific and reproducible, likely dictated by the susceptibility of specific proteins (e.g., regulatory site cysteine thiols) to reaction with the existing enone electrophile of curcumin or with electrophilic moieties in metabolites formed upon oxidative transformation (71, 83, 84). For example, curcumin blockade of NF-κB, a transcription factor that is a master regulator of inflammation, appears attributable to adduct formation with the Cys179 residue of IKKβ, the upstream kinase controlling NF-κB activation (85). This tendency to form covalent protein adducts causes some plant-derived compounds, such as curcumin or flavonoids (74, 76), to be "frequent hitters" in screening assays, leading some to suggest that these compounds should be avoided in drug discovery or, indeed, biomedical research (86, 87). However, covalent modification is a pharmacologic strategy employed by many FDA-approved drugs (88, 89), most notably kinase inhibitors (90), or widely used drugs like proton-pump inhibitors (91), anti-thrombotics targeting the platelet P2Y12 receptor like clopidogrel (92), and the cyclooxygenase inhibitor aspirin (93). Thus, it can be argued that the clinical evaluation of botanicals, such as curcuminoids, that specifically, albeit not exclusively, target proteins physiologically relevant to their ethnobotanical use via this mechanism (e.g., blockade of NF-κB via covalent kinase inhibition), can be justified, particularly when the scientific premise is further supported by pre-clinical evidence of *in vivo* efficacy without "off target" toxicities (14, 16).

Given the major effect that *in vivo* metabolism of botanicals can have on bioavailability and bioactivity, this not only complicates the design and interpretation of relevant pharmacokinetic assays, but also raises questions about possible pharmacogenomic differences between subjects in botanical clinical trials that could alter clinical outcomes. For example, in the case of polyphenols, such as curcumin, that rapidly undergo phase II metabolism, genetic variations in endogenous conjugation (e.g., defective conjugation due to UGT1A1 mutations [Gilbert Syndrome], affecting almost 10% of adults) and/or deconjugation capacity may be important determinants of botanical bioavailability, and thus bioactivity,

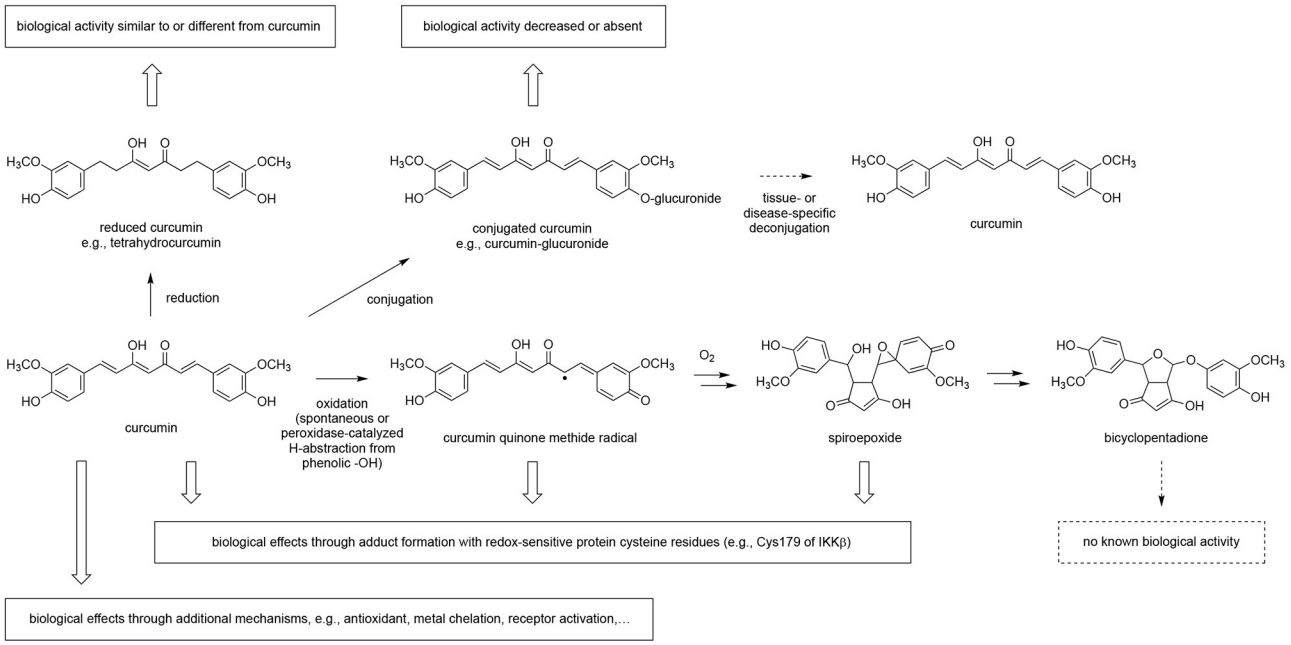

FIGURE 5 | Metabolism of curcumin and the effect on biological activity. Curcumin is reduced and conjugated *in vivo* as shown by the detection of the corresponding metabolites in plasma samples. Deconjugation of circulating and inactive curcumin-glucuronide by β-glucuronidase may contribute to a tissue- or disease-specific effect. Oxidation of curcumin is prominent in buffer and in cultured cells but awaits to be proven *in vivo*. Adduction of curcumin to protein has been described, and at least in part depends on oxidation of curcumin to a quinone methide or other electrophilic oxidation product, i.e., spiroepoxide, that target redox-sensitive cysteine residues and soluble thiols like glutathione. The quinone methide radical and spiroepoxide are unstable intermediates in the oxidation of curcumin to the stable end-product, bicyclopentadione. Curcumin also exerts biological effects through mechanisms not involving metabolic transformation.

which should be considered in clinical trial design (60, 62, 94–96). Interestingly, separate reports suggest that gender may also be an important determinant of clinical curcumin responses, independent of bioavailability, and that gender may also influence bioavailability, although differences in body weight may have accounted for higher levels documented in women. This finding remains clinically relevant since curcuminoids in clinical trials–and clinical use–are rarely dosed based on weight (50, 97, 98). In addition, for certain botanicals, most notably phytoestrogens (99), but also possibly curcuminoids (100), metabolism by the gut microbiome can also affect bioavailability.

OTHER CRITICAL ELEMENTS AND POTENTIAL BARRIERS TO HIGH QUALITY BOTANICAL CLINICAL TRIAL DESIGN

Design elements driving the quality of any clinical trial are obviously also applicable here, including appropriately powered, controlled, randomized and double-blinded studies with pre-specified analyses (101). However, often these elements are overlooked in botanical clinical trials, or, indeed difficult to achieve, whether due to funding limitations, or other issues specific to a botanical. For example, it can be difficult to blind studies, as is the case with curcumin, due to its unique vibrant orange hue. Placebo composition is therefore an important element of botanical study design (21). In our own experience, optimization of placebo composition, particularly when the botanical product is being obtained from a nutraceutical company with fixed production lines, can be a time-consuming issue that should be considered in planning timelines. Another element to be considered in the US, even when testing an over-the-counter product, is the need to prepare, file and undergo an FDA review of an Investigational New Drug application (IND), following botanical specific guidelines (102), if disease outcomes (i.e., disease treatment) are an endpoint, as well as consideration of whether clinical trial goals could be met with an alternative design (103).

Funding is more limited for botanicals than for pharmaceuticals, given their different marketing and approval pathways. This often places limits on study size and duration that impact clinical and statistical significance. For example, the two published trials assessing curcumin in AD which were only of 6-month duration and involved fewer than 30 treated subjects, perhaps not surprisingly, yielded no significant effects, an outcome attributed in part to low product bioavailability (104, 105). In contrast, larger and longer (e.g., 12-month) studies examining effects of enhanced bioavailability curcumin products on cognitive decline in aged adults have all reported benefits (106–111). In the US, industry-funded clinical trials are disincentivized in general, since nutraceuticals can be sold without evidence of efficacy and cannot be marketed for disease treatment (3). Other market driven forces can impact industry-supported study design in ways that are sometimes not helpful to consumers or researchers. For example anti-arthritic benefits have been reported in separate trials for two turmeric products manufactured by the same company, a unique product

combining curcuminoids with turmeric polysaccharides and a curcuminoid-only product analogous to most commercial turmeric supplements, without an assessment of bioactivity attributable—or not—to polysaccharide content (112, 113).

PRIORITIZING PUBLIC HEALTH AND SAFETY

Due to the large number of traditional medicinal plants, the disparate composition of commercial products for a given plant, and the paucity of botanical clinical trial funding, the task of documenting medicinal benefits of every potentially valuable botanical is daunting and likely not achievable. How can available resources best be used? As previously discussed, strong ethnobotanical and pre-clinical evidence of botanical efficacy are important pillars supporting clinical trial design. The study of lesser-known plants, particularly, for diseases lacking effective treatments, can also yield clear benefits. However, in these cases, considering all the intricacies associated with the study of plant-based medicines as described here, strong pre-clinical pharmacokinetic and pharmacodynamic evidence should first be obtained to guide the appropriate design of subsequent botanical clinical trials.

Prevalence of use is one additional factor to consider; public health benefits can be greater in these cases, not only with respect to efficacy, but also safety. Indeed, because some populations tend to use botanicals for disease treatment even in the absence of cultural traditions or evidence of efficacy, examination of safety becomes a key concern. For example, in our recent observational studies, current turmeric use was reported by one third of individuals with rheumatoid arthritis or breast cancer in the US despite a paucity of efficacy or safety data (11, 114, 115). Botanical safety information is thus important for public health. Consumers tend to falsely equate natural with safe, and, in the US, may also incorrectly assume that the federal government requires commercial botanical products to be vetted for efficacy and safety (116, 117). Examination of possible pharmacogenetic risk factors related to botanical metabolism and/or adverse drug-botanical interactions can therefore be important elements of botanical clinical trial design, particularly for government funded studies (118, 119). This is particularly true when studying populations at higher risk of adverse effects due to underlying chronic disease and/or concurrent use of pharmaceuticals, as, for example, has been reported for concurrent use of certain dietary supplements with breast cancer chemotherapy (11, 120, 121).

One additional safety related concern, unique to botanicals (vs. pharmaceuticals) and attributable to their variable content and lack of regulatory oversight, is the risk of adverse effects due to possible contaminants (2, 4). For example, isolated case reports from our laboratories and others of turmeric- or black cohosh-associated hepatitis highlight potential risks, as well as difficulties in determining the etiology, of adverse botanical effects outside the context of clinical trials (2, 122–124). Thus, consideration of all available product- or plant-specific safety data must guide product selection in order to optimize botanical clinical trial design (4, 122). At the same time, well-designed clinical trials are often the only source of high-quality safety information for a given botanical product. For example, a review of FDA MedWatch reports for turmeric obtained by our laboratories under a Freedom of Information Request in 2017 yielded 107 reports, with turmeric products being equally listed as the possible suspected product (being used in combination with other supplements in half of these cases) vs. concurrent medication, making identification of turmeric product-specific safety issues difficult. Lastly, for publicly funded studies, selection of a product representative of those readily available to consumers may also be a consideration (4).

CONCLUSION

Plants are a rich source of potential therapeutics, whether developed as drugs, or used as complex botanical products. However, the chemical complexity and differential regulation of botanicals provide unique challenges when designing high quality botanical clinical trials, with perhaps the largest public health and medical benefits to be gained by prioritizing the study of botanicals with a high prevalence of use and/or likelihood of ameliorating diseases lacking effective treatments. Turmeric is one such example, being a top selling botanical already in widespread use with demonstrated promise in the treatment of inflammatory conditions associated with obesity, a major health problem worldwide. However, for many published turmeric clinical trials, key clinical study design elements unique to botanicals, as described here, have been lacking. Thus, while turmeric may appear to be overstudied as compared to other botanicals, because of its widespread prevalence of use and the strength of existing ethnobotanical and scientific evidence of medicinal effects, it can perhaps be best described as *ineffectively* studied from the viewpoint of consumers and healthcare providers. Improved botanical clinical trial designs, making the best use of limited resources, are needed to realize the full potential of turmeric and other medicinal botanicals, complementing the experimental evidence of our ancestors with the application of current best clinical research practices.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Pregnancy and Lactation in Sprague-Dawley Rats Result in Permanent Reductions of Tibia Trabecular Bone Mineral Density and Structure but Consumption of Red Rooibos Herbal Tea Supports the Partial Recovery

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During pregnancy and lactation, maternal bone mineral density (BMD) is reduced as calcium is mobilized to support offspring bone development. In humans, BMD returns to pre-pregnancy levels shortly after delivery, shifting from a high rate of bone resorption during pregnancy and lactation, into a rapid phase of bone formation post-lactation. This rapid change in bone turnover may provide an opportunity to stimulate a greater gain in BMD and stronger trabecular and cortical structure than present pre-pregnancy. Providing polyphenols present in red rooibos herbal tea may promote such an effect. *In vitro*, red rooibos polyphenols stimulate osteoblast activity, reduce osteoclastic resorption, and increase mineral production. The study objective was to determine if consuming red rooibos from pre-pregnancy through to 4 months post-lactation resulted in a higher BMD and improved trabecular and cortical bone structure in a commonly used rat model. Female Sprague-Dawley rats ($n = 42$) were randomized to one of the following groups: PREG TEA (pregnant, received supplemental level of red rooibos in water: ∼2.6 g /kg body weight/day in water), PREG WATER (pregnant, received water), or NONPREG CON (age-matched, non-pregnant control, received water) from 2 weeks pre-pregnancy (age 8 weeks) through to 4 months post-lactation. Rats were fed AIN-93G (pre-pregnancy through to the end of lactation) and AIN-93M (post-lactation onwards). BMD and trabecular structure (bone volume fraction, trabecular number, trabecular separation) were improved ($p < 0.05$) by 1- or 2-months post-lactation when comparing PREG TEA to PREG CON, though neither group recovered to the level of NONPREG CON. Cortical outcomes (cortical area fraction, cortical thickness, tissue mineral density) for PREG TEA and PREG CON were reduced ($p < 0.05$) following lactation but returned to the level of NONPREG CON by 2-months post-lactation, with the exception of cortical thickness. The lack of recovery of BMD and key outcomes of trabecular bone structure

was unexpected. While consumption of red rooibos did not result in stronger bone post-lactation, red rooibos did support the partial recovery of trabecular BMD and bone structure following pregnancy and lactation. The findings also provide insight into the timing and dose of polyphenols to study in future interventions.

Keywords: bone, red rooibos, polyphenols, pregnancy, lactation

INTRODUCTION

In humans, there is a decrease in BMD and trabecular and cortical structure during pregnancy and lactation due to an increased demand in mobilized calcium for offspring bone development (1). Reductions in BMD during human pregnancy and lactation, measured using dual energy X-ray absorptiometry (DXA), have been reported to be 5–10% (1, 2) and it is believed that women typically return to their pre-pregnancy BMD after delivery as number of pregnancies and duration of breast-feeding is not considered a risk for developing osteoporosis later in life (3–5). More specifically, to meet the elevated calcium demand by the fetus, intestinal calcium absorption is increased beginning in the first trimester. In humans, intestinal calcium absorption returns to pre-pregnancy levels during lactation (but stays elevated in rodents) (2) while there is a concurrent increase in skeletal resorption to provide calcium for offspring bone growth for both humans and rodents (6). This results in an uncoupling of bone turnover with elevated levels of bone resorption compared to formation leading to reductions in BMD and trabecular and cortical structure observed in both humans and rodents. Despite the reduction in BMD following lactation, it is transient as an uncoupling of bone turnover persists but it is reversed with greater formation than resorption occurring (7–9). These high rates of bone turnover observed during pregnancy, lactation, and recovery may provide a "window of opportunity" to stimulate a greater gain in BMD and stronger trabecular and cortical structure than was present pre-pregnancy.

A key contributing factor to the promotion of bone health is diet. Several nutrients, including calcium and vitamin D, as well as various foods and food components have been studied for their bone promoting or supporting effects (10–13). Tea and its polyphenols–including some herbal teas such as red rooibos (RR)-may promote bone health and mineral production (14–17). Several epidemiological studies in different countries including Australia, Britain, and Taiwan have also identified positive associations between black or green tea consumption and greater BMD later in life (18–20). To date, RR tea has not been studied *in vivo* for potential bone promoting or supporting effects. Also, *in vitro*, a wide variety of teas derived from *Camellia sinensis* and herbal teas from other plants such as RR have been shown to increase osteoblast activity and proliferation (16, 17); while also having the capacity to decrease osteoclast activity and proliferation (21, 22)–possibly by acting as antioxidants and thereby reducing reactive oxygen species (ROS). ROS have been shown to suppress mineralization and increase resorption *in vitro* (23–25). Moreover, metabolism is elevated during pregnancy and lactation, along with ROS, due to the requirements for developing fetal tissues (26). Reduction of ROS by tea and its respective polyphenols may counter these effects leading to greater mineralization by osteoblasts and a reduction of osteoclast resorption.

Commonly consumed teas, such as green and black teas, are known to contain caffeine and thus would not be recommended for pregnant women while red rooibos (RR) tea does not contain caffeine. RR herbal tea originates from the *Aspalathus linearis* plant. It is fully oxidized and has a unique profile of polyphenols-including aspalathin, aspalalinin, and nothofagin-that are not present in other teas. Previously our lab has shown RR to have the capacity *in vitro* to increase mineralization by osteoblasts (Saos-2 cells) in a dose-dependent manner (16) using levels that can be achieved by consuming several cups of RR tea a day through to supplementation. Improved cell activity was also observed. With respect to bone resorption, RR has been shown to reduce osteoclast formation and activity *in vitro* using RAW264.7 cells, and displayed oxidant scavenging activity without any cytotoxic effects (27). Although it is likely that these polyphenols will be altered upon absorption and digestion it is possible that their metabolized forms may also have positive effects on bone. Taken together, an increase in mineralization and concurrent reduction in resorption would lead to an increase in bone formation which may support the acquisition of greater BMD and structure following pregnancy and lactation.

The objective of this study was to determine if continuous consumption of RR tea from pre-pregnancy through to 4 months post-lactation resulted in higher BMD and improved structure of trabecular and cortical bone in the tibia compared to a water control. It was hypothesized that consumption of RR tea during pregnancy, lactation, and recovery would result in greater BMD and improved structure of the tibia compared to consumption of only water.

MATERIALS AND METHODS

Animals and Diets

Forty-two female and fourteen male Sprague-Dawley rats (5 weeks of age) were purchased from Charles Rivers Laboratories (St. Constant, QC, Canada). Rats were singly housed under a controlled environment (20°C, and 12 h light and dark cycles) and supplied with water and diet (AIN-93G, Envigo; Indianapolis, IN, USA) *ad libitum*. Each rat had access to physical enrichment in their cage including crinkle nest and a red rat retreat to provide shelter and lower stress. Body weight was measured weekly while diet and water intake was measured bi-weekly using an electronic scale.

Experimental Design

Several methodological aspects were considered in planning and conducting the study to help ensure reproducibility (**Supplementary Material 1**). Following 1 week of acclimatization to handling and the environment of the animal facility, female rats were randomly assigned to one of three groups ($n = 14$/group): a pregnancy and lactation group receiving RR tea prepared in water (PREG TEA), a pregnancy and lactation group receiving only water (PREG CON) or an age matched control group that was not mated (NONPREG CON). An *a priori* sample size analysis was conducted using findings from previous literature that assessed the effects of green tea polyphenols on bone in response to LPS induced chronic bone inflammation and bone loss with a primary outcome of tibial BV/TV used (28)-a sample size of 6 was calculated to be necessary for the study (**Supplementary Material 2**). An additional 8 rats per group were included if pregnancy was not achieved in all rats, a healthy litter with a minimum of 10 pups was not delivered, or if there were complications with longitudinal scans (i.e., death due to anesthetic). As well, pregnancy does not exert as much systematic stress on the skeletal system as LPS induced inflammation so it was anticipated that differences between intervention groups and control could be attenuated compared to this previously published trial. Rats in the PREG CON and NONPREG CON groups had *ad libitum* access to AIN-93G diet and water, while those in the PREG TEA group received AIN-93G diet and RR tea *ad libitum* (concentration of approximately 2.6 g of RR/kg of body weight per day). This concentration is comparable to consuming approximately 12 cups of RR tea daily and was calculated through the conversion of a human equivalent dose (HED) to an animal equivalent dose (AED) (29) (**Supplementary Material 3**). This concentration was chosen based on previous research demonstrating greater concentrations of RR tea eliciting greater levels of mineralization *in vitro* (16). For the following 2 weeks, rats were further acclimatized to the facility and their respective group while water intake was measured to ensure rats in PREG TEA did not have an aversion to the taste. After a 3 week period of acclimatization to diet and environment, half of the rats from each of the PREG TEA and PREG CON groups were mated one to one with males of the same age. The remaining rats from each of these groups were mated 2 weeks later with the same group of males to ensure any potential paternal differences were equally distributed between groups (**Supplementary Material 4**). The staggered mating also helped with time management, allowing for the longitudinal *in vivo* scans throughout the study. The time involved with *in vivo* scanning would have made it challenging to study rats as one cohort. Of the initial 14 rats randomized to the PREG CON group, one rat did not become pregnant after 2 estrous cycles and was removed from the study and one rat did not recover from anesthesia following their post-lactation scan (missing values were replaced by mean imputation). This resulted in the following sample sizes (n): PREG TEA = 14, PREG CON = 13, and NONPREG CON = 14. At postnatal day 3 (PND 3), litters were culled to 5 male and 5 females to normalize milk production among dams. Pups were selected to be culled based on their weight in comparison with the average weight of their litter–with pups that had the greatest deviation from the group mean being culled. Litter weight and average pup weight was measured at PND 3 (prior to culling), and post-culling at PND 7, 14, and 21. Pups were weaned at PND 21. From the end of lactation through 4 months post-lactation, dams were fed AIN-93M (Envigo; Indianapolis, IN, USA). Due to the lack of a litter in rats from NONPREG CON and the need to protect the RR tea from light by wrapping drinking bottles in aluminum foil, experimenters were not blinded to groups during the *in vivo* portion of the trial. The protocol (#18-03-02) was approved by the Animal Care Committee at Brock University.

Preparation of Red Rooibos Herbal Tea and Measurement of Total Polyphenol Content

Loose leaf RR tea was prepared twice weekly (every 3 or 4 days) following manufacturer's recommended steeping time and temperature. RR tea was weighed to the appropriate amount and transferred to tea bags (∼5g of tea/bag) to mimic what would normally be consumed in humans. Tea bags were then placed in glass beakers and steeped for 5 min in water that was 96°C at the onset of steeping. Following 5 min of steeping, tea bags were removed and the resulting RR was cooled to room temperature. Once cooled, all beakers of RR were combined to ensure a homogenous mixture. To ensure rats received approximately 2.6 g of RR/kg of body weight, the concentration of RR tea was constantly adjusted depending on both average body weight and water intake from the previous week's measurements. This allowed a consistent intake of RR tea relative to body weight throughout the study despite any changes in water intake while also allowing rats *ad libitum* access. An example scenario and calculation is shown in **Supplementary Material 5**. Total polyphenol content (TPC) of RR tea was measured throughout the study using Folin-Ciocalteau's reagent and gallic acid as a standard according to ISO 14502-1 as previously reported (30).

In vivo μCT Scanning of Tibia

The right tibia of rats were scanned at 6 time points using high resolution *in vivo* micro computed tomography (μCT) (SkyScan 1176, Bruker microCT, Belgium): prior to mating (5–7 days before the initiation of mating), immediately following the end of lactation (within 48 h), and at 1, 2, 3, and 4 months post-lactation (denoted as Pre, PL, 1PL, 2PL, 3PL, and 4PL, respectively, or at an identical age for NONPREG CON group). Prior to scanning, rats were anesthetized with isoflurane. Rats were placed in an induction chamber with a steady flow rate of approximately 2% isoflurane and anesthesia was confirmed by the absence of a response to a toe pinch. Rats were then transferred and placed in supine position on the scanning bed and isoflurane was given by nose cone to ensure adequate anesthesia to help prevent movement during the scan (31). All scans were performed with parameters that have previously been shown by our lab to be safe for longitudinal measurements–both in terms of the recovery of the rat from anesthetic between scans and without causing radiation damage to bone structure (18 μm voxel size, 1 mm aluminum filter, 700 ms exposure time, 60 kV of voltage, 200 uA of amperage, a rotation step of 0.5° over a 360° scanning frame) (31, 32). Monthly, *in vivo*

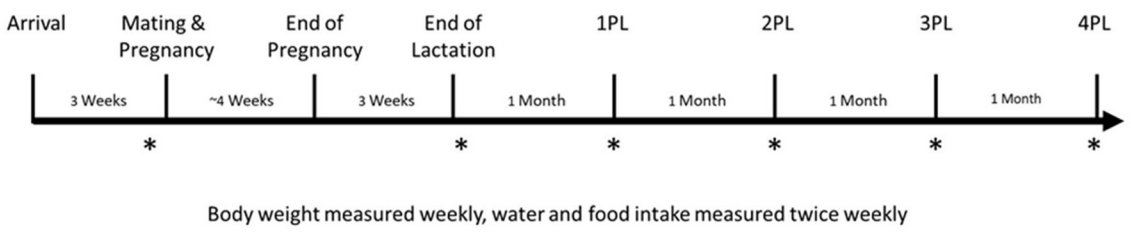

FIGURE 1 | Study design. Female Sprague-Dawley rats arrived at 5 weeks of age and were randomized to either a PREG TEA, PREG CON, or NONPREG CON group ($n = 14$) following 1 week of acclimatization. Rats were then further acclimatized for another 2 weeks to their respective groups. Following acclimatization, females were mated for a duration of 2 estrous cycles (5 days per cycle). *In vivo* scans for measurement of bone structure and quantity of the right proximal tibia using μCT are denoted by an asterisk (*) and were completed at pre-pregnancy (Pre), immediately after lactation (PL), and 1, 2, 3, and 4 months post-lactation (1PL, 2PL, 3PL, 4 PL). Rats from NONPREG CON were scanned at the same time points to provide an age-matched control. Necropsy and organ collection occurred approximately 1 week following the 4PL scan.

scans of rat tibias using the same machine have previously shown no detrimental effects of repeated irradiation to BMD or bone structure (32). At each time-point, scanning order was determined by alternating intervention groups until all scans were complete to minimize the potential for any variability in the X-ray source when scanning. Following the 4PL scan (or age matched equivalent) rats were anesthetized with isoflurane in an induction chamber and euthanized by CO_2 asphyxiation. Tibias were collected, weighed by digital scale, and stored. Organ weights were measured as a preliminary sign of the possibility of any toxicological effects. Study design is shown in **Figure 1**.

Image Reconstruction and Analysis

Following acquisition of all scans, images were reconstructed using a Gaussian filter under the same parameters to ensure accurate comparisons. The region of interest (ROI) for analysis of trabecular bone began 150 slices (2.64 mm) distal from the point where the growth plate and the metaphysis of the tibia met and spanned 75 slices (3.96 mm) distally. The ROI for cortical bone began 400 slices (7.04 mm) distal from the point where the growth plate and the metaphysis of the tibia met and spanned 100 slices (8.80 mm) distally. Within each ROI, the distinction between trabecular and cortical bone was performed manually by the same individual (MDM) and saved as a distinct ROI for analysis. Images were first binarized using global thresholding (trabecular bone: 42–255, cortical bone: 63–255). Following binarization, images underwent several morphological operations to ensure that only bone tissue was being analyzed. Trabecular bone was analyzed for the following structure outcomes: bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N), and bone mineral density (BMD). Cortical bone was analyzed for the following structure outcomes: cortical area fraction (Ct.Ar/Tt.Ar) periosteal perimeter (Ps.Pm), cortical thickness (Ct.Th), endocortical perimeter (Ec.Pm), marrow area (Ma.Ar), and tissue mineral density (TMD). Specific task lists for analysis of trabecular and cortical bone are shown in **Supplementary Materials 6**, **7**, respectively.

Statistical Analysis

The effect of group (three levels: PREG TEA, PREG CON, and NONPREG CON), time, and the interaction on food and water intake, body weight, and bone outcomes were evaluated through a mixed ANOVA with repeated measures using SPSS Statistics (v. 26, IBM). Differences between means were deemed significantly different if $p < 0.05$. When a significant interaction was identified a Bonferroni *post-hoc* was performed to test the main effects between PREG TEA, PREG CON, and NONPREG CON at each time point. In the case of missing values (μCT data for rat which did not recover from anesthesia following post-lactation scan), series mean imputation was performed. Potential differences in litter characteristics were assessed by *T*-tests using GraphPad Prism™ V5 (La Jolla, CA, USA).

RESULTS

Food and Water Intake, Body and Organ Weights, and Litter Characteristics

A significant interaction ($p < 0.001$) was observed for food intake with a significant increase during pregnancy and lactation for PREG TEA and PREG CON (**Figure 2A**). There was a significant interaction ($p < 0.001$) and main effects for time ($p < 0.001$) and group ($p < 0.001$) for water intake (**Figure 2B**). At weeks 4 and 6 through 10 of the study there was a significant increase in water intake for PREG TEA and PREG CON when compared to NONPREG CON. The average intake of RR was calculated to be 2.66 g/kg of body weight per day for rats in the PREG TEA group and the measured TPC of RR tea was determined to be 12.20 ± 0.69 mg gallic acid equivalents/g of tea ($n = 25$). For body weight there was a significant interaction ($p < 0.001$) and main effect of time ($p < 0.001$). Significant increases in body weight occurred at weeks 5 and 6 (during lactation) and a significant reduction was observed at week 9 (following delivery) (**Figure 2C**). There were no significant differences in kidney weight (left or right) or liver weight between the two groups at endpoint when normalized to body weight (**Table 1**). For litter characteristics, there were no differences in litter sizes, the proportion of males and females within each litter, and average pup weights at 3, 7, 14, and 21 days of age (**Table 1**).

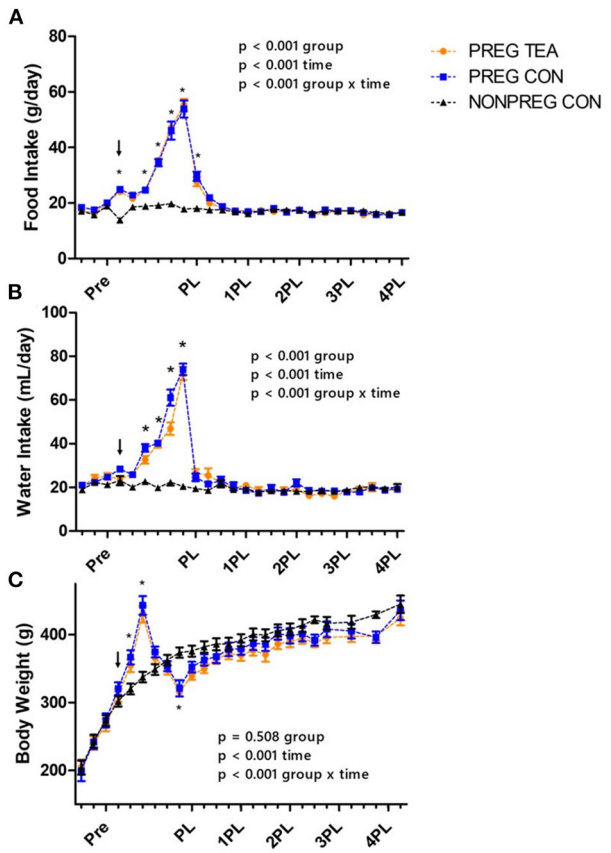

FIGURE 2 | Group daily food and water intake and average body weights; **(A)** mean daily food intake, **(B)** mean daily water intake, and **(C)** body weight of female Sprague-Dawley Values are mean ± SEM, $n = 13$ (PREG CON) or 14/group (PREG TEA and NONPREG CON). Significant differences ($p < 0.05$) between pregnancy and lactation groups (PREG TEA and PREG CON) and NONPREG CON within a time point are denoted by *. The arrow above data points represents the onset of pregnancy. Pre, pre-pregnancy; PL, post-lactation; 1PL, 1 month post-lactation; 2PL, 2 months post-lactation; 3PL, 3 months post-lactation; and 4PL, 4 months post-lactation.

In vivo Measurements of Tibia Trabecular BMD and Structure

There was a significant interaction ($p < 0.05$) for all trabecular outcomes measured (**Figures 3**, **4**). As a result of pregnancy and lactation, BV/TV was significantly reduced ($p < 0.05$) in both PREG TEA and PREG CON groups for the remainder of the study compared to NONPREG CON; while at 2PL and 4PL rats in PREG TEA had significantly greater ($p < 0.05$) BV/TV than PREG CON (**Figure 4A**). Tb.Th was significantly ($p < 0.05$) reduced in PREG TEA and PREG CON following pregnancy and lactation but had recovered to the levels of NONPREG CON by 2PL, with PREG TEA recovering more rapidly (**Figure 4B**). Trabecular separation (Tb.Sp.) was significantly ($p < 0.05$) increased post-lactation in PREG TEA and PREG CON which persisted till the end of the study. Tb.Sp was significantly lower in PREG TEA than PREG CON at 1, 3, and 4PL (**Figure 4C**). Trabecular number (Tb.N.) was reduced ($p < 0.05$) in PREG TEA and PREG CON following lactation compared to NONPREG CON and only partially recovered by the end of the study; while PREG TEA was significantly ($p < 0.05$) greater than PREG CON at 2 and 4PL (**Figure 4D**). BMD was significantly ($p < 0.05$) reduced in PREG TEA and PREG CON beginning at PL and persisted for the remainder of the study, while BMD for PREG TEA was significantly ($p < 0.05$) greater than PREG CON at 1, 3, and 4PL (**Figure 4E**).

TABLE 1 | Litter characteristics and maternal organ weights at 4 months post-lactation.

	PREG TEA	PREG CON	*p*-value
Litter Size (*n*)‡	13 ±2	15 ±2	0.114
# of males *(% of total)*	7 ±2*(54)*	7 ±2*(47)*	0.854
# of females *(% of total)*	6 ±2*(46)*	8 ±2*(53)*	0.086
Average Pup Weight−3 Days Old (g)	13.09 ±0.49	12.71 ±0.35	0.539
Average Pup Weight−7 Days Old (g)	23.74 ±0.75	22.64 ±0.66	0.284
Average Pup Weight−14 Days Old (g)	41.41 ±1.24	41.83 ±1.34	0.819
Average Pup Weight−21 Days Old (g)	65.37 ±1.62	66.95 ±1.97	0.540
L. Kidney Weight (mg/g of body weight)	2.90 ±0.22	2.84 ±0.27	0.477
R. Kidney Weight (mg/g of body weight)	2.95 ±0.27	2.89 ±0.26	0.559
Liver Weight (mg/g of body weight)	32.21 ±1.92	34.18 ±3.34	0.072

‡ *Litter size at delivery.*

In vivo Measurements of Tibia Cortical Tissue Mineral Density (TMD) and Structure

Ps.Pm, Ec.Pm, and M.Ar were similar between all groups at all time points (**Figures 5**, **6A,B,E**). However, there were significant ($p < 0.05$) reductions in Ct.Th, Ct.Ar/Tt.Ar, and TMD in PREG TEA and PREG CON following pregnancy and lactation (**Figures 6C,D,F**). More specifically, Ct.Ar/Tt.Ar and TMD recovered to levels of NONPREG CON by 2PL while Ct.Th remained reduced ($p < 0.05$) in PREG TEA and PREG CON for the remainder of the study.

Discussion

There were two key findings from this study. The first was that reductions in BMD and structure of trabecular bone following pregnancy and lactation remained at 4 months post-lactation. This finding was unexpected as the majority of rodent studies have reported a complete recovery of BMD following pregnancy and lactation within 4 weeks (1, 33, 34). The few studies that have shown an incomplete recovery in terms of trabecular bone structure measured rats for a shorter recovery period post-lactation (6 weeks) (35, 36) and scanned the tibia weekly - prior to mating through 6 weeks post-lactation–with similar findings to the present study in which BV/TV, Tb.N, and Tb.Sp did not fully recover when compared to non-pregnant control rats (35). The other key finding was that consumption of RR tea consumption supported the recovery of trabecular BMD and structure following pregnancy and lactation though not to the level of non-pregnant control group, but significant improvements in trabecular BMD and structure (BV/TV, Tb.N,

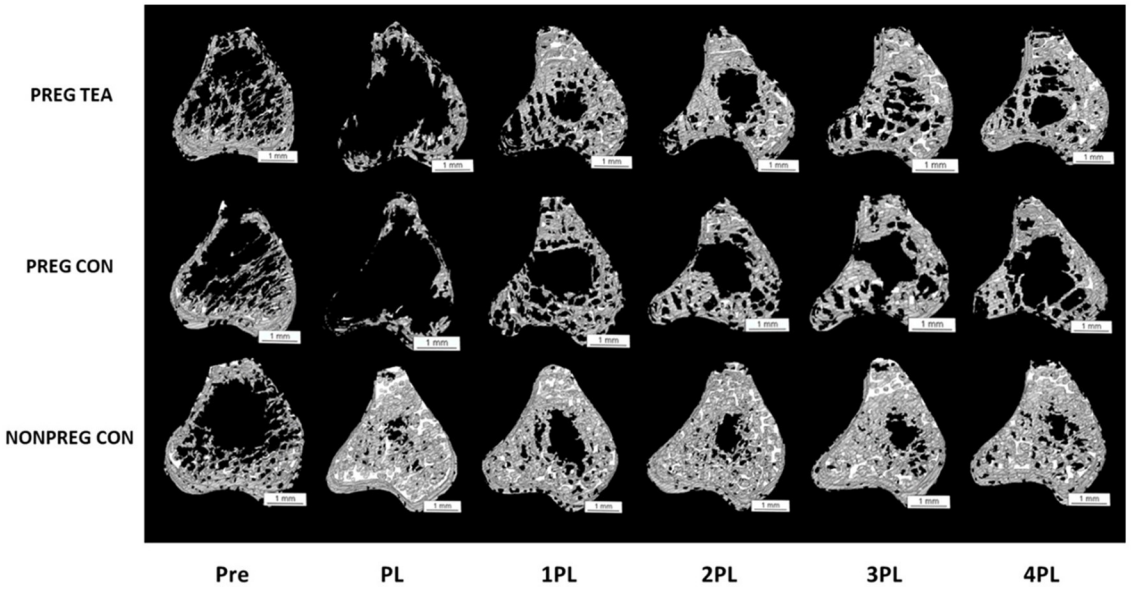

FIGURE 3 | Representative 3D images of trabecular bone from right proximal tibia in female Sprague-Dawley rats pre-pregnancy, post-lactation, and 1, 2, 3, and 4 months post-lactation. Representative scans for each group are of the same rat at each time point and images were chosen by selecting the rat with the closest value for BV/TV to the group mean. Pre, pre-pregnancy; PL, post-lactation; 1PL, 1 month post-lactation; 2PL, 2 months post-lactation; 3PL, 3 months post-lactation; and 4PL, 4 months post-lactation. The white bar under each scan represents a length of 1 mm.

and Tb.Sp) were evident by 1 or 2-months post-lactation when comparing PREG TEA to PREG CON. However, our hypothesis that RR tea intervention would result in higher BMD and improved bone structure post-lactation was not proven.

There are several potential reasons why the present findings differ from earlier studies in terms of recovery of trabecular bone. One potential reason is the differing imaging techniques used between the current study and the majority of previous studies. DXA and bone ash weights were formerly the primary methods used which include assessment of the entire bone-both cortical and trabecular portions (37). As well, ash weight is an endpoint measure making it impossible to observe longitudinal changes in bone. The present study used μCT that allowed for the separate analysis of trabecular and cortical bone. As trabecular bone represents approximately 20% of the total skeleton (with cortical bone comprising the remaining 80%) it is possible that significant reductions in trabecular bone will be masked if whole bone BMD is measured. Evidence for this explanation includes similar findings of permanently reduced trabecular bone structure following lactation for other studies in older rats which have also measured bone structure using μCT without reliance on DXA or ash weights to determine BMD or bone mineral content, respectively (35, 36). Another potential reason for the finding of an incomplete recovery of trabecular bone could be the age at which the rats underwent pregnancy and lactation. In the current study, mating began at 8 or 10 weeks (56 or 70 days) of age, which is likely comparable to early adulthood (38). Our findings demonstrated that non-mated rats had increased trabecular BMD and cortical TMD at the PL scan at 15 or 17 weeks of age (compared to their PRE scan at 8 or 10 weeks of age) demonstrating that they were still growing during this period. However, no significant increases in trabecular BMD or cortical TMD occurred after this scan suggesting that maximal mineral content (BMD, TMD) had been reached between the PRE (8 or 10 weeks of age) and PL (15 or 17 weeks of age) scans. Rats may have been challenged in terms of recovery as lactation-induced resorption may have been simultaneously occurring with growth, leading to greater reductions in bone quantity and structure than may have occurred if the rats were older and had reached maximal mineral content. Previously, our lab measured trabecular BMD and cortical TMD by *in vivo* μCT starting at 13 weeks of age through to 25 weeks of age (at 4 week intervals) in female Sprague-Dawley rats and no differences in BMD or TMD were detected between any of the 4 weeks intervals indicating that maximal mineral content (BMD, TMD) had been reached and occurred prior to 13 weeks of age (32). The age at mating (8 or 10 weeks old) in our study was selected as it reflected the age that rats are often bred at commercial and research facilities.

RR tea consumption stimulated a greater recovery of trabecular BMD and bone structure outcomes (BV/TV, Tb.N, and Tb.Sp) by 1 or 2PL, and for most of these outcomes this benefit persisted compared to the group not receiving tea. Though, it is important to consider that outcomes remained lower than the growth control and disproved the original hypothesis. Previously, RR herbal tea has been shown to contain a large quantity of unique polyphenols (aspalathin, aspalalinin, nothofagin) with antioxidant capacity (30, 39). Antioxidants have been shown to decrease ROS supporting osteoblast proliferation, preventing osteocyte apoptosis and inhibiting osteoclast activity (25, 40, 41). This increased quantity of antioxidants provided by polyphenols

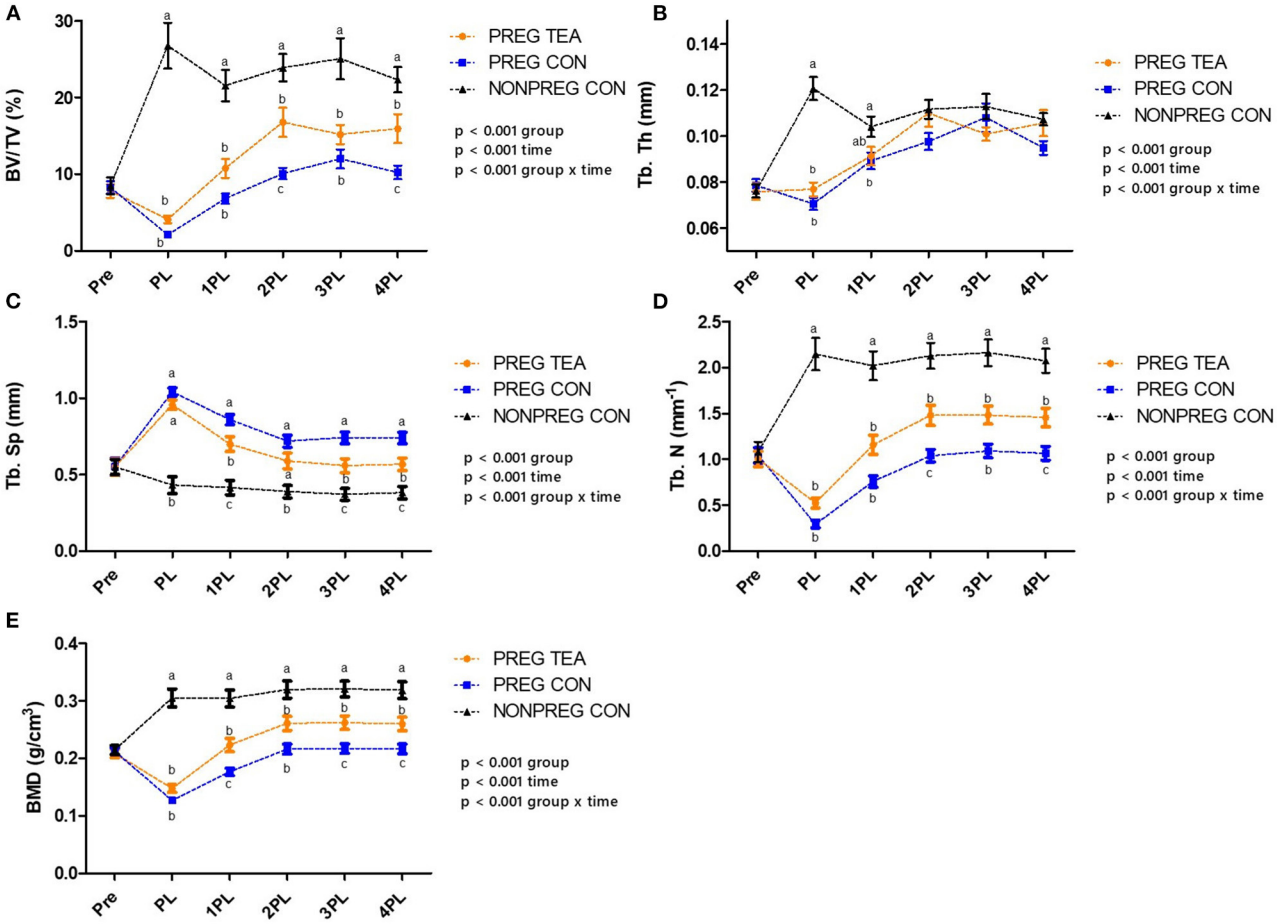

FIGURE 4 | Comparisons of trabecular outcomes for PREG TEA, PREG CON, and NONPREG CON; **(A)** BV/TV, **(B)** Tb. Th, **(C)** Tb. Sp, **(D)** Tb. N, and **(E)** BMD. Values are mean ± SEM, $n = 13$ or 14/group. Differing letters denote a significant difference among groups within a time point. Pre, pre-pregnancy; PL, post-lactation; 1PL, 1 month post-lactation; 2PL, 2 months post-lactation; 3PL, 3 months post-lactation; and 4PL, 4 months post-lactation.

within RR herbal tea may be able to attenuate the rise in ROS which occurs as a result of pregnancy and lactation leading to greater rates of bone formation, reduced rates of osteoclast resorption, and an overall improved recovery in bone structure and quantity. Although no mechanisms were investigated in this study, previous *in vitro* studies have demonstrated significantly reduced osteopontin gene and protein expression by osteoblasts in response to RR tea which may increase mineralization (16, 17). There were no significant differences in the timing of recovery of cortical outcomes between PREG TEA and PREG CON. This discrepancy is likely due to trabecular bone being more metabolically active making it responsive to acute alterations while cortical bone is less metabolically active and more resilient to acute changes. In a recent study, researchers measured trabecular structure of two distinct sites (tibia and vertebrae) in Sprague-Dawley rats prior to mating through to post-weaning and related trabecular structural outcomes to the proportion of a typical load (36). Trabecular structure (BV/TV, Tb.N, Tb.Sp) in the tibia were greatly reduced but was also found to be responsible for bearing a significantly lower mechanical load than the vertebrae which did not have as large a reduction in BMD and trabecular structure. As well, the researchers observed no difference in compressive properties (peak load, stiffness, and energy to failure) of lumbar vertebrae between rats who did not undergo pregnancy and lactation and rats who were 6 weeks post-lactation, signifying a complete recovery of mechanical strength. The authors propose that these findings may support and explain findings of decreased BMD without any alterations in fracture risk as areas which are more mechanically loaded do not have as severe bone loss and are able to retain their mechanical properties whereas much of the bone loss is localized to areas that are not as mechanically loaded (i.e., tibia).

Despite the need for the tea to be highly concentrated to reach levels which would be comparable to a human drinking approximately 12 cups a day there was no aversion to the taste as *ad libitum* water intake was similar between PREG TEA and PREG CON groups. Organ weight is frequently used as an indicator of overall organ health and safety (42)–and the liver and kidney weights–did not differ between PREG TEA and PREG CON. Moreover, pup weights were similar between PREG TEA

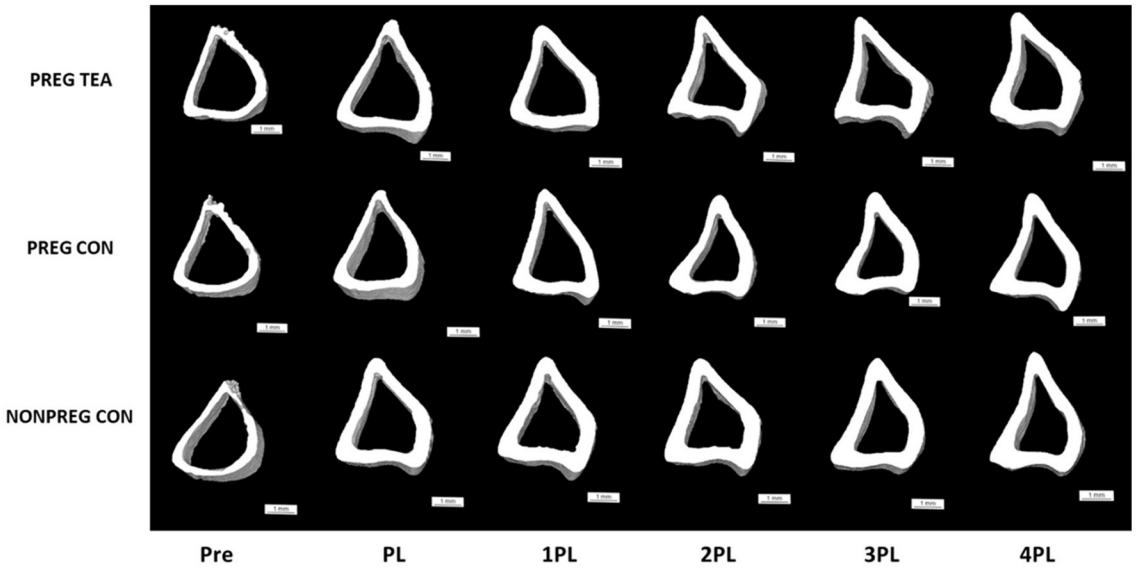

FIGURE 5 | Representative 3D images of cortical bone from right proximal tibia in female Sprague-Dawley rats pre-pregnancy, post-lactation, and 1, 2, 3, and 4 months post-lactation. Representative scans for each group are of the same rat at each time point and images were chosen by selecting the rat with the closest value for Ct.Ar/Tt.Ar to the group mean. Pre: pre-pregnancy, PL, post-lactation; 1PL, 1 month post-lactation; 2PL, 2 months post-lactation; 3PL, 3 months post-lactation; and 4PL, 4 months post-lactation. The white bar under each scan represents a length of 1 mm.

and PREG CON during lactation at 3, 7, 14, and 21 days of age. These findings suggest that the intake of RR at the level studied was safe though more detailed analyses would be needed before making a definitive conclusion about safety.

