frontiers | Research Topics

Medicinal Cannabis: Evolution of therapeutic use, future approaches and other implications, volume II

Edited by
Paola Brusa, Francesca Baratta, Shimon Ben-Shabat and Massimo Collino

Published in
Frontiers in Pharmacology

ISSN 1664-8714
ISBN 978-2-8325-4017-6
DOI 10.3389/978-2-8325-4017-6

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Medicinal Cannabis: Evolution of therapeutic use, future approaches and other implications, volume II

Topic editors

Paola Brusa — University of Turin, Italy

Francesca Baratta — University of Turin, Italy

Shimon Ben-Shabat — Ben-Gurion University of the Negev, Israel

Massimo Collino — University of Turin, Italy

Citation

Brusa, P., Baratta, F., Ben-Shabat, S., Collino, M., eds. (2023). *Medicinal Cannabis: Evolution of therapeutic use, future approaches and other implications, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4017-6

Table of contents

frontiers | Frontiers in Pharmacology

TYPE Editorial
PUBLISHED 13 November 2023
DOI 10.3389/fphar.2023.1322404

OPEN ACCESS

EDITED AND REVIEWED BY
Filippo Drago,
University of Catania, Italy

*CORRESPONDENCE
Francesca Baratta,
francesca.baratta@unito.it

RECEIVED 16 October 2023
ACCEPTED 06 November 2023
PUBLISHED 13 November 2023

CITATION
Baratta F, Ben-Shabat S, Brusa P and Collino M (2023), Editorial: Medicinal *Cannabis*: evolution of therapeutic use, future approaches and other implications, volume II.
Front. Pharmacol. 14:1322404.
doi: 10.3389/fphar.2023.1322404

Editorial: Medicinal *Cannabis*: evolution of therapeutic use, future approaches and other implications, volume II

Francesca Baratta[1]*, Shimon Ben-Shabat[2], Paola Brusa[1] and Massimo Collino[3]

[1]Department of Drug Science and Technology, University of Turin, Torino, Italy, [2]Department of Clinical Biochemistry and Pharmacology, Ben-Gurion University of the Negev, Be'er Sheva, Southern, Israel, [3]Department of Neuroscience "Rita Levi Montalcini", University Hospital of the City of Health and Science of Turin, Turin, Toscana, Italy

KEYWORDS

***Cannabis*, formulations, effectiveness, medical use, clinical trials**

Editorial on the Research Topic
Medicinal Cannabis: evolution of therapeutic use, future approaches and other implications, volume II

Cannabis has been historically used in the oldest traditional medicines. Nevertheless, in the last century, a negative vision has prevailed and *Cannabis* has for a long time been banned and declared illegal in many countries.

The recent marketing authorization of some products for medical use resulted in that *Cannabis*-derived products are gaining increasing attention. These compounds are emerging as potential treatments for a variety of medical conditions. Increasingly in recent years, scientific studies have contributed to provide a broader view of the different aspects related to the therapeutic use of cannabinoids.

Given the growing interest in medical *Cannabis*, the second volume of this Research Topic focused on the in-depth analysis of many aspects related to the medical use of *Cannabis*-based formulations, reporting original data and highlighting innovative perspective. For instance, Cairns et al. documented an increase in the prescription of *Cannabis*-based medicines in Australia for anxiety disorders, sleep-wake disorders, trauma- and stress-related disorders, and neurodevelopmental disorders, as well as for attention-deficit/hyperactivity disorder. The authors rightly underline that there is a dramatic lack of evidence-based clinical guidance on the use of *Cannabis*-derived products in psychiatry and, thus, most of the prescriptions are for pathologies for which there is no definitive clinical evidence. Besides, the high prevalence of prescribed THC (tetrahydrocannabinol)-containing products may rise concerns on their safety concerning (Cairns et al.). In fact, as documented by Stith et al. in real-time *Cannabis* consumption sessions, patients feeling "high" is often associated with improved symptom relief, but it also leads to dangerous increase in negative side effects.

Concerns regarding *Cannabis* side effects, legality and limited availability of information are also pointed out in the manuscript of Albert Garcia-Garcia-Romeu et al. who described the perceived advantages and challenges encountered by medicinal *Cannabis* users, concluding that the majority of participants reported benefits from *Cannabis* use for

various conditions in the cases where conventional treatments were ineffective or undesirable (Garcia-Romeu et al.).

Rojas-Valverde and Fallas-Campos analysed the literature to investigate the use of CBD (cannabidiol) in athletes. CBD appears to have anti-inflammatory, neuroprotective, analgesic, anxiolytic and potentially recovery-inducing properties in athletes, but further scientific evidences are needed to confirm these effects. Furthermore, more consideration should be given to adopting a clearer and more comprehensive administrative policy for the use of *Cannabis* in sports (Rojas-Valverde and Fallas-Campos).

The use of non-psychoactive *Cannabis*-derived compounds such as CBD and CBG (cannabigerol) as chemotherapeutic agents requires further investigation. For this reason, the study conducted by Yüksel et al. explored the potential therapeutic synergy of a triple combination including CBD/CBG, curcumin and piperine in colon adenocarcinoma using HCT116 and HT29 cell lines. Curcumin and piperine have benn selected considering that clinical and epidemiological evidences, along with experimental results, suggest that these micronutrients may offer a safer approach to prevent tumour formation and its recurrence. The authors of this study demonstered a synergy in anti-tumorigenic effects between the investigated molecules (Yüksel et al.).

Del Rio et al. specifically directed their research on CBD as a potential therapy for fibrotic disorders. The antifibrotic effects of CBD in the skin were investigated *in vitro* and *in vivo* using NIH-3T3 fibroblasts, human dermal fibroblasts, and a bleomycin-induced skin fibrosis model. Moreover, non-alcoholic liver fibrosis was induced and investigated in mice. These experiments showed the potential role of the cannabinoid medicinal use in the management of fibrotic conditions, including systemic sclerosis and non-alcoholic fatty liver disease (Del Rio et al.).

Cajiao-Manrique et al. established a mouse model to investigate the neurobiological basis of cannabinoid addiction. In particular, they developed a model to study the neurobiological factors associated with resilience or susceptibility in the development of cannabinoid addiction. This model includes chemogenetic inhibition of neuronal activity in the pathway from the medial prefrontal cortex to the nucleus accumbens (Cajiao-Manrique et al.).

Regardless of the condition for which *Cannabis* extracts are intended to be used, it is essential to know their active molecule content. In this regard, Dei Cas et al. aimed to represent the Italian panorama of Cannabis oils, which were analysed to determine their cannabinoids content from 2017 to 2019. This study could be useful considering that the Italian law states that, in order to ensure the quality of the oil-based *Cannabis* preparation, the titration of the active substance(s) should be carried out. The quantification can be considered as the initial step for pharmacists to evaluate both the correct execution of preparation procedures and the quality of the extracts (Dei Cas et al.). In this field, also Pigliasco et al. developed their research taking into consideration the importance of developing suitable analytical methods useful to understanding the medicinal effects of *Cannabis*-derived products. Therefore a simple and rapid volumetric absorptive micro-sampling method combined with ultra-high-performance liquid chromatography coupled with mass spectrometry *in tandem* has been developed. This analytical method, which use a minimally invasive micro-sampling technique, could be useful for quantifying CBD, THC and their metabolites of relevant interest in patients with epilepsy treated with *Cannabis*-based preparations (Pigliasco et al.).

Overall, the articles included in the Research Topic offer new insights on mechanisms of action, potential risks and pharmacological properties of the components present in the *Cannabis* phytocomplex, confirming the significant interest clearly emerging in the evident potential of *Cannabis* in the medical field. At the same time, these findings confirm the need to further extend knowledge on the efficacy and safety profile of *Cannabis*-based preparations as well as in the development of suitable analytical methods to be applied in this field.

Author contributions

FB: Conceptualization, Writing–original draft. SB-S: Conceptualization, Writing–original draft. PB: Conceptualization, Writing–original draft. MC: Conceptualization, Writing–original draft.

Conflict of interest

The 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.

Publisher's note

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 13 November 2020
doi: 10.3389/fphar.2020.570616

Phytocannabinoids Profile in Medicinal Cannabis Oils: The Impact of Plant Varieties and Preparation Methods

*Michele Dei Cas[1], Eleonora Casagni[2], Antonella Casiraghi[2], Paola Minghetti[2], Diego Maria Michele Fornasari[3], Francesca Ferri[2], Sebastiano Arnoldi[2], Veniero Gambaro[2] and Gabriella Roda[2]**

[1]Department of Health Sciences, Università Degli Studi di Milano, Milan, Italy, [2]Department of Pharmaceutical Sciences, Università Degli Studi di Milano, Milan, Italy, [3]Department of Medical Biotechnology and Traslational Medicine, Universitá degli Studi di Milano, Milan, Italy

OPEN ACCESS

Edited by:
William Chi-Shing Tai, Hong Kong Polytechnic University, Hong Kong

Reviewed by:
Maurizio Servili, University of Perugia, Italy
Sonia Gomes, University of Trás-os-Montes and Alto Douro, Portugal
Federica Pollastro, University of Eastern Piedmont, Italy
Francesco Paolo Busardò, Sapienza University of Rome, Italy

***Correspondence:**
Gabriella Roda
gabriella.roda@unimi.it

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *10 June 2020*
Accepted: *06 October 2020*
Published: *13 November 2020*

Citation:
Dei Cas M, Casagni E, Casiraghi A, Minghetti P, Fornasari DMM, Ferri F, Arnoldi S, Gambaro V and Roda G (2020) Phytocannabinoids Profile in Medicinal Cannabis Oils: The Impact of Plant Varieties and Preparation Methods. Front. Pharmacol. 11:570616. doi: 10.3389/fphar.2020.570616

Cannabis (*Cannabis sativa* L.) is a highly promising medicinal plant with well-documented effectiveness and growing use in the treatment of various medical conditions. Cannabis oils are mostly used in galenic preparations, due to their easy adjustment of the administration dose, together with the enhanced bioavailability of its active compounds. As stated by the Italian Law (9/11/2015, 279 Official Gazette), "to ensure the quality of the oil-based cannabis preparation, the titration of the active substance(s) should be carried out." This study aims to represent the Italian panorama of cannabis oils, which were analyzed (8,201) to determine their cannabinoids content from 2017 to 2019. After application of the exclusion criteria, 4,774 standardized cannabis oils were included, which belong to different medicinal cannabis varieties and prepared according to different extraction methods. The concentration of the principal cannabinoids was taken into account dividing samples on the basis of the main extraction procedures and cannabis varieties. According to this analysis, the most substantial variations should be attributed to different cannabis varieties rather than to their extraction protocols. This study may be the starting point of preparatory pharmacists to assess the correct implementation of the preparation procedures and the quality of the extracts.

Keywords: cannabinoids, medical cannabis, chemometrics methods, pharmaceutical chemistry, phytochemistry

INTRODUCTION

The therapeutic benefits of cannabis are more and more recognized at the scientific level (Bar-Lev Schleider et al., 2018; Freeman et al., 2019; Levinsohn and Hill, 2020), and regulation have to consider the evolution of its use (Zaami et al., 2018; Corli et al., 2019; Brunetti et al., 2020). There are several listed medical indications in Italy, which should be treated accordingly with different cannabis varieties containing tetrahydrocannabinol (THC), cannabidiol (CBD), or both of them (Law 9/11/2015, 279 Official Gazette; Ministero della Salute, 2017; EMCDA, 2018).

Cannabis with high THC levels (Bedrocan) is used to treat conditions, such as Tourette's syndrome (Black et al., 2019), glaucoma (Novack, 2016; Panahi et al., 2017), and nausea (Schussel et al., 2018). Pain reduction and muscle spasm (Whiting et al., 2015) should be handled with a

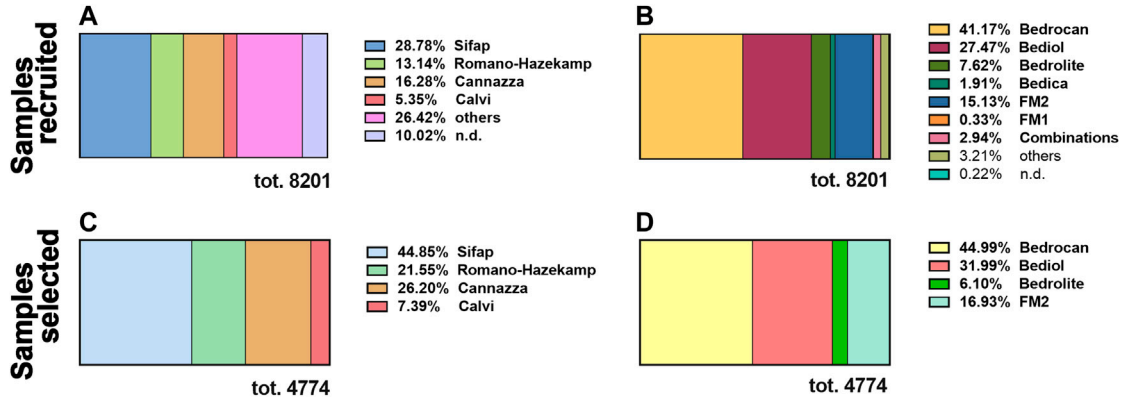

FIGURE 1 | The distribution, between 2017 and 2019, of the total amount of cannabis oil extracts recruited by our laboratory (8,201) by preparation methods **(A)** and varieties of *Cannabis sativa* **(B)**. The distribution of standardized cannabis oil extracts selected for this study (4,774) by preparation methods **(C)** and varieties of *Cannabis sativa* **(D)**. n.d. not determined since those details were not indicated in the sample's addendum. For details on preparation methods, see the following references: Method A (Romano and Hazekamp, 2013), Method B (Citti et al., 2016), Method C (Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018), and Method D (Calvi et al., 2018).

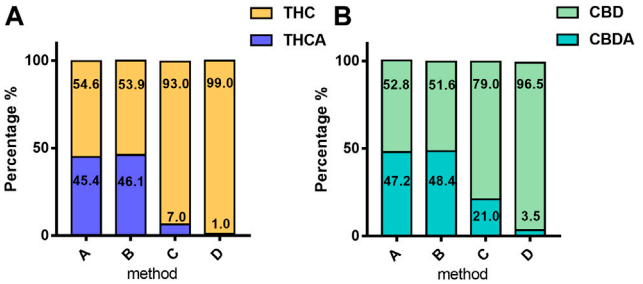

FIGURE 2 | Mean percentage of acidic and neutral form of phytocannabinoids in 4,774 samples according to the extraction method: **(A)** THC and THCA; **(B)** CBD and CBDA. The values are expressed as mean normalized to 100: % acidic form = $[Mean_{acid}/(Mean_{acid} + Mean_{neutral})] \times [100/(Mean_{acid} + Mean_{neutral})]$; % neutral form = $[Mean_{neutral}/(Mean_{acid} + Mean_{neutral})] \times [100/(Mean_{acid} + Mean_{neutral})]$. For details on preparation methods, see the following references: Method A (Romano and Hazekamp, 2013), Method B (Citti et al., 2016), Method C (Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018), and (Method D (Calvi et al., 2018).

combination of THC and CBD, which occur in Bediol. CBD reduces pain, inflammation, and psychoactive side effects of THC (Boyaji et al., 2020). Bedrolite mainly contains CBD and is employed in the treatment of various forms of epilepsy (Rosenberg et al., 2015; Gaston and Friedman, 2017; Brodie and Ben-Menachem, 2018; Office of Medicinal Cannabis, 2019).

Cannabis oil is the preparation form receiving more attention recently (Pacifici et al., 2017; Carcieri et al., 2018; MacCallum and Russo, 2018; Pacifici et al., 2018; Bettiol et al., 2019; Deidda et al., 2019; Mudge and Brown, 2019; Pacifici et al., 2019; Pegoraro et al., 2019) due to its easy adjustment of the needed individual administration dose along the treatment period, together with the enhanced bioavailability of its active compounds.

As stated by the Italian Law (9/11/2015, 279 Official Gazette) "to ensure the quality of the oil-based cannabis preparation, the titration of the active substance(s) should be carried out with sensitive and specific methodologies, such as liquid or gas chromatography coupled with the mass spectrometry and the extraction method must be authorized in accordance with of the legislation in force (Law 9/11/2015, 279 Official Gazette).

In this framework, considering the activity of our laboratory in the field of drugs of abuse in particular cannabis derivatives, synthetic cannabinoids and cathinones (Valoti et al., 2012; Cannizzaro et al., 2016) we were interested in studying the Italian panorama of cannabis oils (n. 8201 samples from 2017 to 2019), which were analyzed by our laboratory to determine their cannabinoids content. These oil samples belonging to different cannabis varieties, here intended as chemotypes (Dei Cas et al., 2020), principally contain THC (chemotype I: Bedrocan) or CBD (chemotype III: Bedrolite) or both of them (chemotype II: FM2 and Bediol). Italian pharmacists prepared them according to different extraction methods present in the scientific literature [Romano and Hazekamp, 2013; Citti et al., 2016; Società Italiana Farmacisti Preparatori (SIFAP), 2016; Calvi et al., 2018; Casiraghi et al., 2018]. The crucial step in the preparation method is the decarboxylation to transform THCA and CBDA, present in the plant material, in the corresponding neutral forms THC and CBD. The need for optimizing and standardizing decarboxylation procedures is dictated by pharmacological reasons because the acidic and neutral cannabinoids have different pharmacodynamic and pharmacokinetic properties that will influence the pharmacological profile of the final product, according to the relative amount of the two compounds. A striking pharmacokinetic difference between THCA and THC concerns the passage through the blood–brain barrier (BBB). As THCA is a substrate of P-glycoprotein (P-gp/abcb1) and breast cancer resistance protein (Bcrp/abcg2), its penetration into the CNS is limited (Spiro et al., 2012). Both abcb1 and abcg2 belong to the ATP-binding cassette (ABC) family of efflux transporters and are critical to BBB function, where they impede the passage of their

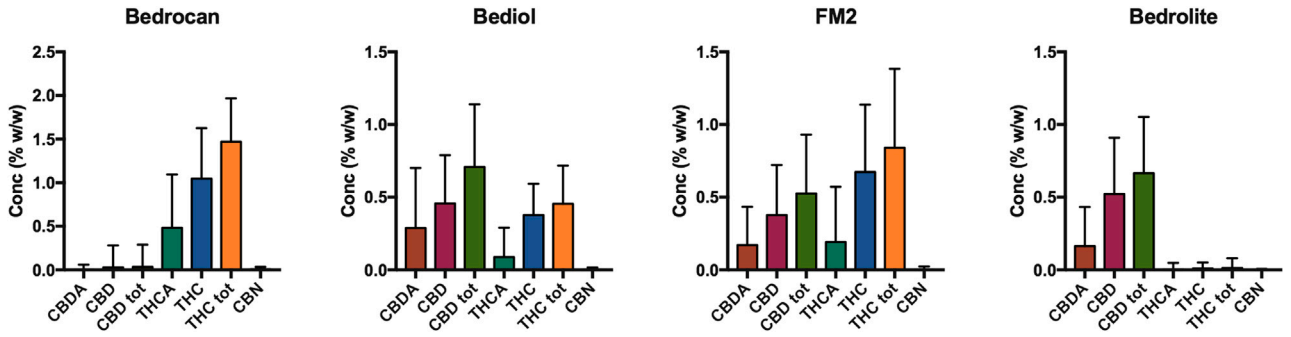

FIGURE 3 | Distribution of phytocannabinoids among *Cannabis sativa* varieties (4,774, mean ± SD).

substrates into the brain (Agarwal and Elmquist, 2012). Thus, the pharmacological activity of THCA would mainly rely on peripheral effects, as already suggested by the lack of psychoactive properties. This is not in contrast with the supposed antiemetic properties of THCA because some peripheral mechanisms of cannabinoids have been described. However, other proposed pharmacological effects of THCA, strictly related to central activities, such as muscle relaxation, should be reconsidered or refused (Russo, 2018).