Sprague-Dawley rats are cost effective (compared to clinical models), easily accessible, and provide a good model for initial *in vivo* studies (43). However, there are some key similarities and differences between the current preclinical model implemented and humans which should be stated. Rat litters contain significantly more pups and as a result the demand for calcium is also substantially increased (44). While humans lose 5–10% of their BMD following pregnancy and lactation, rats commonly lose 25–35% (1). Although this does not directly represent the human situation the greater magnitude of change provides an exaggerated model in which potential interventions can be tested. As well, during pregnancy and lactation there are extensive physiological adaptations to provide calcium to offspring and these adaptations are largely similar between humans and rats with the exception of intestinal absorption of calcium during lactation. In humans, skeletal resorption is able to provide enough calcium for their offspring during lactation but due to the greater demand placed on rats by larger litter sizes, intestinal calcium absorption stays elevated during lactation (2, 45).

Strengths of this study include specific methodological aspects which are summarized in **Supplementary Material 1**. For example, having the same males equally represented in both pregnancy groups, aided in controlling for the influence of paternal genetics. This is important as previous research has demonstrated that the paternal genetics can influence the amount of mineral accrual *in utero* making it an important aspect to be controlled for (46, 47). Furthermore, every dam in each group was mated with a different male reducing the genetic contribution that any one male may have within a group to give a more diverse and realistic group of litters. Additionally, longitudinal *in vivo* measurements within the same rat provided greater statistical power for the study as it controls for many of the variables between subjects. Longitudinal measures can also identify when the ideal timing of an intervention (i.e., consumption of RR tea) may be to elicit the greatest effects. As well, the relatively long duration of the study allowed for statistically significant differences to be observed between the two groups in terms of trabecular recovery. Another strength of this study was the mating strategy used. By staggering the mating, we were able to perform scans at precise times around pregnancy and lactation. For the delivery of tea, we adjusted the concentration on a weekly basis in relation to body weight and intake to allow for *ad libitum* access while still ensuring rats received the appropriate concentration daily to mimic a real-life scenario more closely. Other methodological strengths included culling litters to ensure normalized calcium demand and milk production by dams as well as scanning rats in alternating group order to ensure that if there were any inconsistencies in X-ray transmissions that it would be evenly spread among the groups.

There are also limitations of the present study. Rats were mated at a time in which they were continuing to accumulate mineral and thus may have been particularly challenged in terms of recovery with the lactation-induced resorption occurring simultaneously with growth. Another limitation to this study is that only one concentration of RR tea was used, and that the level administered would require supplementation. Consuming RR tea and/or polyphenols in the form of a supplement would alter its food matrix and may affect their digestion and absorption, changing the compounds which are interacting with bone.

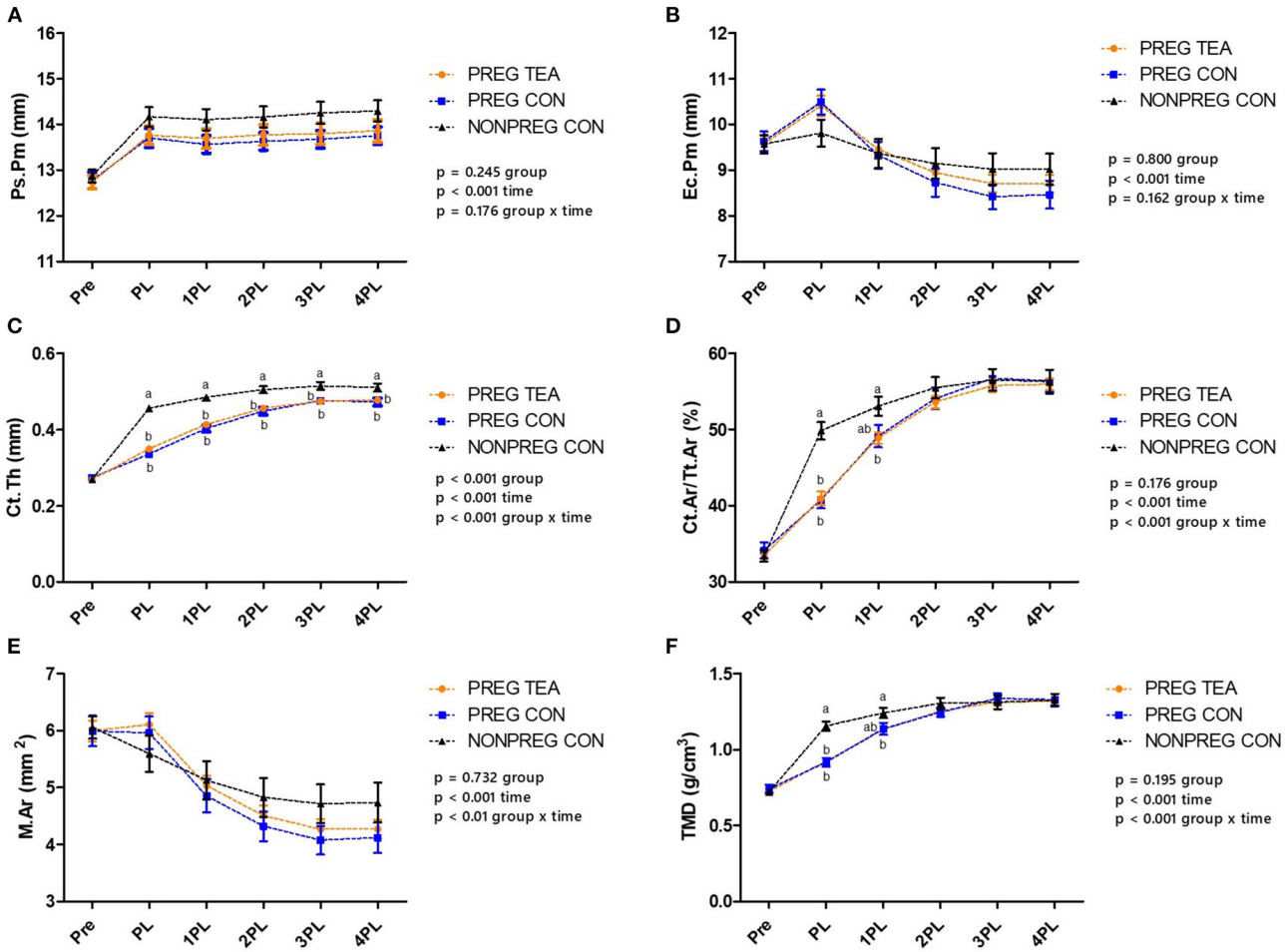

FIGURE 6 | Comparison of cortical bone outcomes between PREG TEA, PREG CON, and NONPREG CON; **(A)** Ps.Pm, **(B)** Ec.Pm, **(C)** Ct.Th, **(D)** Ct.Ar/Tt.Ar, **(E)** M.Ar, and **(F)** TMD. Values are mean ± SEM, $n = 13$ or 14/group. Differing letters denote a significant difference among groups within a time point. Pre: pre-pregnancy, PL, post-lactation; 1PL, 1 month post-lactation; 2PL, 2 months post-lactation; 3PL, 3 months post-lactation; and 4PL, 4 months post-lactation.

However, new techniques are also being developed to increase the bioavailability of polyphenols from supplements including encapsulation of polyphenols within a shell of polysaccharides, cellulose, starch, or proteins to increase bioavailability and possibly their efficacy (48). Although a beneficial effect of consumption was observed at the concentration studied, future studies should assess other concentrations of RR tea to determine if higher concentrations would elicit greater benefits or what the lowest effective concentration would be.

In conclusion, findings from this study demonstrate that consumption of RR tea supported the ability of bone to recover post-lactation in a rat model but did not result in greater BMD and improved structure as hypothesized. Moreover, our unexpected finding that significant reductions in trabecular BMD and structure persisted at 4 months post-lactation provides a basis for more fully understanding the rat model of pregnancy and lactation in terms of bone formation and resorption and should be evaluated further in other rodent models as well as other ages of Sprague-Dawley rats.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Brock University Animal Care Committee (Protocol #18-03-02).

AUTHOR CONTRIBUTIONS

MM and WEW contributed to the conception and design of the study. MM and JY performed the *in vivo* study, sample collection, and analyses, as well as data collection. MM performed the data analysis, statistical analysis, and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.798936/full#supplementary-material

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REVIEW
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The Importance of Reference Materials and Method Validation for Advancing Research on the Health Effects of Dietary Supplements and Other Natural Products

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Insufficient assessment of the identity and chemical composition of complex natural products, including botanicals, herbal remedies, and dietary supplements, hinders reproducible research and limits understanding mechanism(s) of action and health outcomes, which in turn impede improvements in clinical practice and advances in public health. This review describes available analytical resources and good methodological practices that support natural product characterization and strengthen the knowledge gained for designing and interpreting safety and efficacy investigations. The practice of validating analytical methods demonstrates that measurements of constituents of interest are reproducible and appropriate for the sample (e.g., plant material, phytochemical extract, and biological specimen). In particular, the utilization of matrix-based reference materials enables researchers to assess the accuracy, precision, and sensitivity of analytical measurements of natural product constituents, including dietary ingredients and their metabolites. Select case studies are presented where the careful application of these resources and practices has enhanced experimental rigor and benefited research on dietary supplement health effects.

Keywords: dietary supplement (DS) analysis, reference material (RM), method validation, chemical characterization, natural product (NP)

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INTRODUCTION

Dietary supplements (DS) and other natural products (NPs) are repeatedly investigated in cellular systems, observational studies, and randomized controlled trials due to their prevalent use for potential health benefits (1–3). However, outcomes from clinical studies on botanical-derived NP efficacy for health maintenance and disease risk reduction often yield mixed results, driven in part by the varying composition of the experimental interventions investigated (4). Clinical trials studying the effect of *Echinacea* species in respiratory infections, for example, investigate varying doses and different species and plant parts (5–7). Since the early 2000's, seminal papers have described the need for sufficient reporting in clinical research on botanicals, dietary supplements, and traditional medicines, including elaborations on the Consolidated Standards of Reporting Trials (CONSORT) guidelines (8–11). However, even though recent studies have found an overall improved reporting quality in studies of certain NPs, there is still an indication that insufficient

reporting details on methodology and characterization continue to be an issue. For example, an assessment of randomized trials of Asian ginseng *(Panax ginseng* C.A. Meyer) and North American ginseng (*Panax quinquefolius* L.) deemed that <40% of trials conducted from 1980 to 2019 adequately addressed CONSORT criteria for methodology reporting, and <15% provided sufficient details on intervention composition to allow for experimental replication (12). Such insufficient characterization of an investigational DS or NP chemical composition reduces the capability for data analysis, limits research reproducibility, and impedes the continuity of scientific progress (4, 13, 14).

Analytical characterization of DS and NP interventions is essential for rigorous basic and clinical research on their health effects. Detailed chemical characterization improves research reproducibility, as investigators' ability to replicate and build upon studies is substantially increased the more that is known about an intervention's composition. Sufficient characterization also facilitates more meaningful comparisons of experimental design and data interpretation across studies, as it can be difficult, if not impossible, to interpret the public health relevance of a study on NP efficacy using a preparation with poorly understood composition (15). The insight gained from investigations of a NP's mechanism(s) of action, pharmacokinetics, or herb/drug interactions are significantly expanded through a better understanding of its chemical composition. In addition to better understanding the basis for positive health effects, NP chemical characterization is necessary for adequate safety assessment, a prerequisite for any clinical investigation, and particularly important for botanical-derived interventions where adulteration of source materials is a known issue (16, 17). This review describes the importance of utilizing reference materials and validated methods to address these analytical challenges and enhance natural product research rigor and reproducibility. A discussion of important considerations in method validation, an assessment of available certified reference materials, and case studies provide guidance and good practices for advancing research on the health effects of DS and other NPs. Comprehensive tables index examples where reference material use has facilitated innovative method development and/or supported novel research.

KEY CONSIDERATIONS FOR DS AND NP CHARACTERIZATION

The major characterization parameters for complex NPs such as DS include confirmation of identity/authenticity, quantification of known or putative bioactive constituents or marker compounds, an assessment of purity/composition, and safety evaluation (4, 18). For certain vitamins and minerals, it is important for investigators to consider whether the isoforms or chelation states are appropriate to the research hypothesis and experimental design. For example, vitamin E comprises a group of eight chemical isoforms that have varying biological activities, while chelates of trace minerals may alter bioavailability. For botanicals, it is essential to verify plant species identity and authenticity, confirm the correct plant parts were used, and test for the presence of harmful compounds, microbes, pesticides, toxins, and toxic elements (e.g., cadmium, mercury, lead, and arsenic). Batch-to-batch reproducibility and stability of experimental NP interventions should also be confirmed. Investigators should assess these key characterization parameters to the extent practicable to best support advances in NP biomedical research.

Well-designed research must control as many variables as possible to support replicable results. Exploratory and molecular/cellular studies should characterize NP interventions to the extent feasible to increase the potential for gaining insight into underlying biological mechanisms. Animal models and clinical studies of DS and other NPs should utilize test materials that have been standardized to the extent possible. For botanical-derived DS, standardization starts with species verification and continues through chemical identification and quantification of specific compounds which are known or hypothesized to produce a biological activity. Often the particular compound or compounds responsible for an activity are not known, and marker compounds must instead be chosen for standardization. The interpretation of pre-clinical investigations of metabolic pathways and safety, critical to assess before moving to translational research in human subjects, also rests on accurate compositional characterization (19).

Throughout all these considerations, accurate, precise, and reliable analytical measurements are needed to assess the consistency and quality of NPs obtained from various suppliers, confirm the repeatability of product preparation schemes, and assure safety by detecting toxic constituents. DS products are prepared in numerous formulations, including powders, liquids, tablets, capsules, and chewable gels ("gummies"), which adds unique analytical challenges to homogenization, extraction, and reproducible quantification. A limited number of contract research organizations and independent laboratories have the capability and experience to analyze DS (20). Therefore, it is incumbent upon biomedical researchers to assess in-house controls and demonstrate the accuracy of their analytical methods.

BENEFITS OF METHOD VALIDATION AND RMs IN NP BIOMEDICAL RESEARCH

The requisite level of characterization and standardization for DS and other NP research is facilitated by using validated analytical methods and matrix-matched reference materials for accurate quantification of nutrients, minerals, phytochemicals, metabolites, and toxic analytes. Analytical methods employed in NP authentication and characterization must be carefully selected and controlled to ensure the accuracy and precision of quantitative measurements for phytochemicals, nutrients, and possible contaminants (21). Methods should be fit for purpose, meaning the measurements are sufficiently reliable and appropriate for the sample matrix (e.g., ground plant part, liquid extract, and capsule formulation). Formal validation studies of analytical methods are the means to demonstrate

fitness for purpose and reliability through the determination of measurement performance parameters, including precision, accuracy, selectivity, specificity, limit of detection, limit of quantitation, and reproducibility. Therefore, utilizing a validated method is an optimal approach for demonstrating unbiased and reliable measurements, transferring projects or analyses to new lab members, and comparing research results across multiple labs.

Through the processes of method development and validation, it is paramount that researchers confirm their methods are generating the correct answers for analyte quantification or material authentication, and this is where reference materials play a vital role. The use of reference materials to assure the quality of analytical measurements is well-defined in analytical chemistry, particularly in environmental, clinical, and food analyses. A review by Ulberth summarizes the international terminology for the types of reference materials and their use (22). A reference material (RM) is a "material, sufficiently homogeneous and stable for one or more specified properties, which has been established to be fit for its intended use in a measurement process."[1] A certified reference material (CRM) is defined as a "RM characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability" (see text footnote 1). For example, a St. John's Wort (*Hypericum perforatum* L.) CRM could comprise a homogenized powder prepared from authenticated aerial parts, with quantified values for hypericin. CRMs of known quantities of analytes in solution are intended for use as calibration solutions, as described later.

The inherent complexity of natural product preparations and the resulting analytical challenges, such as accounting for extraction efficiency and interfering compounds, are best addressed by matrix-based reference materials. Compared to the myriad NPs and botanicals used worldwide there is a comparatively small number of matrix based RMs available. However, it is important for analysts and researchers to consider that RMs are often not intended to be representative of every possible matrix, nor are they intended to represent a "gold standard" for an ingredient or formulated product. Rather, RMs are meant to be representative of the analytical challenges encountered with similar matrices, e.g., isoflavone extraction from a leaf material. Depending on the purpose of the RM and the analytical question being asked, an exact matrix-matched RM is not necessarily required for method development and validation or NP characterization in research. As such, the limited number of currently available matrix based RMs can be applicable to the characterization of a much larger number of matrices, and they should be used wherever possible when quantification of marker compounds and/or toxic metal contaminants is required. CRMs can be used as quality control (QC) materials to determine bioactive/marker compound content, detect contamination, or assign values to verify in-house QC materials. Researchers using botanical supplements in clinical studies can, and wherever possible should, verify the accuracy of any chemical characterization of the experimental intervention by using CRMs as QC materials.

GOOD PRACTICES IN ANALYTICAL METHOD VALIDATION

Standard-setting organizations and regulatory agencies have provided detailed guidance and workflow schema on how to conduct formal validation studies of analytical methods specifically for NPs and dietary ingredients[2,3,4]. These validation guidance documents define parameters that should be assessed for qualitative or quantitative methods and outline procedures for establishing a measurement's linearity range and reliability. While there is not a consensus agreement of which analytical parameters must be assessed to constitute a formal validation, there are commonalities across the guidelines of different organizations. A quantitative method's selectivity and specificity, accuracy, precision, recovery, limit of detection, limit of quantification, repeatability, and reproducibility are key parameters that should be assessed in a formal validation. A qualitative method, for example one that is intended for botanical identity or authenticity, should also assess specificity, selectivity, and limit of detection, as well as false positive/negative rates. Validation guidelines specific to identification methods also describe a statistical modeling procedure, termed the probability of identification, as a key parameter to assess a qualitative method's reliability (23).

Validated methods for DS ingredients and products can be leveraged in both industry and academic settings to enhance measurement confidence and reproducibility. Biomedical researchers focused on delineating the connections between dietary ingredients, their metabolism, mechanisms of action, and health outcomes have used validation to establish the accuracy and reproducibility of their measurements. As examples, published validation studies of a liquid chromatography—mass spectrometry (LC-MS) quantification of soy sphingadienes under investigation for chemopreventive activity (24), a gas chromatography (GC)-MS characterization of a complex grape seed flavanol mixture studied in models of stress resilience (25), a high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) determination of cholecalciferol in clinical serum and plasma samples (26), and an ultra-high performance liquid chromatography (UHPLC)-MS/MS quantification of red clover isoflavones and milk thistle flavonolignans in supplements and human serum (27) all contribute significantly to experimental rigor in subsequent

[1]ISO/Guide 30:2015 - Reference Materials: https://www.iso.org/obp/ui/#iso:std:iso:guide:30:ed-3:v1:en.

[2]AOAC International Official Methods of Analysis, Appendix K: http://www.eoma.aoac.org/app_k.pdf.

[3]FDA Bioanalytical Method Validation Guidance for Industry: https://www.fda.gov/media/70858/download.

[4]FDA Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products: https://www.fda.gov/media/121751/download.

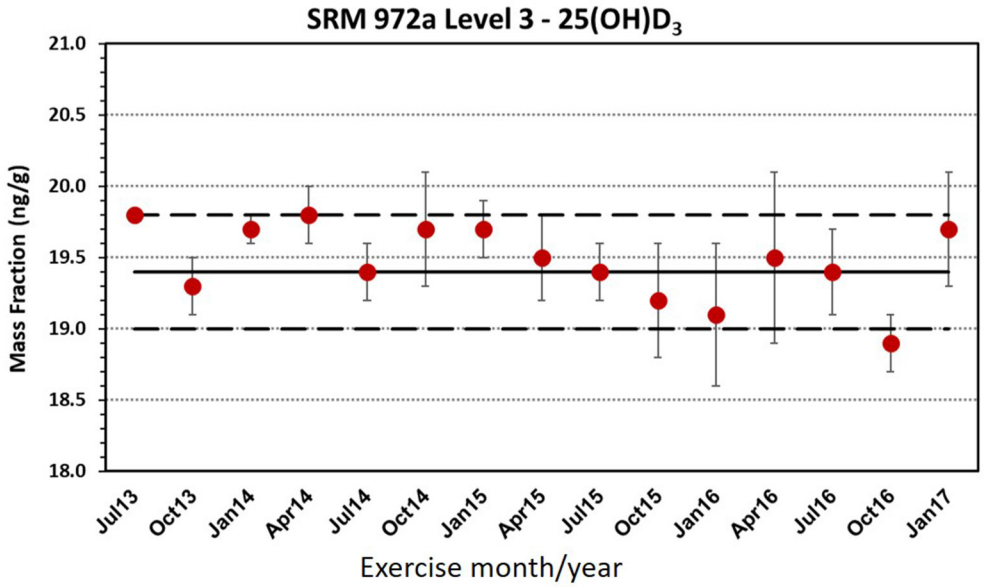

FIGURE 1 | Control chart for the NIST determination of 25(OH)D_3 in SRM 972a for DEQAS exercises from July 2013 to January 2017. Error bars are ± SD for duplicate analyses of SRM 972a. Solid line is the certified value and dashed line is the uncertainty of the certified value. Burdette et al. (35), adapted by permission of AOAC International (aoac.org).

hypothesis-driven investigations and help build confidence in translational NP research.

GOOD PRACTICES IN THE USE OF MATRIX REFERENCE MATERIALS

There are three types of CRMs with different complexity and uses (22, 28): (1) pure substance (neat chemical), (2) calibration solution/mixture containing one or more constituents, and (3) natural matrix materials. Pure substance CRMs and solution CRMs are closely related in that the pure substances are generally intended as primary standards (of known purity) for use in preparing calibration solutions, and solution CRMs are typically intended for use directly as a calibration solution, i.e., eliminating the step of the analyst preparing a solution from a pure substance. These solution CRMs are typically used to calibrate analytical instruments and, in the case where chromatographic separations are involved, confirm retention times and determine detector response for the analytes of interest. Natural matrix CRMs, which ideally should be compositionally similar to the real-world samples analyzed, are used to evaluate the complete analytical measurement process, including dissolution or solvent extraction of the matrix, clean-up of the extract, isolation and/or enrichment of the constituents of interest, and the final instrumental analysis including chromatographic separation, detection, and quantification. Matrix RMs play a critical role in validating the complete analytical method and assessing the accuracy and comparability of results among different laboratories over time.

Matrix CRMs are intended for the following applications: (1) analytical method development and method validation, i.e., to assess accuracy or trueness of measurement results, (2) to serve as QC materials, (3) to assign values for in-house QC materials, and (4) to provide metrological traceability of measurement results. Practical guides to the use of CRMs are provided by several European Reference Materials Application Notes (29, 30) and by Sharpless et al. (31). Matrix CRMs should not, however, be used for the calibration of analytical instruments. The use of matrix CRMs that are similar in matrix to the actual samples analyzed is critical in new method development and validation for assessing the accuracy of the complete analytical measurement process, e.g., extraction and dissolution, clean-up, and finally, chromatographic separation and detection. Several excellent studies from the environmental measurement area have been reported that illustrate the value of using a CRM to evaluate the efficacy of solvent extraction techniques (32–34). CRMs can be used as QC materials during routine measurements through their inclusion in each batch of actual samples to assess the accuracy or trueness of the results and by preparing control charts to monitor the quality of the measurements over time. An example of using a CRM in a control chart is illustrated in **Figure 1**, where a CRM for 25-hydroxyvitamin D_3 in human serum was analyzed quarterly over a period of 5 years as part of an international external quality assurance program, i.e., Vitamin D External Quality Assessment Scheme (DEQAS) (35). Due to the cost of CRMs, laboratories may prepare in-house QC materials and analyze the CRM with the in-house QC materials to assign values (i.e., traceability) and then use the in-house QC material for routine measurements (31).

Finally, CRMs have proven useful in novel, exploratory and hypothesis-driven research because they are homogeneous and stable materials that are widely available to the research community, characteristics which also facilitate collaboration and comparison of results. For instance, if researchers characterize different ginseng materials as part of a study to identify new marker compounds (i.e., not the constituents with values assigned by the CRM producer), ginseng CRMs can be included as part of the study and the published results for the content of these novel marker compounds in the CRM can be referenced and compared to other laboratory analyses in the future. An excellent example of researchers analyzing CRMs for the determination of new analytes is work by Zhu and Hites, where they used a newly-developed method for the determination of the emerging environmental contaminant polybrominated diphenyl ethers (PBDEs) in several existing National Institute of Standards and Technology (NIST) marine and freshwater tissue matrix CRMs (36). Zhu and Hites published the first report of concentrations of PBDEs in these CRMs and stated, "Given the availability and homogeneity of the NIST SRMs, we suggest that these materials can be used for the interlaboratory calibration of PBDE concentrations" (36). Years later, NIST assigned certified values for selected PBDE congeners in these SRMs (37), further increasing their analytical utility.

CURRENT AVAILABILITY OF REFERENCE MATERIALS FOR DIETARY SUPPLEMENTS

Within the dietary supplement industry, the term reference material typically refers to "authentic reference standards" for compounds and/or ingredients found in supplement products. In the early 2000's a limited number of reference standards for botanicals or other DS ingredients were commercially available. However, they typically had limited information on the purity of the material, and quantitative information on the chemical content of natural matrix RMs for botanical dietary supplement ingredients was intended for testing against a limited number of compendial standards (38). In 2002 the U.S. National Institutes of Health Office of Dietary Supplements (NIH-ODS) established the Analytical Methods and Reference Materials (AMRM) Program,[5] with a mission to enhance rigorous dietary supplement research support and quantitative analysis (39). To meet industry and research needs for quantitative reference materials that were not tied to compendial testing, NIH-ODS collaborated with NIST to develop CRMs for dietary ingredients and supplement products.[6] CRMs issued by NIST are called Standard Reference Materials (SRMs), the majority of which are characterized for chemical composition. NIST is the national metrology institute (NMI) within the U.S.; while most matrix CRMs for chemical content are produced by NMIs in their respective countries, they are often distributed worldwide. Commercial suppliers, such as Cerilliant/MilliporeSigma,[7] may also produce CRMs and RMs for certain constituents of dietary supplements.

In addition to utilizing CRMs, which are the major focus of this review, DS and NP researchers can also take advantage of available standards from multiple sources to aid in botanical identification and authentication. The United States Pharmacopeia (USP) develops pure chemical and matrix-based reference standards for use in conjunction with their formal documentary standards (monographs) for the characterization and quality assessment of therapeutics, foods, and DS. These USP analytical tools are intended to aid the verification of identity and composition of ingredients used in drug formulations and DS products, and their catalog of reference standards includes vitamins, minerals, phytochemicals, and complex botanical preparations (40, 41). The American Herbal Pharmacopeia produces qualitative monographs and matching authenticated reference standards with a focus on botanicals and herbal medicines.[8] Some commercial reagent suppliers, such as ChromaDex,[9] PhytoLab,[10] Alkemist,[11] and Extrasynthese[12] also produce nutrient, phytochemical and/or plant material reference standards which can be used to support method development and chemical characterizations of raw materials or experimental interventions. While these types of reference standards and vouchered specimens may not offer the same level of quantitative values for specified bioactive constituents or other properties, their use can play a significant role in providing a well-characterized control material that can readily be used to benefit a research collaboration, compare results across studies, and support experimental replication.

NIH-ODS/NIST Supported Development of Reference Materials

Initial efforts of the NIH-ODS/NIST collaboration focused on the development of authentic botanical ingredient-containing SRMs and RMs with values assigned for the content of active and/or marker compounds for use in verification of content and manufacturing quality control, particularly to address safety concerns related to contaminants such as toxic elements (42–44). Within the dietary supplement industry, chemical analyses are typically performed on raw materials (plants and extracts of plants) and finished products (e.g., tablets). Thus, NIST SRMs were designed wherever possible to comprise a "suite" of materials for each botanical dietary supplement ingredient consisting of authentic plant material, an extract of the plant material, and the finished product. The intent was to provide

[5]NIH-ODS Analytical Methods and Reference Materials Program: https://ods.od.nih.gov/Research/AMRMProgramWebsite.aspx.

[6]NIST Measurements and Standards for Botanical Dietary Supplements: https://www.nist.gov/programs-projects/measurements-and-standards-botanical-dietary-supplements.

[7]Cerilliant Corp Analytical Reference Standards: https://www.cerilliant.com/products/catalog.aspx.

[8]AHP-Verified Botanical Reference Materials: https://herbal-ahp.org/botanical-reference-materials/.

[9]ChromaDex Reference Standards: https://www.chromadex.com/standards-overview/.

[10]PhytoLab Reference Substances: https://www.phytolab.com/en/our-services/reference-substances-phyproof/.

[11]Alkemist Labs Reference Materials: https://www.alkemist.com/reference-materials/.

[12]Extrasynthese Botanical Materials and Quantified Reference Extracts: https://www.extrasynthese.com/.

TABLE 1 | Currently available botanical dietary supplement matrix CRMs.

Source	CRM No.	CRM description	Certified values	Reference values	Total values
NIST	3246	Ginkgo (*Ginkgo biloba*) leaves	Flavonoids (4); ginkgolides (1); toxic elements (3); DNA sequence (identity)	Flavonoids (4); ginkgolides (4); bilobalide (1)	**18**
NIST	3247	Ginkgo (*Ginkgo biloba*) extract	Flavonoids (4); ginkgolides and bilobalide (6); toxic elements (1)	Toxic elements (2)	**13**
NIST	3248	Ginkgo-containing SODF	Flavonoids (4); ginkgolides (3); toxic elements (1)	Ginkgolides (2); bilobalide (1); toxic elements (3)	**14**
NIST	3250	Saw palmetto (*Serenoa repens*) Fruit	Phytosterols (3), fatty acids (14)	Phytosterols (3), fatty acids (4), free fatty acids (16)	**40**
NIST	3251	Saw palmetto (*Serenoa repens*) Extract	Phytosterols (3); fatty acids (17); carotenoids (1); vitamins (1)	Phytosterols (3); fatty acids (3); free fatty acids (17); tocopherol (1); carotenoids (2); cycloartenol	**48**
NIST	3274	Botanical Oils Containing Omega-3 and Omega-6 Fatty Acids	Fatty acids (35)	Fatty acids (33)	**68**
NIST	3274-1	Borage oil (*Borago officinalis*)	Fatty acids (9)	Fatty acids (8)	**17**
NIST	3274-2	Evening Primrose oil *(Oenothera biennis*)	Fatty acids (10)	Fatty acids (8)	**18**
NIST	3274-3	Flax (*Linium usitatissimum*) Seed	Fatty acids (9)	Fatty acids (7)	**16**
NIST	3274-4	Perilla (*Perilla frutescens*)	Fatty acids (7)	Fatty acids (10)	**17**
NIST	3281	Cranberry (*Vaccinium macrocarpon*) Fruit	Organic acids (1); elements (9)	Proximates (5); sugars (3); elements (2); anthocyanidins (3)	**25**
NIST	3282	Low-Calorie Cranberry Juice Cocktail	Organic acids (3); elements (6)	Organic acids (6); anions (2); sugars (3); elements (2)	**22**
NIST	3283	Cranberry (*Vaccinium macrocarpon*) Extract	Organic acids (3)	Organic acids (6); anions (2)	**14**
NIST	3284	Cranberry-Containing SODF	Organic acids (3)	Organic acids (4); anions (2)	**12**
NIST	3285	Mixed Berry-Containing SODF	Organic acids (2)	Organic acids (6); anions (2)	**12**
NIST	3287	Blueberry (*Vaccinium corymbosum*) Fruit	Organic acids (1); vitamins (4); elements (8)	Organic acids (5); proximates (6) and fiber; sugars (3): elements (1); amino acids (16); anions (2)	**50**
NIST	3291	Bilberry extract (*Vaccinium myrtillus*) Extract	Organic acids (3)	Organic acids (3); anions (2)	**11**
NIST	3254	Green tea (*Camellia sinesis*) leaves	Catechins (5); caffeine and theobromine; toxic elements (4); DNA sequence (identity)	Catechins (2), gallic acid, L-theanine, elements (5)	**21**
NIST	3255	Green tea (*Camellia sinesis*) extract	Catechins (7); caffeine and theobromine; toxic elements (2)	Catechins (2), gallic acid, L-theanine, theophylline, elements (5)	**21**
NIST	3256	Green tea-containing SODF	Catechins (6); caffeine and theobromine; gallic acid; toxic elements (4)	Catechins (1), L-theanine, theophylline	**16**
NIST	3234	Soy Flour	Elements (8); vitamins (2)	Elements (1); isoflavones (5); proximates (5) and fiber; amino acids (18)	**39**
NIST	3235	Soy Milk	Elements (8); vitamins (5)	Elements (1); vitamins (4); proximates (6), sugars (1); fatty acids (11); amino acids (16)	**52**
NIST	3236	Soy Protein Isolate	Isoflavones (6)		**6**
NIST	3237	Soy Protein Concentrate	Isoflavones (1)	Isoflavones (2)	**3**
NIST	3238	Soy-Containing SODF	Isoflavones (5)	Isoflavones (1)	**6**
NIST	3262	St John's Wort (*Hypericum perforatum*) Aerial Parts	Toxic elements (3); DNA sequence (identity)	Flavonoids/naphthodianthrones (5); chlorogenic acid; toxic elements (1)	**10**
NIST	3232	Kelp (*Thallus laminariae*) Powder	Elements (15) including toxic elements (4) and iodine	Elements (5), arsenic species (2), arsenosugars (3), vitamin K_1 (3), proximates (5)	**33**
NRCC	GINX-1	North American Ginseng (*Panax quinquefolius*) Root Extract	Ginsenosides (7); elements (9)	Elements (9)[a]	**25**

(Continued)

TABLE 1 | Continued

Source	CRM No.	CRM description	Certified values	Reference values	Total values
NIST	3253	Yerba Mate (*Ilex paraguariensis*) Leaves	Polycyclic aromatic hydrocarbons (PAHs) (5)	PAHs (13); proximates	**23**
NIST	3299	Turmeric (*Curcuma longa*) Rhizome	Curcuminoids (3); toxic elements (3)	-	**6**
NIST	3300	Curcumin Extract of Turmeric (*Curcuma longa*) Rhizome	Curcuminoids (3)	-	**3**
NIST	3398	Ginger (*Zingiber officinale*) Rhizome	Toxic elements (3)	Gingerols (3) and shogaols (3); arsenic species (3)	**12**
NIST	3399	Ginger (*Zingiber officinale*) Rhizome Extract	Toxic elements (3)	Gingerols (3) and shogaols (3);	**9**
NIST	8650	Kudzu (*Pueraria Montana* var. *lobata*) Rhizome	-	Isoflavones (3); toxic elements (3); DNA sequence (identity)	**6**
NIST	3268	Kudzu (*Pueraria Montana* var. *lobata*) Rhizome Extract	Toxic elements (3); nutritional element (1)	Isoflavones (3)	**7**
NIST	3269	Kudzu-Containing SODF	-	Isoflavones (3)	**3**
NIST	3384	Asian Ginseng (*Panax ginseng*) Rhizome	Toxic elements (2)	Ginsenosides (7); toxic elements (1)	**10**
NIST	3385	Asian Ginseng (*Panax ginseng*) Rhizome Extract	Ginsenosides (6); DNA sequence (identity)	Ginsenosides (1); toxic elements (3)	**10**

[a]*Information values for four elements.*

TABLE 2 | Currently available non-botanical dietary supplement matrix CRMs.

Source	CRM No.	CRM description	Certified values	Reference values	Total values
NIST	3280	Multivitamin/multielement tablets	Vitamins and carotenoids (13); elements (18)	Vitamins and carotenoids (4); elements (9)	**43**
NIST	3278	Tocopherols in edible oils	Tocopherols (4)		**4**
NIST	3275	Omega-3 and omega-6 fatty acids in fish oil	Fatty acids (31)	Fatty acids (23)	**54**
NIST	3275-1	Concentrate high in DHA	Fatty acids (9)	Fatty acids (7)	**16**
NIST	3275-2	Anchovy oil (high in DHA and EPA)	Fatty acids (11)	Fatty acids (7)	**18**
NIST	3275-3	Concentrate containing 60% long chain omega-3 fatty acids	Fatty acids (11)	Fatty acids (9)	**20**
NIST	3530	Iodized salt (Iodide)	Iodine (as iodide)	-	**1**
NRCC	VITA-1	Low-level multivitamin	Elements and element species (16); vitamins (1)	Elements (5)[a]; vitamins (7)[a]	**29**
NRCC	VITB-1	Elevated-level multivitamin	Elements and element species (16); vitamins (1)	Elements (5)[a]; vitamins (7)[a]	**29**
NIST	3279	Chromium-Containing Solid Oral Dosage Form	chromium	vanadium	**2**
NIST	8037	Krill Oil	-	fatty acids (22)	**22**

[a]*Informational values available for three elements and seven vitamins.*

the various matrices encountered in the market and used in research that may yield distinct analytical challenges, e.g., different concentrations of constituents of interest, differences in extractability of constituents from the matrix, and various potential interferences.

Certified reference materials for ephedra was the first high priority of the NIH-ODS/NIST collaboration and a suite of SRMs was developed, including aerial plant parts, extracts, solid oral dosage form (SODF), and ephedra-containing protein powder. Despite the discontinuation of this SRM suite following the U.S. Food and Drug Administration (FDA)'s ban of ephedra, the experience provided the model for the botanical matrix SRMs that have been developed subsequently (45). Botanical dietary supplement matrix CRMs, almost all of which are SRMs developed by NIST in collaboration with NIH-ODS during the past 20 years, are summarized in **Table 1**. In 2009, the portfolio of dietary supplement SRMs expanded to non-botanical supplements with the development of SRM 3280 Multielement/Multivitamin Tablets (46) as shown in **Table 2** and later included tocopherols in oil, chromium-containing SODF, and iodized salt. Several botanical dietary supplement NIST SRMs that are currently in development are listed in **Table 3**.

TABLE 3 | Selected in-development dietary supplement NIST materials.

Candidate SRM description	Proposed constituents for value assignment
Yohimbe (*Pausinystalia johimbe*) containing SODF	Yohimbine
Black Cohosh (*Actaea racemosa*) rhizome	Triterpene glycosides and toxic elements
Black Cohosh (*Actaea racemosa*) rhizome extract	Triterpene glycosides and toxic elements
Eluethero (*Eleutherococcus senticosus*) root	Eleutherosides and toxic elements
Eleuthero (*Eleutherococcus senticosus*) root extract	Eleutherosides and toxic elements
Ashwagandha (*Withania somnifera*) root	Withanosides and withanolides
Ashwagandha (*Withania somnifera*) root extract	Withanosides and withanolides
Kava (*Piper methysticum*)	Kava lactones

TABLE 4 | Currently available pure material and calibration solution CRMs for botanical dietary supplement marker compounds.

Source	CRM No.	CRM name	Certified values
NIST	SRM 3389	Ginsenosides calibration solution[a]	Ginsenosides (6)
NRCC	MIGS-1	Multi-component ginsenoside calibration solution	Ginsenosides (7)
NRCC	BERB-1	Berberine chloride[b]	Berberine and berberine chloride purity[c]
NRCC	CANA-1	Canadine[d]	Canadine[c]
NRCC	HYDR-1	Hydrastine[b]	Hydrastine
Cerilliant	G-013	*Ginkgo biloba* terpene lactones mix	Ginkgolides (4) and bilobalide
Cerilliant	G-014	*Ginkgo biloba* flavonoids mix	Flavonoids (3)
Cerilliant	G-015	Ginseng ginsenosides mix	Ginsenosides (8)
Cerilliant	G-016	Green tea catechin mix	Catechins (7); caffeine
Cerilliant	G-027	Ginger gingerols and shogaols mix[a]	Gingerols (3); shogaols (3)
Cerilliant	K-007	Kava kavalactone mix[a]	Kavalactones (9)

[a] *Developed in collaboration with NIH-ODS.*
[b] *Berberine and hydrastine are naturally occurring isoquinoine alkaloids in several plant dietary supplement ingredients, including goldenseal.*
[c] *Information values for trace impurities.*
[d] *Canadine is a benzylisoquinoline alkaloid present in plants from the family Papaveraceae.*

While the primary focus of the NIH-ODS/NIST collaboration has been to provide matrix SRMs for the dietary supplement community, several calibration solution SRMs were also developed by NIST between 2011 and 2016 for catechins, hypericin, organic acids, and isoflavones. Currently, the only botanical dietary supplement ingredient calibration solution available from NIST is SRM 3389 Ginsenosides Calibration Solution (47). Another calibration solution CRM for ginsenosides is also available from the National Research Council of Canada (the NMI for Canada) as well as three pure reference standards for constituents associated with plants used as ingredients in dietary supplements[13] (see **Table 4**). In an effort to expand the availability of calibration CRMs for botanical dietary supplement ingredient markers, the AMRM Program engaged the private sector to produce these analytical resources, with CRMs for key constituents of kava (*Piper methysticum* G. Forst.) and ginger (*Zingiber officinale* Roscoe) recently available from Cerilliant/MilliporeSigma (48). **Table 4** summarizes currently available, select calibration solution and pure chemical CRMs from NMIs and commercial sources, including those developed with support from NIH-ODS.

ANALYTICAL PRINCIPLES FOR CHEMICAL COMPOSITION VALUE ASSIGNMENT

To provide true values for chemical content, CRM producers typically use orthogonal analytical methods, meaning the methods are based on different measurement principles, an approach that NP researchers can apply for greater confidence in their chemical characterizations. The assignment of certified values for the chemical composition of CRMs at NIST is based primarily on the agreement of results from multiple independent analytical methods. The development of the multiple independent methods concept for assigning certified values for trace elements in matrix SRMs has been described, along with a discussion of the importance of independence in the physical principle upon which the measurement is based and in sample preparation, standards, and calibration (49). For the determination of elements in matrix SRMs, the concept of using multiple independent methods is relatively straightforward because there are a variety of analytical techniques that are based on different measurement principles [e.g., inductively coupled plasma-optical emission spectroscopy (ICP-OES), ICP-MS, neutron activation analysis] and different sample preparation approaches are available (e.g., direct analysis of a solid sample or dissolve the matrix and analyze the resulting solution). For the determination of trace organic constituents, independence in the analytical method is achieved in the sample preparation (i.e., extraction, clean-up, and isolation of the compounds of interest) and the final chromatographic separation and detection (e.g., GC vs. LC and UV/fluorescence detection vs. MS or MS/MS detection) (28, 50). Independence is also incorporated in the quantification approaches used, including the use of isotopically labeled internal standards [i.e., isotope dilution (ID) approach] for both elements and organic constituents. If the results from the multiple independent methods agree, the possibility of undetected bias in the resulting certified value is minimized. Epstein summarized the historical development of the multiple independent methods approach for trace element determination (49), and Wise et al. have discussed the application of this approach for trace organic constituents (28, 50). In 2000 NIST

[13] NRC Canada CRMs: https://nrc.canada.ca/en/certifications-evaluations-standards/certified-reference-materials.

TABLE 5 | Examples of reported use of SRM 3280 multivitamin/multielement tablets.

References	Uses			Comments
	Method dev./valid.	QC	Novel Research	
Roseland et al. (20)		X		Used as QC material to evaluate laboratory capabilities and measurement performance
Chen et al. (53)	X			Single-laboratory validation of HPLC-DAD method for water soluble vitamins in multivitamin tablets; SRM 3280 used for reproducibility assessment
Avula et al. (54)	X			Validation of ICP/MS method for 21 elements in dietary supplements; results for SRM 320 reported
Avula et al. (55)		X		Analyzed as control for determination of 16 elements in multivitamin supplements using ICP/MS
Bhandari and Van Berkel (56)	X			Validation of flow-injection MS/MS method for high-throughput determination of B vitamins in supplements
Matsumoto et al. (57)	X			Validation of LC-UV/visible method for vitamin B_{12} in MVM
Sullivan and Zywicki (58)	X			Results for the determination of iodine in SRM 3280
Bhandari et al. (59)	X			Validation of flow-injection MS/MS method for ascorbic and folic acid in multivitamin tablets; results compared with SRM 3280
Christopher and Thompson (60)	X			Determination of cadmium using ID-ICP/MS
Murphy and Vetter (61)	X			Determination of cadmium in dietary supplements
Raju et al. (62)	X			Method development for vitamin B_{12} using IC-ICP/MS
Yilmaz et al. (63)	X			Validation of solid phase extraction of Cu ions from high salt matrices prior to determination by flame atomic absorption spectrometry (FAAS); no results reported
Andrews et al. (64)		X		Investigated variability of vitamin D content in MVM products
Wolle et al. (65)	X			Extraction method development for determination of arsenic in dietary supplements using IC-ICP/MS
Kakitani et al. (66)	X			Validation of LC-MS/MS method for water soluble vitamins in dietary supplements and beverages; results reported for comparison
Pehrsson et al. (67)		X		Used for QC for determination of iodine content in food and dietary supplements
Qiu et al. (68), Novakova et al. (69)	X			Validation for flow-injection TiO_2-mediated UV-photochemical volatile species generation atomic absorption spectroscopy (AAS) method for determination of selenium in supplements; comparison results reported
D'Ulivo et al. (70)	X			Validation of ID-LC-MS/MS method for determination of cyanocobalamin (vitamin B_{12})
Andrews et al. (14)		X		Used as QC material for analyses used to provide data for the Dietary Supplement Ingredient Database
White et al. (71)	X			Method development for cadmium in multivitamin supplements using ID-ICP/MS with coprecipitation schemes
Qiu et al. (68)	X			Single-laboratory validation study for vitamin B_{12} (cobalamin) using RPLC with DAD; results reported and compared
Begu et al. (72)	X			Validation of ICP/MS method for determination of arsenic and cadmium in salt matrix of multivitamin supplements using sequential coprecipitation
Crighton et al. (73)	X			Investigated the application of Direct Sample Analysis (DSA)-TOF for screening adulterated dietary supplements

Uses include method development and/or validation and quality control (QC).

formalized the approaches, or modes, for assigning values and established a hierarchy of values (denoted as certified, reference, and information) and associated confidence in their accuracy based on the various approaches used (51). This document was recently updated with numerous examples illustrating the implementation of various certification modes (52).

CASE STUDIES OF RESEARCH USING DIETARY SUPPLEMENT MATRIX CRMs

A number of research studies investigating the composition and health effects of dietary supplements have utilized RMs and CRMs to support measurement rigor and reproducibility. The case studies and figures described below describe selected examples of where the utilization of specific RMs facilitated innovative method development or empowered investigations by playing a key role in generating and interpreting novel research data.

Method Development and Population Studies for Assessing DS Content and Exposure

NIST SRM 3280 Multivitamin/Multielement Tablet has been extensively used for method development applications and measurement verification of nutrient exposure assessments since its initial 2010 availability (**Table 5**). Van Berkel and coworkers used SRM 3280 in method development/validation studies for rapid and high-throughput determination of vitamins B_1, B_2,

TABLE 6 | Values for vitamin B_{12} in SRM 3280 from different method development studies.

References	Method	Value (mg/kg)	n^a	Comments
Chen et al. (74)	LC/UV	6.02 ± 0.05	15	Part of single laboratory validation study for method precision
Sander et al. (46)	Microbiological assays	4.9 ± 1.9	3,2	Value assignment based on two interlaboratory studies of 3 and 2 laboratories using microbiological assays
Wise and Phillips (75)	LC-ICP/MS	4.51 ± 0.38	10	Results used to update certified value
COA updated 2011		4.8 ± 1.0		Combined microbiological assay and LC-ICP/MS results for updated certified value
Matsumoto et al. (57)	LC-Visible	4.64 ± 0.11	24	
Raju et al. (62)	LC-ICP/MS	4.38 ± 0.05	2	
D'Ulivo et al. (70)	ID-LC-MS/MS	5.41 ± 0.18	4	Method used to certify two new MVM CRMs
Qiu et al. (68)	HPLC-DAD	4.28 ± 0.06	4	Single-laboratory validation study for vitamin B_{12} method

[a] *n = number of replicate measurements used to determine the value.*

B_3, B_5, and B_6 (56) and ascorbic acid and folic acid (59) using flow injection MS/MS without chromatographic separation and demonstrated good agreement with the certified values. Kakitani et al. validated an LC-MS/MS method for 15 water-soluble vitamins in dietary supplements and beverages by comparing results from the analysis of SRM 3280 (66). Chen et al. developed a single-laboratory validated method using LC with three different detection modes, i.e., diode array detection (DAD), fluorescence detection (FLD), and MS for the determination of seven B vitamins. SRM 3280 was used in the validation of the repeatability and ruggedness of the method; however, since this study preceded certification of the values for the vitamins, the method's accuracy was not assessed (53).

The development of non-microbiological assays to determine vitamin B_{12} (cyanocobalamin) in foods and DS has been the focus of several studies that utilized SRM 3280 to verify measurement accuracy. Chen et al. developed an LC-UV/Visible method to determine vitamin B_{12} in multivitamin/multielement DS (MVM) with improved efficiency through on-line sample clean-up, and they assessed accuracy, precision, recovery, limit of detection, and limit of quantification using SRM 3280 (74). Subsequent method development and validation by Matsumoto et al. analyzed SRM 3280 along with samples representative of multivitamin DS with or without elements and other NP ingredients like coenzyme Q10 to assess the accuracy and uncertainty range of their HPLC-UV detection method (57). D'Ulivo et al. developed a novel ID method for cyanocobalamin using an isotopically enriched $^{13}C^{15}N$ cyanocobalamin as the internal standard and validated the LC-MS/MS method using SRM 3280 (70). The validated ID-LC-MS/MS method was then employed to certify the content of cyanocobalamin in two multivitamin CRMs recently issued by the National Research Center of Canada (NRCC), i.e., VITA-1 and VITB-1. Qiu et al. utilized SRM 3280 in a single-laboratory validation study to demonstrate their HPLC-DAD method for determination of cobalamin in dietary ingredients and DS products (i.e., tablets, capsules, and chewable gels) achieved AOAC International Standard Method Performance Requirements (68). All of these method validation studies to determine vitamin B_{12} in SRM 3280 have provided valuable information toward assessing the true value, as shown in **Table 6**. The value for vitamin B_{12} in SRM 3280 was initially assigned using only results from two interlaboratory studies using microbiological assays and denoted as a reference value with a relatively large associated uncertainty (39%). However, this value was revised to 4.8 ± 1.0 mg/kg and upgraded to a certified value based on the combination of results from a NIST LC-ICP/MS method (4.51 ± 0.38 mg/kg). In the five method development studies in **Table 6**, the value for vitamin B_{12} ranged from 4.28 mg/kg to 6.02 mg/kg, with all methods reporting low Relative Standard Deviations (RSD). These results illustrate the analytical challenge associated with vitamin B_{12} measurements in MVM and emphasize the need to use a CRM to assess the accuracy of the analytical method.