The authors would like to highlight possible relationships among cannabis varieties, the effects of the extraction method, and the cannabinoids profile to better understand pharmacological activity of cannabis oils in clinical trials, as a function of oil composition, because very little information in the literature is reported about them. Moreover, it could be helpful for pharmacists, involved in the preparation of these medicines, to check the quality of their preparations. In fact, due to a lack of a single and standard preparation procedure, pharmacists very often ask for preprocessed cannabinoids concentrations to deal with.

MATERIALS AND METHODS

Chemicals and Reagents

Methanol (MeOH), toluene, O, N-bis (trimethylsilyl) trifluoroacetamidetrimethylchlorosiloxane (BSTFA-1% TMCS), methyl oleate (99% purity), THC 1 mg/ml in MeOH (purity ≥ 95.0%), CBD 1 mg/ml in MeOH (purity ≥ 95.0%), and CBN 1 mg/ml in MeOH (purity ≥ 95.0%) were purchased from Sigma-Aldrich. The acidic forms of cannabinoids, such as THCA 1 mg/ml

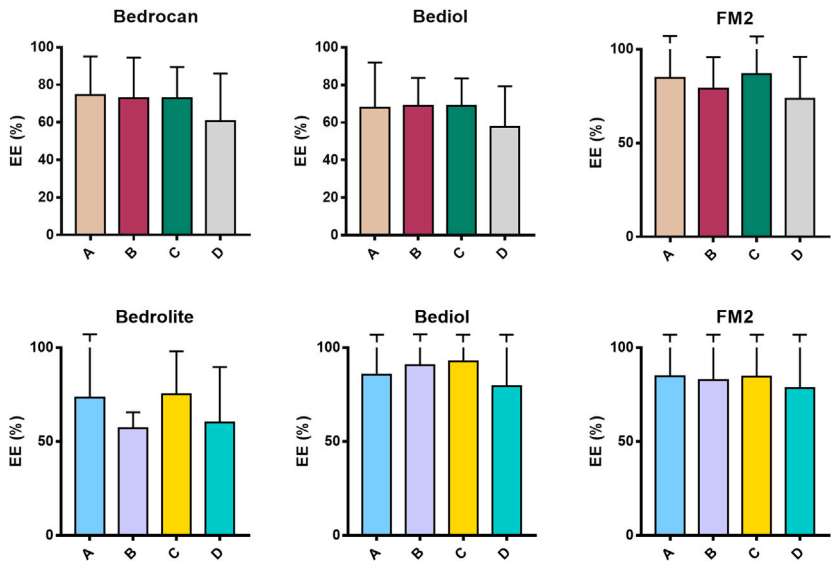

FIGURE 4 | Extraction efficiency (EE%) of THC (**up**) and CBD (**down**) measured in cannabis oil samples (4,774) obtained using different cannabis varieties and preparation methods. The error bars that exceed the axis limit are represented as clipped. The theoretical extraction rate was set as the mean of the declared range content as follows: Bedrocan THC 2.05 (% w/w); Bediol THC 0.65 (% w/w), CBD 0.75 (% w/w); FM2 THC 0.65 (% w/w); CBD 1.05 (% w/w); and Bedrolite CBD 0.85 (% w/w). For details on preparation methods, see the following references: Method A (Romano and Hazekamp, 2013), Method B (Citti et al., 2016), Method C [Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018)], and Method D (Calvi et al., 2018). The values are expressed as mean ± SD and calculated according to the equation EE% = (conc. Exp/conc. Theo) × 100.

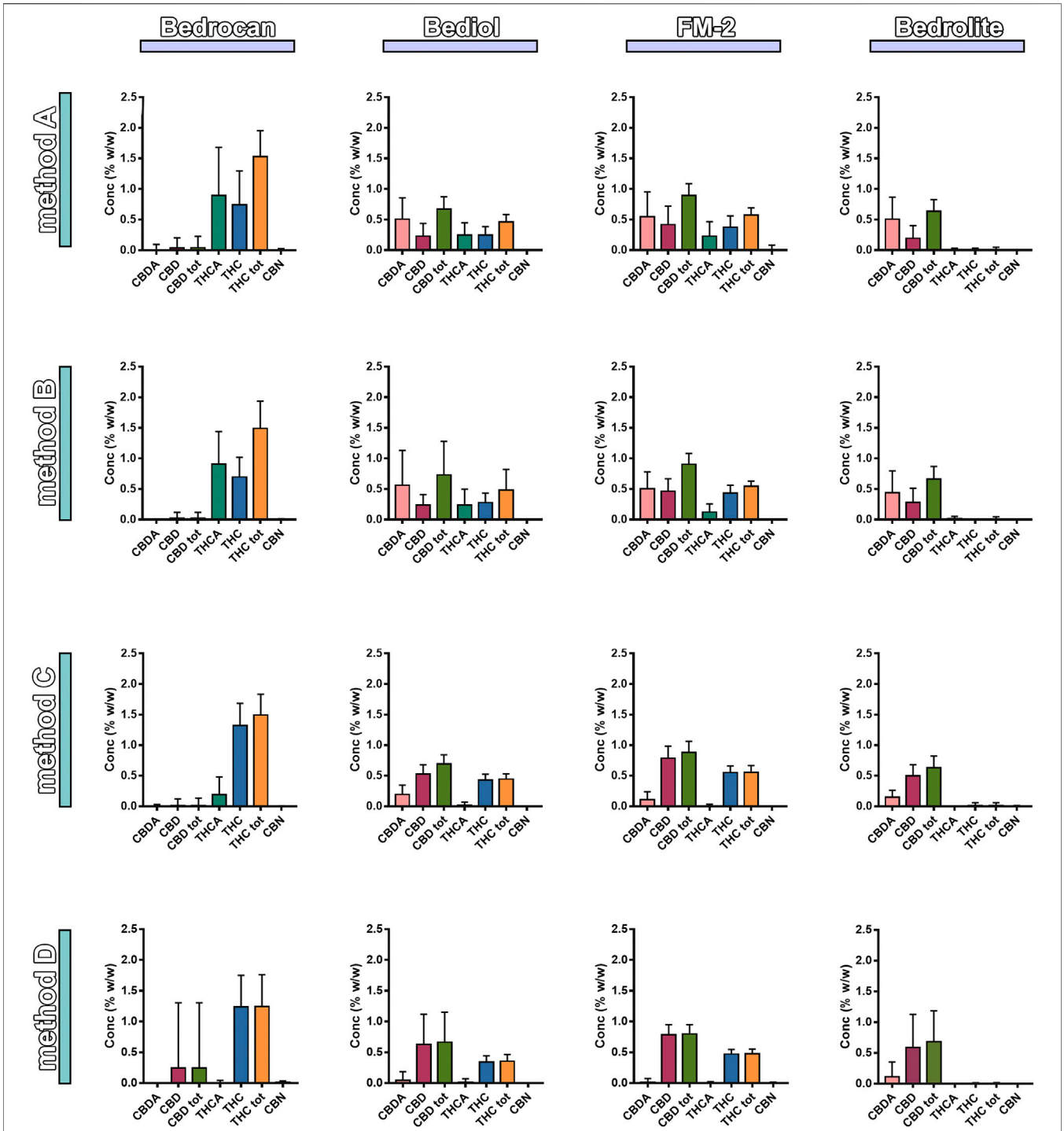

FIGURE 5 | Distribution of phytocannabinoids among extraction methods from plant materials and varieties (4,774, mean ± SD). The columns represented the cannabis sativa varieties (sx to dx) Bedrocan, Bediol, FM2, and Bedrolite and the rows the Method of extraction (up to down) [Method A (Romano and Hazekamp, 2013), Method B (Citti et al., 2016), Method C (Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018), and Method D (Calvi et al., 2018)].

in acetonitrile (purity ≥ 95.0%) and CBDA 1 mg/ml in acetonitrile (purity ≥ 95.0%), were obtained from Cayman Chemical Company.

Galenic Preparations

Cannabis oil galenic preparations were delivered for cannabinoids determination to our laboratory between

TABLE 1 | Cannabinoids concentrations, expressed as both mean ± SD and 25–75th percentile range, as a function of preparation methods and varieties.

Cannabis products	n	THC tot (% w/w)		CBD tot (% w/w)	
		Mean ± SD	Range (25–75th)	Mean ± SD	Range (25–75th)
Bedrocan	2,148	1.47 ± 0.466	1.30–1.68	0.41 ± 0.313	—
Method A	515	1.53 ± 0.425	1.34–1.74	0.04 ± 0.185	—
Method B	682	1.49 ± 0.445	1.33–1.68	0.02 ± 0.096	—
Method C	800	1.49 ± 0.340	1.32–1.66	0.01 ± 0.119	—
Method D	151	1.24 ± 0.519	1.15–1.44	0.07 ± 0.544	—
Bedrolite	291	0.01 ± 0.091	—	0.66 ± 0.351	0.49–0.71
Method A	62	0.01 ± 0.036	—	0.64 ± 0.189	0.55–0.70
Method B	25	0.01 ± 0.034	—	0.66 ± 0.202	0.59–0.73
Method C	151	0.01 ± 0.045	—	0.63 ± 0.191	0.54–0.70
Method D	53	0.01 ± 0.011	—	0.68 ± 0.502	0.41–0.68
Bediol	1,527	0.45 ± 0.262	0.40–0.50	0.70 ± 0.445	0.60–0.76
Method A	253	0.46 ± 0.122	0.40–0.51	0.67 ± 0.203	0.58–0.75
Method B	350	0.48 ± 0.338	0.42–0.50	0.73 ± 0.552	0.64–0.74
Method C	838	0.44 ± 0.087	0.41–0.49	0.69 ± 0.149	0.62–0.79
Method D	86	0.35 ± 0.112	0.29–0.40	0.67 ± 0.486	0.46–0.64
FM-2	808	0.54 ± 0.120	0.47–0.63	0.89 ± 0.294	0.76–1.01
Method A	199	0.57 ± 0.118	0.50–0.65	0.89 ± 0.192	0.78–1.03
Method B	194	0.54 ± 0.085	0.51–0.60	0.91 ± 0.176	0.79–1.00
Method C	352	0.56 ± 0.111	0.49–0.63	0.88 ± 0.183	0.75–1.02
Method D	63	0.47 ± 0.077	0.42–0.52	0.80 ± 0.151	0.72–0.89

For details on preparation methods see the following references: Romano-Hazekamp [Method A (Romano and Hazekamp, 2013)], Cannazza [Method B (Citti et al., 2016)], Sifap [Method C (Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018)], and Calvi [Method D (Calvi et al., 2018)].