The use of SRM 3280 for the determination of minerals and toxic elements (As, Cd) for both method development and validation has been reported by several researchers using ICP/MS (54, 55, 60, 71, 72) and AAS (63, 69). Wolle et al. used SRM 3280 for extraction method development in the determination of arsenic in dietary supplements (65). Sullivan and Zywicki used SRM 3280 in a single-laboratory validation study for an ICP/MS method for the determination of total iodine in various foods and DS (58). To assess precision and accuracy, they reported the individual measurements for 20 replicates ranging from 86 to 102% recovery resulting in a mean result of 125 ± 11 μg/kg (95% confidence interval) compared with the certified value of 132.7 ± 6.6 μg/kg; ruggedness was assessed by comparing results from a second analyst for six replicates [mean of 137 μg/kg (RSD = 3.8%)], and combined results were 128 μg/kg (RSD = 5.9%, $n = 26$).

The consumption of DS contributes significantly to vitamin and mineral intake in U.S. populations, and clinical studies of dietary interventions should take this baseline exposure into account when designing trials and interpreting results. The Dietary Supplement Ingredient Database (DSID), a collaborative effort between NIH-ODS and U.S. Department of Agriculture (USDA), supports research on DS health effects

by providing analytically determined estimates for the nutrient and phytochemical content of representative DS products marketed to certain populations, such as MVMs for adults or children or prenatal supplement products. DSID investigations routinely use NIST SRMs to verify laboratory measurements of vitamins, minerals, fatty acids, toxic elements, and botanical phytochemicals. DSID studies have found wide ranges of ingredient content variability compared to DS product label claims and trends of concentration overages (14, 64, 76), and have investigated if certain constituents, such as caffeine, may reach levels of concern (77). By using CRMs, DSID researchers thus established measurement confidence in their conclusions on whether exposures from DS may approach Tolerable Upper Intake Levels for nutrients or otherwise cause safety concerns for NP constituents.

Vitamin D and 25-Hydroxyvitamin D_3

A USDA-coordinated study to assess the measurement capabilities for vitamin D_3 and its primary metabolite, 25-hydroxyvitamin D_3 [25(OH)D_3], in food and dietary supplement matrices (78) provides an excellent example of using existing matrix RMs to assess analytical methods for analytes that do not already have value assignments. Vitamin D_3 and vitamin D_2 are metabolized in animals to 25(OH)D_3 and 25(OH)D_2, respectively, which may be present in animal tissues along with unmetabolized vitamin D_3 and vitamin D_2. Studies suggest that 25(OH)D_3 may be more potent than vitamin D in elevating serum levels of total 25(OH)D, which is the sum of 25(OH)D_3 and 25(OH)D_2 and the common clinical marker for vitamin D status (78). Therefore, accurate measurement of 25(OH)D in food and dietary supplements is critical to provide reliable estimates of vitamin D intakes. The goal of the USDA study was to assess the capabilities of selected laboratories to measure 25(OH)D in food and dietary supplement matrices and the potential use of these materials as reference materials [i.e., assign values for vitamin D and 25(OH)D]. Although no DS matrix RMs were included in the study, three existing food matrix SRMs that did not have values assigned for vitamin D or 25(OH)D were included, namely bovine liver (SRM 1577c), whole egg powder (SRM 1845a), and meat homogenate (SRM 1546a). Results from this study were later used by NIST, in conjunction with results from an ID-LC-MS/MS method at NIST, to assign values for vitamin D_3 and 25(OH)D_3 in these food matrix SRMs, the first CRMs with values assigned for 25(OH)D_3 (79).

Green Tea SRMs in Metabolomic Studies

Excellent examples of the use of CRMs in botanical research studies are found in recent studies leveraging metabolomics in characterizing *Camellia sinensis* (L.) Kuntze preparations and assessing variability among products. Punyasiri et al. (80) used NIST SRM 3254 (*C. sinensis*) Green Tea Leaves to aid their development of optimized sample preparation approaches for metabolic profiling that mitigated degradation of key flavonoids; the matrix SRM's certified values for major catechins enabled confirmation of their measurement accuracy and precision. Kellogg et al. (81) used SRM 3254 in a study comparing conventional solvent maceration vs. accelerated solvent extraction for metabolomic characterization; however, rather than comparing their measurements to certified values, they instead used the SRM as one of four samples to evaluate differential catechin extraction by the two different methods. In a study by Tian et al., SRM 3254 was included as one of five green tea samples to demonstrate the feasibility of a biochemometric approach that combined metabolic profiling with a bio-fractionation assay to identify intestinal UDP-glucuronosyltransferase inhibitors in green tea, and the results for the SRM were found to be qualitatively similar to the other samples (82).

In a study that compared metabolomic approaches to evaluate phytochemical variability, Kellogg et al. used three NIST green tea SRMs (leaves, extract, and SODF) as "positive controls" as part of their evaluation of untargeted UPLC-MS, targeted quantitative UPLC, and untargeted ^{1}H nuclear magnetic resonance spectroscopy (NMR) to assess chemical similarity and variability among 34 different commercial green tea products (83). Additionally, SRM 3254 was used to demonstrate catechin extraction reproducibility and evaluate hot water vs. methanol extraction efficiency. All three green tea SRMs were analyzed as part of the sample set to determine a set of 16 targeted marker compounds. Principal component analyses (PCA) of the three metabolic approaches found that the various green tea samples clustered with the respective matrix-matched SRMs (**Figure 2**) and demonstrated that the untargeted MS-based metabolomics was more effective in discriminating among the classes of green tea products than either untargeted UPLC-MS or ^{1}HNMR metabolomics. Another key finding was that had they relied on PCA analysis based solely using targeted marker compounds, which represent only a subset of the chemical diversity of the various green tea samples, they might have overlooked the actual chemical dissimilarity of the various products. Leveraging the full NIST suite of green tea SRMs, which represented the three categories of products (ground leaf, extract, and SODF), was instrumental in confirming this conclusion.

In a follow-up study from Cech and colleagues, Clark et al. employed the same green tea commercial samples and SRMs to conduct an interlaboratory comparison of untargeted MS data sets to investigate underlying causes for measurement variability (84). To determine if there was experimental value in performing replicate extractions and replicate injections of the same botanical extract, the authors extracted metabolites from SRM 3254, and the samples were analyzed in two different laboratories using the same LC column, LC gradient, and mass spectrometer acquisition parameters but on different mass spectrometer platforms. The study showed that a significant portion of the features detectable in single replicate measurements are not observed in two out of the three replicates, suggesting that there is little value to conducting replicate injections of replicate extracts if features that appear only once are omitted from analyses. Clark et al. hypothesized that variations in the generated composition feature lists between the two instruments were driven by differences in feature formation for the same molecule sets. To address this, they expanded their study to the larger sample set of 37 green tea samples, including the three NIST green tea SRMs. The results are summarized in **Figure 3** with a Venn diagram and

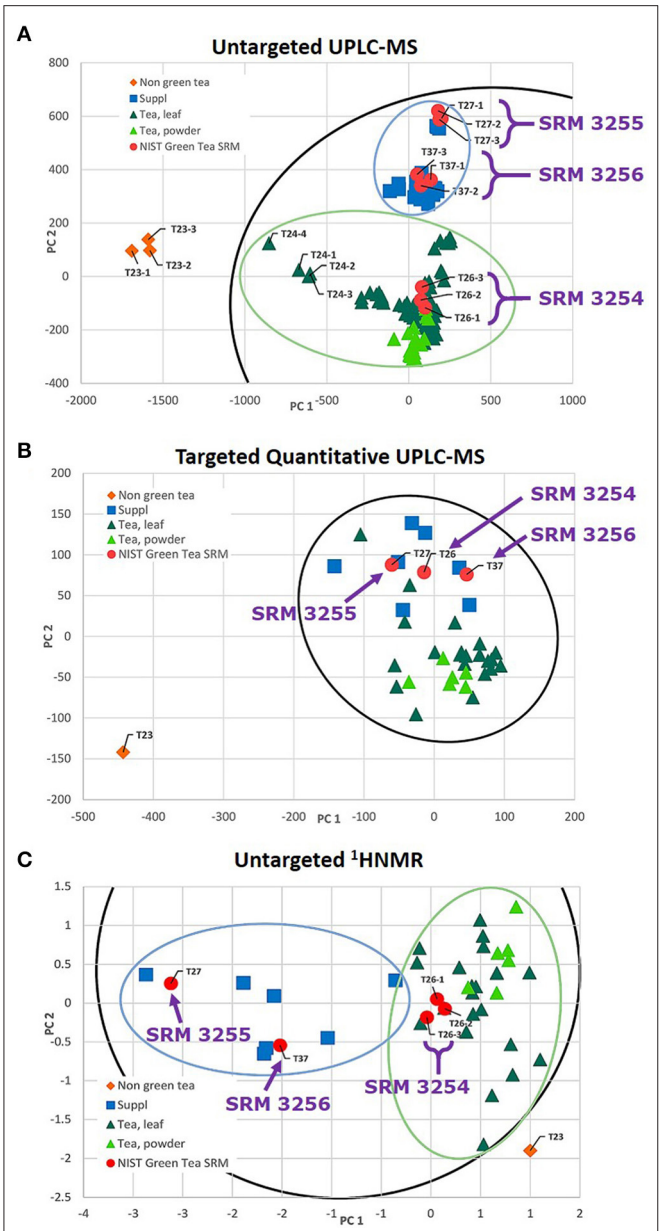

FIGURE 2 | Principal component analysis (PCA) score plots of green tea samples drawn with Hotelling's 95% confidence ellipse for **(A)** untargeted UPLC-MS, **(B)** targeted quantitative UPLC-MS, and **(C)** Untargeted ^{1}HNMR. Data points representing triplicate green tea samples were closely clustered, and distinct clusters were observed between green tea supplements (denoted as Suppl), green teas (leaf and powder), and the negative control (turmeric-ginger T23 denoted as non-green tea). Representative samples are highlighted, including NIST SRM 3254 (T26), SRM 3255 (T27), and SRM 3256 (T37), to demonstrate the reproducibility of the extraction and analytical protocol. Adapted with permission from Kellogg et al. (83); further permissions related to this excerpted material should be directed to the American Chemical Society.

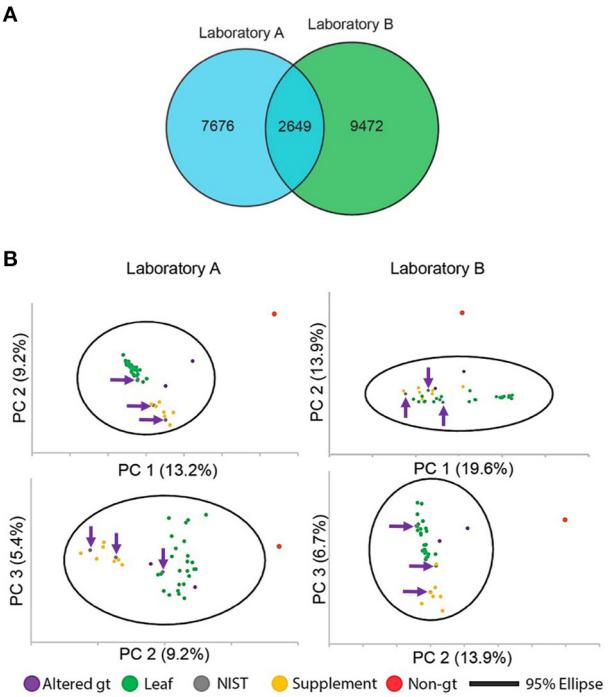

FIGURE 3 | Comparison of MS features derived from untargeted MS analyses by two independent labs for green tea [*C. sinsensis* (L.) Kuntze] samples and unknowns, including 27 leaf or powder ingredients, seven supplement products, a non-green tea turmeric-ginger tea (non-gt), and three NIST green tea SRMs (SRM 3254 leaf, SRM 3255 extract, and SRM 3256 SODF). **(A)** Venn diagram of unique feature list counts and shared features between laboratories A (blue) and B (green). **(B)** Principal component analysis (PCA) score plots drawn with Hotelling's 95% confidence ellipse from Laboratory A (left) and Laboratory B (right). Top plots are PC1 vs. PC2; bottom plots are PC2 vs. PC3. Green tea ingredients (leaves and powders) were qualitatively separated from DS products, and NIST SRMs (gray dots, highlighted with purple arrows) were plotted within matrix matched clusters. Reprinted (adapted) with permission from Clark et al. (84). American Chemical Society.

PCA score plots. As in the previous study, the PCA score plots were successful in discriminating among the three sample types with each of the three SRMs in the expected matrix category. The study of Clark et al. emphasizes that "untargeted metabolomics feature lists are not a description of the chemical composition of the sample, but rather an instrument-specific snapshot of how the chemical entities in the sample respond to the analysis by the particular mass spectrometer" (84). This conclusion supports the need for metabolomic researchers to use commonly available and well-characterized samples, such as the NIST SRMs, to facilitate the comparison of results among laboratories.

Fish and Botanical Oil SRMs for Determination of Fatty Acids

The determination of fatty acids using GC with flame ionization detection (FID) and GC-MS are relatively mature measurement techniques in food and dietary supplement analysis, and as a result, matrix-based CRMs to support these analyses have been available since the late 1990's (75). With the importance of

fatty acids in nutrition studies, particularly omega-3 and omega-6 compounds, SRM 3274 Botanical Oils Containing Omega-3 and Omega-6 Fatty Acids and SRM 3275 Omega-3 and Omega-6 Fatty Acids in Fish Oil were developed as suites of four and three different mixtures of botanical and fish oils, respectively, to provide different levels and different ratios of the individual fatty acids. A krill oil material, which represents a different matrix used as dietary supplements and has mass fractions of fatty acids a factor of 1,000 greater than the fish oil materials, was issued in 2020 as RM 8037. The relative mass fractions of the botanical and marine oil SRMs and RM are shown in **Figure 4** (85).

SRM 3275 has found wide use to validate methods and serve as a QC material as demonstrated by Srigley and coworkers (86–89) at the FDA reporting several studies using SRM 3275 for validation of methods. In a study to evaluate the overall fatty acid composition of 46 marine oil omega-3 supplements, Srigley and Rader analyzed SRM 3275 (all three levels) using GC-FID on a novel ionic liquid phase column (90). In addition to comparisons of their results for 21 fatty acids to the assigned values in SRM 3275, with excellent agreement, the authors also showed GC-FID chromatograms for SRM 3275 on this novel GC stationary phase, thus providing valuable information for comparison with separations on other stationary phases and with other researchers. Li and Srigley (87) later validated a novel method for the quantification of long-chain omega-3 polyunsaturated fatty acids in gummy dietary supplements using SRM 3275 and compared their results for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with the certified SRM values with agreement in all three levels within 5%. Karunathilaka et al. used the fish oil SRM to validate a method for rapid classification and quantification of marine oil omega-3 supplements using Attenuated total reflection—Fourier transform infrared spectroscopy (ATR-FTIR), Fourier transform—near infrared spectroscopy (FT-NIR), and chemometrics (86, 88). In an evaluation of four ionic liquid columns for rapid analysis or the improved resolution of long-chain methyl and ethyl esters of omega-3, omega-6, and additional positional isomeric and stereoisomeric blends, Weatherly et al. used SRM 3275 for method validation (91). Khoomrung et al. compared results from a new fast preparation of fatty acid methyl esters by microwave-assisted derivatization with the certified values from SRM 3275-2 for method validation (92).

Ahn et al. from the Korea Research Institute of Standards and Science used SRM 3274 to validate their ID-GC-MS candidate reference method for the determination of three essential fatty acids (linoleic, α- and γ-linolenic acid) in supplement oil products (93). The results for the three fatty acids in the borage, evening primrose, and flax oil were in excellent agreement with the SRM certified values. Although not intended specifically for fatty acid determinations, two saw palmetto [*Serenoa repens* (W. Bartman) Small] matrix SRMs have been available for over a decade (SRM 3250 Saw Palmetto Fruit and SRM 3251 Saw Palmetto Extract) with values assigned for phytosterols and fatty acids (94, 95). As an oil extract, SRM 3251 has been used for QC in the determination of both phytosterols and fatty acids in saw palmetto supplements (96) and in virgin olive oil (97).

Authentication of Black Cohosh

The value of matrix RMs in the development of novel methods for botanical characterization and identification is demonstrated in studies using chemical fingerprinting approaches for the authentication of black cohosh (*Actaea racemosa* L.). Investigations led by the USDA Methods and Application of Food Composition Laboratory utilized authenticated reference materials from multiple sources, including NIST candidate SRMs, for *A. racemosa* and four related species (*A. dahurica*, *A. pachypoda*, *A. podocarpa*, and *A. foetida*) in the development of methodological approaches for authentication that used chemical analyses such as LC, MS, or NMR combined with PCA and soft independent modeling of class analogy (SIMCA) (98–100). These data demonstrated an effective approach to differentiate *A. racemosa* roots and rhizomes from those of other related species and possible contaminants using statistical models built from the characterization of the authenticated RMs (98). Detailed assessments of the chemical fingerprints lead to the identification of ester and amide derivatives of hydroxycinnamic acids as novel marker compounds for authentication (99, 100). Therefore, using authenticated plant material RMs in these innovative statistical models supported rigorous non-targeted examinations of commercially available *Actaea* ingredients. These models for *Actaea* species discrimination by hydroxycinnamic acids provided good sensitivity and accuracy for plant materials. Notably, using these models for authenticity predictions for finished supplement products required targeted profiling of stable marker compounds, since processing into final dosage forms introduces additional chemical variation. Furthermore, while the related *Actaea* species could be differentiated from one another, analyses of the different RMs for *A. racemosa* rhizomes did not result in a single cluster in the PCA modeling. Instead, several factors such as growing location, harvest conditions, handling, or post-harvesting processing or storage conditions were suggested to result in sufficient chemical variation even among authenticated RMs. These studies serve to illustrate important points. First, where possible, employing orthoganol methods is a powerful approach for botanical authentication. Second, phytochemical variation is to be expected in botanical preparations and must be accounted for by any model that attempts to establish authenticity. Third, an authenticated RM or standard that is broadly available to the research community can help validate techniques that identify the genus and species or plant part. Researchers should be mindful, however, that any given single RM does not represent all possible inherent biological, environmental, and processing variability.

Assessing Botanical Variability, Product Composition, and Formulation Performance

In addition to the identification and authentication of NPs, matrix-matched RMs are also valuable in validating quality control measurements and the detection of adulteration

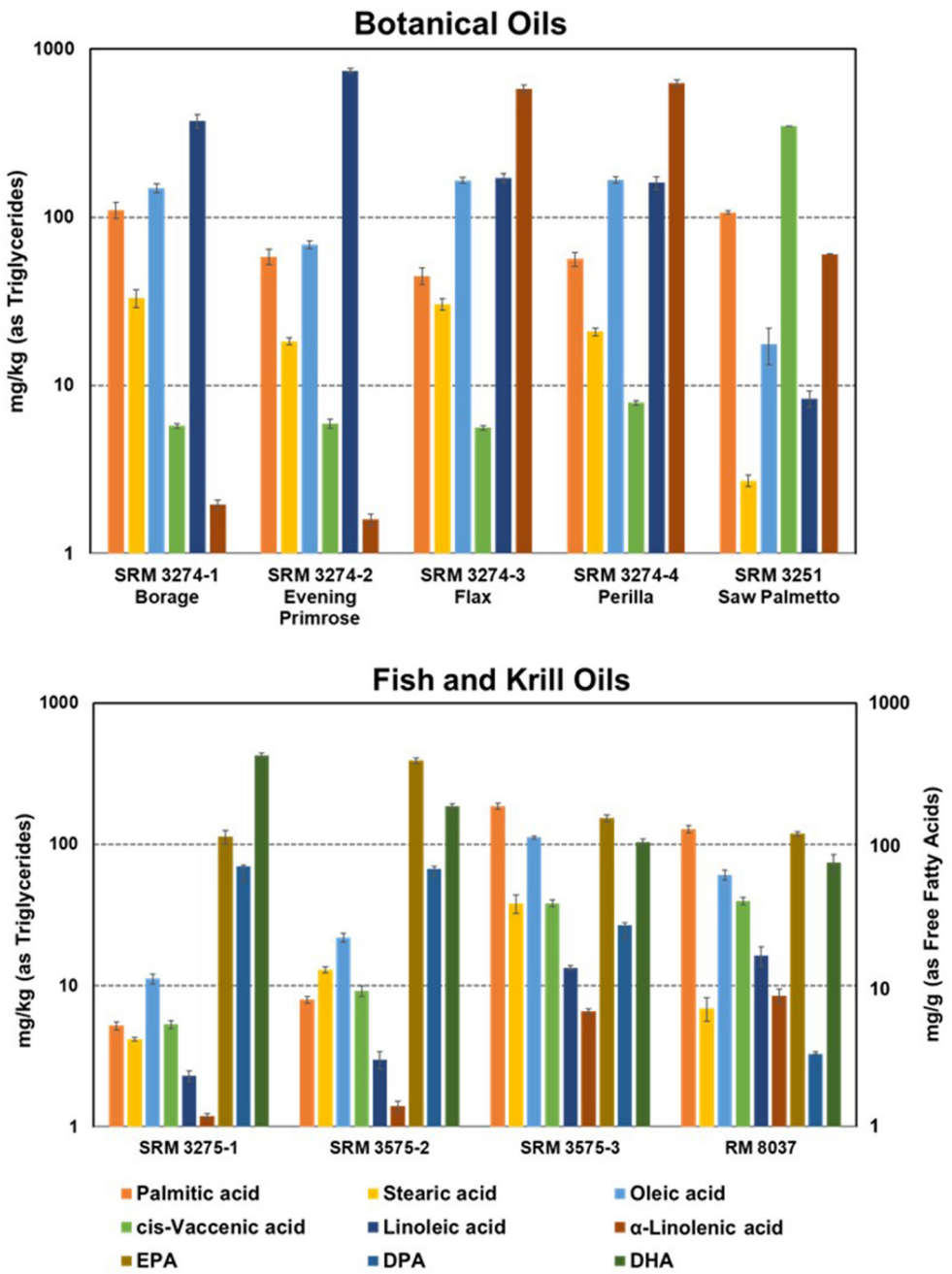

FIGURE 4 | Bar graphs of the distribution of mass fractions of selected fatty acids (as triglycerides) in marine and botanical oil SRMs and RMs. Note the logarithmic scale for the mass fractions, and for NIST RM 8037 Krill Oil the units are mg/g (as free fatty acids). Error bars are the expanded uncertainties of the certified and reference values with 95% confidence. Adapted and reprinted with permission from Springer, *Anal. Bioanal.Chem.*, Wise and Phillips (75).

or contamination, essential considerations when designing intervention studies of dietary supplement health effects. Chromatographic profiling of phytochemicals, whether LC or high-performance thin-layer chromatography (HPTLC), is a well-established approach to querying the quality of botanical preparations, and RMs are routinely employed as comparators to experimental samples with an unknown provenance and/or quality. For example, pharmacopeial standards and CRMs were used as references in an investigation of *Ginkgo biloba* L. leaf and extract products that found the majority of samples contained elevated levels of quercetin and/or rutin, or low levels of marker metabolites when compared with chemically well-characterized reference standards (101). Importantly, the ability to match *G. biloba* leaf products to a leaf CRM and the extract products

to an extract CRM fostered a higher level of confidence in the phytochemical profile comparisons.

Recently there has been an increased push for the application of more non-targeted and orthogonal methods in natural product quality control since assessments focusing on a limited number of chemical constituents or based on insufficiently distinct profiles can be prone to false conclusions. Here again, matrix-based CRMs offer valuable benefits to researchers. Beyond their role in confirming the presence and appropriate amounts of known compounds, RMs can support the systematic establishment of distinct chromatographic profiles for specific botanical preparations. For example, Napolitano et al. utilized NIST SRM 3255 (Green Tea Extract) to evaluate a novel quantitative ^{1}H NMR (qHNMR) method and an orthogonal traditional LC-MS/MS method for multi-targeted determination of major catechins (102). Comparison *via* the SRM demonstrated agreement of the two methods for catechin measurements, and overall the study highlighted potential benefits of incorporating qHNMR analyses into natural product authentication and characterization. In another example, Harnly and colleagues used pharmacopeial standards and CRMs in their determination of chromatographic profiles of *G. biloba* leaves and processed materials that included over 40 flavonoids and terpene lactones, more than 20 of which were newly identified as *G. biloba* constituents (103). In a subsequent study, CRMs were included among authenticated samples to create a one-class SIMCA modeling approach that could easily detect adulteration with isolated phytochemicals like rutin and quercetin (104). Furthermore, these studies illustrated how the content of certain *G. biloba* phytochemicals, specifically biflavones, is differentially affected by processing during extraction and product manufacture and how excipients in formulations can prohibit the detection of adulterants by some analytical methods.

DSID studies used the suite of NIST green tea SRMs as analytical quality control materials to assure rigor and reproducibility in measurements of 32 different commercial products that determined percent differences between labeled and actual catechin content (105). DSID studies also leveraged the certified values for catechins in NIST green tea SRMs as measurement controls in experiments that quantified the extent to which different dosage forms disintegrated and how they affected catechin dissolution (106). These DSID studies highlight the importance of assessing formulation performance and considering the bioavailablity of the constituents when designing clinical intervention studies of dietary supplements.

Dietary Supplement Laboratory Quality Assurance Program

In conjunction with the NIH-ODS, NIST established a Dietary Supplement Laboratory Quality Assurance Program (DSQAP)[14] in 2007 to assist laboratories in improving measurements of active and marker compounds, nutritional elements, toxic elements, organic nutrients (e.g., vitamins and carotenoids), and contaminants in DS and food matrices. In the DSQAP, participating laboratories analyze unknown DS and food samples provided by NIST, and the results are then compared with either NIST assigned target values or a laboratory consensus value from the study (107, 108). NIST conducted 15 exercises from 2007 through 2017, typically consisting of five or six studies each, and thus covered over 90 different analyte/matrix combinations. In 2017, DSQAP was incorporated into a restructured Health Assessment Measurements Quality Assurance Program (HAMQAP)[15], which expanded focus to include analysis of samples representative of both human intake (i.e., food and dietary supplements) and output (i.e., blood, serum, urine). From 2017 through 2021, HAMQAP conducted six exercises which have included ∼34 studies that have increased emphasis on analyte/DS matrix combinations relevant to emerging research on DS metabolism and health effects. By participating in the DSQAP and HAMQAP, laboratories can demonstrate and assess their measurement capabilities and accuracy by comparison with NIST assigned or study consensus results.

In the majority of the QAP studies, NIST SRMs or candidate SRMs (i.e., an SRM currently in progress that may or may not have values assigned) are distributed for analysis as unknown or known samples. When a candidate SRM is used, the results from the interlaboratory study may be used in combination with NIST measurements to assign the certified values. Several DS matrix SRMs have been used numerous times in these studies, including the multivitamin/multielement tablets, green tea, and fish and botanical oil materials (e.g., see NISTIR[16] 7997, 8203, and 8308). Studies for the determination of toxic elements (arsenic, cadmium, lead, and mercury) in DS, particularly botanical matrices, have high levels of participation with 20–30 laboratories typically submitting results, in part because such analyses are routinely required to verify the safety of raw materials intended for use in the manufacture of dietary supplements. Representative results from a DSQAP study for the determination of arsenic in ginger rhizome (SRM 3398) are illustrated in **Figure 5** with laboratory results presented from lowest to highest with color-coding to denote the different analytical techniques used (the majority use ICP/MS) with the consensus value with tolerance limits indicated.

A similar plot is shown in **Figure 6** for the determination of curcumin in turmeric extract (SRM 3300) and demonstrates excellent agreement of the study consensus value (820 ± 22 mg/g) and the NIST target value (822 ± 22 mg/g). The determination of curcuminoids in turmeric samples was the focus of two studies in DSQAP Exercise M (109) and Exercise O (110), with SRMs 3299 and SRM 3300 analyzed in both exercises. The second study for curcuminoids in turmeric was conducted with the intent of providing reproducibility and accuracy data to support moving the method for quantification of curcuminoids to final action status for AOAC *Official Method of Analysis (OMA)*. A total of eight participants used the AOAC OMA 2016.16

[14]NIST DSQAP: https://www.nist.gov/programs-projects/dietary-supplement-laboratory-quality-assurance-program.

[15]NIST HAMQAP: https://www.nist.gov/programs-projects/health-assessment-measurements-quality-assurance-program.

[16]NIST Interagency/Internal Reports: https://www.nist.gov/nist-pub-series/nist-interagencyinternal-report-nistir.

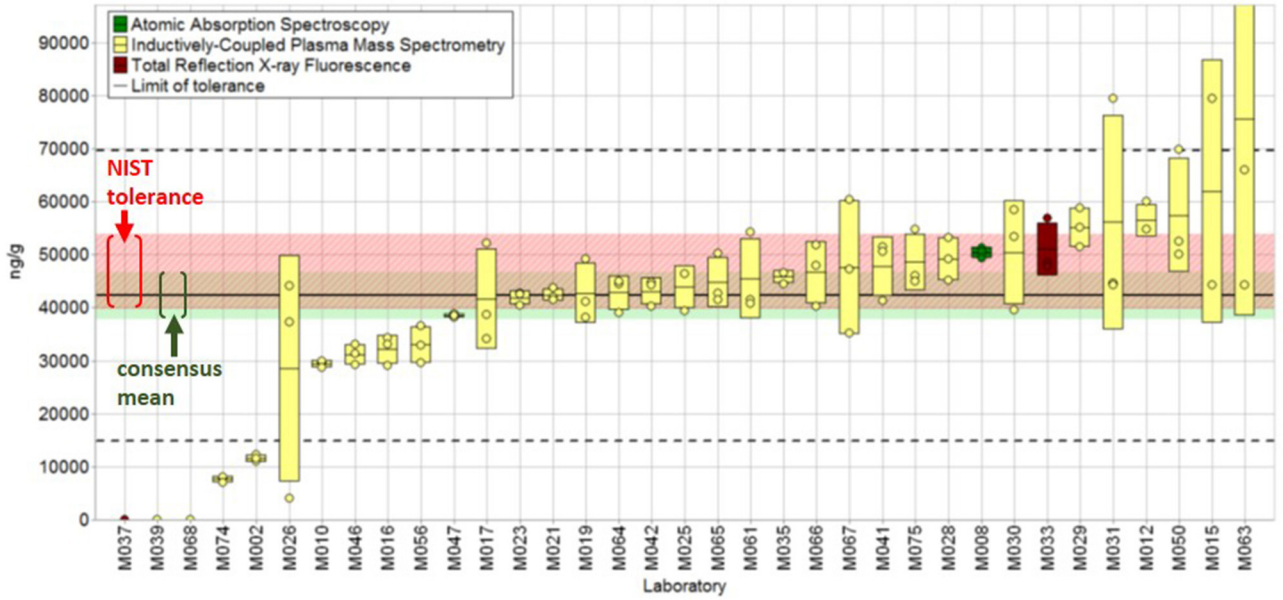

FIGURE 5 | Total arsenic (ng/g) in NIST SRM 3398 Ginger (*Z. officinale* Roscoe) Rhizome. Individual laboratory data are plotted (circles) with standard deviation ($n = 3$, rectangle) in order of increasing magnitude. The color of the data rectangle indicates the analytical method employed. The solid black line is the consensus mean, and the green shaded region represents the consensus mean bounded by twice the consensus standard error. The black dashed lines represent the consensus range of tolerance calculated as the values above and below the consensus mean that result in an acceptable Z'_{comm} score. The red shaded region represents the NIST range of tolerance, which encompasses the NIST-determined value bounded by twice its uncertainty and represents the range that results in an acceptable Z'_{NIST} score. For a detailed discussion of the statistical treatment of the results, see Phillips et al. (109). Adapted with permission from Phillips et al., NISTIR 8203, 2018.

HPLC-DAD method for the quantification of curcuminoids (blue data rectangles in **Figure 6**), and these DSQAP results were reported by Mudge et al. as part of a multi-lab method validation study (111). The results for the two curcuminoid exercises are summarized in **Table 7** and demonstrate the significant improvement in the overall performance of the laboratories as indicated by standard deviations of the consensus values decreasing from 17–20% to 3.6–5.7% for the turmeric rhizome and from 11–34% to 1.9–3.4% for the turmeric extract. A similar improvement is also observed for the results from two studies of the measurement of catechins in the green tea extract (SRM 3254) (see **Table 7**), with improvements from 10 to 72% in the 2012 study (DSQAP Exercise I) to 1–11% in the 2020 study (HAMQAP Exercise 5). In particular, results for the participant labs' measurement of epigallocatechin gallate (EGCG) improved more than 10-fold, as indicated by the reduction in the standard deviation of the consensus means between the two studies.

The QAP exercises provide valuable information regarding the current state of measurement capabilities of laboratories and the need for CRMs for specific analyte/matrix combinations. In addition, laboratories participating in the QAP are introduced to the availability of dietary supplement matrix SRMs and the benefit of using them as part of their laboratory quality control procedures.

Botanical Product Safety Assessments

Perhaps the most direct application of natural product reference materials to public health research is their use in safety evaluations. Whether an experimental natural product preparation will be used in *in vitro* cellular models or administered to animals or human subjects, it is incumbent upon researchers to assess the test article for known contaminants. First and foremost, when used as controls, reference materials enable researchers to confirm that their methods are fit for purpose for the detection of known toxins such as toxic metals, pesticides, or certain microbes and phytochemicals or secondary metabolites. Second, reference materials have been used as comparators for chemical composition in investigations of natural product preparations with putative health risks or unknown hazard profiles.

Analyses for known toxic constituents, whether from natural plant sources (e.g., pyrrolizidine alkaloids) or those incurred from the environment or industrial processing (e.g., mercury or lead), often must be performed quantitatively to assure safety through compliance with regulatory limits. In this context, the use of an appropriate CRM offers a clear advantage over less characterized qualitative standards. Investigations for the presence of toxic elements in NPs are routinely published in the peer-review scientific literature, and the utilization of CRMs with value assignment(s) for the toxin(s) of interest supports increased experimental rigor. A survey on the presence or absence of hazardous constituents in commercial dietary supplement products or traditional medicine preparations, and its subsequent implications on safety, has a higher level of confidence when CRMs are used to confirm measurement accuracy and reliability (112–114).

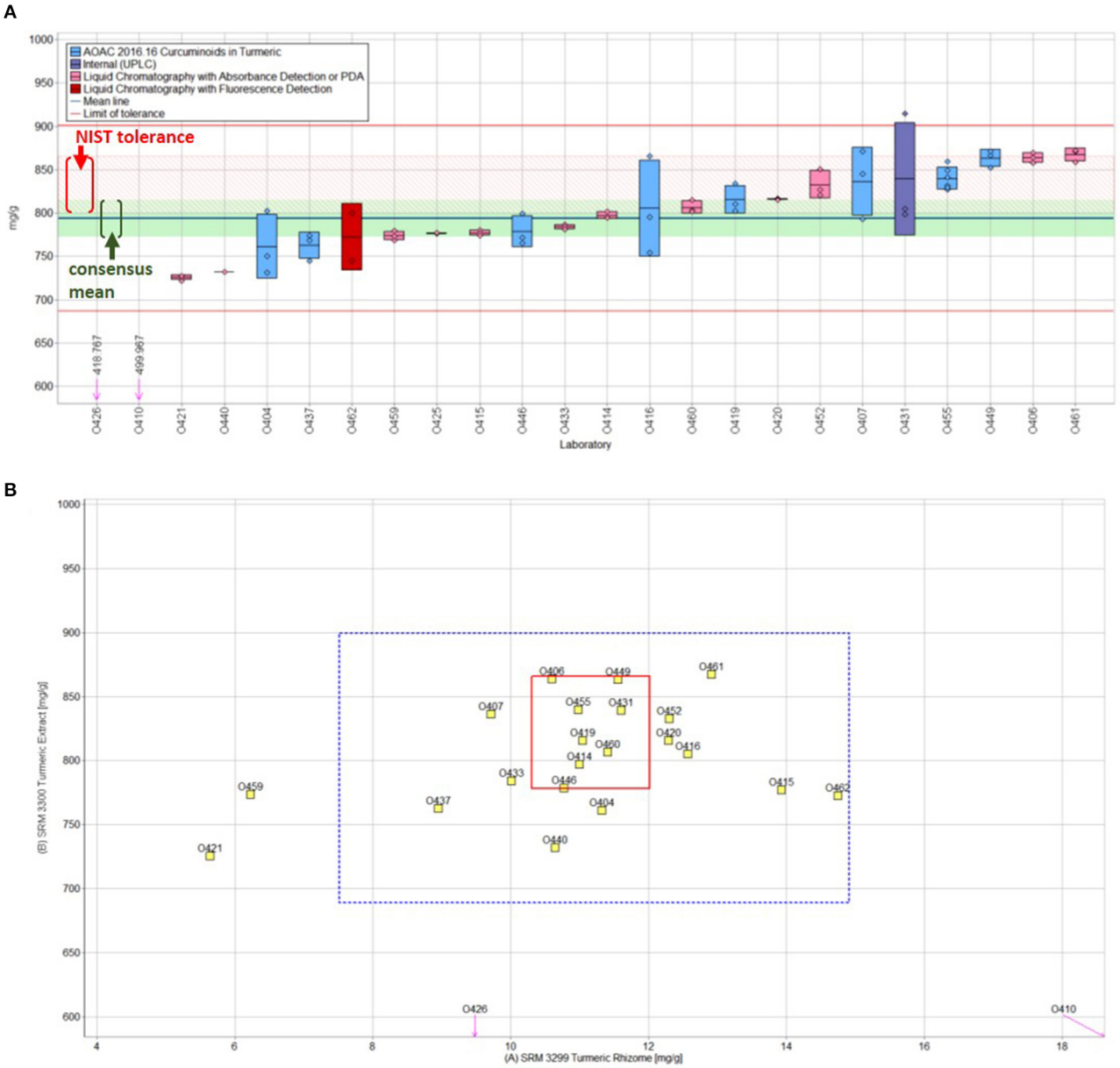

FIGURE 6 | (A) Curcumin (ng/g) in NIST SRM 3300 Turmeric (*C. longa* L.) Rhizome Extract. Individual laboratory data are plotted (circles) with standard deviation (n = 3, rectangle) in order of increasing magnitude. The color of the data rectangle indicates the analytical method employed. The solid blue line is the consensus mean, and the green shaded region represents the 95% confidence interval for the consensus mean. The solid red lines represent the consensus range of tolerance calculated as the values above and below the consensus mean that result in an acceptable Z'_{comm} score. The red shaded region represents the NIST range of tolerance, which encompasses the NIST-determined value bounded by twice its uncertainty and represents the range that results in an acceptable Z'_{NIST} score. **(B)** Laboratory means for curcumin in NIST SRM 3299 Turmeric Rhizome and SRM 3300. The laboratory mean for the turmeric rhizome is compared to the mean of the extract for each laboratory. The solid red box represents the NIST range of tolerance for the two turmeric SRMs, rhizome (x-axis) and extract (y-axis), as the dotted blue box represents the consensus range of tolerance described for **(A)**. For a detailed discussion of the statistical treatment of the results, see Barber et al. (110). Adapted with permission from Barber et al., NISTIR 8266, 2019.

Beyond the presence of known toxic constituents, questions of safety for a complex natural product may arise, such as whether consumption of a particular botanical species or extract thereof has long-term deleterious effects. Matrix-based CRMs have been leveraged in safety investigations in evaluating the identity and composition, and thus experimental relevance, of test articles. For example, safety studies conducted by the U.S. National Toxicology Program (NTP) attempt to

TABLE 7 | Results for determinations of curcuminoids in turmeric and catechins in green tea in multiple QAP exercises.

Curcuminoids	**Target**		**DSQAP exercise M (*n* = 17–23)**		**DSQAP exercise O (*n* = 22–25)**	
	SRM 3299	**SRM 3300**	**SRM 3299**	**SRM 3300**	**SRM 3299**	**SRM 3300**
Bisdesmethoxycurcumin	3.390 ± 0.054	18.25 ± 0.49	3.23 ± 0.66 (20)	16.2 ± 5.5 (34)	3.16 ± 0.16 (5.7)	17.3 ± 0.58 (3.4)
Desmethoxycurcumin	3.634 ± 0.64	117.1 ± 1.2	3.26 ± 0.54 (17)	116 ± 13 (11)	3.63 ± 0.13 (3.6)	117 ± 2.2 (1.9)
Curcumin	11.17 ± 0.21	822 ± 11	11.6 ± 2.1 (18)	801 ± 123 (15)	11.20 ± 0.43 (3.8)	822 ± 22 (2.7)
Catechins	**Target**		**DSQAP exercise I (*n* = 17–28)**		**HAMQAP exercise 5 (*n* = 6–12)**	
			SRM 3255			
Catechin	8.88 ± 0.90		9.84 ± 2.37 (24)		7.95 ± 0.88 (11)	
Epicatechin	45.8 ± 6.5		43.2 ± 5.8 (13)		38.3 ± 2.1 (5.5)	
Epicatechin gallate	97.2 ± 7.6		95.1 ± 13.4 (14)		94.9 ± 3.5 (3.7)	
Epigallocatechin	79.2 ± 6.3		62.9 ± 34.8 (55)		82.4 ± 6.6 (8.0)	
Epigallocatechin gallate	409 ± 18		408 ± 39 (10)		406 ± 2.5 (0.6)	
Gallocatechin	21.3 ± 1.6		28.0 ± 20.2 (72)		19.8 ± 1.9 (9.6)	
Gallocatechin gallate	37.8 ± 1.9		43.1 ± 10.6 (25)		42.8 ± 1.5 (3.5)	

Target (NIST-assigned) values and participant consensus (QAP exercise) values are listed for distributed SRMs. The range for the number of labs which reported data for individual analytes is noted (n); the uncertainty for each result is the standard deviation; the percent RSDs of the consensus values for each analyte are indicated in parentheses.

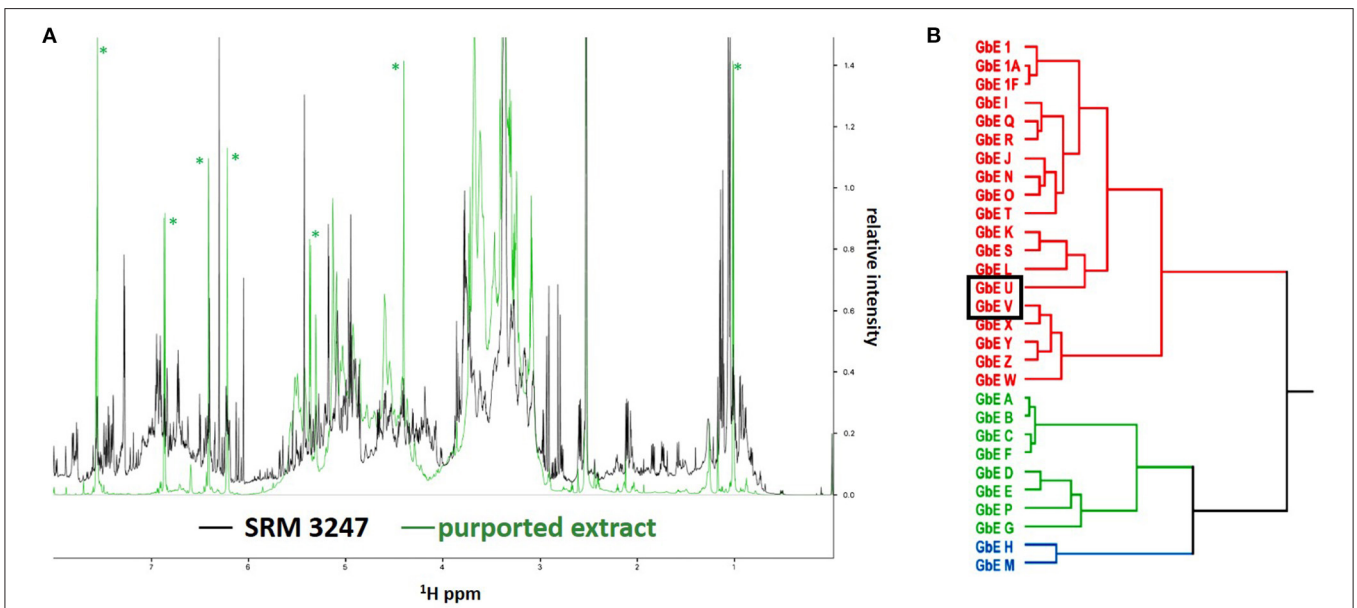

FIGURE 7 | (A) Overlay of NMR spectra analyses of NIST SRM 3247 Ginkgo (*G. biloba* L.) extract (black trace) vs. a product obtained from the market purported to be Ginkgo extract (green line). Differences in peak frequencies and intensities were noted in the aromatic and aliphatic regions (6–8 and 1–3 ppm, respectively); green asterisks indicate rutin peaks. **(B)** Dendrogram analysis of the hierarchical clustering resulting from unsupervised analyses of NMR spectra of various Ginkgo products. Non-targeted NMR profiling of Ginkgo extracts (GbE) was conducted on NTP test articles (1, 1A, 1F), products procured from the U.S. market claimed as containing Ginkgo (A-T), NIST SRM 3247 Ginkgo extract (U), and NIST SRM 3248 Ginkgo SODF (V). Samples with spectra characteristic of Ginkgo clustered together (red), including NIST SRMs (black box), while samples with very low levels of flavonols and terpene trilactones were clustered separately (green and blue). Adapted with permission from Collins et al. (116), under Creative Commons license (https://creativecommons.org/licenses/by/4.0/legalcode).

identify potential harm of short- and long-term exposure to certain botanicals.[17] NTP safety studies are designed to test botanical preparations that represent what the general public will be exposed to *via* the dietary supplement market. NTP researchers use authenticated reference materials in quantitative measurements of key bioactive or marker compounds as well as in qualitative non-targeted profiling to thoroughly

[17]NTP Botanical Dietary Supplements: https://ntp.niehs.nih.gov/whatwestudy/topics/botanical/index.html.

TABLE 8 | Examples of reported uses of NIST botanical matrix SRMs.

SRM	References	Uses			Comments
		Method dev./ valid.	QC	Novel research	
Ephedra					
3243 3244	Andrews et al. (77)		X		SRMs analyzed as controls for the determination of caffeine
Green tea					
3254 3255 3256	Castro et al. (122)	X			Method development for quantification of caffeine and catechins using LC-particle beam/electron ionization MS
3255	Napolitano et al. (102)	X			Compared qNMR and LC-MS/MS methods for quantification of catechins using SRM for assessment of accuracy
3254	Punyasiri et al. (80)	X			SRM used to validate a new sample preparation method involving freeze drying of the samples prior to extraction and analysis
3254 3255 3256	Andrews et al. (105)		X		Used as QC material to evaluate laboratory capabilities and measurement performance for catechins and caffeine
3254	Kellogg et al. (81)	X			Comparison of conventional and accelerated-solvent extraction for catechins from green tea; SRM used as one of four samples evaluated; not compared to certified values
3254 3255 3256	Kellogg et al. (83)			X	Comparison of metabolomic approaches for assessing variability of botanical green tea preparations including SRMs as reference samples
3254	Tian et al. (82)			X	SRM used in studies of intestinal UDP-glucuronosyltransferase inhibitors in green tea using a biochemometric approach
3254 3248	Crighton et al. (73)			X	Investigating application of DSA-TOF for screening adulterated dietary supplements
3254 3255 3256	Gusev et al. (106)		X		Used in a disintegration and dissolution testing study for green tea dietary supplements to evaluate formulation performance
3254 3255 3256	Clark et al. (84)			X	SRMs used as sample for interlaboratory comparison of untargeted MS to assess variability in metabolomic studies
Ginkgo biloba					
3246	Castro et al. (123)	X			Validation of sample preparation and detection of elements by ICP-OES
3246 3248	Booker et al. (101)	X			SRMs included in study of adulteration of *Ginkgo biloba* products; HPTLC analysis shown in paper includes SRMs
3247 3248	Catlin et al. (117)			X	SRMs used to determine chemical and biological similarity of *Ginkgo biloba* extracts
3247	Collins et al. (116)			X	SRMs used in non-targeted and targeted chromatographic and spectrophotometric studies of 24 commercially available *Ginkgo biloba* extracts
Saw palmetto					
3251	Srigley et al. (97)		X		Used in the analysis of virgin olive oil for determination of desmethlsterols, campesterol, stigmasterol, and β-sitosterol.
3251	Penugonda and Lindshield (96)		X		Used for the determination of fatty acids and phytosterols in commercial saw palmetto supplements
Botanical oils					
3274	Ahn et al. (93)	X			Validation of GC-MS method for fatty acids in food supplement oil products; comparison with certified values
Fish oils					
3275	Khoomrung et al. (92)	X			Validation of sample preparation method for fatty acid methyl esters using microwave-assisted derivation
3275	Srigley and Rader (90)	X			Determination of fatty acids in various fish oil supplements; chromatograms for analysis of SRM 3275 and comparison to certified values using a novel ionic liquid stationary phase for method validation

(Continued)

TABLE 8 | Continued

SRM	References	Uses			Comments
		Method dev./ valid.	QC	Novel research	
3275	Weatherly et al. (91)	X			Evaluated ionic liquid GC phases for separation of fatty acids; compared results for three fatty acids
3275	Karunathilaka et al. (86)	X			Validation of portable FTIR Device for prediction of fatty acid content in marine oil omega-3 dietary supplements
3275	Karunathilaka et al. (88)	X			SRMs used to validate ATR-FTIR and FT-NIR chemometric method for quantification of fatty acids
3275	Trbovic et al. (124)		X		Used for QC in GC-FID method for fatty acids in fish tissue and feed
3275	Li and Srigley (87)	X			Validation of GC-FID method for log chain omega-3 polyunsaturated fatty acids in chewable gel dietary supplements
Soy					
3238	Zhang et al. (125)	X			Development and validation of LC-particle beam/electron ionization MS for determination of isoflavones
3234	Kambhampati et al. (126)			X	Method development for protein quantification *via* determination of amino acids
Turmeric					
3299 3300	Mudge et al. (111)	X			Used in multi-laboratory study for determination of curcuminoids in turmeric dietary supplements by HPLC-DAD

Uses include method development and/or validation, quality control (QC), and novel research investigations.

determine the composition of candidate test articles to the extent practicable (115). This approach has been used following the NTP studies of *G. biloba* (**Figure 7**) (116–118), and in advance of studies on *A. racemose*—(119) and *Echinacea purpurea* (L.) Moench (119, 120). Importantly, these characterizations are reported openly, enabling improved research reproducibility and allowing a critical review of the composition of the specific test article chosen for each NTP study. In this way, the merits of botanical safety studies can be assessed in part based on how well the selected test article was representative of products on the market, authenticated reference materials (when available), and consensus quality standards (121).

Notably, these assessments of the chemical similarities and differences between reference materials, commercial ingredients/products, and experimental preparations by NTP researchers underscore how the inherent complexity and variability of NPs can confound research on their safety. For example, investigation of more than a dozen preparations of *E. purpurea* and *A. racemosa* demonstrated how samples could match characteristics of authentic material in orthogonal targeted and non-targeted chemical profile screening and yet yield different or even opposing bioactivities in CYP450 gene expression assays (119). Furthermore, the influence of sample preparation techniques is highlighted by the observation that hydrolyzing glycosides to the corresponding aglycones resulted in a decreased ability to chemically differentiate between authenticated and characteristic *G. biloba* materials and those samples that had been deemed as uncharacteristic (116). When certified reference materials are available, as was the case for *G. biloba* extract, their use can be vital to identifying and confidently addressing these analytical challenges.

DISCUSSION

As described in the above case studies, the use of reference materials and validated analytical methods in NP biomedical research promote accurate and reliable measurements of dietary constituents and their metabolites (**Table 8**). Unsurprisingly, the DS matrix CRMs with the highest prevalence of reported use are those that have been available for nearly a decade (i.e., multivitamin/multielement, fish and botanical oils, Ginkgo, and green tea). However, several important botanical matrix CRMs have been released in recent years or are near completion that significantly broaden the library of available materials. These CRMs, which are listed in **Table 1**, include kelp (*Thallus laminariae*), yerba mate (*Ilex paraguariensis* A.St.-Hil.) leaves, turmeric (*Curcuma longa* L.), ginger (*Z. officinale*), kudzu [*Pueraria montana* var. *lobata* (Willd.) Maesen and S.M. Almeida ex Sanjappa and Predeep], and Asian ginseng (*P. ginseng*). These newly available CRMs should find extensive use in not only method development/validation and quality control applications but also in novel research applications. In addition, several more botanical-derived DS ingredients are currently under development by NIH-ODS/NIST as candidate matrix SRMs, including black cohosh (*A. racemosa*), yohimbe [*Pausinystalia johimbe* (K. Schum.) Pierre ex Beille], eleuthero [*Eleutherococcus senticosus* (Rupr. and Maxim.) Maxim.], and ashwagandha [*Withania somnifera* (L.) Dunal] (**Table 3**). Although still under development, in certain circumstances these candidate DS SRMs may be made available to researchers who are initiating studies involving the use and/or characterization of interventions derived from these botanicals[18].

[18]NIH-ODS AMRM Program RMs for DS Analysis: https://ods.od.nih.gov/Research/AMRMReferenceMaterials.asp

CONCLUSION

Scientists conducting NP research are encouraged to utilize the approaches for analytical method validation and the growing number of RM resources described herein to improve the overall quality of chemical measurements and to expand the knowledge base on the chemical characterization of NPs and DS. The enhanced analytical capacity that comes from leveraging reference materials and validated methods, in turn, optimizes the evidence base for dietary guidelines and healthcare practice as it relates to the use of dietary interventions to help maintain health and reduce illness.

AUTHOR CONTRIBUTIONS

All authors jointly conceived, developed, drafted, and edited this manuscript.

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Chemical, Manufacturing, and Standardization Controls of Grape Polyphenol Dietary Supplements in Support of a Clinical Study: Mass Uniformity, Polyphenol Dosage, and Profiles

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Bioactive dietary polyphenols in grape (*Vitis vinifera*) have been used in Dietary Supplements (DSs) with the aim to prevent numerous diseases, including cardiovascular and neurodegenerative diseases, and to reduce depression and anxiety. Given prior recognition that DSs can be quality challenged from the purity, authentication, adulteration, and actual concentration of targeted bioactives, to ensure consumer health protection as well as the quality and safety of grape polyphenol-based DSs, the present investigation was aimed at establishing a comprehensive quality control (QC) approach for grape polyphenol-based DSs in support of a human clinical study. In this study, the manufactured grape seed polyphenol extract (GSPE) and *trans*-resveratrol (RSV) capsules and Concord Grape Juice (CGJ) along with the corresponding original drug materials were analyzed using the developed different liquid chromatography/UV-visible spectroscopy/mass spectrometry (LC/UV-Vis/MS) methods. The weight variation of GSPE and RSV capsules was also evaluated according to the US Pharmacopeia (USP) tests. The results indicate that the total identified polyphenol content in each grape seed extract (GSE) capsule/CGJ is very similar and all GSE/RSV capsules pass the content/weight uniformity test. Given the complexity of these and many botanical products from the issues of purity, quality, adulteration, consistency, and their coupling to the complex chemistry in each grape-derived botanical, quality assurance and the steps needed to ensure grape-derived DSs being well homogeneous and stable and containing the known and expected bioactives at specific concentration ranges are fundamental to

any research study and in particular to a clinical trial. Each of these issues is essential to provide a solid foundation upon which clinical trials with botanicals can be conducted with the goal of realizing measurable mental health outcomes such as reducing depression and anxiety as well as understanding of their underlying biological mechanisms.

Keywords: botanicals, quality control, grape seed extract (GSE), resveratrol, grape juice, LC/UV-Vis/MS, product quality, authentication

INTRODUCTION

Consumer interest in the use of botanical dietary supplements (DSs) continues to increase. The global DS marketplace was valued at USD 132.8 billion in 2016 and expected to reach USD 220.3 billion in 2022 (1). In particular, the US ranks as the leading country in botanical DS consumption. It is estimated that 77% of US adults consume DSs on a regular basis (2). The consumer demand and growth in the DS industry reinforce the importance of ensuring safe and high-quality DS products.

Vitis vinifera (grape) is one of the most widely cultivated fruit species in the world, and the total production of grapes worldwide is ∼60 million tons (3). Grape and grape-derived products contain a unique mixture of bioactive dietary polyphenols, which have long been reported to have antioxidant and positive health promoting effects and associated with the prevention of numerous diseases, including cardiovascular and neurodegenerative diseases as well as several forms of cancers (4–6). Previous studies have investigated the disease preventative effect of some specific grape polyphenol forms, including resveratrol (RSV), proanthocyanidins, and anthocyanins (7–9). Thus, grapes and their byproducts are the ideal candidates for DSs.

Most grape polyphenols can be found in grape juice after an extraction through pressing, and Concord Grape Juice (CGJ) is one of the main processed products of grapes. Grape seed is also one of the major industrial byproduct of the winemaking process, and more than 70% of grape phenolics can be retained in skins and seeds (10). Therefore, grape seed extract (GSE) is a popular and widely used DS in the USA. *Trans*-RSV (systematically termed as *trans*-3,5,4′-trihydroxystilbene), which is produced by grape berries of *Vitis* varieties in response to UV irradiation, is an antioxidant compound found in the skin of grapes (11). The potential role of RSV in health promotion, such as the prevention and treatment of diabetes, cancer, obesity, pain, inflammation, tissue damage, and even "aging," has made it increasingly popular in recent years as a DS (12).

However, the adulteration of grape-derived botanical products can also be a significant problem. In a study, using liquid chromatography–mass spectrometry (LC-MS) and thin layer chromatography (TLC), researchers found that of 21 commercial GSE products 6 were adulterated and might contain allergens, notably peanut skins (13). Because consumers rely on label claims and other information that are provided directly from the supplier, the adulteration of those DSs, especially with a common allergen, represents a considerable risk to public safety. Therefore, more sophisticated and proper analytical tests are needed to detect such adulteration.

Chemistry, Manufacturing, and Controls (CMC) is an integral part of any pharmaceutical product application to FDA. There is an intrinsic link between the CMC attributes of a pharmaceutical product and the safety and efficacy of clinical therapy (14). The US Pharmacopeia (USP) and the National Formulary (NF) drug substance and excipient monographs, as well as general tests and procedures, are frequently cited in New Drug Applications (NDA) (15), and a summary of pharmaceutical test scheme for pharmaceuticals and DSs is presented in **Table 1**. Because of the coupling of CMC to the recognition that some commercial botanical products on the marketplace were quality challenged, to ensure consumer health protection as well as the quality and safety of grape-based DSs, the present research was aimed at establishing a clear and comprehensive quality control (QC) approach for the grape-based DS that would be used for clinical trials. In this study, GSE and RSV capsules and CGJ were analyzed using our optimized high-performance liquid chromatography coupled with UV coupled with electrospray ionization tandem mass spectrometry (HPLC-UV/Vis-MS) and ultra-high-performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC-QQQ/MS) methods. The weight variation of GSE and RSV capsules was also evaluated according to the USP tests.

MATERIALS AND METHODS

Chemical Reagents

US Pharmacopeia reference standard compounds, including *trans*-RSV, (+)-catechin, (–)-epicatechin, gallic acid, 3-hydroxytyrosol, isochlorogenic acid, 3, 4-dihydroxyphenylacetic acid, 4-methyl gallic acid, 3, 4-dihydroxyphenylacetic acid, 3-hydroxybenzoic acid, caffeic acid, 4-hydroxybenzoic acid, vanillic acid, dihydromyricetin, syringic acid, resveratrol-3-glucoside, dihydroferulic acid, sinapic acid, taxifolin, ferulic acid, 3-hydrocinnamic acid, phenylacetic acid, and *trans*-2-hydrocinnamic acid, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary analytical standard (Grade P) compounds, including procyanidin B2, procyanidin C1, quercetin, and cyanidin-3-glucoside, were purchased from ChromaDex (Irvine, CA, USA). The HPLC grade water, acetonitrile (ACN), methanol (MeOH), formic acid (FA), and trifluoroacetic acid (TFA) were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA).

TABLE 1 | Pharmaceutical test scheme for pharmaceuticals and dietary supplements (DSs).

Pharmaceuticals	Dietary supplements
<301> Acid-neutralizing capacity	<1216> Tablet friability
<701> Disintegration	<2040> Disintegration and dissolution of dietary supplements
<711> Dissolution	<2091> Weight variation of dietary supplements
<724> Drug release	<2750> Manufacturing practices of dietary supplements
<785> Osmolarity	
<905> Uniformity of dosage forms	
<1087> Apparent intrinsic dissolution—dissolution testing procedures for rotating disk and stationary disk	
<1088> *In vitro* and *in vivo* dissolution evaluation of dosage forms	
<1090> Assessment of drug product performance—bioavailability, bioequivalence, and dissolution	
<1216> Tablet friability	

Note: Numbers in angular brackets refer to chapter numbers in the General Chapters section from US Pharmacopeia (USP) 41 and National Formulary (NF) 36.

Drug Material Sourcing

Three kinds of grape-based DSs were analyzed.

MegaNatural® grape seed polyphenol extract (GSPE) was purchased from Polyphenolics Company (Madera, CA, USA). The grapes were grown in California, USA and certified by Halal (IFANCA), and the final GSPE product was processed using hot water extraction at a ratio of 30:1 or −50:1 (dry seed: extract).

Synthetic *trans*-RSV was purchased from BannerBio Nutraceuticals, Inc. (Nanshan District, Shenzhen, China).

The CGJ concentrate was provided by Welch Foods, Inc. (Westfield, NY, USA). The CGJ concentrate was squeezed from Concord grapes grown and harvested in the Eastern USA growing region and processed in Westfield, NY, USA, within 8 h of harvest, and were pasteurized to achieve a 5-log pathogen reduction and commercial sterility.

Manufacturing of GSPE and RSV Capsules and CGJ

Original GSPE and RSV materials were delivered to Eagle Nutritionals (Carlstadt, NJ, USA) to manufacture the final products using encapsulation at specific concentrations required for planned clinical trials in a NIH-funded U19 study. Briefly, for GSPE capsules, 450 mg of GSPE powders and 50 mg of silica were filled into #0 purple/white hard gelatin capsules. For RSV capsules, three different weight levels (150, 300, and 450 mg) of RSV powders were encapsuled using #0 green capsules.

Concord Grape Juice is reconstituted from the CGJ concentrate to single strength 100% CGJ. The general process flow was shown in **Figure 1**. Briefly, 129.8 kg of distilled water was transferred into a 50-gallon batch tank and warmed up to room temperature, and 50.2 kg of the CGJ concentrate was added to the tank. The mixer was warmed to 30°C and gently mixed for 10–15 min to ensure proper mixing. A mixer sample was analyzed for pH and adjusted with remaining water as needed to achieve the target of 16.1°brix and to achieve and confirm the final pH of 3.5–3.7. The final product was transferred to an original product holding tank in a thermalization room adjacent to the Microthermics. Finally, the CGJ was hot filled into 8 oz PET bottles, and following cooling to <40°C bottles it was removed from a bath, dried, and inspected.

QC of Original Drug Materials of GSPE, RSV, and CGJ Concentrate

QC of Original GSPE Materials

The container and inside package of the GSPE original material product were opened and from five distinctly different physical locations subsampled for chemical profiling in Eagle Nutritionals using standard industry subsampling procedures. The five subsamples were carefully placed into ziplock plastic bags, labeled, and then transported to Rutgers University for chemical analysis in our lab. For each of the five subsamples, three replicates were made in parallel for the QC process. Approximately 30 mg of GSPE original material was accurately weighted and prepared in 10 ml 70% MeOH with 1% FA, vigorously vortexed, and sonicated for 10 min. An aliquot of 200 μl of the extract was diluted by mixing with 0.8 ml 70% MeOH with 1% FA and centrifuged at 16,000 rpm for 10 min, and then the supernatant was injected for the LC-UV/Vis-MS analysis.

For the preparation of reference solutions of gallic acid, (+)-catechin, (−)-epicatechin, procyanidin B2, and procyanidin C1, *ca.* 5 mg of each standard was accurately weighed and diluted to 10 ml using 70% MeOH with 1% FA. Each standard stock solution was sonicated for 10 min, and was allowed to cool down to room temperature. Next, 2 ml of each standard stock solution was allowed to combined together with each other and sonicated for 10 min to mix well to form a standard mixture of gallic acid, (+)-catechin, (−)-epicatechin, procyanidin B2, and procyanidin C1. Further serial dilutions up to 100∼0.1 μg/ml were made using the same solvent.

An Agilent 1290 Infinity II UHPLC (Agilent Technology, Palo Alto, CA, USA) equipped with a diode array detector (DAD) and 6546 quadrupole time-of-flight (Q-TOF) MS with electrospray ionization source (ESI) (Santa Clara, CA, USA) was used for chromatographic separation. Nitrogen generated from the Parker Balston NitroFlow60NA nitrogen generator was used for MS electrospray ionization. MassHunter Workstation software Data Acquisition (version B.08.00) was used for data processing. An Agilent Polaris Amide-C18 (250 × 4.6 mm, 3 μm) column was used for compound separation. For the LC part, the mobile phase A was water with 0.1% FA, and mobile phase B was ACN with

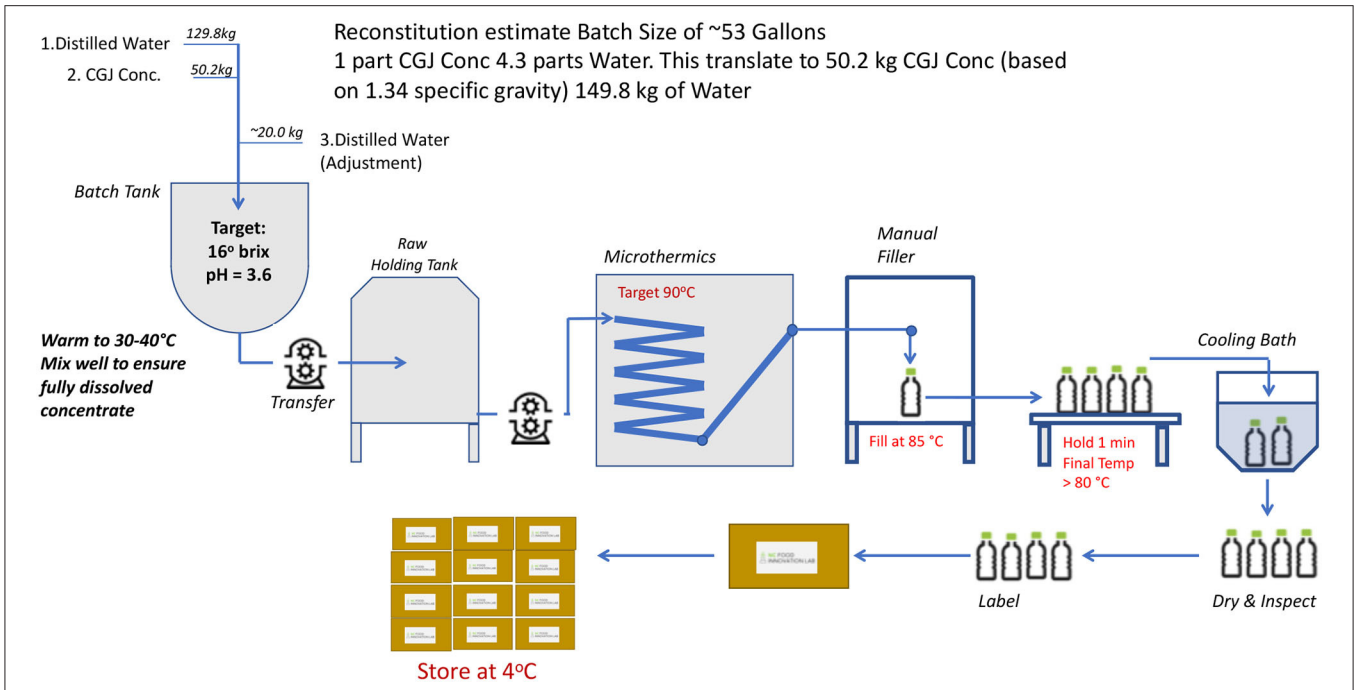


FIGURE 1 | Concord Grape Juice (CGJ) processing flow.

0.1% FA. The gradient was 2% B at 0 min and held for 3 min, raised to 15% B at 15 min and held until 25 min, then raised to 35% B at 50 min, 60% B at 51 min, and held until 55 min. The column was equilibrated with 2% B for 3 min between injections. The flow rate was 0.8 ml/min. The column was set at 40°C, and an autosampler was maintained at 4°C. The injection volume was 2.5 μl. A diode array detector (DAD) was set at 280 nm, with the bandwidth at 4 nm. The reference wavelength was 400 nm, with the reference bandwidth at 10 nm. For the MS condition, the gas was dried at 300°C with a flow rate of 12 L/min. Sheath gas was dried at 250°C with a flow rate of 12 L/min. The nebulizer pressure was 30 psi. The VCap was 4,000 V, and the nozzle voltage was 500 V. Fragmentor voltage was set at 180 V, skimmer was 65 V, and Oct 1 RF Vpp was 750 V. The scan ranged from 150 to 1,700 m/z. Acquisition rate was 6 spectra/s.

Gallic acid, catechin, and epicatechin were quantified with the calibration curve of corresponding reference standards. Procyanidin dimers (P_2) were quantified based on the calibration curve of procyanidin B2, and procyanidin trimers (P_3) were quantified based on the calibration curve of procyanidin C1 as shown in Supplementary Table S1.

QC of Original RSV Materials

The container and inside package of the RSV original material product were opened and from three different physical locations subsampled for chemical profiling in Eagle Nutritionals. Reference standard and RSV samples were prepared under dark conditions, and opaque Falcon® tubes and brown Eppendorf® tubes were used. For each of the three subsamples, three replicates were made in parallel for the QC process. *ca.* 50 mg was accurately weighed and dissolved in 10 ml 70% MeOH with 1% FA. An aliquot of 100 μl of the extract was diluted by mixing with 9.9 ml 70% MeOH with 1% FA, and then 100 μl of each diluted sample was further diluted to 1 ml with the same solvent. The solution was centrifuged at 12,000 rpm for 5 min, and then an aliquot of 2.5 μl of the supernatant was injected into UHPLC for analysis.

For the preparation of reference solutions of *trans*-RSV, *ca.* 5 mg of the standard was accurately weighed and diluted to 10 ml using 70% MeOH with 1% FA. The stock solution was sonicated for 10 min and was allowed to cool down to room temperature. Further serial dilutions up to 200∼0.1 μg/ml were made using the same solvent.

Agilent 1290 Infinity II UHPLC equipped with DAD and 6546 Q-TOF MS with ESI were used for chromatographic separation. The column used for the RSV QC test was Kinetex™ (Torrance, CA 90501-1430 USA) C_{18} column (CO, USA), the particle size was 2.6 μm, and the size was 100 × 2.1 mm. For the LC condition, water with 0.1% FA was used as mobile phase A, and ACN with 0.1% FA was used as mobile phase B. The gradient was 25% B at 0 min, then raised to 60% B at 4 min, held until 4.5 min, then dropped to 25% B at 5 min. The flow rate was 0.35 ml/min, and the column was equilibrated with 25% B for 1 min between injections. The column was set at 40°C, and an autosampler was maintained at 4°C. The injection volume was 2.5 μl. The DAD was set at 210 nm (as the wavelength for general impurities), 280 nm (as the absorption maximum of *trans*-RSV), and 305 nm (for the possible degradation product *cis*-RSV). *Trans*-RSV in the RSV capsule was quantified based on the reference standard calibration curve under 280 nm. The calibration curve parameters are presented in **Supplementary Table S1**.

QC of CGJ Concentrate

Determination of Anthocyanidins and Flavonols Using LC-UV/Vis-MS

Three replicates of the CGJ concentrate were made in parallel for the QC process. To prepare the CGJ concentrate, 2.5 ml of the CGJ concentrate was diluted in 7.5 ml water. All samples were centrifugated at 12,000 rpm for 10 min, and the supernatant was directly injected into UHPLC. For the preparation of reference solutions of cyanidin-3-glucoside and quercetin, *ca.* 10 mg of each standard was accurately weighed and diluted up to 10 ml using 70% MeOH with 1% FA. Then, the standard stock solution was sonicated for 10 min and allowed to cool down up to room temperature. Afterward, 0.5 ml of all standard stock solutions were combined together and sonicated for 10 min to mix well to form a standard mixture. An aliquot of 200 μl stock solution was then spiked into 0.8 ml 70% MeOH with 1% FA to make the first working solution. Further serial dilutions of 100∼0.1 μg/ml were made using the same solvent.

The Agilent 1290 Infinity II UHPLC equipped with DAD was used for chromatographic separation, and the column Agilent Polaris Amide-C18 (250 × 4.6 mm) was used for compound separation. The mobile phase A was water with 0.4% TFA, and B was ACN with 0.4% TFA. The flow rate was 0.8 ml/min. The gradient was 10–20% B from 0 to 20 min; 20–30% B from 20 to 35 min; isocratic elution at 30% B from 35 to 40 min; 30–60% from 40 to 50 min; and kept 60% from 50 to 55 min, then dropped to 10% B in 0.5 min. The column was equilibrated with 10% B for 2 min between injections. The column was set at 40°C, and an autosampler was maintained at 4°C. The injection volume was 2.5 μl. The DAD was set at 370 nm (as the absorption maximum of most flavonols) and 520 nm (as the absorption maximum of most anthocyanidins) with the bandwidth of 4 nm, and the reference wavelengths were set at 500 and 360 nm, respectively, with the reference bandwidth at 10 nm.

Anthocyanidins were quantified based on the calibration curve of cyanidin-3-glucoside at 520 nm, and the quantity was further adjusted based on the molecular weight ratio. Flavonols were quantified based on the calibration curve of quercetin at 370 nm, with a further adjustment of the quantity based on the corresponding molecular weight ratio. The calibration curve parameters are shown in **Supplementary Table S1**.

Determination of Other Phenolic Compounds Using UHPLC-QQQ/MS

To prepare the CGJ concentrate, 2.5 ml of the CGJ concentrate was diluted in 7.5 ml water. Then, the diluted CGJ concentrate samples were further diluted using 1% FA acidified 70% MeOH solution (1:10). The prepared samples were centrifugated at 12,000 rpm for 10 min, and the supernatant was directly injected into UHPLC. Three replicates were made in parallel for the QC process.

For the preparation of reference solutions of 3-hydroxytyrosol isochlorogenic acid, 3,4-dihydroxybenzoic acid, 4-methyl gallic acid, catechin, procyanidin B2, epicatechin, 3-hydroxybenzoic acid, caffeic acid, 4-hydroxybenzoic acid, vanillic acid, dihydromyricetin, syringic acid, resveratrol-3-glycoside, dihydroferulic acid, 3-hydroxycinnamic acid, taxifolin, sinapic acid, ferulic acid, phenylacetic acid, and *trans*-2-hydroxycinnamic acid, *ca.* 10 mg of each standard was accurately weighed and diluted to 10 ml using 70% MeOH with 1% FA. Each standard stock solution was sonicated for 10 min, and was allowed to cool down to room temperature. About 0.5 ml of each standard stock solution was combined together with each other and sonicated for 10 min to mix well to form a standard mixture. An aliquot of 100 μl stock solution was spiked into 10 ml 70% MeOH with 1% FA to make the first work solution. Further serial dilutions up to 5,000∼0.1 ng/ml were made using the same solvent. For the preparation of samples, nine replicates were made in parallel for the QC process. All samples were diluted using 1% FA acidified 70% MeOH solution (1:10). The prepared samples were centrifugated at 16,000 rpm for 10 min, and the supernatant was directly injected into UHPLC.

The instrument used for chemical analysis was an Agilent 1290 Infinity II UHPLC (Agilent Technology, Palo Alto, CA, USA) hyphenated with 6470 triple quadrupole MS with ESI (Santa Clara, CA, USA). Agilent MassHunter Optimizer (version B.07.00) for standard compound-related parameter optimization and MassHunter Workstation software Data Acquisition (version B.08.00) and Quantitative Analysis (version B.07.01) for data processing were used. The column used for this section separation was an Agilent SB-AQ RRHD UHPLC column, the particle size was 1.8 μm, and the size was 150 × 2.1 mm with an SB-AQ guard column (2.1 × 5 mm, 1.8 μm). Nitrogen generated from Parker Balston NitroFlow60NA nitrogen generator was used for MS electrospray ionization. For the LC parameters, the mobile phase A was 0.1% FA in water, and mobile phase B was 0.1% FA in ACN. The flow rate was 0.2 ml/min, and the injection volume was 2.5 μl. The gradient was 4% B to 40% B in 6 min, and raised to 60% B from 6 to 10 min, then held at 60% B for 0.5 min, and dropped to 4% B in 0.5 min. The column was equilibrated with 4% B for 1 min between injections. The column was thermostatted at 30°C, and an autosampler was set to 4°C. Nitrogen was used as the nebulizing and drying gas. The nebulizer was set to 30 psi and the drying gas was set to 300°C with a flow rate of 13 L/min. The sheath gas was set to 250°C with a flow rate of 12 L/min. In the scan mode, dynamic multiple reactions of monitoring (dMRM) were optimized using MassHunter Optimizer as priorly reported, with the parameters presented in **Table 2**.

All calibration curves based on 8–15 points and the calibration curve parameters, coefficient of determination (r^2), linear range, lower limit of detection (LLOD), and lower limit of quantification (LLOQ) of all target analytes are shown in **Supplementary Table S2**.

QC of GSPE, RSV Capsules, and CGJ

Determination of Weight Uniformity of RSV and GSE Capsules

The mass uniformity of the individual unit dosages contained in each RSV and GSE capsule was performed according to USP, 2091 (19). The calibration of the balance was confirmed prior to the start of the study and at the conclusion of the study. Briefly, 20 intact capsules of each kind of DSs were individually weighed using an electronic balance, and the mass of each capsule

TABLE 2 | The information for dynamic multiple reactions of monitoring (dMRM) parameters.

Compound	Retention time (min)	MS/MS transition (dMRM)		Fragementor voltage (V)	Collision energy (V)
		Presursor ion (*m/z*)	Production (*m/z*) (quantifier/qualifier)		
3-hydroxytyrosol	4.95	153.1	122.4/123.1	95	23/15
isochlorogenic acid	5.14	353.1	191.0/179.0	105	16/16
3,4-dihydroxybenzoic acid	5.34	153.0	109.0/108.1	86	12/28
4-methyl gallic acid	5.67	183.0	168.0/124.1	90	8/16
Catechin	5.94	289.1	245.2/123.1	120	12/36
procyanidin B2	5.97	579.2	127.0/287.1	120	33/37
Epicatechin	6.25	289.1	245.2/203.1	134	12/20
3-hydroxybenzoic acid	6.40	137.0	93.1/N.D.	88	8/N.D.[a]
caffeic acid	6.56	179.0	135.1/89.1	88	16/36
4-hydroxybenzoic acid	6.64	137.0	93.1/65.2	76	16/36
vanillic acid	6.66	167.0	152.0/108.1	80	12/16
Dihydromyricetin	6.75	319.0	193.0/301.0	100	4/8
syringic acid	6.78	197.0	182.0/167.0	90	13/17
resveratrol-3-glycoside	7.03	389.1	227.0/185.0	140	13/37
dihydroferulic acid	7.26	195.1	136.1/121.1	100	11/27
sinapic acid	7.73	223.1	208.0/193.0	90	9/21
Taxifolin	7.73	303.0	285.0/177.0	110	9/9
ferulic acid	7.75	193.1	134.1/N.D.	88	16/N.D.
3-hydroxycinnamic acid	7.82	163.0	119.1/91.1	94	12/28
phenylacetic acid	8.13	135.0	91.2/N.D.	50	4/N.D.
trans-2-hydroxycinnamic acid	8.18	163.0	119.1/117.0	80	12/36

N.D[a], not detected.

content was recorded to 1/10 of a milligram (0.1 mg). After that, the average mass and its SD were calculated. Moreover, the requirements are met if the individual weights lie within the range of 90.0–110.0% of the average weight, and the relative SD (RSD) is ≤6.0%.

For any capsules falling within the aforementioned limits, the contents of each capsule should be removed and the emptied shells need to be weighed individually. The net weight could be calculated by subtracting the weight of the shell from the respective gross weight, and the average net content could be determined from the sum of the individual net weights. After that, the difference between each individual net content and the average net content should be determined. The requirements are met if no more than two differences are >10% of the average net content, and in any case the difference does not exceed 25%.

Determination of Content Uniformity of the GSPE Capsule

For the preparation of GSE capsules, nine replicates were made in parallel for the QC process. The contents of each capsule were removed with the aid of a small brush or pledget of cotton and dissolved in 50 ml 70% MeOH with 1% FA, vigorously vortexed, and sonicated for 10 min. An aliquot of 100 μl of the extract was diluted by mixing with 0.9 ml 70% MeOH with 1% FA centrifuged at 16,000 rpm for 10 min, and then the supernatant was injected for the LC-UV/Vis-MS analysis.

For reference solutions of RSV capsules, *ca.* 10 mg of *trans*-RSV standard was accurately weighed and diluted to 10 ml using 70% MeOH with 1% FA. The standard stock solution was then sonicated for 10 min and was allowed to cool down to room temperature. An aliquot of 100 μl stock solution was spiked into 0.9 ml 70% MeOH with 1% FA to make the first working solution. Further serial dilutions up to 100∼0.1 μg/ml were made using the same solvent. The LC-MS conditions might be the same as mentioned in section QC of Original GSPE Materials.

Determination of RSV Capsule Purity and Content Uniformity

For the preparation of RSV capsule samples, nine replicates were made in parallel for the QC process. The contents of each capsule were removed with the aid of a small brush or pledget of cotton and dissolved in 50 ml 70% MeOH with 1% FA. The solution was vigorously vortexed and sonicated for 10 min. An aliquot of 100 μl of the extract was diluted by mixing with 0.9 ml 1% FA in 70% MeOH solution. The solution was centrifuged at 16,000 rpm for 10 min, and then the supernatant was injected for the LC-UV/Vis-MS analysis. The reference standard solution preparation and LC-MS conditions might be the same as described in section QC of Original RSV Materials.

Determination of Content Uniformity of CGJ

Determination of Anthocyanidins and Flavonols Using LC-UV/Vis-MS

For each of the samples, nine replicates were made in parallel for the QC process. All samples were analyzed without dilution and centrifugated at 12,000 rpm for 10 min. The supernatant was

directly injected into UHPLC. All other experimental conditions might be the same as described in section Determination of Anthocyanidins and Flavonols Using LC-UV/Vis-MS.

Determination of Other Phenolic Compounds Using UHPLC-QQQ/MS

For the preparation of samples, nine replicates were made in parallel for the QC process. All samples were diluted using 1% FA acidified 70% MeOH solution (1:10). The prepared samples were then centrifugated at 16,000 rpm for 10 min, and the supernatant was directly injected into UHPLC. The reference standard solution preparation and LC-MS conditions might be the same as described in section Determination of Other Phenolic Compounds Using UHPLC-QQQ/MS.

Preliminary Stability Study of CGJ

Concord Grape Juice and CGJ placebo bottles were stored in a refrigerator cooler, Heat Craft Unit—Compressor Model #CDT-501H2, Model #CHL 450, with the average storage temperature maintained between 0.6 and 2.22°C at the FDA-approved Rutgers Food Innovation Center, Bridgeton, NJ, USA. From the 36 bottles of CGJ, 3 CGJ samples were randomly selected at each time point as shown in **Supplementary Table S10**, and for each of the samples, three replicates were made in parallel for the QC process. About 5 ml of CGJ sample was mixed with 5 ml MeOH, vortex for 10 s. Aliquots of 1 ml were transferred to Eppendorf tubes, stored in a paper box, and put it into a −20-degree freezer. While this study was originally designed as a longer-term stability study, for this work described here all the samples were analyzed at the month 6 time point as described in section CGJ Preliminary Stability Study at Month 6 Time Point.

Dissolution Study of GSPE and RSV Capsules

Grape seed extract and RSV capsules were tested for dissolution based on the recommendations of the FDA and USP 39 general chapters <2040> and <711> (16, 17). Briefly, the two dissolution media, 0.1 N hydrochloric acid (pH 1.2) and 0.05 M acetate buffer (pH 4.6), were evaluated with USP Apparatus 2, 100 rpm rotation speed, and 900 ml dissolution medium. Dissolution profiles were generated over 120 min. Gallic acid, catechin, procyanidin B2, and epicatechin were the marker compounds for GSE capsules, and *trans*-RSV was a marker compound for RSV capsules. Each of these marker compounds was quantified using UHPLC-QQQ/MS. A detailed experiment and the results will be described in a separate report (18).

Statistical Analysis

Raw UV and MS data were processed using MassHunter Workstation software Data Acquisition (version B.08.00) and Quantitative Analysis (version B.07.01). Data analysis and the production of graphs were performed using R software (version 4.0.5), R studio (version 1.3.959), and Microsoft® Excel for Mac (version 16.49).

RESULTS AND DISCUSSION

Weight Uniformity Test

The primary purpose of the USP is to provide guidelines for pharmaceutical dosage forms through a series of QC tests, such as identification, dissolution, uniformity of dosage units, assay, moisture, and heavy metal determinations to confirm the products' identity, content, and purity as well as various other chemical, physical, and biological properties. The term "uniformity of dosage unit" is defined as the degree of uniformity in the amount of the drug substance among dosage units (19). Mass uniformity results are presented in **Supplementary Table S3**, and the GSE capsule weight ranges from 593.70 to 607.90 mg, and for the RSV capsule, the weight range is 551.20–637.20 mg. From the results, all of the observed GSE and RSV capsules within the range of 90.0–110.0% of the average weight had an RSD of 0.56 and 5.30%, respectively, which satisfies the guidelines of the USP<2091> (19).

RSV Purity and Content Uniformity

Method Validation

Numerous studies have reported interesting properties of RSV such as the prevention and treatment of diabetes, cancer, obesity, pain, inflammation, tissue damage, and even aging (7, 12, 20, 21). However, during storage, photochemical and photocatalytic degradation of *trans*-RSV become a problem largely due to the *cis*-isomerization, which occurs when the *trans*-isomer is exposed to sunlight or to artificial or natural UV radiation at the wavelengths of 254 or 366 nm (22–26). Moreover, RSV exists naturally as both *cis*- and *trans*-isomers in nature foods and plants (26). Many reports and data indicate that *cis*- and *trans*-RSV may have different biological effects (21, 27–29). Therefore, we contend that it is necessary to determine the presence and/or absence of *cis*-isomers in RSV capsules. Although HPLC is a popular method to identify and quantify pure compounds, due to the difficulties of isomer separation on the HPLC column, conventional HPLC-UV/Vis method is often not adequate. Thus, a preliminary study was performed to validate the HPLC-UV/Vis method and to prove the *cis*- and *trans*-isomers peaks that were not overlapped and could be clearly separated.

To prepare *cis*-RSV, the photochemical degradation experiment was performed in our lab based on previous studies (26, 30). Briefly, 2 ml of *ca.* 1 mg/ml *trans*-RSV standard stock solution was stored in a colorless glass vial. The vial was then kept under the sun for the whole day because *trans*-RSV is more easily degraded when irradiated using the entire spectral range rather than using UV and near-UV to visible light (30). The solution from the glass vial (solution A) and the freshly prepared *trans*-RSV standard solution (solution B) both were injected into the HPLC separately, and the chromatographs are presented in **Figure 2A**. As shown in **Figure 2**, two major peaks appeared in solution A under 280 and 305 nm, while only one peak (peak b) appeared in solution B, which was the *trans*-RSV peak. The second peak in solution A shares the same retention time and same MS, suggesting that it was also *trans*-RSV. The first peak (peak a) has similar MS, and has the optimum absorbencies at 286 nm, indicating that the new peak

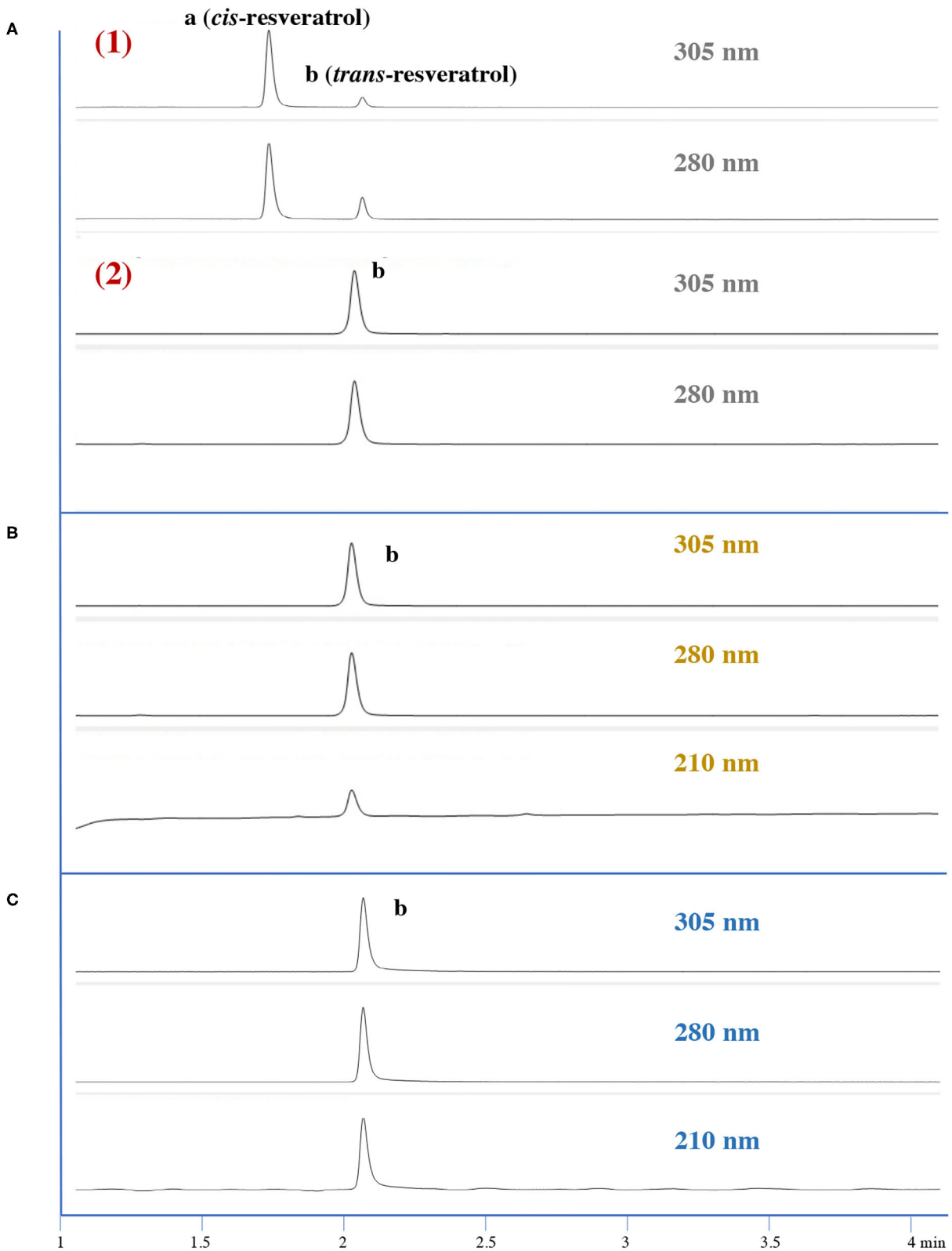
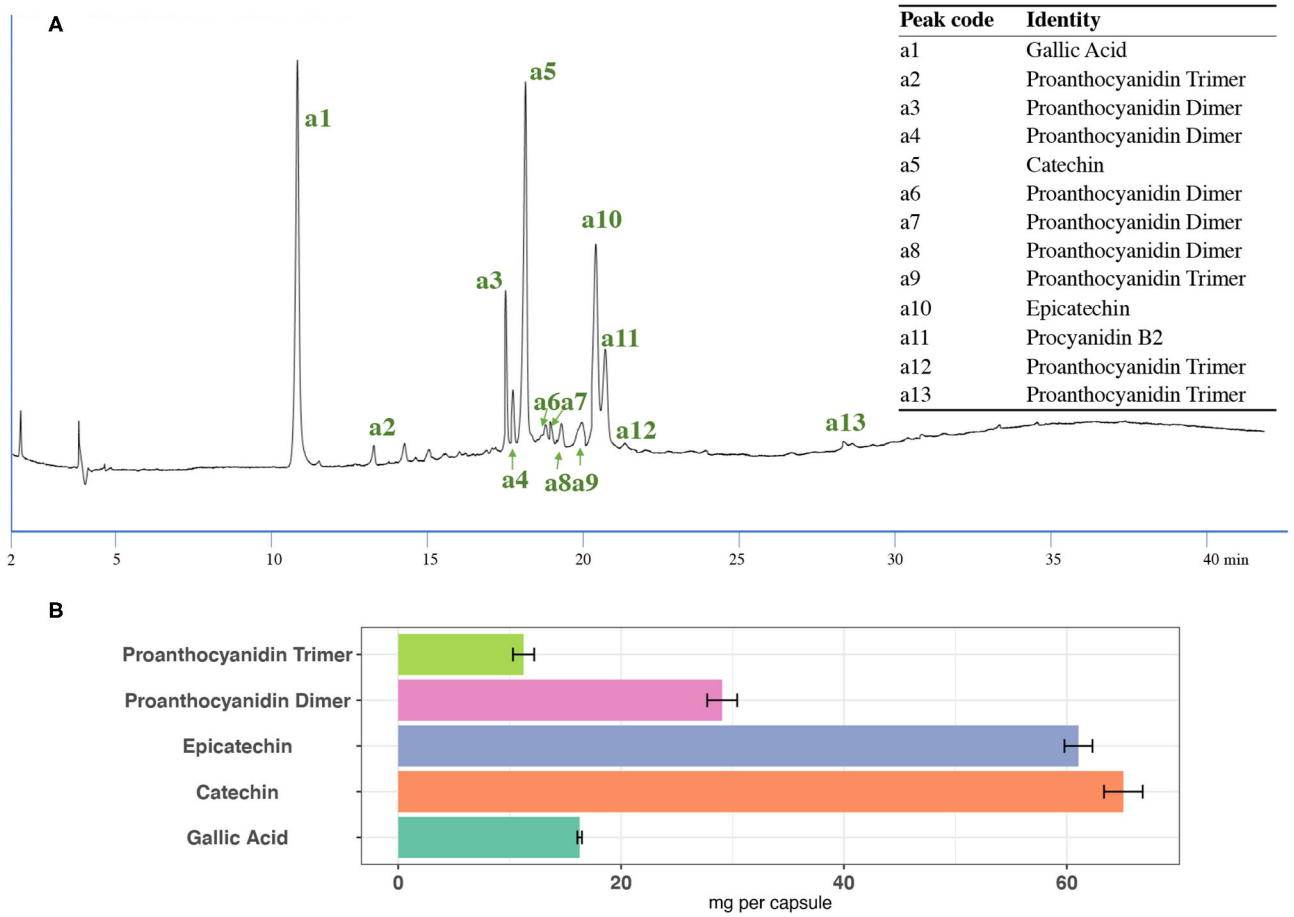


FIGURE 2 | UV chromatographs of **(A)** *trans*- and *cis*-resveratrol (RSV); **(B)** RSV capsules. Peak a, *cis*-RSV, Peak b, *trans*-RSV. **(A)**-(1) UV chromatographs of solution A as described in section RSV Purity and Content Uniformity: Method Validation under 305 and 280 nm; **(A)**-(2) UV chromatographs of solution B under 305 and 280 nm; **(B)** UV chromatographs of the RSV original material under 305, 280, and 210 nm; and **(C)** UV chromatographs of the RSV capsule under 305, 280, and 210 nm.



Peak code	Identity
a1	Gallic Acid
a2	Proanthocyanidin Trimer
a3	Proanthocyanidin Dimer
a4	Proanthocyanidin Dimer
a5	Catechin
a6	Proanthocyanidin Dimer
a7	Proanthocyanidin Dimer
a8	Proanthocyanidin Dimer
a9	Proanthocyanidin Trimer
a10	Epicatechin
a11	Procyanidin B2
a12	Proanthocyanidin Trimer
a13	Proanthocyanidin Trimer

FIGURE 3 | (A) UV chromatographs of the Grape Seed Extract (GSE) capsule under 280 nm and the tentative identifies and **(B)** the content of proanthocyanidin trimer, proanthocyanidin dimer, epicatechin, catechin, and gallic acid in the GSE capsule using the optimized extraction method.

is *cis*-RSV. Therefore, this preliminary experiment indicated that our HPLC-UV/Vis is able to separate *trans*- and *cis*-RSV.

Purity and Content Uniformity of RSV Original Materials and Capsules

Using our rapid and validated HPLC-UV/V method, our analysis shows that all RSV original materials and capsules were free of *cis*-RSV as well as other impurities and the chromatographs (see **Figures 2B,C**). The *trans*-RSV content in the RSV capsule is between 97.77 and 102.83% of the average, with a RSD of 1.50%. While we analyzed with the three dosages of 150, 300, and 450 mg; we present only the data with the highest dosage of 450 mg as similar results were obtained for the other two lower dosages. The average content of trans-RSV in RSV capsules was 453.71 mg, which is 0.8% excess of the labeled content (450 mg). The data are presented in detail in **Supplementary Table S4**.

GSPE Content Uniformity

Grape seed is a byproduct in the winery and grape juice industry, and contains lipids, proteins, carbohydrates, and 5–8% polyphenols (31). Phenolic compounds in grapes and grape-derived products can be divided into two groups: (a) phenolic acids and related compounds and (b) flavonoids. The most abundant phenolic substances in grape seeds are catechins (catechins, epicatechin, and proanthocyanidins) and their polymers (32). The antioxidant capacities of grape seed proanthocyanidins and natural secondary products have been exhaustively studied. Several reports indicated that grape seed proanthocyanidins have a wide array of positive health effects, including antioxidant, antimicrobial, antiobesity, antidiabetic, anti-neurodegenerative, anti-osteoarthritis, anticancer, and cardio- and eye-protective properties (33). For this reason, almost all GSE DSs on the market claim to have a specific "dosage" of proanthocyanidins. Hence, in this study, the total polyphenol content in the GSPE capsule was first tested, and then a HPLC-UV/Vis-MS method was developed and used to tentatively identify and quantify proanthocyanidin compounds in GSPE capsules.

Extracting Solvent Optimization

Phenolic compounds, including polyphenols and proanthocyanidins, vary between the extracts obtained by different solvents. Therefore, a pre-experiment was first

performed to compare the extraction efficiency of water and MeOH. Briefly, the content of one GSE capsule was dissolved with 1 L 70% MeOH acidified by 1% FA, and 1 L water acidified by 1% FA. The solvent was sonicated for 30 min. An aliquot of 1,000 μl of the extraction solvent was then centrifugated at 16,000 rpm for 10 min, and the supernatant was directly injected into HPLC. Three replicates were made in parallel for obtaining more accurate results. Gallic acid, epicatechin, procyanidin B2, and epicatechin were quantified using our abovementioned method, and the results are shown in **Supplementary Figure S1**. For all the four marker compounds, 70% MeOH with 1% FA was more efficient than water with 1% FA. This might be possible because the solubility of those phenolic compounds in water is fairly low, and more water-soluble polysaccharides or other components are extracted as well (34). Hence, 70% MeOH acidified with 1% FA was used to prepare all grape DS products (including RSV and GSE capsules and CGJs) as well as the reference solution. Using this specific solvent to prepare and dilute the standard solution, a high coefficient of determination (r^2) for all the standards was achieved.