2017 and 2019 and account for 8,201 samples. However, after the initial data collection and laboratory analysis, samples were excluded on the basis of 1) the absence, in the detailed sheet, of pharmaceutical-grade *Cannabis sativa* varieties; 2) the use of pharmaceutical-grade *Cannabis sativa* varieties diverse from Bedrocan, Bediol, Bedrolite, and FM2; and 3) a nonstandardized preparation method. Consequently, this study was limited to 4,774 samples standardized for both pharmaceutical-grade cannabis varieties and the extraction methods. Preparation methods are mainly based on maceration of vegetable materials in olive oil at high temperature, at about 100°C or more [Methods A (Romano and Hazekamp, 2013) and B (Citti et al., 2016)]. Both of them do not require a preliminary decarboxylation of the vegetal matrix. A preliminary decarboxylation step is performed with Method C [Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018] or Method D (Calvi et al., 2018). All these methods were used by pharmacists, based on medical prescriptions, to obtained cannabis oils by different varieties of medicinal grade plant material: the Dutch Bedrocan, Bediol, Bedrolite, and the Italian FM2. After decarboxylation, where planned, the cannabis decoctions in oil were mainly carried out with a weight-to-volume ratio between plant material and oil of 1: 10 (usually 5 g in 50 ml) (Baratta et al., 2019). Mainly, pharmacopeia grade olive oil, usually virgin or refined according to the European Pharmacopoeia (Ph. Eur.), was used as extraction solvent. This oil can minimize the formation of large amounts of aldehydes and ketones that can also influence the digestibility of the macerated oil (Pavlovic et al., 2018).

Analytical Samples Preparation From Cannabis Oils

Cannabis oil preparation (50 mg weighted) was added to 5 ml of methanol. The mixture was extracted by vortex and centrifuged (1789 × *g*, 5 min). Then, 50 µl of the supernatant was withdrawn and added with 50 µl of the internal standard solution (methyl oleate, 175 µg/ml in MeOH). The solvent was evaporated, then 50 µl of BSTFA-1% TMCS and 50 µl of toluene were added. The mixture was mixed and heated at 70°C for 30 min, to allow the derivatization.

Analysis of Cannabinoids by GC/MS

The analyses were performed on a 5973 Hewlett Packard GC system, with a split–splitless injection system and an MS detector (Hewlett Packard) operated in the electron ionization (EI) mode (70 eV), as already described elsewhere (Casiraghi et al., 2018). Briefly, the GC was equipped with a capillary column Rxi-5ms (30 m × 0.25 mm, i.d. 0.25 mm, Restek). The GC/MS conditions were as follows: helium was used as the carrier gas at a flow rate of 1.2 ml/min, splitless mode (0.25 min); injector temperature 280°C; interface transfer line 300°C; ion source 230°C; and oven temperature program: initial 70°C, 40°C/min up to 180°C, then 10°C/min up to 300°C (6.25 min). The total analysis time was 21 min. The MS detector was operated in selected ion monitoring (SIM) acquiring characteristic ions in prefixed temporal windows each corresponding to a peculiar cannabinoid: IS methyl oleate at 8.5 min (264 m/z); CBD-2TMS at 9.7 min (390 m/z); THC-1TMS at 10.7 min (386 m/z); CBN-1TMS at 11.4 min (367 m/z); CBDA-3TMS at 11.7 min (491 m/z); and THCA-2TMS at 12.9 min (487 m/z). Throughout this article, the concentrations of phytocannabinoids were expressed as

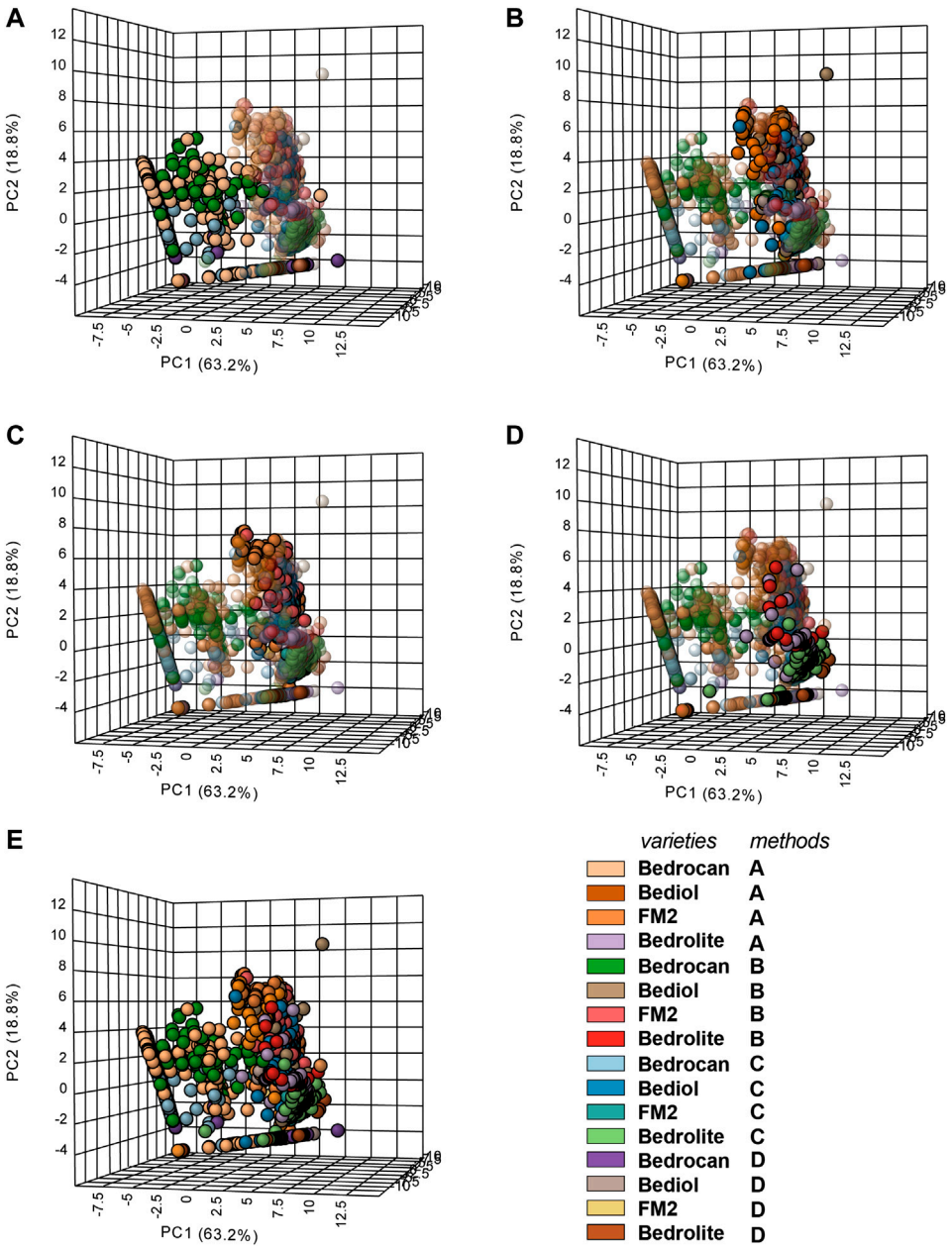

FIGURE 6 | 3D Principal component analysis (PCA) plot of cannabis oil extracts divided into groups according to the plant varieties and extraction method (4,774). In the panel, the plant varieties are evidenced, whereas the extraction adopted was color coded (according to the legend). In the panel, **(A)** Bedrocan, **(B)** Bediol, **(C)** FM2, **(D)** Bedrolite, and **(E)** the entire data set overview are evidenced. For details on preparation methods, see the following references: Method A (Romano and Hazekamp, 2013), Method B (Citti et al., 2016), Method C [Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018], and Method D (Calvi et al., 2018).

percentage weight per weight (% w/w, weight of cannabinoid/weight of oil preparation).

Statistical Analysis

Descriptive statistics was investigated using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). In order to find out potential discriminating features between the groups, a series of univariate and multivariate analyses were performed using the software MetaboAnalyst 4.0. The groups were designed considering cannabis varieties (Bedrocan, Bediol, FM2, and Bedrolite) and the extraction protocol [Methods A (Romano and Hazekamp, 2013), B (Citti et al., 2016), C (Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018), and D (Calvi et al., 2018)]. Data were checked for integrity, filtered by interquartile range, log-transformed (generalized log transformation), and mean centered. PCA and hierarchical

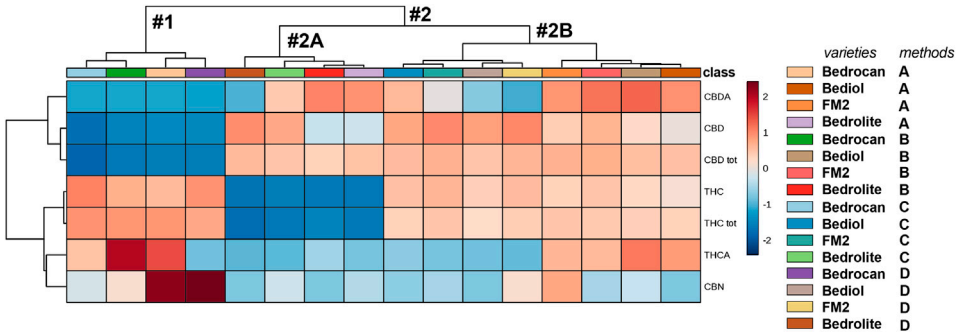

FIGURE 7 | A heatmap overview (showing only group average) with hierarchical clustering of the 4,774 cannabis oils. The first cluster (#1) included Bedrocan variety and the second one (#2) the other varieties, which in particular consisted of (#2A) Bedrolite and (#2B) Bediol and FM2. In respect to other varieties, Bedrocan displayed a lower concentration of CBD (tot, neutral, and acid) and Bedrolite of THC (tot and neutral). The color-scale differentiates values as high (red), mid (gray), and low (blue). For details on preparation methods, see the following references: Method A (Romano and Hazekamp, 2013), Method B (Citti et al., 2016), Method C [Società Italiana Farmacisti Preparatori (SIFAP), 2016; Casiraghi et al., 2018], and Method D (Calvi et al., 2018).

clustering with heatmap were used for considering all variables in the data set simultaneously. In the heatmap analysis, the clustering algorithm was set to Ward, and the distance measure to Euclidean. VIP scores, resulting from the supervised PLS-DA analysis, were used as a cutoff (>1) to include variables with discriminatory power. Further investigations were completed by ANOVA coupled to post hoc Fisher's LSD test to highlight the significant variables with a threshold p-value of <0.05.

RESULTS

From 2017 to 2019, 8,201 samples of cannabis olive oils were delivered to our laboratory for cannabinoid level determination. Samples were time-distributed as follows: in 2017, 1,349 (16.5%), in 2018, 2,281 (27.8%), and in 2019, 4,571 (55.7%). Cannabis oils were divided by preparation methods (**Figure 1A**) and varieties of *Cannabis sativa* (**Figure 1B**).

The most used maceration technique for the oil extraction of cannabinoids was Method C (28.8%), followed by Method B (16.3%) and Method A (13.1%). The more prevalent medical cannabis chemotypes comprised Bedrocan (41.2%), Bediol (27.4%), and the Italian FM2 (15.1%).

All the further statistical analysis were restricted only to a well-characterized subpopulation made of 4,774 (58% of the entire population of 8,201) excluding samples (42%, 3,457) that were not accompanied by a detailed sheet or are not standardized as regard cannabis varieties and method preparation. In the same way, the selected population was divided by preparation methods (**Figure 1C**) and varieties of *Cannabis sativa* (**Figure 1D**). The subpopulation sampled maintains the same distribution of the preparation methods and plant varieties with respect to the total.

The main differences in the cannabinoid profile are due to the decarboxylation step and especially to the heating time and temperature applied. These differences are directly related to the percentage of acidic forms (**Figure 2**) of cannabinoids.

These forms, at high temperatures, are subjected to decarboxylation to respective neutral forms. Methods A and B showed a higher content of the acidic forms compared with the neutral ones from 90 to 50% of the total content of cannabinoids (THC + THCA; CBD + CBDA). In particular, the extraction without a decarboxylation step (Method A: 98°C for 1 h and Method B 110°C for 2 h) leads to a highly variable ratio of acidic/neutral cannabinoids, thus reducing the reproducibility of the extraction procedure.

On the contrary, Methods C and D described a decarboxylation step (respectively, in the oven at 115°C for 40 min and 145°C for 30 min) before oil maceration with a full conversion of the acidic to neutral forms. Then, in Method C, the decarboxylated cannabis is extracted in oil heated by means of a water bath (100°C for 40 min), whereas in method D the extraction is carried out by ultrasound (35 kHz 30 min). In Method C, neutral forms of both THC and CBD were prevalently valued at 93% and 79%, respectively. Moreover, in Method D, the neutral forms covered almost the totality of the cannabinoids, THC 99%, and CBD 96.5%.

The distribution of phytocannabinoids among varieties (**Figure 3**) was further investigated. The detailed samples list separated by varieties and processing methods can be found in the **Supplementary Tables S1–S4**. Bedrocan displayed the highest content of total THC (mean ± SD, 1.47 ± 0.47), followed by FM2 (0.54 ± 0.12) and Bediol (0.45 ± 0.26), whereas Bedrolite, as expected, showed very low amounts of this cannabinoid (0.01 ± 0.09). The situation was the opposite when considering total CBD, in which the highest content was found in FM2 (0.89 ± 0.30), followed by Bediol (0.70 ± 0.45) and Bedrolite (0.66 ± 0.35). Bedrocan displayed, as expected, a slight concentration of CBD (0.04 ± 0.31).

In the different cannabis varieties, the total amount of THC and CBD (**Supplementary Table S5**) are similar to those declared in the literature (Uso medico della cannabis - Ministero della Sanità, 2016; Office of Medicinal Cannabis, 2019) and in labeled content. Some samples deviated from the expected values due to

the variability in both the not strictly standardized preparation protocols and the employed plant matrix.

Samples were also analyzed taking into consideration the efficiency of extraction of total THC and CBD depending on varieties and the preparation method (**Figure 4** and **Supplementary Table S6**). Among all samples analyzed, a reduced number of results showed coherence among the preparation method and declared content of cannabinoids. As result, the extraction efficiency (EE%) ranges (min–max) were from 57.6 to 86.3 for THC and from 57.1 to 92.8% for CBD. **Figure 5** and **Table 1** illustrate the concentration of cannabinoids within main cannabis oil varieties (columns) processed with the most common methods (rows). Being confirmed that the total extracted content of THC and CBD is not significantly different with respect to the extraction method, it is interesting to note that, on the contrary, the relative content of the acidic or neutral form is strictly related to preparation method condition. Samples prepared according to Methods C and D showed a high level of neutral active THC form, whereas methods A and B results were in favor of THCA. The relative content of the two forms is essential for the expected pharmacological effect.

Multivariate analysis (**Figures 6** and **Supplementary Figure S1**) showed only an appreciable separation between Bedrocan and other varieties, Bediol, Bedrolite, and FM2, which were not well detached among them.

The same conclusion can be found in **Figure 7**, which shows a heatmap coupled to hierarchical clustering, in which the cannabinoids profile is graphed against plant varieties and extraction protocol. The map is color coded to three concentration levels (blue = low, gray = middle, and red = high range). Hierarchical clustering is a frequently used method to identify similarities or differences between each individual. We noted the presence of two different and well-divided clusters, represented as dendrogram: one including Bedrocan variety and the second one included other varieties. The latter consisted of two other clusters: Bedrolite and Bediol + FM2. In respect to other varieties, Bedrocan displayed a lower concentration of CBD (tot, neutral, and acid) along with a higher concentration of THCA and CBN, whereas Bedrolite presented a weaker concentration of THC (total and neutral). As clearly demonstrated (**Figures 6**, **7** and **Supplementary Figure S1**), the formation of subgroups within the data set can only be done based on the variety of cannabis inflorescence and not by the extraction methods. PCA is not always able to properly separate the variations produced by each factor, and the results can be somehow problematic to read. In order to avoid this scenario, univariate and supervised statistical tests were also performed. The use of a more conservative method (ANOVA, *post hoc* Fisher's LSD) demonstrated that all the considered cannabinoids should be capable ($p < 0.05$) of discriminating against groups. THC, which showed a VIP score of 1.71 and a p value <0.05, was therefore proposed as the best phytocannabinoid able to discriminate between cannabis oils extracted by different methods and coming from different varieties (**Supplementary Figure S2**). However, as mentioned above, the most substantial variations should be attributed to the different cannabis varieties rather than to their extraction protocols. Further considering the extraction method results, different amplitudes of variability can be observed: higher values were reported in Methods A and B with respect to Methods C and D. The more strictly standardized preparation protocols of the latest are therefore useful.

DISCUSSION

Medical cannabis has been effectively used for treating symptoms from a variety of disorders. Commonly, it is prescribed when first-choice treatments and medicines are not effective enough or have severe side effects. Despite the growing popularity of cannabis-based medicinal oils (Pacifici et al., 2017; Carcieri et al., 2018; Pacifici et al., 2018; Bettiol et al., 2019; Deidda et al., 2019; Mudge and Brown, 2019; Pacifici et al., 2019; Pegoraro et al., 2019), at the moment, there are no studies in which the cannabinoid composition has been strictly defined considering the variety of the plant and the extraction method. However, a notable contribution in this research field comes from the National Institute of Health in Italy, who was involved in the determination of long-term stability of cannabinoids in standardized cannabis oils to assure their quality and therapeutic properties (Pacifici et al., 2017; Pacifici et al., 2018; Pacifici et al., 2019). The relevance of these studies lies in ensuring a conscious prescription by the physicians, who should take into consideration both the composition and stability of cannabis oils. Nevertheless, from a pharmacological point of view, the composition of the final product in THCA and THC content is critical, being the THCA activity mainly based on peripheral effects and, therefore, much less impressive in the majority of situations. Our results stated that cannabinoid content are significantly linked to cannabis varieties (i.e., Bedrocan, Bedrolite, Bediol, and FM2), among which pharmacists and physicians can choose the most suitable. Moreover, there is a clear trend in cannabinoid content with respect to the preparation methods. It is interesting to note that total THC and CBD extracted amounts were in the same range, whereas those methods with the preliminary decarboxylation step (Method C and D) allowed obtaining oils richer in the active neutral form. For these reasons, this study may be the starting point for compounded oils in pharmacies to assess the correct implementation of the preparation procedures and the quality of the extracts. However, there are still many aspects to be improved, including the standardization of raw inflorescences and oil extraction procedures.