Content Uniformity and Proanthocyanidin Content in GSPE Original Materials and Capsules

The major polyphenols in the GSPE include gallic acid and proanthocyanidins, with monomers of catechin and epicatechin and oligomers, were detected under UV 280 nm. The tentative identification of proanthocyanidin compounds in GSPE was done based on the MS data and published work (6). **Figure 3A** shows a representative UV chromatogram of the GSPE capsule at 280 nm. The tentative identities and retention times for individual compounds are also listed in **Figure 3A**. Based on the analysis of MS and UV data and their comparison with the authenticated standards and reported data (6), a total of 13 compounds were simultaneously identified, including gallic acid, catechin, epicatechin, 4 proanthocyanidin trimers, and 6 proanthocyanidin dimers. All tentatively identified proanthocyanidin compound values in the GSPE are reported in **Supplementary Table S5** and illustrated in **Figure 3B**.

For the GSPE original material, the results show that the total identified polyphenols together with gallic acid content in the five subsamples are very similar, with a mean value of 33.37% and a RSD of 3.24%. These results suggest that the GSPE original material was well-homogenous. For the GSPE capsules, the results show that the content of total identified proanthocyanidin compounds together with gallic acid in each capsule is also very close, with a mean value of 182.75 ± 4.07 mg and an RSD of 1.43%. Moreover, catechin and epicatechin are the major proanthocyanidin compounds in the GSPE capsules. Each capsule contains 65.07 ± 2.01 mg catechin and 61.05 ± 1.76 mg epicatechin, as well as 166.45 ± 3.70 mg of all tentatively identified proanthocyanidin compounds, which ensured the daily taking of 95 mg of proanthocyanidins (35). All these results suggest that GSPE capsules show high quality and homogeneity.

CGJ Content Uniformity

Concord Grape Juice contains a variety of phenolic compounds, including anthocyanins and proanthocyanidins and relatively high levels of total phenolics (10). Anthocyanins include red, blue, or purple plant pigments (9). Many *in vitro* and *in vivo* studies have indicated that grape anthocyanins appear to exert health benefit effects, including the prevention of various diseases, such as neuronal and cardiovascular illnesses, cancer, and diabetes, in which reactive radical species are integral to disease development and progression (4–6). Therefore, the total polyphenol content in CGJ was tested, a HPLC-UV/Vis method was used to tentatively identify and quantify the anthocyanidin and flavanol, and a UHPLC-QQQ/MS methodology was used to quantify phenolic compounds in CGJ.

Content of Anthocyanins, Flavonols in CGJ

The representative UV chromatograms at 520 nm (for anthocyanins) and 370 nm (for flavonols) of CGJ are illustrated in **Figures 4A,C**. The identification is done based on the UV data and published report (6), and a total of 13 anthocyanins (delphinidin glucoside, cyanidin glucoside, petunidin glucoside, malvidin glucoside, peonidin glucoside, petunidin acetyl glucoside, delphinidin coumaroyl diglucoside, malvidin coumaroyl diglucoside, delphinidin coumaroyl glucoside, petunidin coumaroyl glucoside, cyanidin coumaroyl glucoside, malvidin coumaroyl glucoside, and peonidin coumaroyl glucoside), and 5 flavonols (rutin, myricetin glucoside, quercetin glucuronoyl, and quercetin) were tentatively identified. The content of all identified anthocyanins and flavonols in CGJ samples is presented in **Figures 4B,D**, and the details of data are provided in **Supplementary Tables S7, S8**. From these results, the total concentration of the identified anthocyanins was 177.39 ± 6.67 μg/ml with an RSD of 3.91%, for the flavonols, the value was 69.82 ± 4.66 μg/ml with an RSD of 5.49%. These data suggest that CGJ is a rich source of grape anthocyanins and flavonols, and that CGJ is well-homogeneous. Among all the identified compounds, rutin is the most abundant one in CGJ, with a content of 32.34–43.90 μg/ml. Rutin is a common dietary flavonoid and has been reported to possess diverse pharmacological activities, including antioxidant, anti-inflammatory, anticancer, antidiabetic, antimicrobial, and neuroprotection effects (36). However, due to the low aqueous solubility, poor stability and limited membrane permeability, bioavailability of rutin is very poor, and the observed effects *in vitro* do not always translate into clinical outcomes (36, 37). Hence, those observations and connections indicate a need to improve CGJ DSs to enhance the bioavailability of rutin and other flavonoids.

Content of Other Phenolic Compounds in CGJ

Even though HPLC-UV/Vis is generally used for the identification and quantification of phenolic compounds from grapes and their products, some compounds are difficult to be effectively separated and accurately identified using the methodology due to their insufficient peak capacity and the accumulation of analytes (38). UHPLC coupled with triple quadrupole MS (UHPLC-QQQ-MS/MS) based on HPLC using a small particle diameter column and mass spectrometer allows rapid screening of a large number of phytochemicals using the information on the characteristics of molecular ions (38, 39).

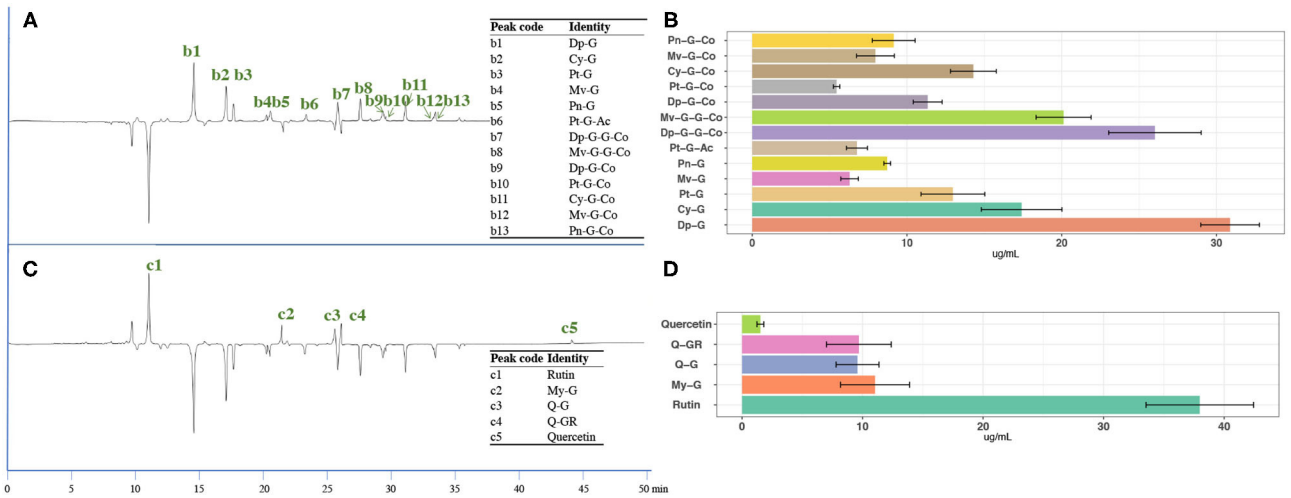


FIGURE 4 | (A) UV chromatographs of CGJ under 520 nm and the tentative identification; **(B)** the content of detected anthocyanins in CGJ; **(C)** UV chromatographs of CGJ under 370 nm and the tentative identification; and **(D)** the content of the detected flavonols in CGJ. Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Mv, malvidin; Pn, peonidin; Pt, petunidin; My, myricetin; Q, quercetin; G, glucoside or other hexoside; Ac, acetyl; Co, coumaroyl; GR, glucuronoyl.

Multiple-reaction monitoring (MRM) mode of QQQ-MS/MS is a highly specific and sensitive MS technique that can selectively quantify the compounds within complex mixtures. It selects specific analytes and absolute quantitation of proteins, peptides, metabolites, and lipids in the fields of biochemistry, drug metabolism, and plant studies (39, 40, 44). In contrast to the UV chromatograms of GSE capsules, the CGJs are more complicated. In part, this may be due to the loss of some phenolic compounds because of the solubility during the extraction process, while the removal of extraction solvents may meanwhile destroy the thermal-labile compounds. Taking into account that the sensitivity of this methodology is higher than that of the UV/Vis detector, and the complexity of CGJ, the MRM mode of UPLC-QQQ-MS/MS was next used to analyze all CGJ samples.

Due to highly priced commercial phenolic compound standards, we first screened the CGJ using our published UHPLC-QQQ-MS/MS method (41), and identified 21 phenolic compounds in CGJ DS samples, including 3-hydroxytyrosol isochlorogenic acid, 3,4-dihydroxybenzoic acid, 4-methyl gallic acid, catechin, procyanidin B2, epicatechin, 3-hydroxybenzoic acid, caffeic acid, 4-hydroxybenzoic acid, vanillic acid, dihydromyricetin, syringic acid, resveratrol-3-glycoside, dihydroferulic acid, 3-hydroxycinnamic acid, taxifolin, sinapic acid, ferulic acid, phenylacetic acid, and *trans*-2-hydroxycinnamic acid. Then, the UHPLC-QQQ-MS/MS method was optimized based on 21 phenolic compounds. A well-separated peak for each compound standard was achieved as shown in **Figure 5A**. Standard curve linearity, detection limits, precision, and recovery of phenolic compounds are shown in **Supplementary Table S1**.

Determination of the contents of each of the targeted phenolic compounds in the CGJ and CGJ concentrate and the results are presented in **Supplementary Table S9**, **Figure 5B**. Among the 21 phenolic compounds, the most abundant compound in CGJ is *trans*-2-hydroxycinnamic acid, with a concentration of 10.46 ± 0.50 μg/ml. Moreover, the CGJ DS also has a high concentration of caffeic acid and 3-hydroxycinnamic acid, with the values of 8.90 ± 0.24 and 7.72 ± 0.25 μg/ml. All three compounds belong to the hydroxycinnamic acid, which is an important class of polyphenolic compounds originated from the Mevolanate-Shikimate biosynthesis pathways in plants and possess potent antioxidant and anti-inflammatory properties (42). Recent publications and data have confirmed the important role of those kinds of hydroxycinnamic acid class compounds in the prevention and treatment of obesity, diabetes, and associated disorders (43). The total content of all the identified phenolic compounds using our optimized UHPLC-QQQ-MS/MS was 42.21 ± 1.28 μg/ml with an RSD of 2.10%. Overall, our results clearly revealed that the CGJ DS is a rich source of phenolic compounds and CGJ is well-homogeneous, suggesting that the manufacturing system meets the USP standardization.

CGJ Preliminary Stability Study at Month 6 Time Point

While this part of experiment with CGJ is still in progress, the data in detail are presented in **Supplementary Table S11**. For CGJ, we found that under proper cold storage conditions (4°C), with the bottled product kept in the packaged and shipping cartons and in absence resulted in a stable shelf-life for anthocyanidins, flavanols, and phenols up to month 6, at which time these compounds decreased to 85.70, 95.52, and 94.98%, respectively, of their original contents. The total concentration of all the identified compounds also decreased to 89.20%. Taking into account that anthocyanidins are more unstable than flavanols and phenols, the degradation rate of anthocyanidins was higher as expected than the others. More research is needed to fully understand the degradation profile of CGJ and to identify the strategies to further extend its shelf-life in storage. Our data show that under our experimental conditions the CGJ DS is stable until month 6 after which a new batch

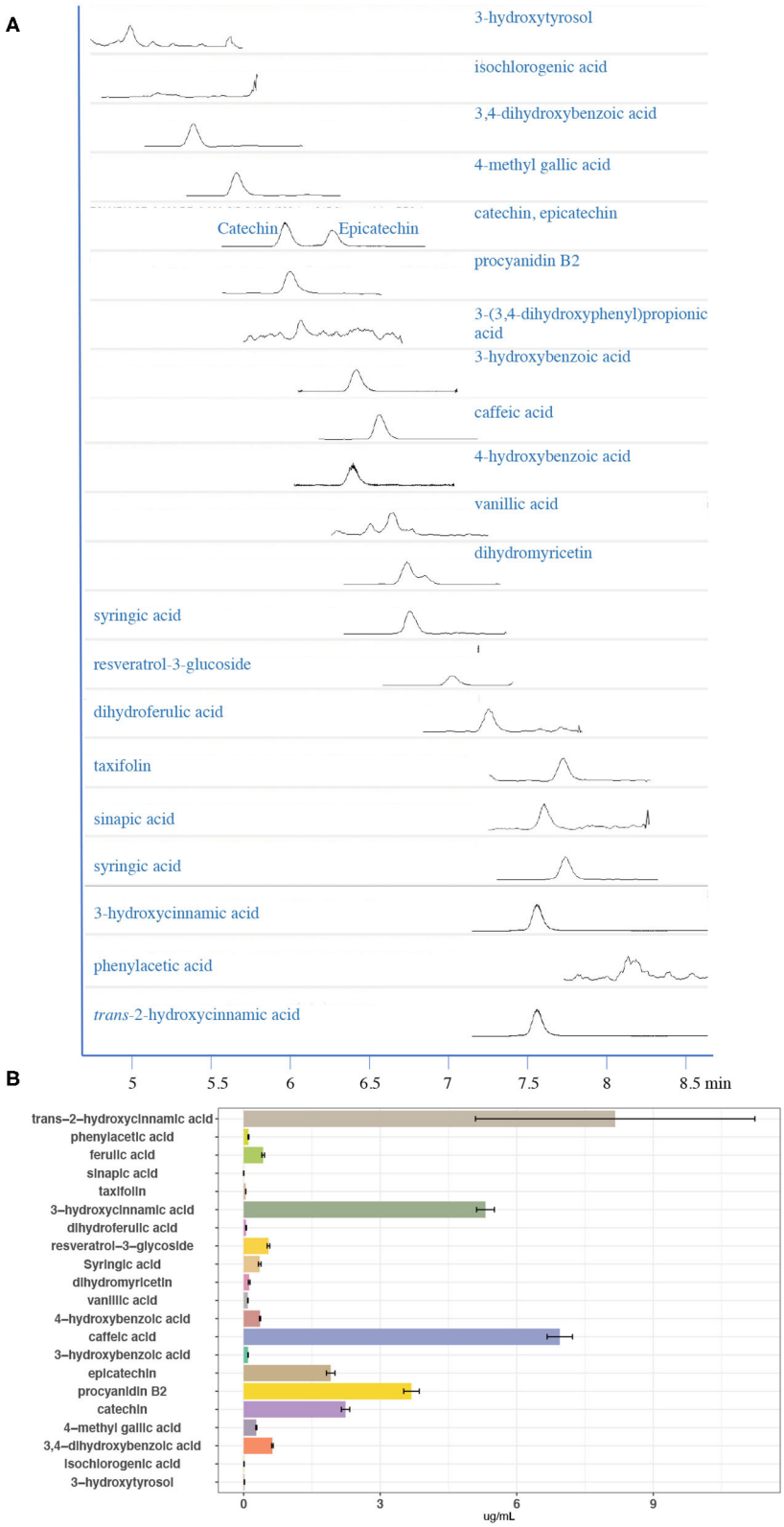


FIGURE 5 | (A) Mass spectrometry (MS) chromatographs of CGJ and **(B)** the content of targeted compounds in CGJ.

with the same needed chemical profile should the clinical trial continue beyond that time point.

CONCLUSION

In the present study, we extensively analyzed three *V. vinifera*-(grape-) based DSs, including GSE and RSV capsules, and CGJ, using our optimized LC-UV/Vis-MS and UHPLC-QQQ/MS methods. The weight variation of GSE and RSV capsules was also evaluated according to the USP tests. The total polyphenol content in the three products was also tested. From the results, all RSV and GSE capsules satisfy or meet the guidelines of the USP<2091> (19). Moreover, GSE capsules and CGJ both possessed a high polyphenol content according to the total polyphenol content test. All RSV capsules were free of *cis*-RSV as well as other impurities from our optimized HPLC-UV/Vis-MS, and the average content of *trans*-RSV in RSV capsules only exceeded the labeled content by 0.8%. Meanwhile, the chemical fingerprinting using the HPLC-UV/Vis-MS method displayed that the content of total identified proanthocyanidin compounds together with gallic acid in each GSE capsule is very similar, and the GSE capsule is a good resource of catechin and epicatechin, with 65.07 $\pm$ 2.01 mg and 61.05 $\pm$ 1.76 mg per capsule, respectively. Thirteen anthocyanins and five flavonols were identified and quantified using the HPLC-UV/Vis-MS methodology, with the total concentration of the identified anthocyanins and flavonols being 177.39 $\pm$ 6.67 μg/ml and 69.82 $\pm$ 4.66 μg/ml. Finally, the optimized UHPLC-QQQ/MS method was used to quantify 21 phenolic compounds in CGJ, and this DS showed a high concentration of *trans*-2-hydroxycinnamic acid, caffeic acid, and 3-hydroxycinnamic acid. The present study provides a comprehensive overall QC for grape-derived DSs, and the results show that a careful strategic approach to the authentication of each botanical ingredient to be used in clinical trials needs to follow the NIH guidelines on natural product integrity to avoid the issues of adulteration (13). Given the complexity of these and most botanical products from the issues of purity, quality, adulteration, consistency, and coupled to the complex chemistry found in grape-derived botanicals, such an approach is required to ensure that each of the materials used is homogeneous and stable and contain specific concentrations and profiles of bioactives to provide the needed solid foundation upon which clinical trials are conducted with the goal of realizing measurable mental health outcomes such as reducing depression and anxiety and understanding of their underlying biological mechanisms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

WL, JS, and QW designed the experiment. WL executed the experiment, performed the data analysis, and drafted the manuscript. JS and QW supervised the overarching project. WL, DR, MF, GP, and JM discussed the original concept and overall objectives. DR and MF provided key insights into botanical ingredients and formulation. GP and JM provided criteria under which the botanicals were needed for clinical trial applications. All authors contributed to reviewing, editing, and strengthening of the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: DR was employed by company Eagle Nutritionals.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest..

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frontiers in Nutrition

METHODS
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Designing, Conducting, and Documenting Human Nutrition Plant-Derived Intervention Trials

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Best practices for designing, conducting, documenting, and reporting human nutrition randomized controlled trials were developed and published in Advances in Nutrition. Through an example of the randomized clinical trial on blueberries and bone health funded by the National Institutes of Health, this paper will illustrate the elements of those best practices that apply specifically to plant-based intervention clinical trials. Unique study design considerations for human feeding interventions with bioactive plant compounds include the difficulty of blinding the intervention, background nutritional status of participants, carry-over effects of the intervention, benefits of a run-in period, lack of safety/tolerability data, and nutrition-specific regulatory policies. Human nutrition randomized controlled trials are the gold standard for establishing causal relations between an intervention and health outcome measures. Rigorous studies and documentation define the quality of the evidence-base to inform public health guidelines and to establish personalized dietary recommendations for the health-promoting plant components.

Keywords: best practices, human nutrition, clinical trials, plant-derived interventions, study design

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INTRODUCTION

A comprehensive resource on best practices for designing, conducting, documenting, and reporting human nutrition randomized controlled trials (RCT) for everyone involved in the clinical trials research enterprise was published as a series of manuscripts in the American Society for Nutrition journal, Advances in Nutrition (1–5). A two-part training workshop on these articles was offered by the American Society for Nutrition in July 2021 with plans for future repeat offerings of the workshop. The National Institutes of Health (NIH) Clinical Trials Award (CTSA) program and the American Society for Nutrition appointed members of a working group called Nutrition InteRvention ReSearcH (NURISH) to develop best practices and train researchers, institutional representatives, research sponsors, and regulators to improve rigor of human nutrition research that provides the evidence-base for making policy decisions regarding diet with the ultimate goal of improving human health.

The articles that described best practices for human nutrition RCTs cover general considerations unique to nutrition interventions such as the difficulty of blinding the intervention, baseline nutritional status of participants, carry-over effects, run-in periods, and safety of the intervention. The aim of this perspective is to discuss these best practices in the context of human plant-derived interventions. Key concepts are illustrated with examples from an NIH-funded RCT of the dose-response effects of freeze-dried blueberry powder on bone calcium

retention in postmenopausal women (NIH/NICCH grant: R01 AT008754; ClinicalTrials.gov ID: NCT02630797). This RCT employed novel bone labeling technology and was conducted in healthy participants, thus, results are more generalizable compared with trials in patient populations. The trial required careful monitoring of participant well-being to avoid attrition and presented many considerations relevant to the design and conduct of plant-derived interventions. Although our primary outcome of net bone calcium retention in postmenopausal women is a specific outcome, the methodologies described herein are relevant to other aging and chronic disease related outcomes including cardiovascular, cardiometabolic, cognitive, inflammatory, and gastrointestinal outcomes.

DESIGN OF RANDOMIZED CONTROLLED TRIALS

Identifying a research question that is important, novel, and feasible to address with the available methods informs the study aims, hypotheses, design, and procedures. RCTs are considered to provide the most reliable evidence on the effectiveness of interventions, because they minimize the risk of confounding by other factors, and thus, help to establish causal relations between the exposure and health outcome measures.

Plant-derived interventions have unique challenges. For example, bioactive components such as polyphenols are not under the same homeostatic control mechanisms as nutrients. Bioavailability studies of these bioactive compounds show that absorption is limited, but we know little about their metabolism, distribution, and excretion. They undergo extensive metabolism by the gut microbiota, which complicates the causal pathway. Their concentration in plasma is usually low and they may be unstable; thus, their quantification requires highly sensitive and selective analytical techniques (i.e., high-performance liquid chromatography and mass spectrometry).

The characteristics of the example study on blueberries and bone are presented in the format of the CONsolidated Standards Of Reporting Trials (CONSORT) guidelines (6) (**Table 1**), which were published in 2010 with the aim of improving quality of clinical trial reports. The blueberry and bone RCT used the gold standard of a randomized, crossover trial design as illustrated in **Figure 1** and investigated changes in net bone calcium retention (a measure of bone loss and our primary outcome) in response to a dietary intervention with freeze-dried blueberry powder. Changes in net bone calcium retention were quantified with the use of a rare, long-lived radiotracer, ^{41}Ca, measured in urine by accelerator mass spectrometry (AMS) (7). The use of this ultra-sensitive measurement method and the ability to make within-subject comparison of treatment vs. control over time greatly increased the power to assess the efficacy of our intervention, thereby decreasing sample size and intervention duration compared with trials using the traditional bone density measurement approach. The AMS method also allows for a relatively more rapid screening of several interventions than is feasible with bone mineral density or fracture outcomes. The equilibration period, which is necessary to allow bone to be labeled with the rare isotope, also serves as a run-in period to determine participant commitment to the protocol. In the blueberry and bone RCT described in **Table 1** and **Figure 1**, we tested three doses of blueberry powder in 13 participants over 1.5 years compared to a typical parallel arm study in two groups of $\geq$60 each requiring up to 4 years to establish intervention-related changes in bone mineral density using densitometry. The multiple studies conducted to validate our study design were described by Weaver et al. (7).

A limitation of the crossover design is a potential carry-over effect from one intervention period to the next. In our previous studies of bone health, only the intervention with bisphosphonates (osteoporosis treatment drug that is retained in the skeleton) precluded urinary ^{41}Ca:Ca ratios from returning to baseline after a 50-day washout period (8). Thereafter, positive control long-acting drugs were given as the last intervention rather than in a randomized order. Our protocol is most feasible for small efficacy studies with limited generalizability compared to effectiveness studies. Effectiveness studies in a "real world setting with a more generalized population" typically follow an efficacy study before policy is developed.

PARTICIPANTS

General participant considerations were discussed in Lichtenstein et al. (2). The choice of study population influences the generalizability of the study results. The more diverse the population, the more generalizable the results, although responses to a plant-derived intervention are likely to vary even in a relatively uniform sample due to mediating factors, which are difficult to control even in an RCT (e.g., background diet, physical activity, gut microbiota). The likelihood of high variability requires a larger sample size, which imposes a higher cost of the study. Patients with a specific disorder of interest may be more responsive to the intervention than a generally healthy population. However, recruitment of patients requires careful screening of medical histories to exclude participants with medications or conditions potentially confounding to the outcome of interest. Participants in our blueberry and bone RCT were healthy women stable to menopause. Postmenopausal women are most vulnerable to bone loss, and therefore, most likely to benefit from dietary interventions that would ameliorate bone loss due to estrogen deficiency. We selected women at least 4 years menopausal because rapid and inconsistent bone loss during the perimenopausal period would shift urinary ^{41}Ca:Ca ratios independent of diet effects. To control fluctuation of two nutrients known to influence bone loss, we provided calcium and vitamin D supplements throughout the study. We also monitored serum 25(OH)D to ensure that status did not change throughout the study. Vitamin D status can also affect immune function, and thus indirectly the health outcome. Sex differences were not determined in our RCT, but preclinical studies of blueberries showed important sex differences of bone in response to blueberry feeding (9). Determining sex differences should be part of the study design for plant-derived interventions whenever possible.

TABLE 1 | CONSORT guidelines applied to Blueberries and Bone randomized controlled trial.

Section/topic	Guideline
Title	Dose-response effect of blueberries on net bone calcium retention in postmenopausal women: a randomized controlled trial
Abstract	Structured summary of trial design, methods, results and conclusions
Introduction	
Background	Preclinical studies have shown a benefit of blueberry consumption on bones.
Objective	To evaluate the dose response effects of blueberry consumption on bone calcium retention in humans
Hypothesis	Increasing dose of freeze dried whole blueberry powder will decrease ^{41}Ca excretion from bone in postmenopausal women.
Methods	
Trial design	Randomized, crossover; changes to trial design: intervention periods reduced from 50 to 42 d
Participants	Healthy postmenopausal women ($\geq$4 years postmenopause) aged 45–70 years, not on osteoporosis treatment medication or other medicines that influence bone loss for $\geq$6 months prior to study initiation, not osteopenic or with a history of bone fractures, and willing to discontinue self-selected natural products
Study settings	Free living with clinical visits at the University Clinical Research Center
Intervention	Three doses of freeze-dried whole blueberry powder, i.e., low (17.5 g equivalent to 0.75 cup fresh berries), medium (35 g equivalent to 1.5 cups fresh berries), and high (70 g equivalent to 3 cups fresh berries)
Outcomes	The primary outcome measure was urinary ^{41}Ca excretion from pre-labeled bone (equilibrated for $\geq$150 days post dose for soft tissue ^{41}Ca to be excreted) expressed as % net bone Ca retention compared to non-treatment periods of baseline and washout. Secondary outcomes included diet analysis, polyphenolic content of blood and urine, and bone turnover biomarkers.
Changes to outcomes	None
Sample size	Eighteen participants were enrolled, 16 initiated the study, and 13 completed the entire study giving us 80% power to detect a 0.9% improvement in ^{41}Ca retention based on effect size and retention in previous similar studies conducted by our research group.
Interim analyses and stopping guidelines	Our *a priori* rules were to stop the intervention if adverse events or new information raised safety concerns or if recruitment failed.
Randomization	The dose sequence was generated by a random generator program by the study statistician. The products were coded according to dose by the Clinical Research Center kitchen staff. The Study Coordinator recruited participants, managed the clinical visits, and supervised sample preparation for analysis.
Blinding	Products were prepared by kitchen staff to vary only the dose of blueberries. Research staff and participants were blind to the product codes according to dose.
Results	
Participant flow	Of the 16 participants who enrolled and began the study, 13 completed the entire study and constituted the sample for analysis.
Losses and exclusions	Seventeen were found ineligible on screening, three dropped out before the first intervention, and one completed two of the three phases before she moved out of the area.
Reason for stopping trial	The IRB suspended the trial for 2 months because of an adverse event.
Baseline data	Baseline and clinical characteristics were collected for the participants.
Outcomes	Will be reported elsewhere.
Discussion	Limitations and interpretation will be reported elsewhere.
Generalizability	This was an efficacy, not an effectiveness trial, in a small group of reasonably similar postmenopausal women.

INTERVENTION, BACKGROUND DIET, RANDOMIZATION, AND ADHERENCE

A plant-derived intervention can be provided in the form of a food, an ingredient, a supplement, or an extract. When selecting the intervention, form and dose level, safety, acceptability, and practicality are key considerations. Testing several doses within the safety limit is useful when the dose-response relationship is not established, as is the case for many plant bioactives. Single vs. repeated dosing can also alter bioavailability and pharmacokinetic response to polyphenols (10).

In our blueberry and bone RCT, the intervention consisted of three dose levels (low: 17.5 g/d, medium: 35 g/d, and high: 70 g/d) of freeze-dried whole blueberry powder incorporated into three products: a drink, a spread, and granola bites (cubic bars) consumed as part of a self-selected diet. Considerable product development efforts were undertaken to formulate products that did not require heat for preparation (high temperatures may degrade certain bioactive constituents), were palatable at the provided doses of blueberry powder (equivalent to 0, 1.5, and 3 cups of whole blueberries), and practical for consumer use with minimal preparation and storage requirements. Importantly, we also verified stability of the polyphenol profile in the freeze-dried

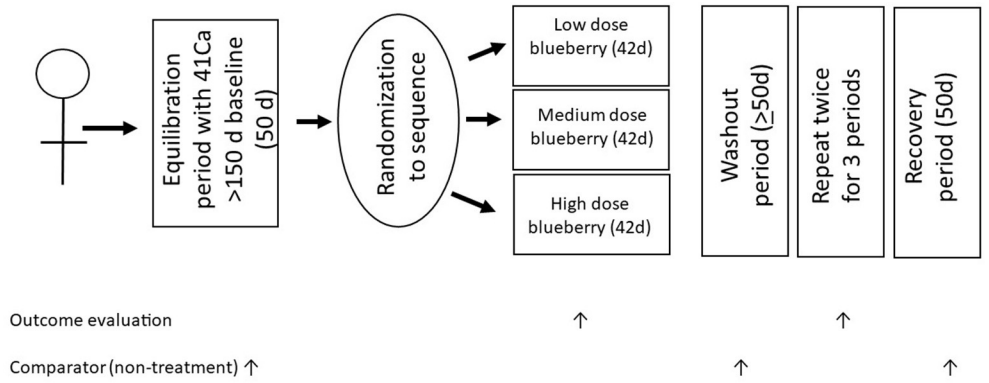

FIGURE 1 | Randomized crossover study design for blueberries and bone RCT.

blueberry powder and the intervention products throughout the study. The concentration of total polyphenols in freeze-dried powder was 35.2 ± 0.6 mg/g, which was consistent with data provided by the manufacturer. The concentration of total polyphenols in the intervention products ranged from 522 to 613 mg per each low-dose serving indicating that processing lowered the concentration by <20%. Participants taste tested all products prior to study initiation and selected two products to consume daily, one in the morning and one in the evening. Our choice of a self-selected diet is more generalizable than the use of a controlled diet. Moreover, when we compared an intervention of a prebiotic as part of a controlled diet (11) vs. a self-selected diet (12) on calcium absorption efficiency in a cross-over study in adolescents, results did not differ. However, the self-selected diet could introduce potential confounding effects of nutrient and bioactive compound intakes that blunt the effect of the intervention.

Formulating a comparator control or placebo can be challenging in plant-derived human intervention trials, especially if the intervention is rich in pigment, as with blueberries. Both participants and researchers may be able to infer treatment and/or dose by color if the products are compared side by side. The choice of comparator product or placebo is further complicated by the potential interaction of plant-derived compounds with other product components (e.g., proteins) or the interaction of placebo components (e.g., fibers) with gut microbiota. In a prior trial of prebiotic fibers (11), we have used maltodextrin as the comparator, but there is some concern that maltodextrin alters the gut microbiome, which acts as the mediator for health outcomes.

In our blueberry and bone RCT, double blinding was implemented by coding of products by research kitchen staff prior to dispensing them to the study coordinator who delivered them to participants. Minimally, single blinding can usually be accomplished if sample analysis is performed by researchers blinded to the intervention.

In crossover trials, the use of comparator or placebo may be substituted by adding a control period, during which participants undergo the same procedures as during the experimental period without consuming the intervention product. In our blueberry and bone RCT, multiple untreated periods (baseline, washout, and recovery periods) served as the control periods. We opted not to use a placebo based on our previous RCT of hesperidin using the same protocol, which demonstrated that results of the placebo period were indistinguishable from those of the untreated periods (13). Eliminating the placebo also reduced participant burden by shortening the study by one placebo period and the subsequent washout (∼100 days).

The randomized schedule for the sequence of interventions was provided by our statistician. More complex studies that assign participants to different groups may randomize by blocks or clusters to minimize bias, ensure that groups have similar baseline characteristics, or minimize contamination of the intervention as discussed in Lichtenstein et al. (2).

A common approach to monitor adherence and limit confounding by other nutrients/dietary bioactives is to provide participants with a list of polyphenol-rich foods to avoid and/or limit. In the blueberry and bone RCT, we instructed participants to limit the consumption of polyphenol-rich foods and collected diet records to quantify polyphenol intake. In addition to the self-reported diet records, the study coordinator kept a record of returned uneaten foods (<2% returned) and spot and 24-h urine samples were analyzed for polyphenols. To minimize attrition, e-mail reminders and calendars with all study visits were sent to participants on a weekly basis. A run-in period is also useful to determine participant commitment to the study protocol, but it may alter baseline measures (2).

OUTCOME MEASURES

When selecting outcome measures, biomarkers for outcomes of interest should be on the causal pathway and validated for predicting the end condition. In the blueberry and bone RCT, changes in the primary outcome, i.e., urinary ^{41}Ca:Ca, have been validated against changes in bone mineral density (14). Bone mineral density is also a biomarker for fracture risk, the health outcome of interest, and is one of several biomarkers approved by the FDA. Other FDA approved qualified biomarkers for specific

chronic diseases include serum cholesterol and blood pressure for cardiovascular disease, adenomatous colon polyps for colon cancer, and elevated blood glucose and insulin resistance for diabetes (15). An active area of research is identification of validated biomarkers of exposure and predictors of health outcomes. In nutrition intervention studies, the exposure is often estimated by self-reported dietary assessment methods. Best practices for this approach have been reported (16), but more objective approaches are desirable. Davy and Davy (17) make a strong case for controlled feeding studies to reduce variability of exposure. Genetics, metabolomics, and microbiome profiles are some of the approaches being investigated to identify good biomarkers of both exposure and outcomes and to account for potential confounders. In our trial, we measured phylogenetic diversity of bacterial communities using 16S rRNA sequencing. The statistical analyses of microbial taxa, alpha and beta diversity, and correlations with polyphenol metabolites and bone health outcomes are ongoing.

Few studies using plant-derived interventions consider the timing of sample collection relative to the ingestion of intervention for monitoring exposure. Most studies default to collecting fasting urine and/or blood, although serum concentrations of plant-derived bioactives tend to be more variable than in urine. Because the half-life of bioactives is usually short (<12 h), a fasting sample could miss their appearance if consumed the day before. Furthermore, there is more natural variation in urinary excretion of bioactives than para-amino benzoic acid, which frequently serves as a marker of urine collection completeness due to its 100% excretion in urine. In our blueberry and bone RCT, we used 24-h urine samples for both the primary outcome and polyphenol outcomes, as well as for monitoring adherence. The most abundant metabolites recovered from 24-h urine included anthocyanin metabolite delphinidin-3-glucuronide and two phenolic acids, hippuric acid and caffeic acid sulfate. Continuing efforts are focused on estimating the interindividual variation in blueberry polyphenol metabolite excretion as well as the changes due dosage.

Timing of ingestion of the bioactives can influence not only adherence measures but also the outcome measures directly or indirectly by altering mechanisms that influence health outcomes. A study of the effect of morning vs. evening consumption of chocolate showed that, compared to evening consumption, morning consumption decreased *ad libidum* energy consumption, fasting glucose, and waist circumference, increased lipid oxidation, sleep onset variability and temperature rhythms, and altered microbiota composition and function (18).

DOCUMENTATION AND REGULATION

The importance of documentation and meeting regulatory requirements for human nutrition RCTs was described in detail by Weaver et al. (3). A plant-derived intervention may require pre-approval by the Food and Drug Administration (FDA), even if it is a commonly consumed food ingredient or product. For our trial, we assessed multiple varieties of wild and cultivated blueberries using a principal component analysis and selected 6 with the most divergent phenolic profiles. These varieties were then tested for polyphenol bioavailability in a preceding animal study. The material of choice for the human trial was a composite of several low-bush varieties (*Vaccinium angustifolium*) sourced from a number of growing regions including Quebec, Newfoundland, Maine, and Nova Scotia. The composite was prepared by Wild Blueberry Association of North America and freeze-dried by FutureCeuticals, Momence, IL. The powder was packed in multilaminate foil pouches and stored at 4°C. The powder was accompanied by a certificate of analysis to ensure that it passed the safety test, and is also available commercially.

Prior to initiating our blueberry and bone RCT, we obtained a waiver decision by the FDA that an Investigative New Drug (IND) application was not required for trial initiation. An IND is required if the RCT is evaluating diagnosis, cure, mitigation, treatment, or prevention of a disease. This can be off-putting to a commercial supplier of a product who does not wish to have a public record that the FDA is evaluating their product as a drug when they are marketing it as a dietary supplement.

Aside from FDA, the sponsor, safety, and ethical committees may require certain characteristics of the test substance to be reported prior to trial initiation. These may include absorption, distribution, metabolism, and excretion determined in preclinical studies. In our RCT, the funding agency required an analysis of the blueberry polyphenolics, the hypothesized bioactives.

Ensuring participant safety and data integrity in a plant-derived intervention trial may require the oversight of multiple ethics and regulatory committees. An Institutional Review Board (IRB) at the research institution or a commercially contracted IRB reviews the study application. A Data Safety and Monitoring Board (DSMB) or Investigational Monitoring Committee (IMC) may be appointed by the funding agency or the Principal Investigator. The DSMB committee is RCT-specific and its members must have scientific expertise in the topic of investigation and experience conducting similar studies. For our blueberry and bone RCT, an IMC and Data Safety and Monitoring Plan (DSMP) (**Table 2**) were required. The DSMP included safety of the ^{41}Ca method, data protection, integrity, and confidentiality. Although consumption of freeze-dried blueberry powder was not expected to have any adverse effects, a standard process for reporting any adverse events was also included.

During the trial, a participant reported that the blueberry drink irritated a mouth sore, which was resolved by diluting the drink. The IRB suspended the study until we modified the consent form to include a statement that consumption of products with blueberry powder may cause oral irritation and re-consented all participants. The IRB also requested input from the IMC, which subsequently reported that it considered oral irritation to be a minor event and recommended that the study be permitted to resume. The suspension caused extra participant burden for those who were in the middle of an intervention when the study was suspended. Permission to repeat or extend a phase also required IRB approval.

The reporting of the suspension of the RCT by the IRB to the funding agency prompted an external audit by the sponsor. The

TABLE 2 | Data safety and monitoring plan for blueberries and bone RCT.

I. Study identification number

A. NIH/NCCIH study number: R01 AT008754; ClinicalTrials.gov ID: NCT02630797

B. Study title: Blue Berries and Bone

C. *Name of Principal Investigator (PI):* Connie Weaver, PhD

D. *Name and role of Co*-Is: Gorge McCabe, PhD-statistician; Munro Peacock, MD-study physician

II. Study overview

A. Brief description of the purpose of the Study: The overall goal of this study is to evaluate the dose response effects of continuous blueberry consumption over a 50 day period on net bone calcium retention in healthy-post menopausal women.

B. Adherence statement: The Data Safety and Monitoring Plan (DSMP) outlined below for RO1 AT0087541 will adhere to the protocol approved by the Indiana Clinical and Translational Science Institute (CTSI) Research Review Committee and the Purdue University Institutional Review Board (IRB).

III. Confidentiality

A. Protection of subject privacy

During the study, all records associated with each person's participation in the study will be managed using the usual confidentiality standards applicable to medical records. All of the materials collected are for research purposes only, and data will be kept in strict confidence. No information will be given to anyone without permission from the subject. The consent form includes the informed consent statements required by Purdue University. Confidentiality will be ensured by use of identification codes. All data, whether generated in the laboratory or at a clinical visit, will be identified with a randomly generated identification code unique to the subject.

B. Database protection

The database will be secured with password protection. Electronic communication with outside collaborators will involve only unidentifiable information. All paper source documents from all enrolled participants, including lab reports and subject study binders, will be stored in a locked cabinet in a locked storage facility, which is only available to the study staff. Electronic data will be stored in a password protected account.

C. Confidentiality during Adverse Event (AE) reporting

AE reports and annual summaries will not include subject or group identifiable material. Each report will only include the identification code.

IV. Adverse event information

A. Definition

An adverse event (AE) is any untoward medical occurrence in a subject during participation in the clinical study. An adverse finding can include a sign, symptom, abnormal assessment including laboratory test value, vital signs or any combination of these.

A serious adverse event (SAE) is any AE that results in one or more of the following outcomes:

- Death
- A life-threatening event
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity
- A congenital anomaly or birth defect
- An important medical event based upon appropriate medical judgment

B. Classification of AE Severity

AE's will be labeled according to severity, which is based on their impact on the subject. An Ae will be termed "mild" if it does not have a major impact on the subject, "moderate" if it causes the subject some minor inconvenience, and "severe" if it causes a substantial disruption to the subject's well-being.

C. AE attribution scale

AE's will be categorized according to the likelihood that they are related to the study intervention. Specifically they will be labeled definitely unrelated, definitely related, probably related, or possible related to the study intervention.

D. Expected risks

Expected risks to the subject are as follows:

- Radioisotope dose: the lifelong radiation exposure associate with receiving Ca-41 is <1/100,000th of a set of dental x-rays.
- Blood collection: The health risks involved in this study include drawing blood which can lead to bruising and infection. Precautions will be taken to minimize this risk by using sterile technique and applying pressure to the site after the needle is withdrawn. Professional trained staff will be present at all study visits at the Purdue University site to ensure necessary interventions in the event of adverse events. Trained staff at Indiana University School of Medicine (IUSM) will administer the ^{41}Ca.
- Dual energy x-ray absorptiometry: The average absorbed dose of radiation from the bone measurement is 1.424 mRem. In comparison the average exposure from a set of dental x-rays is 1 mRem and from a chest x-ray is 6 mRem.
- We know of no risks associated with consumption of blueberries. However, project personnel will contact each subject at least once during the intervention phases to inquire about such events. The occurrence of adverse events will also be queried during each clinical visit.

E. AE reporting and follow-up

Adverse Event Report Forms are to be completed at each clinical visit.

Individual data will be summarized and reported every 6 months to the Data Safety and Monitoring Committee (DSMC), IRB and other oversight organizations when necessary.

(Continued)

TABLE 2 | Continued

F. SAE reporting

SAEs that are unanticipated, serious, and possibly related to the study intervention will be reported to the DSMC, IRB, Indiana CTSI, FDA, and NCCIH in accordance with requirements.

- Unexpected fatal or life-threatening AEs related to the intervention will be reported to the NCCIH Program Officer within 7 days. Other serious and unexpected AE's related to the intervention will be reported to the NCCIH Program Official within 15 days.
- Anticipated or unrelated SAEs will be handled in a less urgent manner but will be reported to the DSMC, Indiana CTSI, NCCIH, and other oversight organization in accordance with their requirements. In the annual AE summary, the DSMC Report will state that they have reviewed all AE reports.

V. Data quality and safety review plan and monitoring

A. Data quality and management

1. Description of plan for data quality and management

The study staff will review all data collection forms on an ongoing basis for data completeness and accuracy as well as protocol compliance. Someone other than the study staff will enter data into the password protected spread sheets. A summary of the data review will be reported to the DSMC.

2. Frequency of data review

Data will be reviewed by the PI and/or Study Director every 6 months.

B. Subject accrual and compliance

1. Recruitment of subjects and compliance with inclusion/exclusion criteria

During the initial recruitment period the PI will review rate of enrollment and compliance with inclusion and exclusion criteria monthly until enrollment goals are met.

2. Reporting of compliance to intervention

Products to be consumed will be delivered bi-weekly to the participants. Any products that have not been consumed will be returned and the numbers will be recorded on an appropriate spread sheet. Participants will be provided with a calendar that is designed to report the date and time of consumption of the products. The PI and Director will review these records monthly and report to the DSMC if compliance falls below 50%.

C. Justification of sample size

We will use the same ^{41}Ca methodology that we have used in several other studies to evaluate the effects of interventions on net calcium retention. The response variable is the log of the ratio of ^{41}Ca to total Ca in urine samples. Specifically, for each subject a simple linear regression is constructed using all control and recovery periods. This line is then used to estimate values for the treatment periods. Differences between the estimates using the control data and the actual treatment values are averaged for each subject and then combined across subjects. Results are back transformed to obtain estimates of net calcium retention for each treatment. The crossover design is particularly efficient for these studies because the same control information can be used for each of the treatments. Based on our previous data, we will have 80% power to detect a 0.9% improvement in net calcium retention with 13 subjects.

D. Stopping rules

This study will be stopped prior to its completion if: (1) the intervention is associated with adverse effects that call into question the safety of the intervention, (2) difficulty in recruitment or retention that may impact appropriate evaluation of endpoints, (3) any new information becomes available during the trial that necessitates stopping the trial.

E. Designation of a monitoring committee

The PI will designate a DSMC to perform a review of ongoing study progress and safety. The members will not be associated with this research project.

F. Safety review plan

Study progress and safety will be reviewed quarterly. Progress reports will be provided to the DSMC. A summary of details of subject recruitment, retention and AE's will be included. An annual report will include evaluation of recruitment and retention as well as continuation of the study.

G. Study report outline

The study team will develop a plan for writing a study report that will include the following topics:

Study status including issues or problems, a study description including projected timetable, recruitment status, enrollment data, as well as summary of AE's and safety assessment.

VI. Informed consent

Written informed consent will be obtained from each participant before the screening process. A member of the study team will summarize the procedures involved in the study and answer any questions that the subject might have.

The participant will acknowledge their willingness to participate in the study by signing the consent form in the presence of the study staff member.

VII. Reporting changes in study status

Any disruption in the study status as a result of decisions made by FDA, IRB, or one of the study investigators will be reported to the funding agency (NICCH) within one business day.

external audit was conducted over 3 days and involved a review of regulatory documents, consent forms, source documentation, intervention preparation and dispensing records, study data, and a summary meeting with the Principal Investigator and the study staff. The same review standards and assessment criteria were applied as are used in monitoring pharmaceutical trials of substances with unknown and potentially serious side effects. The monitoring visit culminated with a report stating that no corrective action was necessary; however, 13 recommendations were provided for creating additional documents to track compliance with regulations and data integrity, e.g., delegation of authority log, concomitant medication use log, specimen

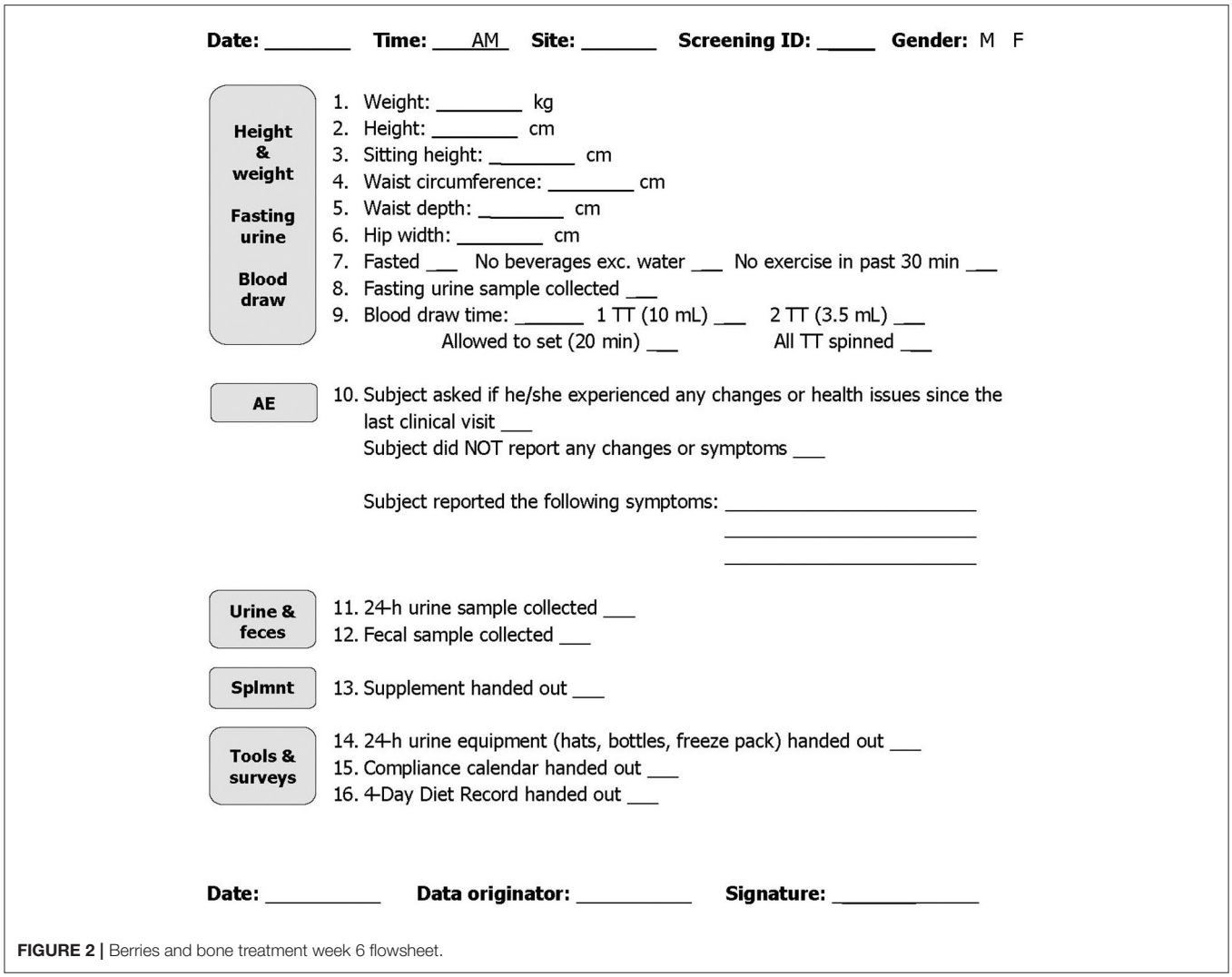

FIGURE 2 | Berries and bone treatment week 6 flowsheet.

tracking log, and internal quality assurance log. Assembling and maintaining such level of documentation may be a challenge in human nutrition trials, which do not usually have the support of multiple clinical research associates and data managers, but is nevertheless recommended by external auditing agencies.

STANDARD OPERATING PROCEDURES

Specific procedures need to be established to estimate and monitor exposures, adherence, safety, and efficacy of the intervention. A standard operating procedure (SOP) for each should be in place prior to trial initiation to assure data quality and improve reproducibility, especially if the trial is a multi-center study. In the blueberry and bone RCT, we used flowsheets outlining the steps of each participant visit (**Figure 2**) to standardize data collection procedures and monitor participant safety during the study. We also developed an SOP of good documentation practice (**Table 3**) to ensure that all study aspects are properly recorded.