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

Conceptualization: MDC and GR. Investigation: FF, SA, and EC. Formal analysis and drafting of the manuscript: MDC. Supervision: GR, VG, and PM. Writing—review and editing: EC, AC, PM, DF, and GR. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGMENTS

MDC was supported by the PhD program in Molecular and Translational Medicine of the Università Degli Studi di Milano, Milan.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.570616/full#supplementary-material

REFERENCES

Agarwal, S., and Elmquist, W. F. (2012). Insight into the cooperation of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) at the blood-brain barrier: a case study examining sorafenib efflux clearance. *Mol. Pharm.* 9, 678–684. doi:10.1021/mp200465c

Bar-Lev Schleider, L., Abuhasira, R., and Novack, V. (2018). Medical cannabis: aligning use to evidence-based medicine approach. *Br. J. Clin. Pharmacol.* 84, 2458–2462. doi:10.1111/bcp.13657

Baratta, F., Simiele, M., Pignata, I., Enri, L. R., Torta, R., De Luca, A., et al. (2019). Development of standard operating protocols for the optimization of cannabis-based formulations for medical purposes. *Front. Pharmacol.* 10, 1–10. doi:10.3389/fphar.2019.00701

Bettiol, A., Lombardi, N., Crescioli, G., Maggini, V., Gallo, E., Mugelli, A., et al. (2019). Galenic preparations of therapeutic *Cannabis sativa* differ in cannabinoids concentration: a quantitative analysis of variability and possible clinical implications. *Front. Pharmacol.* [Epub ahead of print]. doi:10.3389/fphar.2018.01543

Black, N., Stockings, E., Campbell, G., Tran, L. T., Zagic, D., Hall, W. D., et al. (2019). Cannabinoids for the treatment of mental disorders and symptoms of mental disorders: a systematic review and meta-analysis. *Lancet Psychiatry* 6, 995–1010. doi:10.1016/S2215-0366(19)30401-8

Boyaji, S., Merkow, J., Elman, R. N. M., Kaye, A. D., Yong, R. J., and Urman, R. D. (2020). The role of cannabidiol (CBD) in chronic pain management: an assessment of current evidence. *Curr. Pain Headache Rep.* 24, 2–7. doi:10.1007/s11916-020-0835-4

Brodie, M. J., and Ben-Menachem, E. (2018). Cannabinoids for epilepsy: what do we know and where do we go? *Epilepsia* 59, 291–296. doi:10.1111/epi.13973

Brunetti, P., Pichini, S., Pacifici, R., Busardò, F. P., and del Rio, A. (2020). Herbal preparations of medical cannabis: a vademecum for prescribing doctors. *Med. Plus* 56, 237. doi:10.3390/medicina56050237

Calvi, L., Pentimalli, D., Panseri, S., Giupponi, L., Gelmini, F., Beretta, G., et al. (2018). Comprehensive quality evaluation of medical *Cannabis sativa* L. inflorescence and macerated oils based on HS-SPME coupled to GC-MS and LC-HRMS (q-exactive orbitrap®) approach. *J. Pharmaceut. Biomed. Anal.* [Epub ahead of print]. doi:10.1016/j.jpba.2017.11.073

Cannizzaro, C., Malta, G., Argo, A., Brancato, A., Roda, G., Casagni, E., et al. (2016). Behavioural and pharmacological characterization of a novel cannabinomimetic adamantane-derived indole, APICA, and considerations on the possible misuse as a psychotropic spice abuse, in C57bl/6J mice. *Forensic. Sci. Int.* 265, 6–12. doi:10.1016/j.forsciint.2015.12.035

Carcieri, C., Tomasello, C., Simiele, M., De Nicolò, A., Avataneo, V., Canzoneri, L., et al. (2018). Cannabinoids concentration variability in cannabis olive oil galenic preparations. *J. Pharm. Pharmacol.* 70, 143–149. doi:10.1111/jphp.12845

Casiraghi, A., Roda, G., Casagni, E., Cristina, C., Musazzi, U. M., Franzè, S., et al. (2018). Extraction method and analysis of cannabinoids in cannabis olive oil preparations. *Planta Med.* 84 (04), 242–249. doi:10.1055/s-0043-123074

Citti, C., Ciccarella, G., Braghiroli, D., Parenti, C., Vandelli, M. A., and Cannazza, G. (2016). Medicinal cannabis: principal cannabinoids concentration and their stability evaluated by a high performance liquid chromatography coupled to diode array and quadrupole time of flight mass spectrometry method. *J. Pharmaceut. Biomed. Anal.* 128, 201–209. doi:10.1016/j.jpba.2016.05.033

Corli, O., Davoli, E., Medana, C., Garattini, S., Banzi, R., Cervo, L., et al. (2019). Cannabis as a medicine. An update of the Italian reality. *Eur. J. Intern. Med.* 60, e9–e10. doi:10.1016/j.ejim.2018.10.021

Dei Cas, M., Casagni, E., Saccardo, A., Arnoldi, S., Young, C., Scotti, S., et al. (2020). The Italian panorama of cannabis light preparation: determination of cannabinoids by LC-UV. *Forensic Sci. Int.* 307, 110113. doi:10.1016/j.forsciint.2019.110113

Deidda, R., Avohou, H. T., Baronti, R., Davolio, P. L., Pasquini, B., Del Bubba, M., et al. (2019). Analytical quality by design: development and control strategy for a LC method to evaluate the cannabinoids content in cannabis olive oil extracts. *J. Pharmaceut. Biomed. Anal.* 166, 326–335. doi:10.1016/j.jpba.2019.01.032

EMCDA (2018). Medical use of cannabis and cannabinoids: questions and answers for policymaking. Available at: http://www.emcdda.europa.eu/system/files/publications/10171/20185584_TD0618186ENN_PDF.pdf (Accessed May 2020)

Freeman, T. P., Hindocha, C., Green, S. F., and Bloomfield, M. A. P. (2019). Medicinal use of cannabis based products and cannabinoids. *BMJ* 365, 1–7. doi:10.1136/bmj.l1141

Gaston, T. E., and Friedman, D. (2017). Pharmacology of cannabinoids in the treatment of epilepsy. *Epilepsy Behav.* 70, 313–318. doi:10.1016/j.yebeh.2016.11.016

Law 9/11/2015, n. 279 Official Gazette 2015, 16. Available at: https://www.gazzettaufficiale.it/eli/gu/2015/11/30/279/sg/pdf

Levinsohn, E. A., and Hill, K. P. (2020). Clinical uses of cannabis and cannabinoids in the United States. *J. Neurol. Sci.* 411, 116717. doi:10.1016/j.jns.2020.116717

MacCallum, C. A., and Russo, E. B. (2018). Practical considerations in medical cannabis administration and dosing. *Eur. J. Intern. Med.* 49, 12–19. doi:10.1016/j.ejim.2018.01.004

Ministero della Salute (2017). Raccomandazioni per i medico prescrittore di sostanza vegetale cannabis FM2 inflorescenze - Ministero della Salute. Available at: http://www.salute.gov.it/portale/temi/p2_6.jsp?lingua=italiano&id=4589&area=sostanzeStupefacenti&menu=organismo (Accessed May 2020)

Mudge, E. M., and Brown, P. N. (2019). Determination of cannabinoids in cannabis sativa dried flowers and oils by LC-UV: single-laboratory validation, first action 2018.10. *J. AOAC Int.* 103 (2), 489–493. doi:10.5740/jaoacint.19-0197

Novack, G. D. (2016). Cannabinoids for treatment of glaucoma. *Curr. Opin. Ophthalmol.* 27, 146–150. doi:10.1097/ICU.0000000000000242

Office of Medicinal Cannabis (2019). Documents for healthcare professional - Ministry of Health, Welfare and Sports, The Netherlands. Available at: https://english.cannabisbureau.nl/doctor-and-pharmacists/documents (Accessed May 2020)

Pacifici, R., Marchei, E., Salvatore, F., Guandalini, L., Busardò, F. P., and Pichini, S. (2017). Evaluation of cannabinoids concentration and stability in standardized preparations of cannabis tea and cannabis oil by ultra-high performance liquid chromatography tandem mass spectrometry. *Clin. Chem. Lab. Med.* 55, 1555–1563. doi:10.1515/cclm-2016-1060

Pacifici, R., Marchei, E., Salvatore, F., Guandalini, L., Busardò, F. P., and Pichini, S. (2018). Evaluation of long-term stability of cannabinoids in standardized preparations of cannabis flowering tops and cannabis oil by ultra-high-

performance liquid chromatography tandem mass spectrometry. *Clin. Chem. Lab. Med.* 56, e94–e96. doi:10.1515/cclm-2017-0758

Pacifici, R., Marchei, E., Salvatore, F., Guandalini, L., Busardò, F. P., and Pichini, S. (2019). Stability of cannabinoids in cannabis FM1 flowering tops and oil preparation evaluated by ultra-high performance liquid chromatography tandem mass spectrometry. *Clin. Chem. Lab. Med.* 57, E165–E168. doi:10.1515/cclm-2018-1145

Panahi, Y., Manayi, A., Nikan, M., and Vazirian, M. (2017). The arguments for and against cannabinoids application in glaucomatous retinopathy. *Biomed. Pharmacother.* 86, 620–627. doi:10.1016/j.biopha.2016.11.106

Pavlovic, R., Nenna, G., Calvi, L., Panseri, S., Borgonovo, G., Giupponi, L., et al. (2018). Quality traits of "cannabidiol oils": cannabinoids content, terpene fingerprint and oxidation stability of European commercially available preparations. *Molecules* 23, 1–22. doi:10.3390/molecules23051230

Pegoraro, C. N., Nutter, D., Thevenon, M., and Ramirez, C. L. (2019). Chemical profiles of cannabis sativa medicinal oil using different extraction and concentration methods. *Nat. Prod. Res.*, 1–4. doi:10.1080/14786419.2019.1663515

Romano, L. L., and Hazekamp, A. (2013). Cannabis oil: chemical evaluation of an upcoming cannabis-based medicine. *Cannabinoids*. 1 (1), 1–11.