STATISTICAL ANALYSIS PLAN

A statistical analysis plan developed *a priori* as part of the planning of the whole RCT is critical to the success of the study (5). The statistical analysis plan has many of the components of the DSMP described in **Table 2** for the blueberries and bone RCT; i.e., (1) Study descriptor information, (2) Background and rationale for the study, and (3) Study methods and sample population. It also includes a plan for selecting the sample for final analysis (intent-to-treat, per protocol, completers, safety), testable hypothesis with consideration for the primary and secondary outcomes, and specific approach to be used for statistical analysis. In the blueberry and bone RCT, all data were included for participants who completed at least two out of three intervention periods. No provision for populating the

TABLE 3 | Weaver laboratory good documentation practice standard operating procedures.

1.	Original source documents regarding study procedures and subject health (questionnaires, flowsheets, screening lab results, etc.) will be reviewed and filed before subjects move from one phase of a study to another. These documents will be retained after data entry for all studies and stored securely in a locked cabinet.
2.	The source of data (whether by self-report or by data collector) will be captured on all data collection forms. Source documents that require handwriting and that are completed by study staff will be filled-out legibly. A signature log will maintained in the regulatory binder, such that the data originator can be easily identified.
3.	Details of all communications with subjects regarding symptoms and study-related events will be documented in questionnaires completed by subjects and in clinical visit flowsheets. A Concomitant Medication Log will be used for subjects participating in clinical trials.
4.	All e-mails that contain any instructions or clarifications regarding study procedures, clinical visits, questions from subjects, information about concomitant medication use, adverse events and health problems, postponed and missed appointments, consultations with study physician, consultations with the principal investigator, and other study-related information will be filed on an ongoing basis in individual subject folders entitled "Subject (ID#) Correspondence."
5.	Instructions given to subjects either in-person or by e-mail will be transcribed and stored together with other study documents on the university password-protected storage network.
6.	Blank questionnaire items will be reviewed with subjects at the time of study completion to ensure that they were not omitted by mistake. If entries were left blank on purpose, they will be marked with ø symbol, the reviewer initials, and the date of review.
7.	Both Human Subject Protections and Good Clinical Practice training certifications will be on file prior to a staff member's involvement in a clinical trial.
8.	Study-specific Training Logs and meeting minutes will be maintained in the regulatory binder.
9.	Specimen Tracking Logs will be used for the collection, processing, storage, and disposal of all specimens collected from subjects.
10.	Study documents will be updated at the time of each amendment submitted to the IRB.
11.	All pertinent communications with the sponsor will be maintained in the regulatory binder.

Source documents - All information in original records and certified copies of original records or clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial.
Source data - All data contained in source documents (original records or certified copies).

database with missing data or use of covariates were planned. The primary outcome variable, i.e., natural logarithm of urinary ^{41}Ca:Ca ratio, was analyzed with a modified general linear model that included terms for participant, time, the participant $\times$ time interaction, and the intervention period. This model allows the intervention effects to be estimated from the difference between the intervention period and the non-intervention periods. Exponentiating the differences captures the treatment effect. Standard errors for significance tests were calculated using asymptotic methods and bootstrap procedures. SAS software was used for computations. The statistical model, bootstrap procedure, and sample data are available at http://www.stat.purdue.edu/~mccabe/ca41. A *P*-value was considered significant at <0.05. In our blueberry and bone RCT, no interim analysis was planned. However, advances in clinical trial designs with ability to alter sample sizes and analytical approaches are being explored to conserve resources and minimize subject burden. A special issue of Contemporary Clinical Trials featured examples of innovative and adaptive designs (19).

Best practices for reporting clinical trial progress and results were previously outlined in the paper by Petersen et al. (5). The CONSORT checklist provided in **Table 1** covers the fundamental elements of the RCT trial design and statistical analysis plan that need to be reported. A working group convened by the Federation of European Nutrition Societies (FENS) is developing a nutrition extension for the CONSORT checklist to include elements specific to the human nutrition trials (20). Some particular issues that have plagued plant-derived RCTs include not knowing the bioactive constituents or their mechanisms of action, use of biomarkers that do not reflect the health condition of interest, and an enormous placebo effect. In our blueberry RCT, we assumed that the bioactive component responsible for the observed changes in bone turnover were the polyphenols. However, blueberries with different polyphenol profiles have different effects on bone in preclinical studies (9). The bone effect mediated by the endogenous antioxidant and inflammatory pathway was also shown to vary by sex (9). The *in vitro* antioxidant activity of plant compounds does not reflect their physiological activity as previously thought. Thus, both *in vitro* and *in vivo* approaches have been developed to assess the endogenous antioxidant effects (9, 21). Physiological actions could also be mediated by the gut microbiota. Shifts in microbiota suggest that the fiber in the plant material, which serves as a substrate for bacterial metabolism, may also be the bioactive. In a preclinical study of blueberries, a dose-response effect of whole blueberries on the diversity and structure of the gut microbiota was observed; however, there were no significant differences in microbial diversity after feeding blueberry extract (without fiber) (22). Moreover, there were interactions between polyphenol metabolites and shifts in gut microbiota. These potential confounding/mediating factors are important to address in the study protocol and the statistical analysis plan.

Some plant-derived intervention trials have been unsuccessful because of larger than expected placebo effects. This is especially true when the outcome is subjective, as the perception of pain. In a large, multi-center RCT of chondroitin sulfate with and without glucosamine on knee pain, justified by several positive smaller trials, the placebo effect was as large as 60% (23). With such a

large placebo effect, it is nearly impossible to quantify the benefit of a bioactive over a comparator.

OPEN DATA SHARING

The NIH expects researchers and institutions to develop plans for data management and sharing as part of grant applications under a policy effective January 25, 2003 (24). Open sharing of data promotes secondary analyses that advance science and extend the impact of the investment in research including participant efforts. Open data sharing also allows for corrections to the databases thereby increasing the quality of evidence. It is hoped that all funded clinical trial research will adopt open data sharing practices.

Depositing data in a quality data repository generally improves FAIR (findable, accessible, interoperable, and reuseableness) attributes. Multiple data repositories exist for different types of data, with the 20 most frequently mentioned in literature identified by Federer et al. (25). For the blueberry and bone RCT, a National Science Foundation funded platform, Digital Environment for Enabling Data-Driven Science (DEEDS) was used to preserve, document, support, and publish data as online, discoverable datasets (26). To facilitate data sharing and re-use, it would be advantageous to develop one clinical trial repository available to all researchers.

IMPLICATIONS AND RECOMMENDATIONS FOR NUTRITION RESEARCH AND POLICY

Adoption of best practice guidelines for plant-derived interventions in human nutrition RCTs described in this article will increase the rigor of the evidence-base for determining dietary bioactive intake recommendations. Although few countries have attempted to develop dietary guidance for bioactives, a process for using an evidence-based approach for policy makers to establish dietary bioactive intake recommendations based on safety and beneficial health outcomes has recently been published (27). Dietary guidance is only as strong as the strength of the evidence-base. A consistent and transparent evidence-base can facilitate development of robust dietary guidelines for plant-derived compounds, foods, and beverages.

CONCLUSIONS

This paper outlines best practice guidelines for design and conduct of human nutrition RCTs involving plant-derived interventions. These guidelines are intended to promote rigor and transparency of the evidence-base used to establish dietary recommendations for health-promoting plant bioactives. Rigorous and transparent RCTs are needed to allow for causal interpretation of data in diverse populations, across the lifespan, race/ethnicity, and health status variables, and to address the limitations of the current literature for plant bioactives including lack of understanding of the mechanisms, effective and safe doses, and unanticipated effects (28).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CW conceptualized the topic. CW and JH researched and analyzed the literature and wrote the manuscript including interpretations. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Developing a Rational, Optimized Product of *Centella asiatica* for Examination in Clinical Trials: Real World Challenges

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Botanical products are frequently sold as dietary supplements and their use by the public is increasing in popularity. However, scientific evaluation of their medicinal benefits presents unique challenges due to their chemical complexity, inherent variability, and the involvement of multiple active components and biological targets. Translation away from preclinical models, and developing an optimized, reproducible botanical product for use in clinical trials, presents particular challenges for phytotherapeutic agents compared to single chemical entities. Common deficiencies noted in clinical trials of botanical products include limited characterization of the product tested, inadequate placebo control, and lack of rationale for the type of product tested, dose used, outcome measures or even the study population. Our group has focused on the botanical *Centella asiatica* due to its reputation for enhancing cognition in Eastern traditional medicine systems. Our preclinical studies on a *Centella asiatica* water extract (CAW) and its bioactive components strongly support its potential as a phytotherapeutic agent for cognitive decline in aging and Alzheimer's disease through influences on antioxidant response, mitochondrial activity, and synaptic density. Here we describe our robust, scientific approach toward developing a rational phytotherapeutic product based on *Centella asiatica* for human investigation, addressing multiple factors to optimize its valid clinical evaluation. Specific aspects covered include approaches to identifying an optimal dose range for clinical assessment, design and composition of a dosage form and matching placebo, sourcing appropriate botanical raw material for product manufacture (including the evaluation of active compounds and contaminants), and up-scaling of laboratory extraction methods to available current Good Manufacturing Practice (cGMP) certified industrial facilities. We also address the process of obtaining regulatory approvals to proceed with clinical trials. Our study highlights the complexity of translational research on botanicals and the importance of identifying active compounds and developing

sound analytical and bioanalytical methods for their determination in botanical materials and biological samples. Recent Phase I pharmacokinetic studies of our *Centella asiatica* product in humans (NCT03929250, NCT03937908) have highlighted additional challenges associated with designing botanical bioavailability studies, including specific dietary considerations that need to be considered.

Keywords: placebo, translation, *Centella asiatica*, botanical, dietary supplement, reproducible, clinical trials

INTRODUCTION

Botanical products are widely used by the public for their reputed health care benefits. Consumers in the United States (US) spent over $10 billion on herbal supplements in 2020, a record-breaking 17.3% increase from 2019 (1). The popularity of these products arises from familiarity with their folk medicine uses, vigorous commercial advertising, and their ready availability for self-selection through retail outlets. The US Food and Drug Administration (FDA) allows the marketing of botanical products as "dietary supplements" under the 1994 Dietary Supplements Health and Education Act (DSHEA). Notably, under DSHEA botanical products may be marketed without proof of efficacy, but must comply with labeling requirements limiting and qualifying the claims that are made. Nevertheless, consumers take these products with an expectation of a particular pharmacological effect or health benefit. At the same time, the FDA does provide for the development and registration of herbal products as "botanical drugs" for which proof of efficacy is a requirement (2).

While there is a significant body of literature on preclinical studies performed on botanicals, relatively few of these materials have been evaluated in formal, rigorous clinical trials. Additionally, several recent large-scale trials have failed to demonstrate a clinical effect of the botanical product under study (3). Recent articles led by authors at the National Institutes of Health (NIH) Office of Dietary Supplements (ODS) and National Center for Complementary and Integrative Health (NCCIH) have highlighted the particular challenges involved in the clinical evaluation of botanical supplements (3–5). Significant factors include the natural variation in plant materials and the multiplicity of available products of a given botanical. Similar challenges, and the need for standardization, have been noted for translational studies and clinical evaluation of plant foods that may promote health (6–8).

Important guidelines for the conduct of valid clinical evaluation of botanicals have been outlined (3–5). For the product these include: using a preparation method that closely matches traditional use or one that was used in supporting preclinical studies, confirmation that the active compounds of the botanical are present and remain stable throughout the trial period, selecting a dose expected to deliver therapeutic levels of the active compounds, formulating a product that is palatable and acceptable to study participants, and creating a matching placebo for successful blinding. The participants, treatment duration, and study end points selected must also be appropriate for the expected effects. It is now recommended that prior to performing costly efficacy trials, initial studies confirming bioavailability of the active compounds from the trial product, as well as identifying biological signatures in response to the intervention demonstrating relevant, mechanism-related target engagement are needed (3). Ultimately the health benefits of a botanical intervention need to be verified experimentally in efficacy trials (7). Given the inherent variability in raw botanicals and their products, it is acknowledged that it will not be possible to ensure total consistency between products tested in different trials. However, the provision of sufficient chemical analytical information and deposition of voucher samples would allow for comparison between the products used in separate studies (4, 5).

Here we present the process of designing a product made from *Centella asiatica* (L.) Urban (family Apiaceae), following the guidelines described above, for use in clinical trials relating to its potential use in the amelioration of cognitive decline. *Centella asiatica* (CA) is a small, perennial, creeper that grows in swampy areas of tropical and subtropical regions of Asia and Africa including Madagascar and Seychelles (9–11). The medicinal uses of CA can be traced from early documentation by the Indian physician Sushruta (*ca.* 1200 BC), to present worldwide use in commercial topical and oral products for skin and gastrointestinal conditions (12–14). Of particular relevance to our group's work is CA's importance in Ayurvedic medicine as a "medhya-rasayana" herb (i.e., one that has rejuvenating effects, boosts memory, prevents cognitive deficits, and improves brain function) (15–17). In the West, CA and CA dietary supplements sold under its Sri Lankan name "gotu kola" are marketed for its reputed benefits on brain and nerve function. Common preparations include tinctures (hydroethanolic extracts) or capsules containing powdered CA herb or a dried CA extract. A search for "*Centella asiatica*" and "gotu kola" on the NIH ODS labels database yields 657 and 1,476 hits, respectively (18), suggesting that there are around 1,500 dietary supplements containing CA currently available in the US.

The neurotropic and neuroprotective effects of CA have been widely studied and documented (14, 19). The vast majority of studies in the literature report data from preclinical models; however several small clinical studies also report CA's ability to improve memory, mood, or brain function in different population groups, including children (20), young adults (21, 22), middle-aged adults (23), or older adults (24–28). A meta-analysis failed to find a positive effect of CA on cognition (29); but as noted previously, the trials reviewed varied widely in the CA product tested, the level of product details provided, the

subject population, the end points examined, and the quality of the methodology (19, 30) making direct comparisons between studies difficult.

Our group has been studying the mechanisms and active compounds associated with the cognitive effects of a hot water extract of CA, "CAW." We have reported that CAW at doses of 200 to 1,000 mg/kg/day administered in the drinking water improves cognitive function in the Tg2576 (31) and 5xFAD (32, 33) mouse models of Alzheimer's Disease (AD), and also in aged wild-type (WT) mice (34–36). These effects are associated with improved antioxidant responses, mitochondrial activity, and synaptic density in the mouse brain (32, 34, 36, 37) and *in vitro* in neuroblastoma cells (38) and/or mouse primary neurons (39, 40).

CA's biological activity has classically been ascribed to its characteristic triterpene (TT) compounds (**Figure 1**), chiefly asiatic acid (AA) and madecassic acid (MA), and their glycosides asiaticoside (AS) and madecassoside (MS), respectively (12). The International Union of Pure and Applied Chemistry's (IUPAC) names for these compounds are available on PubChem (41). The neurotropic and neuroprotective effects of these TT compounds, particularly AA and AS, are well-documented (19); however, in our preclinical studies, we have found that another group of specialized compounds in CA, mono- and di-caffeoylquinic acids (mono- and di-CQAs), also contribute to CA's neurological effects (19). The nomenclature of these compounds in the literature is inconsistent and IUPAC names are provided in a recent review (42). CAW and equivalent concentrations of di-CQAs (but not TTs) were found to protect neuroblastoma cells from beta amyloid (Aβ) toxicity *in vitro* (43) and improve antioxidant and mitochondrial gene expression in these cells (38). A CAW-equivalent mixture of mono and di-CQAs improved cognition *in vivo* in the 5xFAD mouse model of AD (44). CAW, as well as some TT and CQA compounds in isolation, reverse Aβ related loss of dendritic arborization and spines in mouse primary hippocampal neurons (40). These data led us to conclude that both TT and CQA content would be important to evaluate, optimize, and document for any future clinical trial CA interventional products.

Based on these preclinical studies, CAW appeared to be a good candidate for development as a "botanical drug" for the treatment of cognitive decline, in both normal and pathological aging, notably AD. Since CAW elicits cognitive improvement in aged, but not young, wild-type mice (34), and in mouse models of AD (31–33), an appropriate target population for a botanical drug made from CAW was deemed to be older subjects (age 65 years and over) experiencing cognitive decline in normal or pathological aging.

Due to the unavailability of a commercially prepared product that matched the composition of CAW used in our preclinical trials, and a company willing to comply with FDA reporting requirements, we elected to develop a custom product containing CAW for use in clinical trials. This product will be referred to as *Centella asiatica* product (CAP).

Here we describe our approach to developing CAP including how we encountered and addressed the challenges that arose, in particular those typically associated with botanical products.

MATERIALS AND METHODS

Product Manufacture

Dosage Calculation and Delivery Method

Human doses equivalent to the mouse doses used in our preclinical studies (200–1,000 mg/kg/d) were estimated by interspecies (allometric) scaling (45). An oral delivery method was selected to mimic the preclinical studies. Additional consideration was placed on long-term compliance and convenience of consuming the dosage form in the target population selected.

Raw Material Selection: Identification, Chemical Characterization, and Evaluation of Contaminants

Sourcing of Material

To produce ample investigational product for the translational studies, including stability, bioavailability, safety, and biological signature evaluation, a source of a large quantity of single-batch raw CA herb material was identified. The form of the plant used in traditional herbal medicine (and our preclinical studies) is the dried aerial tops of CA, usually obtained from cultivated sources, so focus was placed on sources of aerial material to maintain ethnobotanical relevance and clinical applicability. Efforts were made to obtain organic material if possible (to minimize exposure to environmental toxins) and plant material that had been dried but had not undergone any other known processing apart from milling. Trade samples (80 g) of six different commercial sources of dried CA aerial parts, raw material (designated CA-1, CA-2, CA-3, CA-6, CA-7 and CA-8) were obtained through the dietary supplements company Oregon's Wild Harvest (Redmond, OR) and underwent quality control (QC) and chemical fingerprinting prior to purchasing a large quantity of raw material for product manufacture.

Identity Tests

First, organoleptic tests to confirm characteristic features (visual appearance, plant part, smell, and taste) and Fourier-transform infrared spectroscopy (FTIR) were performed on each of the six trade samples by the quality control laboratory at the Oregon's Wild Harvest. For organoleptic analysis, focus was placed on confirming the following characteristics: greenish-brown color with tan pieces, leafy, sour, a bit mint-like flavor, and a leafy aroma. FTIR spectroscopy is an established chemometric method for the identification of botanical powders where the spectrum of obtained samples is compared to a composite spectrum of multiple previous batches of the same botanical (46, 47). These crucial cost-effective analyses eliminated false or grossly adulterated products before proceeding with more costly chemical characterization and product manufacture.

To confirm identity of the botanical materials as CA, ethanolic (CAE) and aqueous (CAW) extracts were prepared from each trade sample using previously described methods (48). Extracts were analyzed using thin layer chromatography (TLC) alongside extracts prepared from CA materials used in our preclinical studies (CA-4 and CA-5) and commercial reference standards (Chromadex, Irvine, CA; Sigma Aldrich, St Louis, MO; TransMIT, Gießen, Germany) of the TT and CQA

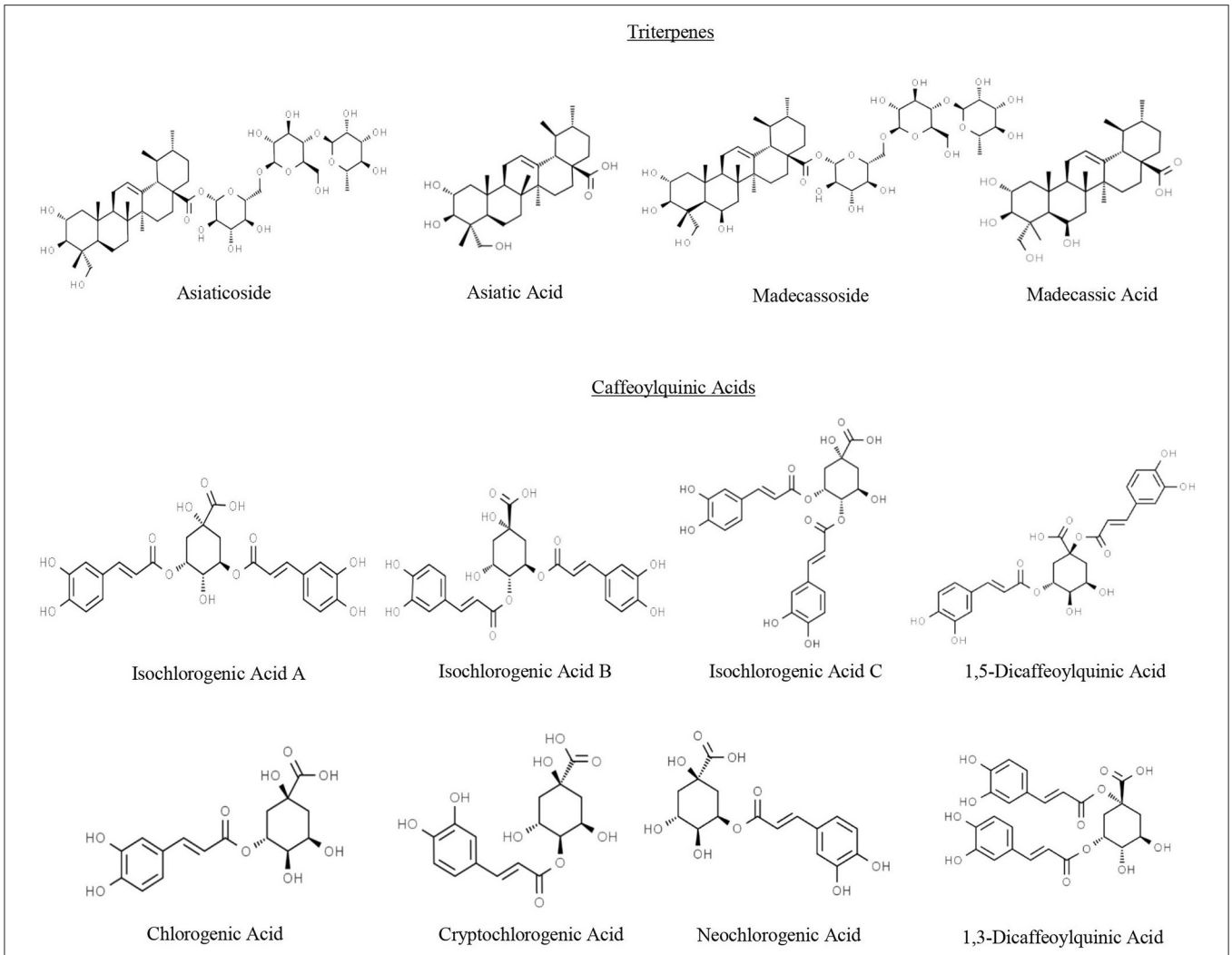

FIGURE 1 | Chemical structures of triterpenes and caffeoylquinic acids found in *Centella asiatica*. Structures were obtained from Chemspider.

compounds shown in **Figure 1**. For TLC, 25 μL of each CAW (10 mg/mL) or CAE (10 mg/mL) extract was spotted onto a silica gel stationary phase (200 μm) plate with fluorescence indicator (particle size 20 μm) on aluminum backing (Lot #3110; Sigma Aldrich Z193291) alongside reference standards (1 mg /mL, 25 μL). The mobile phases used were ethylacetate: formic acid: glacial acetic acid: water (100:5:5:5) for separating the CQA's, chloroform: methanol: glacial acetic acid (90:10:5) to separate the TT aglycones (AA and MA), and chloroform: methanol: glacial acetic acid (75:25:5) to separate the TT glycosides (AS and MS). Visualization of zones was achieved using ultraviolet light (254 and 365 nm) for the CQAs or 1% vanillin/sulfuric acid spray followed by heating for the TTs (49).

Chemical Characterization

For chemical characterization, high-pressure liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) (50) was performed on the trade samples and the CA materials used in preclinical studies to identify and quantify the TT and CQA compounds of interest. Untargeted LC-HRMS of the raw plant materials was also performed. Analytical data on mass, retention time, and peak area of all components detected using both positive and negative electrospray ionization (ESI) were recorded as "fingerprints" of the CA extracts to create an archival record of each material.

Evaluation of Contaminants

To assess the presence of contaminants, the Safe Quality Food Institute (SQFI)-certified quality control laboratories of Oregon's Wild Harvest performed impurity analysis of the sourced CA trade materials. Microbial content (aerobic plate count, yeast, mold, *Escherichia coli*, total coliforms, and *Salmonella*) was determined using the company's standard plating methods (Binary Detection Technology; BDT, AOAC 2005.03 and AOAC 2013.01). Aflatoxin analysis was not required due to the absence of mold in any of the samples. Heavy metal content (lead, arsenic, cadmium) was determined at Oregon's Wild Harvest using atomic absorption spectroscopy, while mercury and pesticide

analyses were performed by a third-party contract laboratory (Columbia Food Laboratories, Portland, OR) using inductively coupled plasma mass spectrometry and gas chromatography coupled to mass spectrometry, respectively. In addition to the raw plant material, heavy metal and pesticide content was also determined in water extracts prepared from these plant materials, as the levels could either be enhanced (for water soluble contaminants) or reduced (for water insoluble contaminants) by water extraction.

Evaluation of Bulk Materials Selected for Manufacture

Identity tests and contaminant evaluation were repeated at Oregon's Wild Harvest on the bulk samples chosen for use in product manufacture, with attention to the use of standard Oregon's Wild Harvest sampling procedures (United States Pharmacopeia (USP) #40; Part 561; Articles of Botanical Origin). In addition, analysis of aflatoxins was performed out of an abundance of caution, even in the absence of mold. Aflatoxin analysis was performed either at Oregon's Wild Harvest (Agri-Screen, Neogen Co.; United States Department of Agriculture (USDA)-Grain Inspection, Packers and Stockards Administration (GIPSA) 2010-006) or by a third-party contract laboratory (Romer Labs, Union, MO) using high-performance liquid chromatography (HPLC). Chemical characterization by LC-HRMS (as in section Chemical Characterization) was also performed on these bulk materials.

Selection of Manufacturing Facilities and Formulation Development

Identification of Certified Facilities

It was essential to perform the product manufacture in facilities with Current Good Manufacturing Practice (cGMP) certification. In addition, such facilities needed to be capable of large-scale water extraction of CA and drying of the extract. Written communications were sent to numerous cGMP certified facilities followed up by phone calls which identified one suitable facility (Ashland Laboratories, previously Pharmachem Laboratories (PCL); Kearny, NJ). Additional facilities were identified (Oregon's Wild Harvest) that could complete product manufacture including blending of the dried extract with excipients and final product labeling and packaging.

Formulation of the Clinical Trial Product and Matching Placebo

In designing the clinical trial product formulation, delivery method, palatability and placebo matching were considered. Due to the oral delivery method selected for the target population, the sticky, hygroscopic nature of the dried CAW, and available facilities for mass manufacture, it was decided to dry the CAW extract onto a suitable matrix to optimize further blending and packaging steps, and aid its efficient dispersion in water for consumption by trial participants. The percent loading of CAW onto the matrix was determined based upon the final product's properties and the amount of matrix determined to be safe for consumption (51). Essentially different ratios of CAW and matrix were mixed in water, dried by lyophilization, and examined for homogeneity, texture, and water dispersibility.

Additional excipients were identified to improve palatability and color matching for a placebo. The recommended maximum daily intake, normal amount or percentage in food, normal daily intake, caloric content, and median lethal dose (LD50) of each excipient were used to identify potential excipients and determine safety and dosing. This information was found using the FDA Code of Federal Regulations, the National Library of Medicine ToxNet Toxicology Data Network, the Global Safety Management, Inc. Safety Data Sheet, the USDA and United States Department of Health and Human Services 2015-2020 Dietary Guidelines for Americans, the certificate of analysis and data sheet provided by the supplier of each excipient, and available journal articles discussing safety and tolerability of each specific excipient.

Each potential excipient evaluated was analyzed independently by the investigators at the Oregon Health & Science University's Bioanalytical Shared Resource/Pharmacokinetics Core Lab (Portland, OR) for TT and CQA content using HPLC-tandem mass spectrometry (HPLC-MS/MS) and excluded from consideration if any TT or CQA were identified as present. For the detection of TTs, selected reaction monitoring was performed on an Applied Biosystems Q-Trap 4000 LC-MS instrument (Framingham, MA). Chromatographic separation was achieved using a Poroshell 120 EC18 column (3 mm id × 50 mm; 2.7 μ), Poroshell ultra high-performance liquid chromatography (UHPLC) guard column, and a methanol:ammonium acetate gradient (Santa Clara, CA). Triterpenes were detected as their ammonium adducts with positive ion mode electrospray ionization using the following MS/MS transitions (m/z): AA (506/453), MA (522/451), AS (976/453; 976/635), MS (992/487; 992/451). Two internal standards were used with chrysin being detected as the molecular ion (255/255) and ursolic acid as its ammonium adduct (474/411; 474/191). For the detection of CQAs and their associated metabolites, HPLC-MS/MS was performed on an Applied Biosystems 5500 QTRAP HPLC-MS instrument (Framingham, MA) using an analytical method modified from that described by Nair et al. (52). Chromatographic separation was achieved using a C8 reversed-phase column (Agilent Zorbax Eclipse plus C8 Rapid resolution 4.6 × 150 mm 3.5 μ; Santa Clara, CA), an Agilent Zorbax Eclipse plus C8 Rapid resolution guard column (4.6 × 12.5 mm 5 μ; Santa Clara, CA), and an acidified acetonitrile:water gradient. CQAs, their metabolites, and internal standards were detected using negative ion mode electrospray ionization and the following MS/MS transitions (m/z): mono-CQAs (353/191); di-CQAs (515/353; 515/191); caffeic acid (179/135); ferulic acid and isoferulic acid (193/134); dihydrocaffeic acid (181/109); $^{13}C_9$-caffeic acid (188/143); $^{13}C_3$-ferulic acid (196/136); d_3-isoferulic acid (196/134); and d_3-dihydro-isoferulic acid (192/135).

Excipients with any reported neurological activity were eliminated from consideration. A total of 19 excipients were evaluated (14 for palatability, 3 for coloring, and 2 as a carrier matrix for dispersability).

Manufacturing Process, Analysis of Intermediates, and Final Product

Extraction and Drying

Selected CA herb batches (CA-3 and CA-6) were purchased in bulk by Oregon's Wild Harvest and shipped from the two original suppliers directly to the identified extraction and drying facility (Ashland Laboratories). Using one of three stainless steel jacketed reactors with stainless steel agitators each with water-cooled condensers and receivers (2 at 500-gallon capacity, 1 at 750-gallon capacity) and an evaporator pfaudler (3' diameter x 8' high; glass lined), CA dried herb was extracted using distilled water under reflux (160 g herb: 2 L water to match the laboratory scale method used in our preclinical studies). The hot extract was cooled from 212 to 150°F, filtered through a 200 mesh screen, then filtered through filter paper to remove particulates, and a 3 L aliquot (free of spray drying carrier matrix) was removed and sent to OHSU for lyophilization. This sample was used for phytochemical and percent loading analyses.

The remainder of the filtered extract was spray dried onto a carrier matrix at a target 66% loading capacity using a Type III A-No. 7 304 gas fired stainless steel APV anhydro spray dryer with centrifugal atomizer, Gaulin Homogenizer feed pump, with Clean in Place (CIP) system. Due to size limitations of the extraction equipment, two separate extractions were performed to obtain the required total amount of dried extract. In the first process, a mixture of CA-3 (14.50 kg) and CA-6 (30.5 kg) was extracted with 562.5 L of hot water and spray dried onto a carrier matrix (Batch 1). In the second extraction, CA-6 (45 kg) was extracted with 562.5 L of hot water and spray dried onto the same carrier matrix (Batch 2). A 200 g sample of spray dried CAW from each batch was sent to Oregon State University for analysis, while the bulk of the spray dried product was shipped to Oregon's Wild Harvest for quality control, blending into the drug product, packaging, and storage.

Analysis of Intermediates

Intermediates in the manufacturing process were evaluated for CAW content based on the concentrations of TTs and CQAs. LC-HRMS data was used to calculate the amount of CAW loaded onto the carrier matrix by comparing relative concentrations of the active compounds (mg/g material) in the Batch 1 and Batch 2 lyophilized samples and their respective spray dried counterparts. For this, the mean loading was calculated from values for all the individual TT and CQA compounds except for AA and MA. The data suggested that AA and MA levels were disproportionately skewed by possible hydrolysis of the more abundant glycosides, AS and MS, during spray drying.

Product Blending and Packaging

Based upon the amount of TTs and CQAs determined to be present in each batch of spray dried material, and the quantity of spray dried product (here referred to as "Gotu Kola Extract Preblend") needed for QC, stability studies, and the proposed trials, Batch 1 spray dried material was blended with Batch 2 spray dried material. The quantity from each batch used in the blend were determined using the desired phytochemical content of the final product.

Because the weights of "Gotu Kola Extract Preblend" corresponding to the different doses of intervention and the placebo would contain different amounts of matrix, additional matrix material was added to the placebo and to the lower CAW dose to equalize the matrix content across all 3 doses. All other excipients (for color and flavor) were added in equal amounts to each dose. The required excipients/additional matrix for each dose of CAP were blended in bulk in quantities sufficient for 200 individual dose sachets per dose. The correct weight and composition of blended excipients for a single dose of CAP was weighed into individual sachets (stand-up metalized barrier pouches, Item 183-60, Associated Bag Company, Sparks, NV) at Oregon's Wild Harvest. The required weight of "Gotu Kola Extract Preblend" was hand weighed and added to each individual sachet, to give the required dose of CAW. The filled sachets were heat sealed. All sachets were stored in the freezer (−20°C) until their use for quality control, stability tests, or dispensing for use in the proposed clinical studies.

Quality Control

All final CAP doses including the placebo were analyzed using TLC to confirm TLC zone profiles characteristic of CAW and to confirm the presence of TTs and CQAs in CAP containing CAW 2 g or 4 g but their absence in placebo. LC-HRMS was used to quantify TT and CQA in CAP 2 g and 4 g and to be able to identify those peaks in the untargeted fingerprint of CAP that belong to the excipients rather than CAW. All products were also analyzed for microbial, pesticide and heavy metal content. Uniformity of weight of the sachets was checked by weighing each sachet to confirm the correct weight for that dose and minimal variation between packages of a given dose.

Product Stability

To comply with regulatory requirements for obtaining Investigational New Drug (IND) status for a manufactured botanical product, an accelerated stability study was conducted in collaboration with the Food Innovation Center (FIC; Oregon State University, Portland, OR). Multiple sachets of each dose of CAP were placed at two different accelerated storage conditions ($25 \pm 2°C/60\% \pm 5\%$ relative humidity and $40 \pm 2°C/75\% \pm 5\%$ relative humidity) for 32 days. In addition, to compare routine, commonly available storage conditions, sachets were placed at −20°C (freezer), 4°C (refrigerator), and ambient temperature (benchtop) for the same duration. All samples were transferred to −20°C after 32 days until analysis by LC-HRMS ($n = 6$ replicates of each dose for −20°C; and $n = 3$ replicates of each dose for other temperatures). The placebo was not assessed for stability since the end point was analysis of levels of TT and CQAs. For each known bioactive compound, the mean level in the samples stored for 32 days under the other 4 conditions were compared to the level seen in the samples stored at −20°C (deemed to be the most stable) to determine degradation. Visual and olfactory inspections were also performed on all samples.

Bioassay to Confirm Biological Activity

An *in vivo* bioassay appropriate to the antioxidant and cognitive enhancing effects of CAW was used to confirm biological

activity of the final formulated product. *Drosophila melanogaster* fruit flies with a mutation in the *sniffer* gene *sni1* allele were determined to be an appropriate model due to an impaired locomotion phenotype consistent with neurodegeneration from oxidative stress (53). Study vials (35 mL, 24.5 × 95 mm) were prepared using standard *Drosophila* food with the addition of CAW (10 mg/mL), CAP (equivalent to 10 mg/mL CAW), excipients alone (equivalent to those in CAP), or deionized water (control). Flies (males and females) were placed in vials containing either control food or experimental food for 7 days before testing. Fast phototaxis assays were conducted as a measure of locomotion and cognitive function as previously described (54, 55) using a countercurrent apparatus (56) and a single light source. Data was analyzed using GraphPad (v.5 for Windows, San Diego, CA) and one-way ANOVA with a Dunnett's post-test to determine significance to the untreated control or to flies treated with the excipients alone.

Regulatory Considerations

Early in the planning of the proposed translational work, exploration was conducted into the necessity of Investigational New Drug (IND) status from the FDA. Written communication was sent to a member of the FDA's Botanical Review Team and, subsequently, an enquiry to the Food, Dietary Supplements and Cosmetics IND Jurisdiction Team (FIJT) was submitted. Following a recommended pre-IND consultation, an IND application including a detailed Chemical and Manufacturing Controls (CMC) document was submitted as per the FDA's published guidance document (2). For documentation of prior human use and human safety, online databases such as the ODS Labels Database (18), and the FDA and CFSAN Adverse Event Reporting System (FAERS and CAERS) databases were used to illustrate widespread use of CA preparations with minimum toxicity. Several previous clinical trials that had been done without notable adverse effects were included; and the use of CA as an edible plant, and its safety when brewed into a tea (similar to CAW preparation), were highlighted. Published animal toxicity studies of CA preparations were also included. Additional approvals for proposed studies were obtained from the clinical trial sponsor (NCCIH) and the OHSU Institutional Review Board (IRB) following receipt of IND status.

RESULTS

Product Manufacture

Dosage Calculation and Delivery Method

Human doses equivalent to the mouse doses (200–1,000 mg/kg/d) used in our preclinical studies were estimated to be 2–10 g CAW per day using interspecies scaling (45). Since robust cognitive improvements in mice had previously been observed at doses of 200 mg/kg/d (31, 32, 34, 36) and 500 mg/kg/d (33), a 10 g/day dose in humans was determined to be unnecessary. For the planned Phase I studies, the dose range was adjusted to 2 g and 4 g of CAW per day based on the highest reported, well-tolerated, human dose of CA triterpenes (240 mg/day) (57). The standard maximum content of a capsule or tablet is 500 mg. Daily doses of 2 g and 4 g CAW translated to 4–8 capsules per day, which was considered unsustainable and inconvenient for an elderly population. Therefore, it was decided to provide the CAW as a powder to be dispersed in one glass (10–12 ounces) of water and consumed orally, closely mimicking the administration of CAW in the preclinical studies. This delivery format required the design of a new formulation with the addition of excipients to improve dispersion and palatability, and coloring agents to mask the placebo.

Raw Material Selection: Identification, Chemical Characterization, and Evaluation of Contaminants

Sourcing of Material

It was challenging to identify sources of a single batch of CA material that was available in the required large quantity (90 kg). All suppliers were willing to provide a trade sample but could not guarantee that the larger amount would still be available after preliminary evaluations had been completed. There was variability in the form of bulk raw materials available including powder, finely milled "tea cut," coarsely milled "cut and sift," and dried whole plant materials.

Identity Tests

Most of the available plant material was a cut or milled version of the dried herb, making formal identification and verification of authenticity through examination against botanical keys not possible. However, all the trade materials evaluated passed organoleptic, FTIR and TLC criteria for identification as CA. Specifically, FTIR provided acceptable matches to prior database spectra and TLC confirmed the presence of TTs and CQAs characteristic of CA (data not presented). The TLC zone profiles were essentially similar, qualitatively, to the preclinical study voucher samples, although quantitative variations in band intensity between the materials were noted.

Chemical Characterization

Targeted analysis was used to evaluate the content of TTs and CQAs in the trade samples, with the goal of identifying material containing within ±10% of active compounds found in CA-4 and CA-5 that had shown biological activity in prior preclinical studies (31–34, 36, 38–40, 43). There was high variability between CA accessions in the content (% w/w in CAW) of total TTs (0.5–11%), total mono-CQAs (0.1–0.4%) and total di-CQAs (0.1–1.4%), as well as in individual members of each class, identified by LC-HRMS (50). Interestingly, the TTs and CQAs did not vary in the same way (i.e., plant materials that had higher levels of TTs did not necessarily also have higher levels of CQAs). No single CA trade sample matched CA-4 or CA-5 (50). Validation of the LC-HRMS method, results of targeted analysis of TTs and CQAs in samples CA-1 to CA-8 and a comparison of their fingerprints using principal component analysis have been published (50). Detailed LC-HRMS data from untargeted analysis of the eight samples has been archived.

Evaluation of Contaminants

All trade materials passed the following acceptance criteria for microbial content established at Oregon's Wild Harvest for their raw materials and products: aerobic plate count <10,000,000

TABLE 1 | Heavy metal and pesticide content of five CA trade samples acquired for possible use in preparing CAP, and their water extracts.

Source	Cadmium (ppm)	Lead (ppm)	Arsenic (ppm)	# Pesticide residues	Highest pesticide residue (mg/kg)
CA-1 Raw	<0.1	1.8	<0.5	Not measured	Not measured
CA-2 Raw	0.3	0.3	<0.5	11	2.5 (Chlorpyifos)
CA-2 Extract	<0.1	<0.2	<0.5	5	1.2 (Quizalofop)
CA-3 Raw	1.2	1.6	<0.5	4	0.059 (Propiconazole)
CA-3 Extract	0.435	0.455	<0.5	1	0.018 (Propiconazole)
CA-6 Raw	0.6	2.5	<0.5	6	0.049 (2,4-D)
CA-6 Extract	0.233	0.358	<0.5	2	0.064 (2,4-D)
CA-7 Raw	5.9	4.3	<0.5	8	0.044 (Carbendazim)
CA-7 Extract	2.444	0.766	<0.5	1	0.073 (2,4-D)
CA-8 Raw	1	4.1	<0.5	None detected	None detected

TABLE 2 | Contaminant content of bulk CA samples selected for mass manufacture.

Plant materials	Microbial levels	Heavy metals (ppm)	Pesticides (mg/kg)
CA-3	Conforms to Oregon's Wild Harvest specifications	Lead (2.088) Arsenic (<0.5) Cadmium 1.13	Diphenylamine (0.028) Propiconazole (0.038)
CA-6	Conforms to Oregon's Wild Harvest specifications	Lead (2.434) Arsenic (<0.5) Cadmium 0.537	Cypermethrin (0.018) 2,4-D (0.039) Hexachlorobenzene (0.010) Permethrin (0.033)

colony forming units (cfu)/g; mold or yeast <100,000 cfu/g; coliforms report cfu/g; *E. coli* <10 most probable number (mpn)/g; *Salmonella* absent in 25 g (**Supplementary Table 1**). Other analyses revealed that even those trade samples that had been certified "organic," had detectable levels of pesticides, and most also contained heavy metals (**Table 1**). Generally, heavy metal and pesticide concentrations were lower in the extracts than the starting material, but two pesticides (quizalofop and 2,4-D) appeared more prominently in the extracts than the raw materials from which the extracts originated.

Evaluation of Bulk Materials Selected for Manufacture

Based upon their phytochemical profile (50) and relatively low contaminant content, CA-3 and CA-6 were selected for the manufacture of CAP. Interestingly, CA-3 was certified organic while CA-6 was not but was listed as not genetically modified (non-GMO). CA-6 had unusually high levels, and CA-3 had unusually low levels, of AS compared to CA-4 and CA-5. The decision was made to mix the two plant materials to yield an extract with TT levels closer to those found CA-4 and CA-5. Bulk quantities of these materials were purchased and analyzed for potential impurities (**Table 2**) and phytochemical profile to confirm that they matched their respective trade samples. Both CA-3 and CA-6 raw materials passed the standards for microbial content (**Supplementary Table 1**) and aflatoxins (absent) and had phytochemical profiles matching their trade sample.

For heavy metals, tolerances were obtained from the United States Pharmacopoeia (USP). The USP #40; Part 561; Articles of Botanical Origin suggests the following guidelines for residual levels of heavy metals: cadmium 0.5 μg/g, lead 5 μg/g, arsenic 2 μg/g. Although the CA-3 material did not conform to this specification, the levels of cadmium reduced following water extraction (**Table 1**), allowing for the continued progression with using this material. When evaluating the acceptability of the pesticide residue levels, measured levels were compared to maximum residue limits (MRL) available in the USP #40; Part 561; Articles of Botanical Origin and the European Pharmacopeia 8.2 (Ph.Eur; section 2.8.13). Cypermethrin, hexachlorobenzene and permethrin were below the USP and Ph.Eur limits of 1, 0.1 and 1 mg/kg, respectively, however MRL values were not available for diphenylamine, 2,4-D, and propiconazole.

Selection of Manufacturing Facilities and Formulation Development

Identification of Certified Facilities

Three facilities with current Good Manufacturing Practice certification (cGMP) were identified that were capable of large-scale water extraction of CA and/or drying the large volume of extract to be generated. Most companies identified in initial searches performed hydroalcoholic but not aqueous extractions and were thus eliminated. Only one facility identified had both capabilities (Ashland Laboratories); however, the method for drying offered was spray drying onto a matrix and not freeze-drying. A trial batch did not show appreciable degradation of TT and CQA following spray drying compared to a lyophilized counterpart (data not shown), so it was decided to contract out the CAW production and drying to Ashland Laboratories. cGMP and SQFI-certified facilities at Oregon's Wild Harvest were identified to be used for final product manufacture (blending of the dried extract and excipients) and packaging.

Formulation of the Clinical Trial Product and Matching Placebo

The three doses of CAP were designed to differ only in CAW content, i.e., 0 g (placebo) or 2 g and 4 g of CAW, but contain identical amounts of excipients, including the spray drying matrix substance. This would ensure any measured biological

activity in a placebo-controlled study is solely attributable to CAW. It was determined that the spray dried product could contain up to 66% CAW (i.e., a 2:1 ratio of CAW to matrix) and this was the target loading aimed for during manufacture, based on the theoretical weight of CAW extracted.

Of the 19 excipients evaluated for taste, color, neurological activity, and phytochemical content, six were eliminated due to TT and/or CQA content and two were reported to have cognitive enhancing properties. Six other excipients were eliminated due to challenges with color matching (**Figure 2A**). In the end, five excipients were selected with one being a food grade colorant, three for palatability, and one as the carrier matrix. The identities of these excipients are not disclosed in this publication due to intellectual property considerations but can be provided under agreement. The three doses of CAP, when reconstituted in water, are shown in **Figure 2B** and were not readily distinguishable by color, smell, or taste.

Manufacturing Process, Analysis of Intermediates, and Final Product

Extraction and Drying

CA-3 and CA-6 were extracted and dried in two separate batches. Batch 1 (CA-3 plus CA-6) was expected to have a lower concentration of AS than Batch 2 (CA-6 only) due to the presence of CA-3 plant material which had low levels of this compound (50). The spray dried product from Batch 1 (10.85 kg) was blended with spray dried product from Batch 2 (6.40 kg) at Oregon's Wild Harvest to yield "Gotu Kola Extract Preblend" (17.25 kg). The quantities were based on the calculated total amount of material needed for QC, stability studies and the proposed clinical trials, and utilized the maximum amount of Batch 1 available to ensure a lower AS level similar to CA-4 and CA-5.

Analysis of Intermediates

Prior to incorporation into the drug product, further quality control tests were performed on "Gotu Kola Extract Preblend." Samples of the lyophilized extract aliquots (without matrix materials) from the two batches were compared by TLC with their spray dried counterparts, "Gotu Kola Extract Preblend," and CA-4. The compounds typical of CA in the test materials were identified in each of the samples. CA's TT and CQA compounds were analyzed in each manufacturing intermediate using LC-HRMS. Using untargeted LC-HRMS in negative and positive ionization mode, the retention time, peak area and high-resolution mass of "Gotu Kola Extract Preblend" was recorded as a fingerprint (data archived).

Despite the targeted loading value of 66%, the actual percent loading of CAW onto the matrix was below this value and differed between the two extraction batches. Batch 1 was calculated to have ∼51% loading of CAW onto the matrix, while Batch 2 had just over 34% loading. By calculation from CAW content in the two spray dried batches, "Gotu Kola Extract Preblend" was estimated to have 7.73 kg CAW in 17.25 kg of material (about 45% loading). Based on this, the amounts of "Gotu Kola Extract Preblend" required for CAP 2 g CAW and CAP 4 g CAW were calculated as 4.4 g and 8.8 g, respectively.

Product Blending and Packaging

Individual sachets containing single doses of 0, 2, or 4 g of CAW and equal amounts of all the excipients (including the carrier matrix) were made and packaged as described in section Product Blending and Packaging.

Quality Control

Product sachets passed Oregon's Wild Harvest criteria for uniformity of weight. Analysis of TT and CQA content of samples of the final CAP products, in comparison to the parent CAW extract, confirmed that the correct levels of CAW had been included in CAP 2 g and 4 g and that these compounds were absent in CAP 0 g (placebo). TT and CQA content in CAP 0, 2, and 4 g are shown in **Figure 3**. When contaminants were assessed, all three products remained within the limits for microbial levels (**Supplementary Table 1**). Heavy metal and pesticide content of all CAP doses are shown in **Table 3**. Cadmium and lead were detected at levels above the limit of quantitation in at least one of the CAP products. Of the various pesticides seen in the starting materials (CA-3 and CA-6) (**Table 2**), only two of them (diphenylamine and 2,4-D) were detected in the CAP (**Table 3**).

Product Stability

Visual and olfactory inspection of the samples stored at different temperatures during stability analyses showed no obvious changes at lower temperatures; however, the samples stored in the 40°C accelerated condition showed some clumping. There was no difference in the levels of TTs and CQAs between the different storage conditions demonstrating stability under all conditions tested (**Figure 4**); however, principal component analysis (PCA) of all LC-HRMS peaks did show a temperature dependent separation of samples stored at the two accelerated storage conditions from those stored at −20°C, 4°C, and ambient temperature (**Figure 5**). Based on this data, it appears that CAP 2 g and CAP 4 g will be stable for at least 1 month if stored at −20°C, 4°C, or ambient temperature. While some changes were observed under accelerated storage conditions using PCA, the main active compounds appeared unaffected (**Figure 4**).

Bioassay to Confirm Biological Activity

Using the fast phototaxis assay, there was no significant difference in transitions toward the light between the control and excipients-treated *sniffer* flies (**Figure 6**). CAW-treated flies showed significantly ($p < 0.001$) greater transitions toward light than control and excipients-treated flies. CAP treatment also significantly ($p < 0.05$) increased transitions toward light compared to control and excipients treatment. CAP and CAW treatment were not significantly different from each other. These data suggests that the formulated CAP product has similar neurological activity to the active component, CAW, and the excipients selected do not have appreciable neurological effects as measured in this model. This bioassay appears to be suitable for use in evaluating CAP biological activity in future studies where additional product manufacture will be necessary.

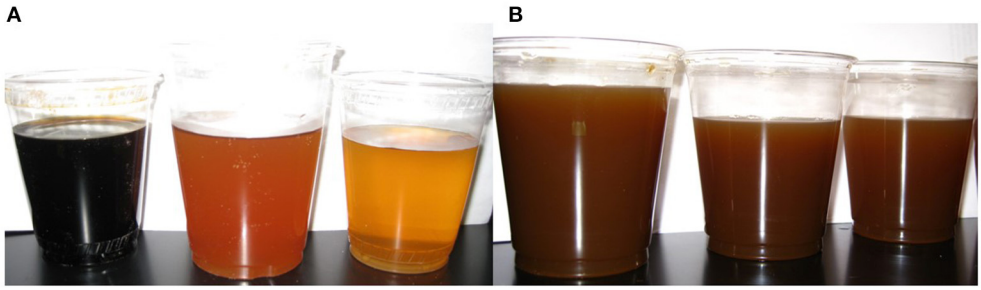

FIGURE 2 | Coloring excipient test and final CAP matching evaluation. **(A)** Three different coloring agents were dissolved in 8 oz. of water and compared against one another for color matching with CAW. **(B)** 0, 2, and 4 g CAP, containing the selected coloring agent and additional excipients for flavor matching, dissolved in 8 oz. of warm water.

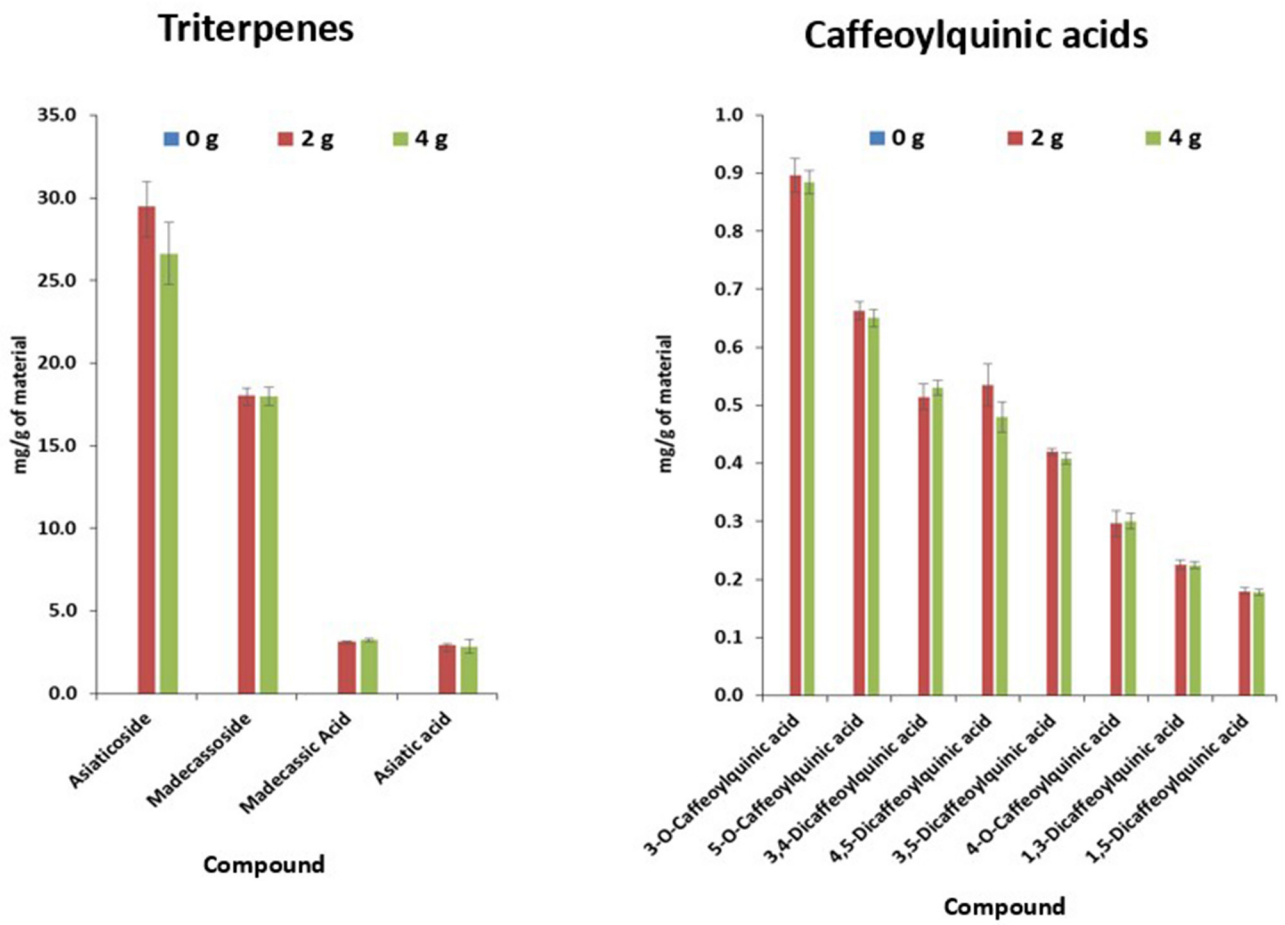

FIGURE 3 | Concentration of triterpenes and caffeoylquinic acids found in 0, 2, and 4 g *Centella asiatica* product as determined by LC-HRMS. Sachets of the *Centella asiatica* water extract products (CAP; $n = 5$ per dose) were extracted with methanol and analyzed for the content of active compounds (triterpenes and caffeoylquinic acids) using LC-HRMS in positive and negative ion mode against commercial reference standards. The content of triterpenes and caffeoylquinic acids per gram of *Centella asiatica* extract was identical for the 2 g and 4 g doses of CAP and showed low variability indicating successful and uniform manufacture of the two doses. None of the specific analytes were detected in the 0 g dose, confirming their absence at detectable levels in the placebo which was comprised solely of the excipients used.

Regulatory Considerations

The FIJT determined that an IND was required for two reasons (a) because the goal of our clinical studies was to develop CAW to mitigate a disease (cognitive decline) bringing it under the category of a drug rather than a dietary supplement, and (b) because CAW was not a currently lawfully marketed drug. After

TABLE 3 | Heavy metal and pesticide residue content in CAP 0, 2, and 4 g.

Heavy metal or pesticide	CAP 0 g		CAP 2 g		CAP 4 g	
	Ppm (μg/g)	Amount per sachet (μg)	Ppm (μg/g)	Amount per sachet (μg)	Ppm (μg/g)	Amount per sachet (μg)
Cadmium	<0.1 loq	(<1.1)	<0.1 loq	(<1.3)	0.26	3.81
Lead	<0.2 loq	(<2.2)	0.27	3.43	0.69	10.15
Arsenic	<0.5 loq	(<5.4)	<0.5 loq	(<6.4)	<0.5 loq	(<7.7)
Mercury	<0.01 loq	(<0.11)	<0.01 loq	(<0.13)	<0.01 loq	(<0.15)
Diphenylamine	<0.01 loq	(<0.11)	0.014	0.179	0.035	0.516
2,4-D	<0.01 loq	(<0.11)	0.024	0.306	0.024	0.354

loq, limit of quantitation.

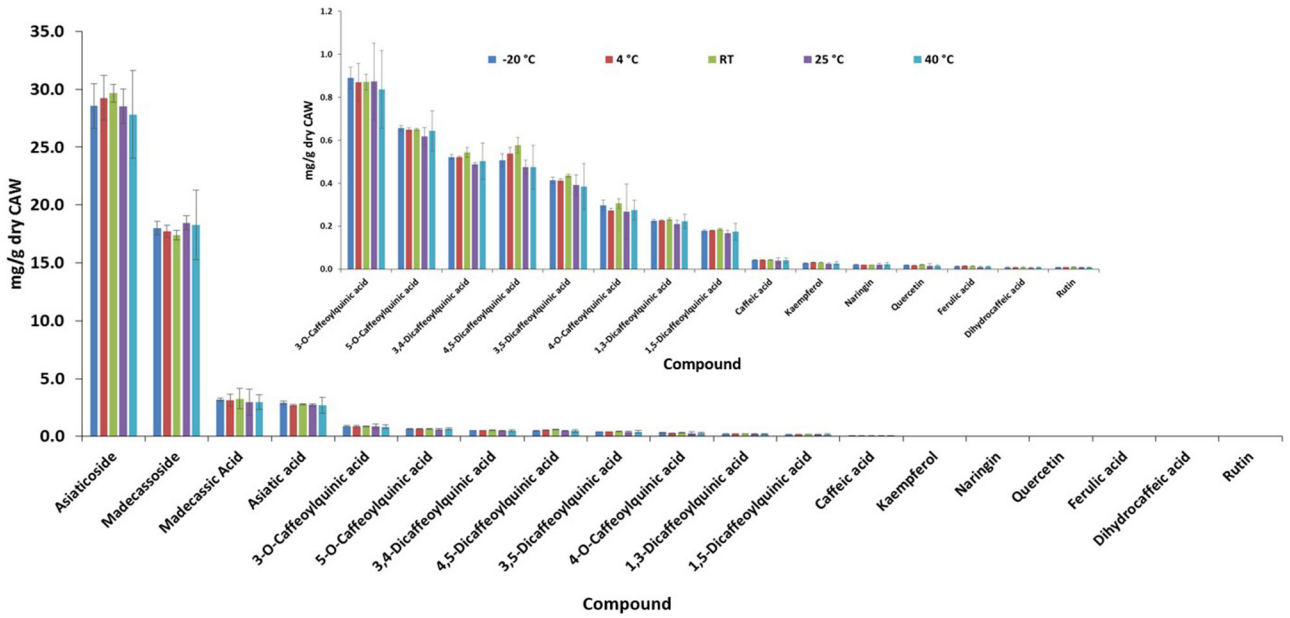

FIGURE 4 | A 32-day stability test of CAP 2 g and 4 g showing unchanged levels of triterpene and caffeoylquinic acid components at all temperatures as determined by LC-HRMS. Storage conditions: 25 ± 2°C/60% ± 5% relative humidity, accelerated 40 ± 2°C/75% ± 5% relative humidity, fridge (4°C), ambient temperature (RT), and freezer (−20°C) (n = 3–6 per condition).

a few amendments to the initial submission, IND status for CAP was awarded. The success of the FDA IND application was pivotal to moving forward with the clinical study; both the study sponsor (NCCIH) and OHSU's IRB required IND status to be obtained before granting their respective approvals for clinical protocols.

DISCUSSION

Chemical Variability and the Importance of Analytical Methods

Our experience with custom manufacture of a CA product for a clinical trial confirmed several of the earlier known challenges relating to botanical research. The first among these is the inherent chemical variability within a single botanical species. Levels of the bioactive TT compounds are reported to vary considerably between accessions of CA (19) and this was confirmed for both TTs and CQAs in the present study (50).

Identification of active compounds in a botanical, followed by chemical fingerprinting and quantification of known bioactives using validated methodology and authenticated reference standards, is an imperative step for rigorous translational studies. Such methods, as we have developed for CA; (50), can be used to identify (1) the material with the closest matching active compound profile to those used in preclinical studies showing biological activity, (2) variability between accessions from the same plant material, and (3) adulterated products. While it is valuable to apply targeted techniques to measure and control the content of known active compounds, plant raw materials and extracts also contain a vast number of other compounds, whose identity and/or contribution to the overall activity of the product may as yet be unknown. Therefore, the use of untargeted analysis to document a comprehensive range of analytical features (e.g., chromatographic retention time and mass spectral information) of both known and additional components is invaluable in

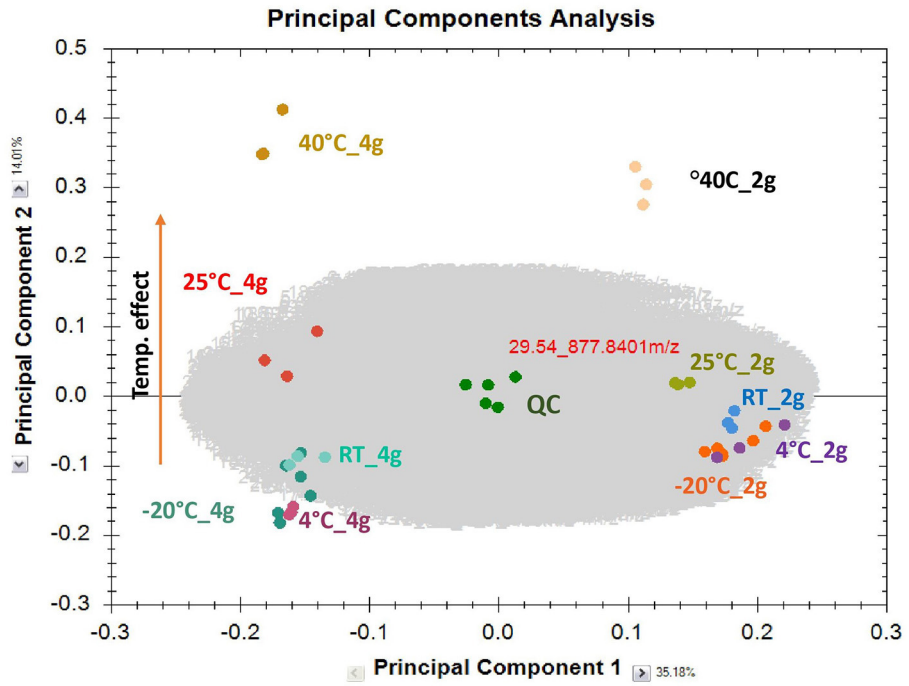

FIGURE 5 | Principal Component Analysis (PCA) of 32-day stability test of CAP 2 g and CAP 4 g. Samples were stored in special chambers held at 25 ± 2°C/60% ± 5% relative humidity or accelerated 40 ± 2°C/75% ± 5% relative humidity, at ambient temperature (RT), in a refrigerator (4°C), or in a freezer (−20°C) (n = 3–6 per condition). Chemical fingerprinting analysis of CAP storage stability samples by untargeted data dependent acquisition was performed using LC-HRMS as described earlier (50). The content of each sachet was suspended in 70% v/v methanol (100 mL) containing formic acid (0.1% v/v). Samples were sonicated for 2 hrs with strong shaking every 30 min at room temperature. The suspension (1 mL) was centrifuged (15,000 rpm, 10 min) and diluted 100 times before injection. QC samples were obtained by pooling equal aliquots of each sample. Principal component analysis was performed in Progenesis QI software (V 2.4). All *m/z*-signals that triggered MS2 experiments (5,849) were used after log-transformation and Pareto scaling. The first principal component represents the maximum variation through the data. Next, another axis representing the next highest variation within the data is added orthogonal to the first one, and is designed as the second principal component. Each marker represents a sample; identically colored markers are replicate samples (n = 6, −20C; n = 5, QC; and n = 3 for all other groups). The PCA plot shows that samples that have a high degree of similarity in their chemical fingerprints cluster closely together. Clustering of the QC samples in the center of the plot confirms LC-MS platform stability. The shift from the left bottom quadrant to the upper left quadrant (4 g sachets) and from right bottom quadrant to right upper quadrant (2 g sachets) indicates that the chemical fingerprints are sensitive to storage temperature. Targeted analyses of the CAP stability samples conducted in parallel (**Figure 4**) indicated CQA and TT levels were unaffected by storage temperature; thus the observed shifts hint that either excipients or other components of CAW were sensitive to storage temperature.

preserving a complete picture of the chemical profile of a botanical product. The overall profile of different materials can be compared using principal component analysis for example (50). These analyses can, and should, be performed on the raw herb, prepared extracts, manufacturing intermediates, the final product(s) upon completion of manufacture, and the final product(s) during storage and use (e.g., in a clinical trial). We have taken this approach for our study.

The identification of sufficient, chemically similar raw material for all the planned studies is an important aspect in botanical translational medicine studies to ensure limited variability in phytochemical content and impurity profile. Ideally, a reliable, reproducible source of plant material with desirable and minimally variable chemical profile should be identified.

Contaminants in Plant Materials

There are several unique environmental contaminants of concern when sourcing raw plant material for use in clinical trials. These include heavy metals, pesticides, microbes, mycotoxins, and polyaromatic hydrocarbons (PAH) (8, 58–60). While several pesticides are banned (57), it is more common that an upper limit of content is specified by regulatory authorities or pharmacopeias for specific pesticide residues, as well as for heavy metals, microbes, and mycotoxins. Limits for PAH content, while established for many food commodities, are still being considered for botanical products (60).

To assess the acceptability of the heavy metal content in the final CAP products (**Table 3**), we compared the content to published maximum daily intake recommendations. The American Herbal Products Association (AHPA) Guidance Policy (2012; current at the time of product manufacture) recommends the following maximum intakes: cadmium 4.1 μg/day, lead 6 μg/day, inorganic arsenic 10 μg/day, and methyl mercury 2 μg/day. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human USE (ICH) Harmonized Guideline for elemental impurities (Q3D; 2014) lists the following values for permitted daily exposure (PDE) by the oral route: cadmium 5 μg/day, lead 5 μg/day,

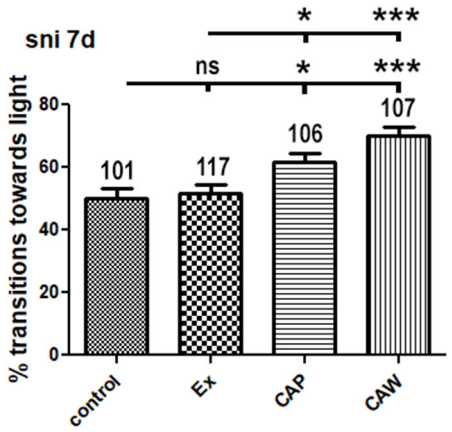

FIGURE 6 | Percentage transitions toward light for *sniffer* flies treated with *Centella asiatica* water extract, *Centella asiatica* water extract product, excipients used in the manufacture of CAP or control food. *Drosophila melanogaster* fruit flies with a mutation in the *sniffer* gene *sni1* allele were fed standard food (control) or standard food supplemented with either *Centella asiatica* water extract (CAW; 10 mg/mL), *Centella asiatica* water extract product (CAP; equivalent to CAW 10 mg/mL), or the matching placebo for CAP containing only excipients (Ex; equivalent to the amount in CAP) for 7 days. Fast phototaxis was performed with flies (data from both sexes combined) and compared to either control or Ex treatment. The number of tested flies is given above the bars and the SEM is indicated. $^{*}p < 0.05$, $^{***}p < 0.001$.

arsenic 15 μg/day, and mercury 30 μg/day. The amounts of cadmium, mercury, and arsenic, delivered per dose of CAP were less than the lower of the two recommended maximum values. However, the lead content in 4 g CAP (10.15 μg; **Table 3**) was higher than daily maximum intake of 5 μg/day for this metal recommended by both AHPA and the ICH. While this was concerning, the ICH guidelines (Q3D, section Bioassay to Confirm Biological Activity) mention that intermittent or short-term (30 days or less) dosing may be justification for allowing impurity levels higher than the established PDE. The FDA has allowed use of this product for a pharmacokinetic (PK) study where each participant only receives a single dose of CAP 4 g. However, a new batch of product with a more acceptable heavy metal profile may be needed for studies involving longer term dosing.

The Code of Federal Regulations (CFR; Part 180) lists maximum recommended level (MRL) values for pesticide residues in several food commodities. For example, for diphenylamine (CFR part 180.190) the tolerances in pears and apples range from 5 to 30 ppm. For 2,4-D (CFR part 180.142), the tolerances in various agricultural commodities range from 0.05 to 50 ppm. However, most botanicals used in dietary supplements are not included in these commodity lists. Information on allowable levels of these contaminants may be found in the USP and Dietary Supplements Compendium (DSC) sections on articles of botanical origin, and the European Pharmacopeia (Ph.Eur.), or guidelines from the ICH or World Health Organization (WHO) and Food and Agriculture Organization (FAO). Some limitations to finding permitted levels of pesticide residues in particular, are that not all pesticides are listed in the pharmacopeias. For example, maximum recommended level (MRL) values were not available in the USP, DSC or Ph.Eur. for diphenylamine and 2,4-D. Hence, it may be more relevant to consult documentation, where available, on maximum allowed daily intake rather than MRL. The FAO and WHO's Codex Alimentarus online pesticide database cites allowable daily intakes (ADI) of up to 0.08 and 0.01 mg/kg body weight for diphenylamine and 2,4-D, respectively, corresponding to 4.8 and 0.6 mg per day for a 60 kg adult. The National Science Foundation (NSF) International Standard/American National Standard organization has published maximum allowable levels per day (MAL) values for many pesticides (61). For diphenylamine, this value at 700 μg/day, is considerably lower than the FAO/WHO value, and at the time of our study, no MAL value was given for the pesticide 2,4-D. Both the residual levels (ppm) and the amount (μg) of diphenylamine and 2,4-D delivered by a single dose of CAP 2 g and CAP 4 g appear well below the available MRL (food commodities), ADI, and MAL values.

Designing Botanical Interventions and Placebos for a Human Trial

The principal factors to consider in designing the product for our clinical trial were similarity to the material showing biological activity in preclinical studies, translation of the mouse doses to human studies, and the development of a suitable dosage form to deliver human doses along with a matching placebo. Our preclinical studies had examined a hot water extract of CA (CAW) that is produced by boiling CA in water under reflux for 2 h followed by filtration, freezing and lyophilization (31–38, 40, 42–44, 48). We were unable to find a commercial product that had been prepared in this way for use in the proposed human trials. We also considered using products made by a different extraction method (e.g., using ethanol), but which may have had similar levels of TT and CQAs. However, some companies that had such products expressed reluctance to provide information on their product to the FDA for an IND application. Consequently, we decided to use a custom-made hot water extract, and, with some difficulty, found a cGMP facility able to perform the extraction and drying. However, they were only able to provide a product spray-dried onto a carrier matrix rather than a lyophilized dried extract. This resulted in additional analytical studies to ensure the TT and CQA were not adversely affected by this process before proceeding with manufacture of CAP. In addition, it was observed that the percentage loading onto the carrier matrix during spray drying was significantly different from the target value of 66% and varied between batches (51 and 34%) despite efforts to maintain batch consistency. This introduces a need for further analytical measurements after drying of every batch to determine actual loading prior to final formulation and blending with excipients. Variability in loading must be monitored and controlled, if possible, to maintain consistent excipient levels between CAP batches. Otherwise, variations would need to be accounted for using batch specific placebo controls.

The human doses of CAW for our proposed study were selected by interspecies scaling (45, 62) and reference to earlier human studies (19) to limit the potential for toxicity at higher doses. The doses calculated were larger than the dose of CA or CA extract provided by most commercial CA products (around 500 mg/capsule). The larger dose (4 g) required that the product be administered as a liquid drink rather than swallowed as a capsule. We therefore had to formulate a product that was palatable and dispersed easily in water. This raised the issue of making a matching placebo, and some considerable effort was required to design one that had a similar taste and color to the CAW-containing product. It was also essential that any agents added to produce CAP were devoid of TT and CQA, safe for human use, and not known to have any neurological effects. It was important to make the placebo and CAW containing products identical in all respects except for CAW content, so that any differences in biological effects between CAW and placebo-treated groups could truly be ascribed to CAW.

Regulatory Aspects

FDA IND status was required for CAW for the reasons mentioned earlier (section Regulatory Considerations). Although we were not required to provide extensive toxicology data on CA for the IND application, we did have to provide evidence of its likely safety. We cited widespread human use of CA as a dietary supplement and edible plant with limited reports of human toxicity, organ toxicity studies in animals, and the relative absence of adverse effects in clinical trials of CA. However, the relevance of this evidence to CAW was complicated by the large variability in the type of CA preparations that are used in dietary supplements, as well as in those used in earlier preclinical and clinical studies (19). For example, although previous studies had examined the effects of CA extracts on CYP drug metabolizing enzymes (63–67), the FDA recommended that we examine and report the drug interaction potential of our CAW water extract specifically (68). Also, since earlier preclinical studies on organ toxicity had been performed using other types of CA extracts (69–72), we obtained organ toxicity data from mice treated with CAW that could be used in the IND application. A point of caution regarding several reports of CA hepatotoxicity in humans (notably in review articles) (73–75)—an examination of the original papers cited showed that some preparations involved were multi-herbal products including CA where direct association could not be established.

Looking Ahead to Clinical Studies

We have embarked on a series of translational studies with the ultimate goal of performing a Phase II trial examining the efficacy of CAW in ameliorating cognitive decline in humans. Important steps to optimize this future Phase II study would be to:

I. identify or develop a CAW product that matches the one used in our preclinical studies and contains an appropriate dose of TTs and CQAs likely to have a biological effect in humans;
II. confirm bioavailability of CA's active compounds by performing a plasma pharmacokinetics (PK) study following acute oral dosing of the CAW product;
III. determine safety and tolerability of the CAW product; and
IV. demonstrate target engagement by evaluating changes in specific biological signatures related to CAW's mechanisms of action that had been observed in preclinical studies.

In the present report, we describe our experience with Step I above. Steps II to IV would need to be performed in the selected target population (older adults of both sexes). Recently, CAP 2 g and 4 g have been used in two pharmacokinetic studies in older adults (NCT03929250, NCT03937908). The multiplicity of active compounds (TTs and CQAs) in CAW has required the development and validation of sensitive bioanalytical methodology to measure these CAW compounds in human plasma and urine at much lower concentrations than found in the plant extracts. For bioavailability studies from botanical extracts it is important to consider potential biotransformation of the phytochemicals during their transportation through the body. For example, in humans the TT glycosides of CA (AS and MS) appear to be hydrolyzed in the gut such that only the aglycones (AA and MA) appear in the plasma (57, 76–78). Similarly, the CQAs undergo extensive gut, Phase I and Phase II metabolism to metabolites such as caffeic acid, dihydrocaffeic acid, dihydroferulic acid, ferulic acid, isoferulic acid, dihydroisoferulic acid and hydroxyphenylpropionic acids, and their glucuronide and sulfate conjugates (79). The biodistribution of any these compounds may be relevant to the biological activity of CA and must therefore be evaluated.

The pharmacokinetic studies also required participants to observe a strict low phytochemical diet for 48-h prior to dosing and for 12 h post dosing. This is because of the ubiquitous distribution of CQAs in common foodstuffs and beverages, as well as the potential to obtain TTs from food sources, although the TTs have a more limited distribution in plants. Participants were instructed to avoid fruits, vegetables, nuts, spices, coffee, tea, whole grains, and whole wheat products for 48 hours prior to the pharmacokinetic studies. They were placed on an identical low phytochemical diet during each study visit to reduce dietary variability. They were asked to fast for 10 h prior to each visit and were not provided food for the first 2 h following CAP administration to account for differences in gastrointestinal transit time and dietary interference. While this may not be practical for studies involving prolonged consumption of CA, it is clearly needed for a pharmacokinetic study. Based on our preclinical *in vivo* studies reviewed earlier, we expect that 4–6 weeks of continuous administration of CAP will be needed to see evidence of target engagement in humans e.g., a reduction in oxidative stress markers or evidence of improved mitochondrial function and neuronal viability from brain imaging studies. Several months of CAP administration may be required to demonstrate a change in the rate of cognitive decline.

Some additional challenges are anticipated in upcoming dose escalation and safety studies. For the product, the success of blinding to placebo, and tolerability and acceptability of the product when consumed daily for extended periods of time

are important parameters to evaluate. It is also important to monitor the stability of the product for the duration of the clinical trial whether stored by the investigators, research pharmacy, or the participants.

Here we present an example of a rigorous science-based approach to translating preclinical botanical studies to clinical trials. Matching the clinical trial material as closely as possible to that used in preclinical studies is important, as well as choosing a dose range for the product based on sound rationale (e.g., interspecies scaling). A valid, matched placebo must also be produced for comparison if appropriate to the study, and attention paid to the possible presence of confounding phytochemicals in the placebo materials or the diet of participants. Good botanical raw materials will contain adequate levels of active compounds and minimal contaminants. Sufficient material of this quality must be secured for the duration of the study to minimize variability in the test material throughout the study. Validated analytical methods specific to the botanical extract under consideration are essential and key to a successful clinical trial, from guiding the selection of raw materials, through product manufacture and validation of product stability during the trial. With the inherent variability of plant materials, the data from even the most carefully performed trial remains product specific. However, the careful documentation of product chemical characteristics will facilitate reproducibility between studies, as well as comparison of data from different trials.

Significance of CAP Clinical Trials to the Use of CA in Traditional Medicines and Food

As well as its use as a traditional medicine, CA is an edible plant consumed regularly in the diet as a vegetable, juice, or tea in several Asian countries (80–84) and in South Africa (85, 86). Indeed CA's popularity in foods is increasing due to growing awareness of its health benefits (81). A question may arise regarding the relevance of clinical trials of CAP to the health benefits of traditional medicines or foods containing CA. Aside from the complicating issue of inherent variability of plant materials, the answer largely depends on how closely the product tested matches the preparation method, and amount of CA consumed. For cognitive benefits, in Ayurvedic medicine CA herb is reportedly prepared as the fresh juice "swarasam," mixed in clarified butter as a "gritham," or given in milk (15). In our preclinical studies, and in CAP, we used a hot water extract of CA (CAW). This extraction method was based, not on traditional preparation methods, but on earlier published studies demonstrating superior cognitive enhancing properties in rats of CA aqueous extract compared to extracts made with other solvents (87, 88). It would be interesting to compare the composition of extracts made using traditional preparation methods to CAW. In the US, as in Asian countries (81, 89, 90), CA is a popular component of herbal teas prepared by extracting dried CA herb with hot water. This extraction process closely mirrors the preparation of CAW and would be expected to yield similar components, although comparative dosing would need to be evaluated.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KMW, AAM, JR, DK, JFS, CSM, JFQ, and AS participated in research design. KMW, AAM, JM, MC, DK, and AS conducted the experiments and performed data analysis. KMW, DK, and AS wrote or contributed to the writing of the manuscript. KMW, JFQ, and AS secured funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.799137/full#supplementary-material

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Designing a Clinical Study With Dietary Supplements: It's All in the Details

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A successful randomized clinical trial of the effect of dietary supplements on a chosen endpoint begins with developing supporting data in preclinical studies while paying attention to easily overlooked details when planning the related clinical trial. In this perspective, we draw on our experience studying the effect of an ethanolic extract from *Artemisia dracunculus* L. (termed PMI-5011) on glucose homeostasis as a potential therapeutic option in providing resilience to metabolic syndrome (MetS). Decisions on experimental design related to issues ranging from choice of mouse model to dosing levels and route of administration in the preclinical studies will be discussed in terms of translation to the eventual human studies. The more complex considerations in planning the clinical studies present different challenges as these studies progress from testing the safety of the dietary supplement to assessing the effect of the dietary supplement on a predetermined clinical outcome. From the vantage point of hindsight, we will outline potential pitfalls when translating preclinical studies to clinical studies and point out details to address when designing clinical studies of dietary supplements.

Keywords: botanical, natural products, dietary supplements, *Artemisia dracunculus*, clinical trial

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INTRODUCTION

Over one-half of all adults in the United States report using a dietary supplement to improve their health (1). This trend coincides with increasing interest in the health benefits of plant-based natural products and a burgeoning number of preclinical studies investigating the therapeutic properties of extracts derived from botanical sources. While animal model-based pre-clinical studies are expected before moving to clinical studies, there is ample data showing these pre-clinical models often do not translate in terms of efficacy or safety to human studies (2). Our view is that reliable information about the safety and efficacy of botanically-based dietary supplements in humans requires clinical studies that build on well-designed preclinical results that are relevant to the targeted human population. To accomplish this goal, the eventual clinical trial should be kept in mind when designing the preclinical study. Our experience studying the metabolic effect of an ethanol extract from *Artemisia dracunculus* L. (termed PMI-5011) points out the myriad details that have a substantial impact on the ability to translate preclinical findings to the clinical setting and the additional details that must be considered when designing the related clinical study. In this perspective, we offer some hard-won advice based on our stumbles and successes with investigating how dietary supplementation with PMI-5011 impacts obesity-induced insulin resistance.

DESIGNING THE PRECLINICAL STUDY: THINK AHEAD TO THE CLINICAL TRIAL

The ability to translate preclinical studies of dietary supplementation with botanical extracts fundamentally begins with quality control of the plant material. Plants grown for preclinical studies of botanical extracts should be cultivated under uniform conditions in a controlled growth environment to yield consistent plant material over time. The growth conditions should be pesticide-free as a safety consideration for eventual human consumption (3). Bioactivity-guided fractionation (4) or DESIGNER (Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources) (5) approaches can be used to define the active fraction and provide a biomarker for evaluating each batch of the plant material to ensure quality control. Our experience is that batch-to-batch variation in the selected biomarker will occur even with quality control practices in place and may be traced back to seed lot, disruptions in the growth conditions, storage conditions, or variability in the extraction method. In addition, the stability of identified bioactives may change over time with fractionation of the complex mixture of phytochemicals when compared to the parent extract. In each case, having a reliable biomarker of the biological endpoint of interest is essential for extract content analysis, interpreting experimental results, and carrying out pharmacokinetic studies in animal models or clinical trials.

Our research with *Artemisia dracunculus* L. provides an example of identifying an extract-derived biomarker that is strongly associated with the biological endpoint of interest. Our studies focus on the ability of botanical extracts to impart resilience to developing risk factors for type 2 diabetes associated with obesity. The metabolic syndrome (MetS) is a prediabetic state characterized by insulin resistance and elevated blood glucose along with elevated triglycerides, reduced high-density lipoprotein (HDL) cholesterol, elevated blood pressure, and abdominal obesity (6). Clinically, MetS is defined by the presence of at least three of the five risk factors. This illustrates the need to determine the important endpoints that are feasible to assay in preclinical studies and are clinically translatable. In our example, a focus on insulin responsiveness during screening and the subsequent activity-guided fractionation experiments offered the best opportunity to capture a preclinical effect (e.g., signaling responses to insulin in skeletal muscle cells *in vitro* and *in vivo*; blood glucose and insulin levels *in vivo*) that is commonly measured in the clinical setting (blood glucose and insulin levels) to evaluate the risk of developing type 2 diabetes. Using these endpoints, bioactivity-guided fractionation of an ethanolic extract of plant shoots obtained from *A. dracunculus* during the flowering stage (termed PMI-5011) identified a fraction containing four potential bioactive compounds (4, 5, 7–9). 4-*O*-methyldavidigenin was subsequently identified as a reliable extract biomarker for the biological activity of interest *in vitro*, including cell culture of human skeletal muscle cells, which reflect *in vivo* biology (10–13).

Moving from cell-based studies to animal models brings another layer of issues to consider. Choice of an animal model has to be made with the human target population in mind. Our interest in obesity-related insulin resistance and type 2 diabetes prompted us to use the KK-A^y genetic model of hyperglycemia in the initial *in vivo* experiments (9, 14). Although the KK-A^y mouse is a robust model to test our supplement, a genetic model of hyperglycemia does not capture the early phases of obesity-related changes in insulin sensitivity that predict developing type 2 diabetes. To better align with the clinical picture of MetS, later preclinical experiments were carried out in a rodent model of obesity-induced insulin resistance (15, 16). However, the insulin resistance phenotype in the C57BL/6 mouse model of obesity-induced insulin resistance occurs in obese male, but not female mice when fed a defined high fat diet (17).

Subsequent studies using both male and female mice show striking sex-dependent differences in response to dietary supplementation (17, 18). Given the evidence that dietary supplements are often consumed by women across the age and health spectrum (1), this points out the importance of using both sexes in pre-clinical rodent studies unless the target population is only men or women. Experimental variability is not greater in fertile female mice compared to male mice (19). This alleviates much of the concern about including female mice in pre-clinical studies, but our experience indicates the two sexes should be studied initially as independent cohorts. Inclusion of female mice also highlights the importance of determining the age of the target population. Metabolic syndrome is becoming more prevalent in younger women due to increasing obesity or changing dietary patterns (20, 21). With that in mind, our studies are carried out in fertile female mice rather than simulating the onset of MetS by ovariectomizing young mice or aging a colony of female mice for up to 2 years. An excellent guide for designing pre-clinical studies of sex differences is provided by Mauvais-Jarvis et al. (22).

Formulation of the botanical dietary supplement will also be an issue. Oral administration is a given, but capsules or pills cannot be given to a mouse, so the supplement is typically formulated in the diet. This points out the importance of establishing bioavailability in the pre-clinical animal model. While dietary supplementation with a botanical extract can be incorporated into a rodent diet at a wide range of concentrations, this will not be feasible in clinical trials with humans. Even if the supplement is ultimately administered to humans in gel or capsule form, low bioavailability in the pre-clinical phase may signal that an excessive number of capsules will be required in the clinical trial. Thus, the pre-clinical studies offer an excellent opportunity to optimize bioavailability based on the identified biomarker. When determining bioavailability in rodents, keep in mind the food intake patterns of nocturnal animals. If bioavailability in a fasted state is important, food should be withdrawn for 4–6 h prior to the test. The time of day should also be noted to account for any circadian effects on bioavailability (23, 24).

Diet composition is another important variable. We typically use a defined 45% kcal high fat diet that contains 17% kcal sucrose (Research Diets) as the standard diet for studies of obesity-related metabolic disease. This has the advantage of increased bioavailability of the lipid-soluble bioactives, but more recent studies use high fat (45% kcal), high sucrose (30% kcal) diets

that more closely mirror the typical Western diet associated MetS (18). While our interest in obesity-induced insulin resistance requires following the extract's effect as obesity develops, the extract may be given in preclinical studies over a short time to determine acute effects or to carry out pharmacokinetic studies. In our studies, we observe that botanical extract efficacy varies with the length of time administered (unpublished observation). An effect present after 2 months consuming the dietary supplement may not be present after 3 months or longer consuming the same diet and dietary supplement even if the supplement composition is unchanged over time. The pre-clinical stage allows for evaluation of whether the dietary supplement should be taken chronically or will be more effective when taken over shorter periods. If the dietary supplement is given for a short period, it is tempting to administer the supplement using gavage. However, this is stressful for the mouse and adaptation to gavage should be carried out using vehicle only prior to initiating supplementation.

An essential part of pre-clinical studies is evaluating the safety of the chosen botanical dietary supplement. This should include measuring body weight, food intake, activity level, tissue weights, mortality rate, liver morphology at necropsy, and clinical evaluation of hepatic function using albumin, bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) serum levels. These findings can be correlated with the bioavailability of the botanical extract. Depending on the disease model of interest, more specific safety questions should also be addressed. Our experience indicating PMI-5011 supplementation with a 45% high fat diet may have adverse effects in female, but not male mice (17) further highlights the need to consider supplement safety in both sexes of the chosen pre-clinical model.

THE CLINICAL STUDY: THE NITTY-GRITTY DETAILS

Clinical trials assessing the impact of nutrition on human health face unique challenges when designing, interpreting, and reporting the study results (25–29). The complexity of botanical extracts increases the challenge of designing clinical studies of dietary supplements, but the ability to build on strong pre-clinical data increases the likelihood of an outcome that provides reliable clinical data. Thus, the first step when considering a clinical trial of the dietary supplement is to ask if there is good evidence from the preclinical studies for moving forward with a clinical study. To answer this question there should be clear evidence of preclinical safety as well as a convincing demonstration of efficacy before transitioning into the clinic. While there are often significant species differences in physiology and pharmacology, any concerns regarding pre-clinical acute and/or chronic toxicity will preclude moving forward to first-time-dosing in humans. Despite a promising ethnopharmacological track record as either a food or natural medicine, pre-clinical botanical safety studies conducted with a well-characterized extract are necessary to allay any concerns when the extract is formulated and administered as a dietary supplement to human subjects.

Start With Safety, Tolerability, and Bioavailability

Central to assessing the safety and efficacy of a botanical dietary supplement in humans is determining the bioavailability of marker compounds that appear key to pre-clinical effectiveness. Unfortunately, for many phytochemicals, poor oral bioavailability is the rule, not the exception (30–33). This stems from two major factors: human physiology and phytochemical physico-chemistry. From an evolutionary perspective, the ingestion of plants has significantly impacted human development, such that, as a species, we readily biotransform phytochemicals, either through enzymatic metabolism in the gut or liver parenchyma, or via gut microflora (30). Many phytochemicals are also substrates for various efflux transporters expressed on the apical surface of intestinal enterocytes, which can preclude their uptake from the gut lumen. Collectively, actions of these enzymes and transporters reduce phytochemical exposure. On the other hand, many phytochemicals are lipophilic, having insufficient water solubility to become bioaccessible. Combined, extensive pre-systemic metabolism and poor bioaccessibility (efflux transport, lipophilicity) render many seemingly "active" phytochemicals inadequately bioavailable.

Formulation of the Product

Most botanical dietary supplements on today's market are gelatin or cellulosic capsules filled with dried extract. Whether the extract was derived using an aqueous or non-aqueous procedure can have a significant impact on the "performance" of the dosage form. Non-aqueous extracts (e.g., hexane, ethylacetate, etc.) will recover more highly lipophilic phytochemicals, while aqueous or ethanolic extracts will recover more hydrophilic components. Lipophilic marker compounds will oftentimes have poorer bioavailability due to inadequate solubility in gastrointestinal fluid, especially if taken on an empty stomach. An assessment of dietary supplement dosage form performance is critical before conducting a clinical study, as poor performance will undoubtedly lead to questionable clinical outcomes. Dosage form performance assessment can be accomplished by conducting a disintegration/dissolution study. An overview of dissolution/disintegration guidelines and recommended equipment can be found in the United States Pharmacopeia (34).

Briefly, dosage form performance investigates whether a tablet, capsule or liquid gel capsule can disintegrate and release its contents into gastrointestinal fluids in a timely manner. Dosage forms that quickly disintegrate and readily release their active components generally exhibit better bioavailability than those with inferior performance traits. In effect, this process is an *in vitro* means of gauging the rate and extent of phytochemical release into simulated gastric or intestinal fluids using standardized conditions and equipment (35). Even capsule composition (i.e., gelatin vs. cellulosic) can impact dosage form performance. Old or outdated gelatin capsules may bleb and not adequately disintegrate, whereas certain phytochemicals may induce cross-linking of polysaccharide chains in hydroxypropylmethylcellulose

capsules which can compromise disintegration and dissolution (36).

It may also be prudent to incorporate biorelevant media into dissolution studies (37, 38). Biorelevant media are designed to simulate fasted and fed states, two conditions that better emulate the gastrointestinal environment phytochemicals may experience *in vivo*. For lipophilic phytochemicals, dissolution media mimicking fed conditions facilitate micelle formation and enhance solubility, whereas those simulating fasted conditions may yield less favorable results. For other phytochemicals, the presence of food may impair absorption. Incorporation of biorelevant media, therefore, can aid in clinical study design; that is, provide guidance as to whether the supplement should be taken with or without food. The simple task of taking the supplement formulation with food, especially a fatty meal, may preclude the need for developing a novel dosage form specifically designed to improve phytochemical bioavailability (e.g., phytosome, nanoemulsion, etc.) (31). If suitable bioavailability was achieved in pre-clinical studies when the extract was incorporated into the diet, then meals served in the clinical setting should try to reasonably match the carbohydrate, fat, and protein percentages within the animal chow. A word of caution, however, regarding vegetables in meals, for clinical studies is that the potential for phytochemical–phytochemical interactions always exists between the supplement and any plant-based food (39).

Choosing the Study Population

When selecting participants for a supplement-based clinical study, it is ideal to recruit those representative of the product's intended target population. If the product is designed for women, athletes, elderly, etc., then individuals representing those groups should be targeted; otherwise, healthy volunteers of both sexes covering a range of ages are typically utilized. Exclusion criteria generally include acute and chronic disease, prescription medications (an exception is sometimes made for oral contraceptives, unless there is evidence the metabolism of select marker compounds may be affected), and dietary supplement use.

Dosing and Timing of Administration

Doses for first-time administration to humans of a botanical extract not currently on the market (i.e., PMI-5011) can be extrapolated from those evaluated in pre-clinical animal studies using allometric scaling. Allometric scaling incorporates appropriate power functions that correlate body surface area and/or weight to various physiological parameters across animal species in order to estimate human equivalent doses (HED). While there is much debate within the scientific literature regarding the best exponent for use in exponential allometry calculations, U.S. Food and Drug Administration (FDA) recommends the following approach: HED = animal NOAEL $\times$ $(W_{animal}/W_{human})^{(1-b)}$, where NOAEL is the "no observed adverse effect level" for the pre-clinical, animal dose scaling study, W is weight in kg, and b is the allometric exponent equal to 0.67. Utilizing this approach, **Table 1** is useful in converting animal doses to HED.

TABLE 1 | Converting preclinical supplement doses to human equivalent doses (HED).

Species	To convert animal dose in mg/kg to HED in mg/kg, either:	
	Divide animal dose by:	Multiply animal dose by:
Mouse	12.3	0.08
Hamster	7.4	0.13
Rat	6.2	0.16
Guinea pig	4.6	0.22
Rabbit	3.1	0.32
Dog	1.8	0.54
Rhesus monkey	3.1	0.32
Mini-pig	1.1	0.95

As an example, the HED for a 50 mg/kg extract dose (NOAEL) in a 0.02 kg mouse would be $50 \times 0.08 = 4$ mg/kg, or 280 mg in a 70 kg adult human. It should be noted that these FDA guidelines were developed to determine the maximum recommended starting dose (MRSD) of experimental drugs in humans. It must be emphasized that an HED is simply a starting point, it may or may not need to be adjusted depending upon the drug/phytochemical and its specific biotransformation pathways. Incorporating additional safety factors, however, are often recommended prior to administering an HED derived from animal NOAEL data. Further reducing an MRSD by a factor of 10 or even 100 may be prudent for first-time-use in a clinical safety study. Additional discussions of dose conversion methods for botanical extracts based upon allometric scaling and safety factors can be found in Wojcikowski and Gobe (40) and Schilter et al. (41).

Planning a Pharmacokinetic (PK) Study

The first-time-in-human dose for a botanical extract should be aimed at determining PK parameters for one or more biologically relevant marker phytochemicals. These parameters include "area under the concentration-time curve" ($AUC_{0-\infty}$), maximum blood/plasma concentration (C_{max}), time to reach C_{max} (T_{max}), elimination half-life ($t_{1/2}$), and if possible, apparent clearance (CL_{app}). To generate these parameters, it is important to collect a sufficient number of blood levels over a defined period to best characterize the AUC and $t_{1/2}$. These time points often mirror those used for the animal study, although subject inconvenience may preclude certain timepoints (i.e., 18 h post-dose). Blood collection times of 0.0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h are fairly common, although these can be modified for subject convenience. To be as efficient and practical as possible, a single dose of extract is administered in the morning (with or without food) and blood sampling will span at least 12 h the first day, with subsequent blood draws at 24 and perhaps 48 h. Given that most phytochemicals have fairly short elimination half-lives in humans, samples obtained after 24 h may not be necessary (30, 32). A clearly defined biomarker as determined by pre-clinical studies is essential for the clinical PK study. Once there is evidence that the marker compound(s) is bioavailable and a

half-life can be determined, a multi-dose administration scheme can be devised to assess PK parameters at "steady state." Marker compound half-lives <6 h will mean that the extract should be dosed at least 3–4 times daily for at least 3–5 days to reach steady state. Longer half-lives will require less frequent dosing but a longer period of administration to achieve steady state.

Dietary Influences

Diet and concomitant drug use may affect phytochemical PK. Prescription drug and botanical dietary supplement (e.g., St. John's wort, goldenseal, licorice, multi-ingredient supplements, etc.) use are often exclusion criteria, while non-prescription drug use is strongly discouraged. Dietary factors (i.e., vegan diet) and certain dietary restrictions should also be considered, as certain fruits and vegetables can modulate exogenously administered phytochemical metabolism. Cruciferous vegetables (e.g., broccoli, brussel sprouts, asparagus, water cress, etc.) and certain citrus fruits and fruit juices (i.e., grapefruit, pomelo, orange) should be avoided (42). Food diaries are highly recommended for use in multi-dose PK or efficacy studies so that any unanticipated dietary influences can be accounted for (42).

Given that many phytochemicals undergo extensive pre-systemic metabolism, especially glucuronidation, it is plausible that phytochemical metabolites may contribute to a botanical extract's efficacy. In fact, active glucuronide metabolites have been identified for many phytochemicals (43–45). While parent phytochemicals may be below detection limits for some analytical assays, their glucuronides—or indirect evidence of glucuronides when samples are treated with β-glucuronidase—may be quantifiable in plasma or urine. Thus, urine collection should also be considered when conducting both phytochemical marker PK and efficacy studies. Urine collection intervals of 0–4, 4–8, 8–12, and 12–24 h are frequently used to characterize the contribution of renal elimination to phytochemical PK and for active metabolite identification and quantification.

Regulatory Considerations

From a regulatory perspective, one must consider the study's ultimate goal. An Investigational New Drug (IND) application may need to be filed with the U.S. FDA if the study's aim is to investigate whether the supplement may be used to "diagnose, treat, cure, or prevent any disease." This verbiage is from the FDA's definition of a drug, which is quite different from the legal definition of a dietary supplement. If, however, the study's goal is to simply characterize the pharmacodynamics (i.e., blood pressure measurement) or pharmacokinetics (i.e., rate and extent of oral absorption) of a phytochemical marker compound(s), then an IND is typically not required. To best determine whether an IND may be needed for a particular study involving a dietary supplement, investigators are encouraged to contact the FDA in advance.