Rosenberg, E. C., Tsien, R. W., Whalley, B. J., and Devinsky, O. (2015). Cannabinoids and epilepsy. *Neurotherapeutics* 12, 747–768. doi:10.1007/s13311-015-0375-5

Russo, E. B. (2018). Cannabis therapeutics and the future of neurology. *Front. Integr. Neurosci.* 12, 1–11. doi:10.3389/fnint.2018.00051

Schussel, V., Kenzo, L., Santos, A., Bueno, J., Yoshimura, E., de Oliveira Cruz Latorraca, C., et al. (2018). Cannabinoids for nausea and vomiting related to chemotherapy: overview of systematic reviews. *Phyther. Res.* 32, 567–576. doi:10.1002/ptr.5975

Società Italiana Farmacisti Preparatori (SIFAP) (2016). Estratto oleoso di infiorescenze femminili di Cannabis. Available at: https://www.sifap.org/procedure/estrazione-oleosa-di-infiorescenze-femminili-di-cannabi (Accessed May 2020)

Spiro, A. S., Wong, A., Boucher, A. A., and Arnold, J. C. (2012). Enhanced brain disposition and effects of Δ 9-tetrahydrocannabinol in P-glycoprotein and breast cancer resistance protein knockout mice. *PLoS One* 7, 3–8. doi:10.1371/journal.pone.0035937

Uso medico della cannabis - Ministero della Sanità (2016). Available at: http://www.salute.gov.it/portale/temi/p2_6.jsp?lingua=italiano&id=4587&area=sostanzeStupefacenti&menu=organismo (Accessed May 2020).

Valoti, E., Casagni, E., Dell'Acqua, L., Pallavicini, M., Roda, G., Rusconi, C., et al. (2012). Identification of 1-butyl-3-(1-(4-methyl)naphtoyl)indole detected for the first time in "herbal high" products on the Italian market. *Forensic. Sci. Int.* 223, 42–46. doi:10.1016/j.forsciint.2012.08.009

Whiting, P. F., Wolff, R. F., Deshpande, S., Di Nisio, M., Duffy, S., Hernandez, A. V., et al. (2015). Cannabinoids for medical use: a systematic review and meta-analysis. *J. Am. Med. Assoc.* 313, 2456–2473. doi:10.1001/jama.2015.6358

Zaami, S., Di Luca, A., Di Luca, N. M., and Montanari Vergallo, G. (2018). Medical use of cannabis: Italian and European legislation. *Eur. Rev. Med. Pharmacol. Sci.* 22, 1161–1167. doi:10.26355/eurrev_201802_14405

Conflict of Interest: The 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.

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 06 September 2022
DOI 10.3389/fphar.2022.965535

OPEN ACCESS

EDITED BY
Daniela Calina,
University of Medicine and Pharmacy of Craiova, Romania

REVIEWED BY
Andrew Lofts Gray,
University of KwaZulu-Natal, South Africa
Piotr Dobrzynski,
Jan Długosz University, Poland
Michelle Sexton,
University of California, San Diego, United States

*CORRESPONDENCE
Albert Garcia-Romeu,
agarci33@jhmi.edu

SPECIALTY SECTION
This article was submitted to Drugs Outcomes Research and Policies, a section of the journal Frontiers in Pharmacology

RECEIVED 09 June 2022
ACCEPTED 18 July 2022
PUBLISHED 06 September 2022

CITATION
Garcia-Romeu A, Elmore J, Mayhugh RE, Schlienz NJ, Martin EL, Strickland JC, Bonn-Miller M, Jackson H and Vandrey R (2022), Online survey of medicinal cannabis users: Qualitative analysis of patient-level data. *Front. Pharmacol.* 13:965535. doi: 10.3389/fphar.2022.965535

Online survey of medicinal cannabis users: Qualitative analysis of patient-level data

Albert Garcia-Romeu [1*†], Joshua Elmore[2], Rhiannon E. Mayhugh[1], Nicolas J. Schlienz[3], Erin L. Martin[4], Justin C. Strickland[1], Marcel Bonn-Miller[5], Heather Jackson[6] and Ryan Vandrey[1]

[1]Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2]University of Colorado Boulder, Boulder, CO, United States, [3]Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States, [4]Medical University of South Carolina, Charleston, SC, United States, [5]Canopy Growth Corporation, Smiths Falls, ON, Canada, [6]Realm of Caring Foundation, Colorado Springs, Colorado, CO, United States

Aim: To characterize perceived benefits and challenges experienced by medicinal cannabis users.

Methods: An anonymous online survey collected demographics, health information, and open-ended responses from medicinal cannabis users regarding perceptions, motivations, and experience of treatment. Qualitative open-ended responses were thematically analyzed.

Results: Respondents (*N* = 808) were predominantly White (79%), female (63%), with a mean (SD) age of 38 (20). Two hundred eighty-four (35%) respondents provided data on a dependent family member (e.g., child; 22% of total sample). Most used cannabidiol (CBD)-dominant products (58%), primarily for neurological disorders (38%) or pain (25%). Primary motivations for medicinal cannabis use were based on beliefs that traditional treatments were ineffective and/or had intolerable side effects (51%), positive scientific or media portrayals of the safety/efficacy of cannabis as a therapeutic (29%), or preference for "natural" treatments over pharmaceuticals (21%). A majority of respondents (77%) attributed positive effects to the medicinal use of cannabis/cannabinoids. These included physical symptom improvements such as reduced pain (28%), improved sleep (18%), and seizure reduction (18%), and mental health improvements including reduced anxiety (22%) and improved mood (11%). Additionally, respondents reported reduced use of other medications (e.g., opioids) (12%), and improved quality of life (14%). Problems associated with use were cited by 41% of respondents, and included unwanted side effects (16%), lack of information or medical support (16%), prohibitive costs (12%), and legal concerns (10%).

Conclusion: Most participants reported benefits from cannabis use for a variety of conditions where traditional treatments were ineffective or unacceptable. Concerns regarding cannabis side effects, legality, lack of information, and cost were raised. Data indicate greater research and education on the safety and efficacy of medicinal cannabis/cannabinoid use is warranted.

KEYWORDS

cannabis (marijuana), CBD-cannabidiol, THC-tetrahydrocannabinol, medical marijuana, qualitative

1 Introduction

The rapid adoption of cannabis and hemp[1] legalization globally has resulted in growing accessibility of a wide variety of cannabis products with purported medicinal benefits (Spindle et al., 2019). Provisions for legal cannabis have spread throughout the United States as well as Canada, Mexico, Uruguay, Luxembourg, Australia, Israel, and others, with more nations in Europe, Asia, and Africa considering similar measures (Hall et al., 2019). However, clinical research on most non-pharmaceutical cannabis products remains limited (Levinsohn and Hill, 2020), emphasizing the need for patient-level data on the impacts of increased access to and use of cannabis for medicinal purposes (Bonn-Miller et al., 2019).

Cannabis products have been used to treat a wide range of health conditions, including pain (Stockings et al., 2018), sleep disturbance (Bachhuber et al., 2019), seizure disorders (Hussain et al., 2015), mental health conditions (e.g., depression, anxiety, post-traumatic stress disorder [PTSD] (Black et al., 2019; Martin et al., 2021; Bonn-Miller et al., 2022), and cancer (Schleider et al., 2018). To date, results surrounding efficacy are mixed at best, and limited by the lack of large controlled clinical trials (Whiting et al., 2015; Pratt et al., 2019). Current clinical data indicate that vaporized cannabis flower can reduce chronic pain (Wallace et al., 2015; Wilsey et al., 2016); and that oral cannabinoids are effective in reducing chemotherapy-induced nausea and vomiting (Meiri et al., 2007), and patient-reported spasticity in multiple sclerosis (Zajicek et al., 2003). Other studies suggest potential sleep improvement associated primarily with oral cannabinoids in patient populations (Serpell et al., 2014; Whiting et al., 2015). Though a comprehensive account of clinical research with cannabis and cannabinoids falls outside the scope of the present manuscript, interested readers can refer to reviews by Whiting et al. (2015), National Academies of Sciences, Engineering, and Medicine (2017), and Pratt et al. (2019). In the United States, because medicinal cannabis use has been legislatively approved in most states but remains a controlled substance at the federal level, large clinical trials are difficult to achieve, which has led to greater reliance on observational research designs (Vandrey, 2018).

In light of this, the current manuscript presents a qualitative thematic content analysis of open-ended survey responses detailing the experiences of 808 medicinal cannabis users. Earlier quantitative analyses of data from the present study sample reported significant health benefits associated with medicinal cannabis use (Schlienz et al., 2020; Martin et al., 2021; Strickland et al., 2021). Schlienz et al. (2020) initially found significantly better self-reported quality of life, health satisfaction, and sleep, and significantly lower pain severity, anxiety, and depression among a sample of 808 medicinal cannabis users compared to a control group of 468 patients with similar health issues and demographics who were not medicinal cannabis users. Additionally, medicinal cannabis users reported significantly less healthcare utilization (i.e., prescription medications, emergency department visits, hospitalizations) than non-cannabis using controls (Schlienz et al., 2020). Secondary analyses from the same dataset found that medicinal cannabis users with anxiety and/or depression (n = 368) scored lower on self-reported depression and pain (but not anxiety), as well as reporting better quality of life and sleep than non-cannabis using controls (n = 170) at baseline (Martin et al., 2021). Furthermore, longitudinal analyses found individuals in the control group who initiated medicinal cannabis use during a follow-up period showed significant reductions in anxiety and depression from baseline that were not evident in those who did not use medicinal cannabis (Martin et al., 2021). Finally, Strickland and others (2021) compared a subsample of patients with epilepsy who used cannabidiol (CBD) products (n = 280) with a control group of individuals with epilepsy who did not use CBD or medicinal cannabis (n = 138), finding better quality of life and sleep, and lower severity of psychiatric symptoms among the CBD users at baseline. No difference was found in self-reported seizures, though this may indicate a floor effect due to the high proportion (>40%) of respondents who reported no past-month seizures (Strickland et al., 2021).