CONCLUSION

Clinical studies of dietary supplements based on botanical extracts are particularly challenging due to the complex nature of the extracts. Ensuring the design of the clinical study accurately tests the experimental question will depend on keeping the clinically relevant endpoints in mind when planning the pre-clinical studies and careful attention to the seemingly minor details that will ultimately determine the success of translating preclinical studies to the clinical setting.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquires can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

ZF and BG wrote the manuscript. DR, IR, DH, and JR critically reviewed the article. All authors gave final approval for all aspects of the work, agreed to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

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REVIEW
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Interpreting Clinical Trials With Omega-3 Supplements in the Context of Ancestry and *FADS* Genetic Variation

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Human diets in developed countries such as the US have changed dramatically over the past 75 years, leading to increased obesity, inflammation, and cardiometabolic dysfunction. Evidence over the past decade indicates that the interaction of genetic variation with changes in the intake of 18-carbon essential dietary omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFA), linoleic acid (LA) and α-linolenic acid (ALA), respectively, has impacted numerous molecular and clinical phenotypes. Interactions are particularly relevant with the *FADS1* and *FADS2* genes, which encode key fatty acid desaturases in the pathway that converts LA and ALA to their long chain (≥20 carbons), highly unsaturated fatty acid (HUFA) counterparts. These gene by nutrient interactions affect the levels and balance of n-6 and n-3 HUFA that in turn are converted to a wide array of lipids with signaling roles, including eicosanoids, docosanoids, other oxylipins and endocannabinoids. With few exceptions, n-6 HUFA are precursors of pro-inflammatory/pro-thrombotic signaling lipids, and n-3 HUFA are generally anti-inflammatory/anti-thrombotic. We and others have demonstrated that African ancestry populations have much higher frequencies (vs. European-, Asian- or indigenous Americas-ancestry populations) of a *FADS* "derived" haplotype that is associated with the efficient conversion of high levels of dietary n-6 PUFA to pro-inflammatory n-6 HUFA. By contrast, an "ancestral" haplotype, carrying alleles associated with a limited capacity to synthesize HUFA, which can lead to n-3 HUFA deficiency, is found at high frequency in certain Hispanic populations and is nearly fixed in several

indigenous populations from the Americas. Based on these observations, a focused secondary subgroup analysis of the VITAL n-3 HUFA supplementation trial stratifying the data based on self-reported ancestry revealed that African Americans may benefit from n-3 HUFA supplementation, and both ancestry and *FADS* variability should be factored into future clinical trials design.

Keywords: polyunsaturated fatty acid, fatty acid desaturase, gene-diet interaction, oxylipins, endocannabinoid, omega-3 supplements, ancestry, omega-3 deficiency syndrome

BACKGROUND

Highly unsaturated fatty acids (HUFA)—polyunsaturated fatty acids (PUFA) with ≥20 carbons and ≥3 double bonds—and signaling metabolites derived from them play key roles in inflammation and thrombosis that contribute to numerous medical conditions including cardiovascular disease (CVD), Alzheimer's disease (AD), type 2 diabetes, autoimmunity, cancer, hypersensitivity disorders, skin and digestive disorders, and infectious disease such as COVID-19 (1, 2). More specifically, the ratios of circulating and cellular levels of omega-3 (n-3) and omega-6 (n-6) HUFA dictate the balance of inflammatory and thrombotic signaling molecules such as eicosanoids and other oxylipins. The proportions of oxylipins, such as eicosanoids and docosanoids, modulate a wide variety of physiological and pathophysiological functions *via* their capacity to mediate numerous biological processes including inflammation and blood clotting (3–5).

The 18-carbon n-3 and n-6 dietary PUFA, alpha linolenic acid (ALA) and linolenic acid (LA) are essential nutrients throughout the animal kingdom. ALA is generated in plants utilizing a methyl-end desaturase Δ-15 desaturase (ω3 desaturase) that adds a double bond to LA between the n-6 double bond and the methyl end of the hydrocarbon chain (6). Once formed and ingested by humans, ALA can be converted to n-3 HUFA, including eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), utilizing several desaturation and elongation steps (**Figure 1**) (2). Similarly, a second series of n-6 HUFA including dihomo gamma linolenic acid (DGLA), arachidonic acid (ARA), and adrenic acid (ADA) can be formed by the same biosynthetic pathway and compete with the corresponding n-3 species for the same enzymes. With few exceptions, ARA is a precursor to pro-inflammatory/pro-thrombotic signaling molecules, and n-3 HUFA are generally metabolized to anti-inflammatory/anti-thrombotic products (3–5).

In 1961, the American Heart Association recommended that dietary PUFA be substituted for saturated fats "as a possible means of preventing atherosclerosis and decreasing the risk of heart attacks and strokes" (7). This recommendation was largely based on data showing that this substitution of predominately the n-6 PUFA, LA, lowered serum total and LDL cholesterol (8, 9). As a result, increased ingestion of LA-containing vegetable oils and processed foods has increased dietary LA dramatically (to 6–9% of daily energy) and has increased the ratio of dietary n-6/n-3 PUFA to >10:1 (10, 11). Concerns have arisen over the rapid increases in dietary LA levels and the resulting imbalance in n-6 to n-3 PUFA, and intense controversy remains over the health and disease implications of these levels of dietary LA (11–20).

For example, studies are consistent that replacing saturated fats with vegetable oils high in LA reduces serum cholesterol. Further, the traditional diet-heart hypothesis would predict that this would lead to lower deposition of cholesterol in arterial walls, attenuation of atherosclerosis resulting in reduced coronary artery events and all-cause mortality. However, not all studies show this replacement reduces coronary heart disease and all-cause deaths (12, 13, 20, 21). In fact, Ramsden et al. demonstrated in analysis of data from the Sidney Diet Heart Study (a single blinded, parallel group, randomized controlled trial, $n = 458$) that selectively increasing LA (to ∼15% of food energy) from safflower oils and safflower margarine increased risk of cardiovascular risk and all cause mortality by 35% and 29%, respectively, compared to diets enriched in saturated fats (to ∼10% of food energy) (20). Similar results were observed in the Minnesota Coronary Experiment, a double blind randomized controlled trial designed to test replacement of saturated fat with vegetable oil rich in linoleic acid (13). In contrast, multivariable-adjusted associations of circulating or adipose tissue LA demonstrate that higher levels of LA (expressed as % of total fatty acids) are significantly associated with lower risks of cardiovascular mortality and all-cause mortality (22, 23).

Collectively, these studies have raised important questions as to why these results are so inconsistent especially as it relates to such a vital health question. In a meta-analysis of randomized clinical trials (RCTs), Ramsden et al. showed that mixed n-3 ALA/n-6 LA vs. n-6 specific LA interventions have significantly different effects on coronary heart disease (CHD) risk with mixed interventions reducing the risk of non-fatal myocardial infarction (MI) and non-fatal MI+CHD death, while specific LA diets increased risk of all coronary heart disease endpoints (12). We have demonstrated that the method used to express levels of PUFA and HUFA can affect the magnitude and direction of associations with blood lipids (24), and it remains to be seen whether this impacts CHD endpoints or mortality risk. Importantly for the purpose of this review, there are no studies on the impact of dietary LA in the context of ancestry, which is a critical missing gap given our knowledge of the role of ancestry-driven genetic variation in HUFA metabolism summarized below.

Up until a century ago, LA in the diet was limited (2, 25–28). Human diets were largely limited to plants, naturally grazing animals, and fish, which all had much lower levels of LA and

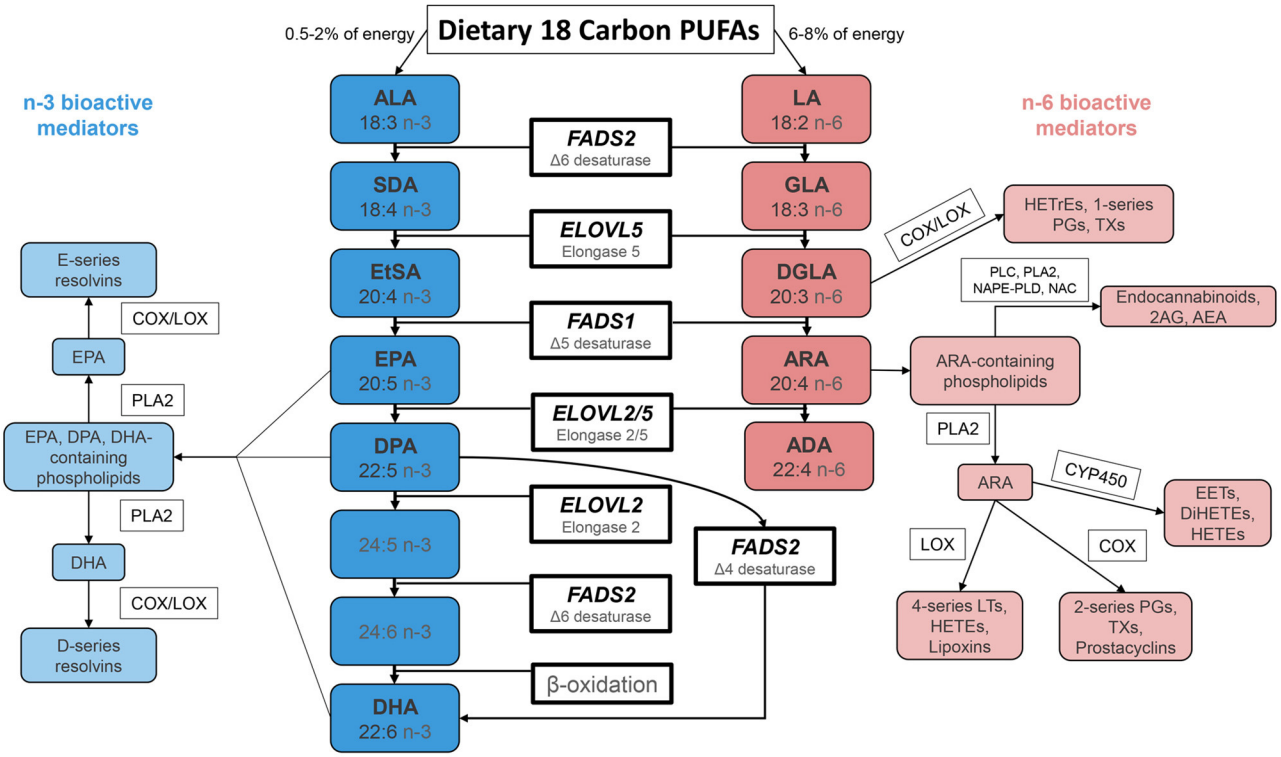

FIGURE 1 | Biosynthesis of n-3 and n-6 Highly Unsaturated Fatty Acids and Lipid Signaling Products. N-6 and n-3 HUFA are synthesized from dietary intake of essential fatty acids ALA and LA, respectively, through a series of enzymatic desaturation (FADS2 and FADS1) and elongation (ELOVL2 and ELOVL5) steps. This pathway gives rise to primary n-3 HUFA and n-6 HUFA such as EPA, DPA, DHA and ARA. These LC-PUFA (as free fatty acids or complex lipids) and lipid signaling metabolites impact a wide range of physiologic and pathophysiologic processes. FADS 1/2, fatty acid desaturase 1/2; ELOVL 2/5, fatty acid elongase 2/5; ALA, α-linolenic acid; SDA, stearidonic acid; EtSA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; ARA, arachidonic acid; ADA, adrenic acid; PG, prostaglandin; TX, thromboxane; LT, leukotriene; HEPE, hydroxyeicosapentaenoic acid; HETrE, hydroxyeicosatrienoic acid, HETE, hydroxyeicosatetraenoic acid; DiHETE, dihydroxyeicosatetraenoic acid; EET, epoxyeicosatetraenoic acid; 2AG, 2-arachidonoylglycerol; AEA, arachidonoyl ethanolamide/anandamide.

an overall dietary ratio of LA to ALA of ∼2:1, as opposed to the >10:1 observed today (10). Available data suggests that dietary LA provided 2 to 3 % of daily energy at most until the late nineteenth and twentieth centuries (27, 29). Additionally, up until that time, the diet contained much more balanced levels of n-3 and n-6 HUFA. LA was dramatically increased in the food supply of western countries as the result of several technological events and dietary recommendations (2). There was the commercial refining of LA-rich seed oils, the invention of hydrogenation that enabled seed oils to be solidified in shortenings and margarines, and the substitution of animal fats with LA enriched oils. These taken together with several dietary recommendations that PUFA (particularly LA) be substituted for saturated fats (8, 9) dramatically increased LA levels an estimated four-fold and markedly increased ratios of LA to ALA and n-6 to n-3 HUFA by reducing n-3 HUFA by an estimated 40% (10).

The effects of this increase in LA have been debated, with one systematic review finding that large changes in LA intake resulted in little to no changes in tissue ARA levels (30). However, given the shared enzymatic steps involved in the metabolism of LA and ALA (**Figure 1**), these PUFA and their intermediates compete in the liver and other tissues for enzymatic reactions that produce biologically active HUFA. Consequently, a marked increase in LA reciprocally deceases levels of all major n-3 HUFA including EPA, DPA and DHA (31–35), while a reduction in LA increases these n-3 HUFA. Additionally, the conversion of PUFA to HUFA reaches a saturation point at which additional PUFA have no effect on HUFA levels (36). Recent studies from our lab reveal that this saturation occurs at the *FADS1* (Δ-5 desaturase) step in the biosynthetic pathway (37–39) and flux through this step is markedly altered by genetics as described in detail below.

Another concern with increased ratios of LA to ALA is related to oxylipins (including eicosanoids and docosanoids) and other signaling molecules that are synthesized from n-6 and n-3 HUFA. ARA-derived eicosanoids compared to EPA and DHA-derived eicosanoids, docosanoids, and specialized pro-resolving lipid mediators typically have opposing biological effects (3–5), and a precise HUFA balance is therefore critical to avoid hyperinflammatory and hypercoagulopathy events in numerous human conditions (1). While the effects of increasing tissue ARA are not completely clear (40), the importance of this balance was demonstrated 20 years ago when selective cyclooxygenase

2 inhibitors were removed from the market after an increase in the number of thrombotic events. This was attributed to an enhanced production of thromboxane A2 *via* the cyclooxygenase 1 enzyme coupled with a reduction in levels of prostacyclin (41). This lack of a "balanced HUFA milieu" has also recently been proposed to be important for the avoidance of the cytokine storm and excess clotting associated with COVID-19 (1). However, it is important to point out that there are a large number of oxylipins derived from n-6 and n-3 PUFA and HUFA that can be produced enzymatically and by autooxidation, and this results in an immense complexity of physiological effects that are not yet fully understood (42).

In summary, the production and human consumption of LA has risen dramatically over the past 100 years (2), and LA now represents over 90% of total PUFA + HUFA intake in Western diets (10). Nutrient deficiencies or imbalances have historically occurred as a result of inadequate food consumption; however, in this case, the amount of PUFA entering the HUFA biosynthetic pathway has been immensely changed by manipulation of food supplies, increases in processed foods, and dietary recommendations. Moreover, in countries such as the US, LA-containing food and oils are being consumed by highly diverse populations representing numerous ancestries and evolutionary histories. The purpose of this review is first to describe the potential impact of PUFA-based gene-diet interactions that lead to ancestry- and gene variation imbalances in n-6 HUFA to n-3 HUFA levels. Second, we will provide a rationale for how these can provide metabolic underpinnings for much of the observed clinical trial heterogeneity with n-3 HUFA supplementation trials. Finally, we describe how precise dietary and supplementation strategies with n-3 HUFA could rebalance HUFA to prevent and manage human diseases especially in certain human populations.

THE BIOCHEMISTRY AND GENETICS OF N-6 AND N-3 HUFA FORMATION

Early studies carried out in European ancestry populations showed that humans had the capacity to convert only a small proportion (3–4% of calories/kilojoules) of ingested LA and ALA into n-6 and n-3 HUFA (36, 43). It was also recognized that this was largely due to the desaturase steps (Δ6 and Δ5), encoded by two genes (*FADS2* and *FADS1*) in the *FADS* cluster on chromosome 11 (chr11q12-13.1) (44). Based on these assumptions, theoretically, recommendations to increase dietary LA to 6–8% of energy should not fundamentally impact HUFA levels, because at most, only 4% of LA could be converted to ARA. Thus, the impact of high dietary LA on ARA signaling products such as oxylipins (including eicosanoids) and endocannabinoids was thought to be limited by the efficiency of conversion of LA to ARA, and this was assumed to be similar across human populations.

Around 2006, candidate gene studies and genome-wide association studies (GWAS) began to show that genetic variation in the fatty acid desaturase cluster, which includes *FADS1*, *FADS2,* and *FADS3,* and also in the fatty acid elongase genes, which include *ELOVL5* and *ELOVL2,* was highly associated with tissue and circulating levels of PUFA and HUFA (45–52). These initial studies, conducted mostly in European-ancestry individuals, revealed variability in the genes associated with HUFA biosynthesis and also demonstrated that this genetic variability impacted the efficiency of the HUFA biosynthetic pathway. Importantly the same variants found in HUFA association studies were also reported in relation to several human diseases including CVD, metabolic syndrome, obesity, atopic dermatitis, and major depressive disorders (1, 44, 53, 54).

In particular, an early study by Martinelli et al. showed that a higher ratio of ARA to LA in individuals with the *FADS* haplotype for efficient conversion of LA to ARA was an independent risk factor for coronary artery disease (55). Additionally, levels of high-sensitivity c-reactive protein, which is an inflammatory marker associated with risk of CVD, increased progressively with the haplotype-dependent ratio of AA to LA. The authors concluded that in a Western diet, "subjects carrying *FADS* haplotypes that are associated with higher desaturase activity may be prone to a proinflammatory response favoring atherosclerotic vascular damage." This study and similar ones began to raise the alarm that "one size fits all" recommendations related to high dietary LA may actually harm certain individuals in a highly diverse population such as the US.

Recently, in a study that combined metabolomic and GWAS analyses, we examined the capacity of *FADS* variants to impact the balance of pro- vs. anti-inflammatory or thrombotic HUFA metabolite balance (38). This study was designed to examine not only the rate-limiting step of HUFA biosynthesis but also regions of the genome that exert genetic control over a large number the HUFA-containing complex lipids and signaling molecules. We examined associations between levels of 247 lipid metabolites (including four major classes of HUFA-containing molecules and signaling molecules) and common and low-frequency genetic variants throughout the genome. Genetic variation within the *FADS* locus was strongly associated with 52 HUFA-containing lipids and signaling molecules, including free fatty acids, phospholipids, lyso-phospholipids, and an endocannabinoid (2-arachidonyl glycerol). The HUFA within these lipids were largely a precursor (such as dihomo gammalinolenic acid [DGLA]) or a product (such as ARA) of the Δ5 desaturase (*FADS1*) step. Perhaps most surprisingly, for over 80% of the *FADS*-associated lipids, there were no significant genetic associations outside the *FADS* locus, offering further evidence of the impact of this single genetic locus. This was unanticipated as many of these HUFA-containing lipids were synthesized by up to five biochemical steps past the *FADS1* desaturation step. These data suggest that *FADS* variation is the critical "control point" in the formation and levels of numerous biologically important, HUFA-containing lipids and signaling molecules, many of which are related to health and human disease outcomes.

Given the role of the *FADS1* step as a central control point of so many biologically important lipids, it is important to also consider the effect of genetic variation on the precursor-product flux across the *FADS1* desaturation step. In particular, it was important to understand the effect of genetic variation on levels of the precursor of the *FADS1* desaturation step, DGLA,

which is converted to anti-inflammatory, anti-thrombotic, and vasodilatory signaling molecules and the product of this step, ARA, which is the precursor to largely pro-inflammatory, pro-thrombotic, and vasoconstricting products. Kothapalli et al. recently modeled the DGLA to ARA flux across the *FADS1* step to be altered by 84% between the DD and II genotype of the *FADS* insertion/deletion variant (indel) rs66698963 (1, 56). We found a similar impact on flux of 82% measuring DGLA and ARA levels when comparing the GG and the TT genotypes for the variant rs174537 (37) near *FADS1/2*. When this relationship is further modeled using LA quantities in Western diets, not only does the ratio of ARA to DGLA shift dramatically between genotypes but the ratio of ARA to the sum of all n-3 HUFA is projected to increase by 47% (1). As highlighted in the section on ancestry below, this becomes highly relevant when population frequencies differ dramatically, and haplotypes in many populations are fixed with almost all individuals harboring the higher flux genotypes.

The importance of circulating and red blood cell levels of n-3 HUFA to human health has been demonstrated in several studies and meta-analyses (57–59). In general, these studies show that higher levels of n-3 HUFA are associated with lower risk of coronary heart disease and all-cause mortality. A recent meta-analysis from 17 prospective studies with a median of 16 years of follow up in 42,466 individuals showed a 15–18% reduction in all-cause mortality when comparing the highest and lowest quintiles (57). An important limitation of these studies is they have not included ancestry-based subgroup analyses that, based on the discussion below, are likely to be very important.

The degree to which such HUFA imbalances affect human disease is unknown, but these studies certainly raise concerns as to whether interactions between the homozygous efficient converter, *FADS* genotypes and current levels of LA in Western diets place certain individuals at greater risk for disease due to elevated n-6 HUFA levels and n-6 HUFA to n-3 HUFA ratios. The above-mentioned analyses also raise the question of whether stratifying individuals by *FADS* genotypes/haplotypes may represent an important opportunity for rebalancing n-6 to n-3 HUFA ratios with n-3 HUFA supplementation. We provide evidence to support this hypothesis in the last section of this paper.

IMPACT OF ANCESTRY AND ASSOCIATED *FADS* GENETIC VARIATION ON HUFA LEVELS AND HUMAN DISEASE

African Ancestry

In 2012, it was discovered by our group that African American (AfAm) and European American (EuAm) populations in the US had dramatic differences in both levels of HUFA and the frequency of more efficient *FADS* variants (60, 61). It was also reported that the substantially higher levels of HUFA found in AfAm vs. the EuAm could be explained in part by significant differences in frequency of *FADS* alleles associated with efficient HUFA biosynthesis in the two populations; notably the effect sizes were similar across populations but the frequencies were not. More specifically, only ∼45% of EuAm were homozygous for the efficient allele for the variant rs174537 vs. ∼80% of AfAm. At about the same time, Ameur et al. identified a set of 28 SNPs in a primary haplotype block that clearly distinguished what was called the "ancestral" and "derived" haplotypes with the derived haplotype having enhanced HUFA biosynthetic capacity (62). Evidence was then provided that these pathway efficient alleles that reside on the derived haplotype in the *FADS* gene cluster were driven to fixation on the African continent ∼85 thousand years ago (kya) by positive selection (63). This in turn would have facilitated the production of circulating and tissue HUFA from plant-based PUFA. As HUFA are critical to brain development/function and innate immunity, this study proposed that the selection event played a key role in "allowing African populations obligatorily tethered to marine sources for HUFA in isolated geographic regions, to rapidly expand throughout the African continent 60–80 kya" (63). It also explained the high frequency of pathway efficient alleles in African ancestry populations.

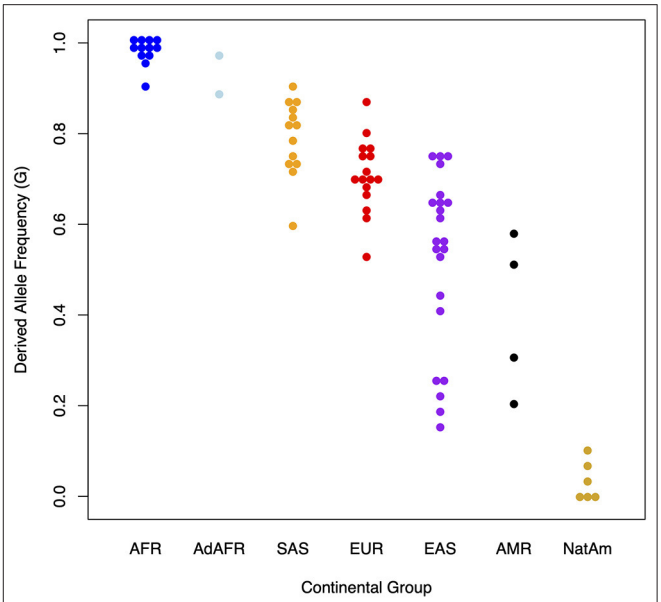

FIGURE 2 | Presence and absence of the derived allele (G) at rs174537 in 80 globally diverse populations. The *Y* axis illustrates the proportion of the derived allele (G) at rs174537 and the X axis shows global populations. AFR, African; AdAFR, Ad Mixed African; SAS, South Asian; EUR, European; EAS, East Asian; AMR, Ad Mixed American; NatAm, Native American.

Figure 2 illustrates the dramatic differences in the frequencies of the derived allele at rs174537 in modern global populations. Looking at 80 globally diverse populations separated broadly by continent of origin, we find a near absence of the ancestral allele in Africa, with various levels of polymorphisms elsewhere. The other exception to this is in the Americas, where we have shown previously an absence of the derived haplotype in many populations (64). Overall, this demonstrates a pattern of repeated natural selection on both the ancestral and derived variants at different times and places across the globe (65).

With regard to African ancestry populations, data from the studies raised questions as to the impact of a Western diet (containing 6–8% LA and a LA to ALA ratio >10:1) on these populations, which have a high frequency of alleles associated with efficient HUFA biosynthesis. Both observational genetic association studies suggesting potential interactions (genetic allele frequencies do not completely explain population differences), and recent clinical trials (offering empirical evidence of gene-nutrient interactions) suggest that this gene-diet interaction would lead to a marked increase in the production of ARA relative to DGLA as well as reducing n-3 HUFA including EPA, DPA and DHA by about 50% in AfAm and other African-ancestry populations (1, 37, 66, 67).

Figure 3 shows the relationship between n-6 and n-3 HUFA levels and global proportions of African and Amerind ancestry in AfAm and Hispanic American participants from the Multi-Ethnic Study of Atherosclerosis (MESA) cohort. **Figure 3E** illustrates the impact of African ancestry on ARA levels in circulating phospholipids. Total ARA levels (expressed as % of total fatty acids) increased as a function of African ancestry by ∼30%. **Figures 3A,C** show that n-3 HUFA, EPA and DHA also increased by ∼30 and ∼50%, respectively. These data reveal the impact of African ancestry in AfAm MESA participants on n-6 and n-3 HUFA levels. **Figures 3B,D,E** (described in detail below) show the inverse impact of Amerind (AI) ancestry on HUFA levels in circulating phospholipids.

Importantly, as discussed above, combined genetic and metabolomic analyses reveal that this shift is not only seen in the HUFA themselves but also HUFA-containing complex lipids and signaling molecules. Additionally, a separate study revealed an association between *FADS* genotype and the ratio ARA/DGLA as well as the biosynthesis of 5-lipoxygenase products produced in whole blood (68). Collectively, these studies show that elevated levels of LA combined with *FADS (*particularly *FADS1)* genetic variability create different mixtures of HUFA that serve as the precursors of critical signaling lipids (oxylipins including eicosanoids and endocannabinoids). These data would lead to the conclusion that African ancestry populations consuming a Western diet with high LA could be impacted more by gene-diet interactions that lead to a HUFA balance predicted to move toward proinflammatory and prothrombotic signaling molecules potentially contributing to disease severity and disease disparities.

Figure 4 (Right Side) illustrates the potential impact of two alleles of derived, pathway efficient variants combined with current levels of LA on levels of n-6 and n-3 HUFA. Here, the biosynthetic efficiency through the FADS (and particularly through the FADS1 [D5 desaturase]) enzymatic step is maximized and produces high, excess levels of n-6 HUFA as well as their signaling metabolites. Under these same circumstances, n-3 HUFA levels and signaling products are measurable but not sufficient to balance the excess quantities of n-6 HUFA synthesized from high quantities of dietary LA entering the highly efficient pathway. With *FADS* variants associated with the derived haplotype, the n-6 HUFA to n-3 HUFA ratio more closely reflects the >10:1 LA/ALA entering the pathway. The mixture of n-6 HUFA to n-3 HUFA serving as substrates for the biosynthesis of signaling molecules is critical as these are typically competitive enzymatic reactions.

European and Asian Ancestry

Approximately 44% of the European ancestry population in the US have two *FADS* alleles associated with the derived haplotype (60, 61). By comparing *FADS* sequencing data from present-day and Bronze Age (5–3k years ago), Mathieson et al. and Buckley et.al showed that selective patterns observed in Europeans were likely driven by a change in the dietary composition of PUFA following the transition to agriculture (75, 76). This transition gave rise to high intake of LA and ALA and lower ingestion of HUFA thus driving the need for more efficient biosynthesis of HUFA. **Figure 2** shows a great deal of variability in the frequency of ancestral and derived variants in Asian populations, with the derived haplotype proportion ranging from ∼0.4 in east Asian to ∼0.8 in south Asian to populations. In south Asia, Kothapalli et al. showed positive selection for an insertion-deletion mutation (rs66698963) in *FADS2* leading to more efficient biosynthesis of HUFA and proposed this too may have been an adaptation to a more vegetarian diet (56).

Amerind Ancestry

In contrast to African ancestry populations, in AI-ancestry Hispanic populations the ancestral haplotype that harbors *FADS* variants associated with limited biosynthetic efficiency is found at high frequency and the alleles are nearly fixed in many Native American populations (**Figure 2**). Fumagalli et al. first found strong signals for the ancestral haplotype in the *FADS* cluster when examining natural selection to cold adaptations in an indigenous Greenland Inuit population (77). The identified variants were also strongly associated with body weight and height, and these findings were replicated in European populations. We confirmed that the ancestral haplotype is fixed in Indigenous Americans in Peru and provided evidence that positive selection possibly continued after the founding of the Americas (64). Our study also demonstrated that the ancestral haplotype is at higher proportions in more northern regions of Siberia, independent of European admixture. Mathieson et al. suggest that the current distribution of the haplotype indicates that Indigenous Americans retained it from Paleolithic Eurasians (65).

The positive selection toward the ancestral haplotype is puzzling given the potential detrimental health effects of a reduced capacity to synthesize HUFA given their role in brain function/development and innate immunity. However, HUFA and particularly n-3 HUFA from cold water marine sources would have likely been the major food source for early Siberian and indigenous American ancestors that remained isolated, possibly in Beringia (78). There is evidence that the ancestral haplotype provided a cold adaptation advantage, but it is not apparent how. As discussed above, it is worth noting that a recent combined genetics and metabolomics study has shown that the *FADS* locus (mainly *FADS1*) is a central control point for signaling lipids such as endocannabinoids, and endocannabinoids are known to impact anthropomorphic and

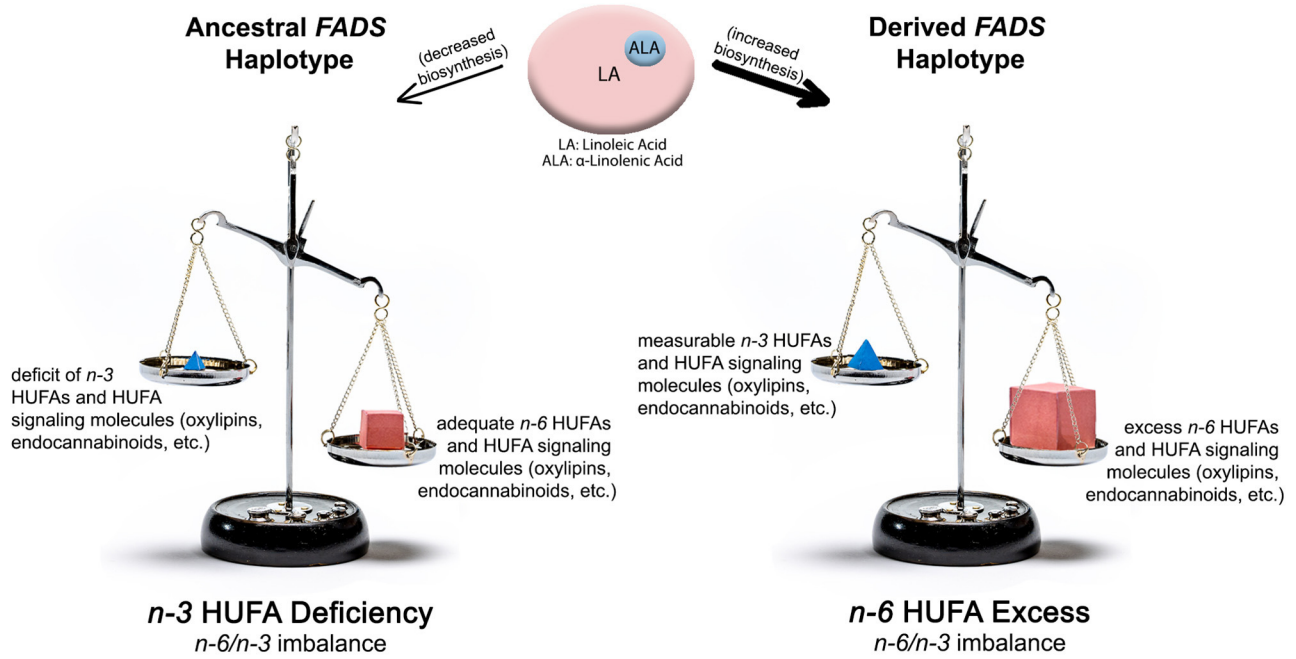

FIGURE 3 | Impact of ancestral and derived *FADS* haplotypes combined with n-6 and n-3 PUFA from western diets on producing n-3 HUFA deficient (Left Side) and n-6 HUFA excess (Right Side) molecular phenotypes. The top of the illustration shows quantities and ratios of dietary PUFA entering the lower efficiency "ancestral" pathway or the higher efficiency "derived" pathway. The lower shows n-6 and n-3 HUFA levels, metabolites and imbalances produced by these gene-diet interactions.

other phenotypic characteristics that could have been critical to cold adaptation (38, 79).

These genetic observations raise questions as to the biochemical and clinical ramifications of the ancestral haplotype for modern populations and particularly those that find themselves ingesting Western diets. In 1997, Okuyama and colleagues made a compelling case that excess LA, generating a dramatic increase in the dietary LA to ALA ratio, would lead to n-3 HUFA deficiency in certain populations and this in turn would increase the risk of CVD, western-type cancers, cerebrovascular diseases, and mental health disorders (80). Regarding our current understanding of the impact of *FADS* haplotype variants, populations with high frequencies of two *FADS* alleles associated with the ancestral haplotype would be expected to have a limited capacity to synthesize HUFA. A gene-diet interaction then would be predicted to produce low (perhaps deficient) levels of n-3 HUFA given a ratio of LA to ALA of 10:1 and restricted flux through the pathway.

We recently tested this hypothesis in Hispanic American participants originating from Central America, South America, Mexico, Dominican Republic, Cuba, and Puerto Rico in the US-based MESA cohort (39). Not surprising, global proportions of genetic ancestry differed markedly, with Central American, South American, and Mexican populations having high AI ancestry compared to those of Dominican, Cuban, or Puerto Rican origin (which have higher African and European ancestry). This was mirrored by a higher frequency of the ancestral haplotype *FADS* alleles in high AI populations vs. low AI populations. Populations from Central America, South America, and Mexico had ancestral allele frequencies ranging from 0.56 to 0.59, while populations from the Dominican Republic, Cuba and Puerto Rico had ancestral allele frequencies ranging from 0.27 to 0.40. The primary hypothesis of this study was that the levels and ratios of LA and ALA found in western diets would be metabolized through a less efficient pathway in a high proportion of AI individuals due to the elevated frequency of two alleles associated with the ancestral haplotype. This in turn would be proposed to produce adequate levels of ARA, but because of the competition between LA and ALA and their intermediates under conditions of limited metabolism, low (inadequate) levels of n-3 HUFA (EPA, DPA and DHA) would be expected as shown in **Figure 4 (Left Side)**. Without adequate n-3 HUFA and in the presence of the imbalance of n-6 to n-3 HUFA, the anti-inflammatory and lipid lowering functions needed to protect against obesity, CVD, and cardiometabolic disease would be reduced. Such data would once again reveal that "one size fits all" dietary interventions are unlikely to be clinically effective for all populations, given the dramatic differences in *FADS* variant frequencies that impact circulating and tissue levels of n-3 HUFA.

Figures 3B,D,F shows that in MESA, AI ancestry was associated with lower levels of n-6 and n-3 HUFA in Hispanic individuals (39). When comparing participants with the highest AI ancestry to those with the lowest AI ancestry,

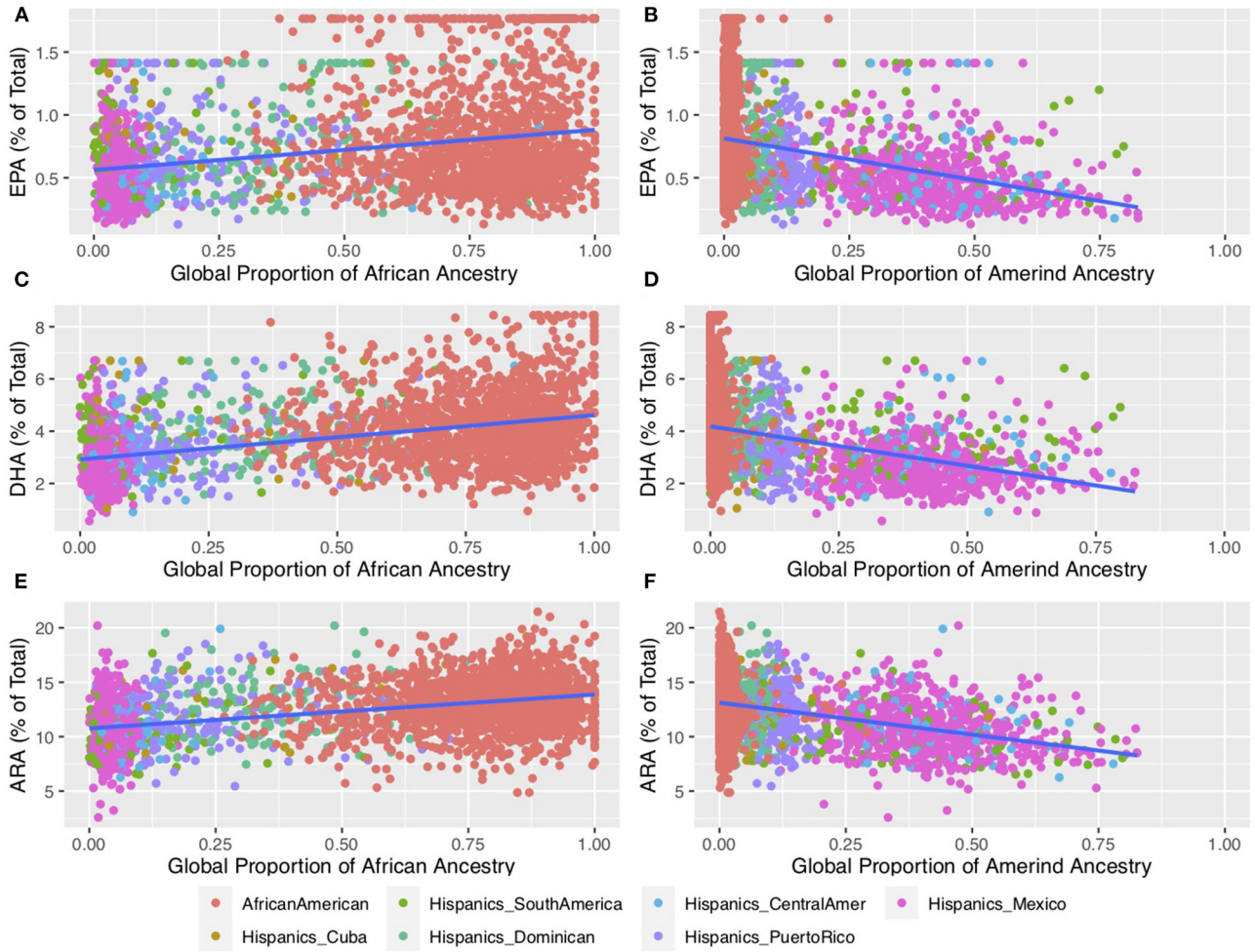

FIGURE 4 | Impact of African and Amerind ancestry on n-6 and n-3 HUFA Biosynthesis. This plot shows HUFA levels for self-reported African American ($n = 1,505$) and Hispanic ($n = 1,102$) participants from MESA (69). Fatty acids were measured using the methods described in Cao et al. (70) and outliers were winsorized to the value of median +/− 3.5 * median absolute deviation (MAD) (39). Proportions of African and Amerind ancestry were estimated using ADMIXTURE (71) using selected Amerind (39) and all African reference samples from the 1,000 Genomes (72) and the HGDP6 (73, 74). **(A,C,E)** illustrate circulating phospholipid levels containing EPA, DHA, and ARA, respectively, as a function of African Ancestry. **(B,D,F)** illustrate circulating phospholipid levels containing EPA, DHA, and ARA, respectively, as a function of Amerind Ancestry. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid.

there was a 60, 47 and 31% reduction in EPA, DHA and ARA, respectively, in circulating phospholipids. These declines gave rise to predicted levels among individuals with 100% AI ancestry of ∼0.3 and ∼2% of total fatty acids for EPA and DHA, respectively, compared to ∼8.6 % for ARA. While it is not possible to determine the levels n-3 HUFA where a deficiency with pathophysiologic impact would occur, these are quantitatively very low concentrations of n-3 HUFA.

The observed inverse relations with AI ancestry and n-3 HUFA levels are to be expected given that the substrate saturation point in the pathway is reduced due to genetic variation associated with the ancestral haplotype. Together, these data reveal that combinations of LA to ALA found in Western diets combined with carrying two alleles associated with the ancestral haplotype and low consumption of preformed dietary n-3 HUFA have the capacity to give rise to Omega-3 Deficiency Syndrome, as proposed by both Okuyama et al. and Lands et al. three decades ago (80, 81).

The potential ramifications of AI ancestry, the presence of the ancestral haplotype, and low levels of n-3 HUFA on critical cardiometabolic and inflammatory risk factors were next examined. AI ancestry is positively associated with levels of circulating triglycerides (TGs) and much of this effect was explained by variation in the *FADS* locus (39, 82–91). The high frequency of the ancestral *FADS* alleles together with their effect size in AI-Ancestry Hispanic populations suggest that *FADS* variation is particularly relevant to TG levels in this population. The liver is important in TG synthesis and deficiencies of n-3 HUFA and imbalances of n-6 relative to n-3 PUFA and HUFA have been associated with elevated TGs and non-alcoholic fatty liver disease (NAFLD) (92). A strong association between the *FADS* variant rs174537 and E-selectin was also observed in Hispanic populations, with higher levels

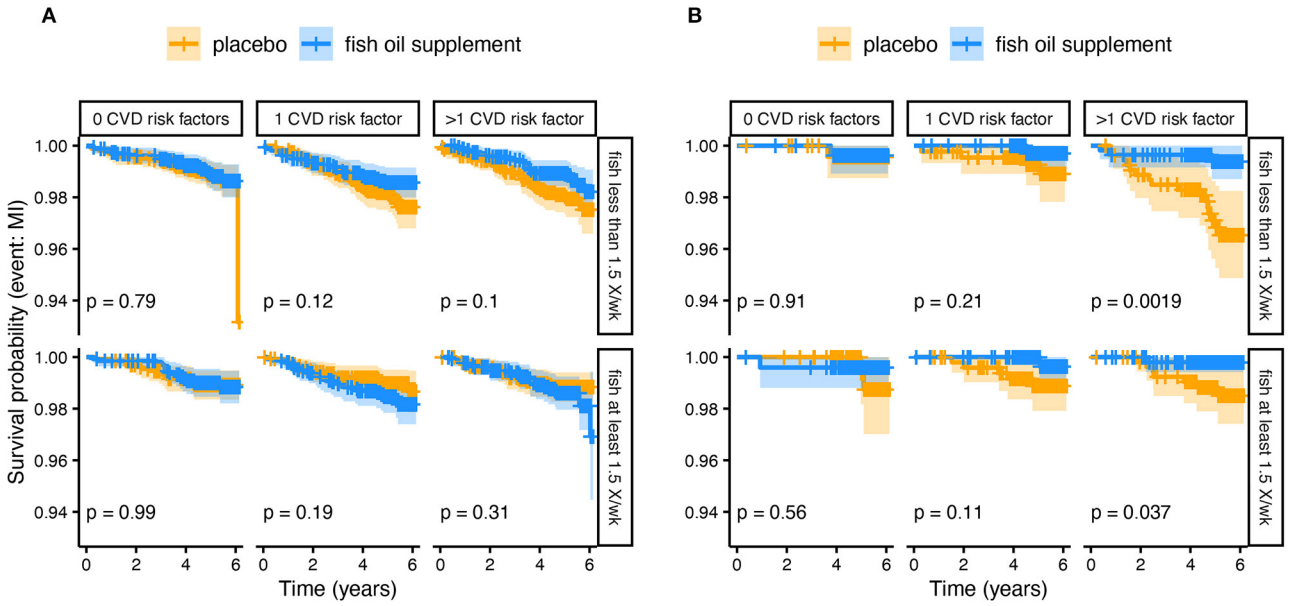

FIGURE 5 | Kaplan-Meier survival curves comparing n-3 HUFA supplementation and placebo from a subgroup analysis of **(A)** White and **(B)** African American Subjects. The plots have been divided by fish intake (at least 1.5× per week) and the number of cardiovascular disease (CVD) risk factors. The plotted lines show the probability of surviving (i.e., not experiencing a myocardial infarction) over time. The lighter shaded regions surrounding the plot lines show 95% confidence regions. The greatest difference is observed in African Americans who did not eat fish and had >1 CVD risk factors, such that those receiving n-3 supplements had many fewer myocardial infarction events over the study period.

in individuals carrying copies of the ancestral T allele (39). Circulating levels of E-Selectin (CD-62E) are elevated in many diseases involving chronic inflammation including obesity (93), cardiovascular disease (94), bronchial asthma (95), and cancer (96, 97).

The original observation by Fumagalli et al. that strong signals for the ancestral haplotype in the *FADS* cluster are associated with anthropometric characteristics such as body weight and height are curious (57). We also examined the effect of rs174537 and rs174557 on the same set of phenotypes and found them to be significantly associated with higher waist-hip circumference ratio, as well as lower height and weight. The rs174537 allele T further demonstrated an association with reduced height and weight in the large Hispanic Community Health Study/Study of Latinos (HCHS/SOL) cohort (n = 12,333) (39). The capacity of this region of the genome to impact anthropometric characteristics likely played a key evolutionary role in cold adaptation for early Siberian and indigenous American populations. Additionally, it may also be vital in impacting key CVD risk factors in modern AI ancestry Hispanic and Native American population. While it is unclear which signaling molecules from *FADS* derived steps are responsible for the anthropometric changes, our recent combined genetic and metabolomic analyses showed the *FADS* locus is a central control point for biologically active HUFA-containing complex lipids that act as signaling molecules. The endocannabinoid, 2-AG, and such endocannabinoids are known to impact anthropometric and other phenotypic characteristics (38).

POST HOC SUBGROUP ANALYSIS OF THE VITAL TRIAL

Imbalance of n-6 to n-3 HUFA could likely be attenuated by n-3 HUFA enriched diets or supplementation thereby preventing or improving serious disease outcomes; however, this relationship needs to be evaluated in the context of *FADS* genetic variants and/or ancestry. Results from randomized clinical trials with EPA and DHA (mostly with European ancestry participants) have shown conflicting results, and thus their efficacy for reducing CVD and cancer remain controversial. The 2019 Vitamin D and Omega-3 Trial (VITAL) is of particular interest here, as it included n = 5,106 African American participants out of n = 25,871 total participants. Overall, they reported that supplementation with either n−3 HUFA (EPA + DHA, provided as ethyl esters) at a dose of 1 g/day or vitamin D3 at a dose of 2,000 IU/day was ineffective for primary prevention of CVD (composite endpoint) or cancer events among their entire study cohort of healthy middle-aged men and women over 5 years of follow-up (98). However, in line with the *FADS* hypothesis outlined above, for African American participants they reported (in the supplemental material) an overall reduction in myocardial infarction of 77% (vs. placebo), and a marked reduction in coronary revascularization and total coronary heart disease with EPA and DHA supplementation.

Using the VITAL study data, we performed additional subgroup comparisons based on the *FADS* framework. **Figure 5** compares the Kaplan-Meier survival curves for the myocardial infarction endpoint, faceted by fish consumption and the number of cardiovascular risk factors, for both

White and African American subjects. These data show the largest impact of n-3 HUFA supplementation on African American subjects who do not consume fish and have existing cardiovascular risk factors, suggesting that this group in particular could benefit from n-3 HUFA supplementation. In line with our expectations based on the mixed distribution of *FADS* haplotypes in European-ancestry populations, we do not see such a response to n-3 HUFA supplementation in the White subjects. These results come with all the usual caveats for *post-hoc* re-analyses of clinical trial data and there is always a risk of finding false positive associations. However, in this case, the pattern of subgroup results agrees with predictions based on *FADS* genetics and resulting HUFA metabolism.

CONCLUSIONS

The human consumption of LA has risen dramatically over the past 100 years (2), due largely to manipulation of food supplies, increases in processed foods, and dietary recommendations. LA-containing food and oils are currently being consumed by highly diverse populations in countries such as the US whose populations represent numerous ancestries and evolutionary histories. Ancestry-influenced *FADS* variability together with current levels of LA in Western diets likely place certain individuals and populations at greater risk for disease due to elevated n-6 HUFA or markedly reduced n-3 HUFA levels and concomitant alterations in n-6 HUFA to n-3 HUFA ratios. These imbalances manifest themselves as alterations in levels of lipid signaling metabolites that impact inflammatory and thrombotic conditions.

Data from the studies of African ancestry populations raise concerns as to the impact of a Western diet (containing 6–8% LA and a LA to ALA ratio >10:1) combined with a high frequency of alleles associated with efficient HUFA biosynthesis. Recent clinical trials (offering empirical evidence of gene-nutrient interactions) suggest that this gene-diet interaction would lead to a marked increase in the production of ARA relative to DGLA as well as reducing n-3 HUFA (1, 37, 66, 67).

Our findings with African Americans in the VITAL trial further highlight the urgent need to consider genetic ancestry (or race as proxy) and/or *FADS* genotypes in n-3 HUFA supplementation trials and dietary recommendations. By focusing on a one-size-fits-all outcome (i.e., all subjects), the impact of *FADS* variation gets obscured, and subgroups who could benefit miss out. In this case, many African Americans who may have benefited from n-3 HUFA supplementation likely did not receive a recommendation or prescription due to reporting of the overall negative result.

This will continue as long as HUFA metabolism and n-3 HUFA supplementation are viewed through a single lens for all people. Instead, future work should expect this diversity of responses and focus on those groups for which placing preformed n-3 HUFA into the diet would be the most helpful. Ultimately, considering individual genotypes at *FADS* and other loci will likely lead to personalized supplementation and dietary recommendations.

AUTHOR CONTRIBUTIONS

AM, CY, MT, and SR carried out the analysis in the manuscript with the MESA data. TO'C analyzed genetic population data and created **Figure 2**. SB and SS help with the genetic and biochemical analyses in the manuscript and created **Figures 1**, **3**. LJ and BH carried out the VITAL *post hoc* subgroup analyses. FC oversaw the various analyses shown in the manuscript. FC, RM, SMS, SB, BH, and AM contributed to writing the manuscript. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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