These data indicate that patients with a wide array of health conditions report notable physical and mental health benefits associated with medicinal cannabis use that are not evident in patients who do not use medicinal cannabis, and that upon initiation of medicinal cannabis use significant improvements are reported across diverse areas such as sleep, mood, and healthcare utilization (Schlienz et al., 2020; Martin et al., 2021; Strickland et al., 2021). To supplement these findings, the current study provides a qualitative account of participants' lived experience as medicinal cannabis users based on open-ended response data. The aim of this qualitative analysis is to systematically document medicinal cannabis users' reported benefits, challenges, and overall perceptions regarding their medicinal cannabis use in their own words. Given increasing access to and use of medicinal cannabis, these data may help inform public policymakers, patients, and healthcare providers regarding the evolving landscape of medicinal cannabis.

1 The term hemp is used here to denote low-THC containing varieties of cannabis, legally defined in the United States as containing ≤0.3% THC by dry weight.

2 Methods

The current study employed a qualitative thematic content analysis (Braun and Clarke, 2006) of medicinal cannabis users' open-ended responses in a large-scale, online study conducted in collaboration with the Realm of Caring Foundation, a 501(c) (3) non-profit organization dedicated to providing evidence-based education and community support to medicinal cannabis users. Respondents were a convenience sample of medicinal cannabis users recruited from the Realm of Caring Foundation's patient research registry and social media postings. This study was approved by the Johns Hopkins University School of Medicine Institutional Review Board. All participants provided informed consent and completed Internet-based surveys via Qualtrics (Provo, UT), a secure online platform, detailing their own medicinal cannabinoid use, or that of a dependent for whom they were a caregiver.

Participants provided demographic and health-related information on their medical conditions, treatments, and medicinal cannabis use. Quantitative data regarding participant health outcomes were previously reported (Schlienz et al., 2020; Martin et al., 2021; Strickland et al., 2021). The current study presents novel data from free-text participant responses. Open-ended items were designed to inquire about participants' perceptions of medicinal cannabis treatment, the ways it may have helped or harmed them, and motivations for use. This comprised three questions asking the following: 1) "Why did the participant choose to begin therapeutic use of cannabinoids?"; 2) "How has therapeutic use of cannabis/cannabinoids helped the participant?"; and 3) "How has therapeutic use of cannabis/cannabinoids harmed or caused problems for the participant?"

TABLE 1 Participant demographics (*N* = 808).

Age: Mean (SD)	38 (20)
Range; *n*, % below age 18	1–86; 175, 22%
Sex: n, %	
Male	298, 37%
Female	510, 63%
Race; n, %	
Caucasian	637, 79%
African American	16, 2%
Hispanic/Latino	38, 5%
Other	75, 9%
Not reported	42, 5%
Education (among age ≥18); *n*, %	
High school or less	106, 17%
Some college	133, 21%
Undergraduate degree	183, 29%
Graduate degree	123, 19%
Trade/technical training	51, 8%
Not reported	37, 6%
Non-therapeutic cannabis use; *n*, %	
Lifetime	250, 31%
Past year	111, 14%
Past month	79, 10%
Primary medical condition; *n*, %	
Neurological	307, 38%
Chronic pain	204, 25%
Neuropsychiatric	146, 18%
Autoimmune	75, 9%
Cancer	59, 7%
Insomnia	6, 1%
Other	11, 2%

Note: For more details see Schlienz et al. (2020).

2.1 Data analysis

Open-ended responses were collated and thematically analyzed by the authors using an iterative, atheoretical approach (Braun and Clarke, 2006). First, responses were organized according to specific items enumerated above, providing an *a priori* thematic structure to examine (A) motivations for initiating medicinal cannabis use, (B) perceived benefits of medicinal cannabis use, and (C) perceived challenges related to medicinal cannabis use. Then, three authors (AGR, JE, and RM) generated a codebook based on recurring patterns in an initial subset of participant responses. Codes were derived both top-down from interview questions (e.g., benefits of medicinal cannabis), and bottom-up from emerging patterns agreed upon in regular research analysis meetings (e.g., 'improved quality of life' as an oft-cited benefit). All responses were then coded using Dedoose qualitative data analysis software (version 8.3.35, 2020, SocioCultural Research Consultants, Los Angeles, CA, United States).

Afterwards, codes were organized into distinct themes, subthemes, and categories encompassing data into a coherent thematic structure, and quantified by relative prevalence. To test for inter-rater reliability, responses from 81 randomly selected participants (i.e., ~10% of the sample) were concurrently coded by the two primary raters (AGR and JE) using the final codebook, and submitted to a pooled Cohen's kappa test (De Vries et al., 2008). Using these methods, open-ended response data were analyzed for patterns concerning how participants had been affected by and perceived medicinal cannabis use. The underlying aim of analysis was to identify common themes across participant responses, and to characterize salient benefits, challenges, and concerns based on firsthand accounts of medicinal cannabis users' experiences.

Additionally, participant responses were examined quantitatively for differences in prevalence of major themes and subthemes of interest between users of primarily

TABLE 2 Major themes and subthemes identified from participants' open-ended responses and observed prevalence among the study sample (*N* = 808).

Major themes/subthemes	*n*, %
Cannabis Products	399, 49%
Factors Driving Use	538, 67%
Medical Conditions	432, 53%
Traditional treatments ineffective/intolerable	415, 51%
Positive scientific or media portrayals	234, 29%
Prefer natural products	170, 21%
Recommended by trusted parties	136, 17%
Last resort	127, 16%
Curiosity or other factors	127, 16%
Good Effects	624, 77%
Physical symptom improvements	446, 55%
Mental health improvements	232, 29%
QOL improvements	116, 14%
Reduced medications or healthcare utilization	93, 12%
Too early or unsure	127, 16%
Issues or Problems	330, 41%
Side effects	130, 16%
Lack of information or support	127, 16%
High cost	97; 12%
Too early or unsure	96, 12%
Legal concerns	81, 10%
Difficult to access	54, 7%
Not fully effective	46, 6%
Social stigma	31, 4%
Other concerns	29, 4%

Note: QOL, quality of life.

CBD-based products compared with users of other (e.g., Delta-9-tetrahydrocannabinol [THC]-containing) products. Two-sided Fisher's exact tests were used to assess between group differences in relative proportions of respondents endorsing major themes and subthemes to explore potential product related variations in medicinal cannabinoid outcomes. These focused specifically on themes and subthemes regarding medicinal cannabinoid efficacy or adverse effects, and were calculated using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA, United States).

3 Results

3.1 Participants

Participants were enrolled between April 2016 and February 2018, including 524 medicinal cannabis users aged 18 or older, and 284 adult caregivers of individuals using medicinal cannabis. Table 1 shows demographics for the study sample (*N* = 808). Respondents reported a mean (SD) patient age of 38 (20). Medicinal cannabis users in the study sample were primarily female (63%), Caucasian (79%), and using medicinal cannabis to treat neurological conditions (38%) or chronic pain (25%).

3.2 Major themes

The final study codebook consisted of 531 unique codes that were divided into four major themes and 21 subthemes. Inter-rater reliability analysis for the 81 randomly selected responses across both raters found a pooled Cohen's kappa of 0.72, indicating good inter-rater agreement (McHugh, 2012). Three major themes were based on *a priori* research questions regarding medicinal cannabis use: Factors Driving Use; Good Effects; and Issues/Problems. An additional major theme that emerged from participant responses included Cannabis Products. Among the first three major themes, each contained a number of subthemes (Table 2), which are described in detail below. Number of participants and proportion of the total sample (*n*, %) endorsing particular themes and subthemes are included below to characterize overall prevalence of each. Excerpts from open-ended responses are presented to illustrate relevant codes in participants' own words, citing participant (ppt.) ID numbers and redacting any personally identifiable information. Some excerpts have been lightly edited for clarity to correct typographical, grammatical, or spelling errors.

3.3 Cannabis products

Participants (*n* = 399, 49%) mentioned a wide range of medicinal cannabis products they were either using or considering using, comprising 35 unique codes. Most prominent among these were cannabidiol (CBD) and CBD-containing products (*n* = 361, 45%), THC (*n* = 53, 7%), cannabis flower (*n* = 32, 4%), and cannabis-based oils (*n* = 18, 2%), e.g., "I read about CBD and joined a support group for people with various problems, using CBD to provide symptomatic relief. I started using THC after a while, when the CBD alone wasn't providing the pain relief I need" (ppt. 1232). Other cannabis products mentioned included edible products (*n* = 6, 0.7%), vape pens (*n* = 4, 0.5%), tetrahydrocannabinolic acid (THC-A; *n* = 4, 0.5%), and transdermal products (*n* = 3, 0.4%). For instance, participant 1060, the parent of a 25-year-old woman with epilepsy said

> The THC-A, which seems to be most beneficial for *A*, is extremely expensive, over $100 a month. The CBD is $250 but lasts for months because we cannot increase it due to increase in seizures. We'd like the THC-A to be more affordable. We keep decreasing it due to cost, but then the intensity of her seizures increases.

3.4 Factors Driving Use

Respondents described a number of factors driving their medicinal cannabis use, which were divided into seven subthemes: medical conditions they were seeking to manage (n = 432, 53%), traditional treatments were ineffective and/or intolerable (n = 415, 51%), cannabis use was motivated by positive scientific or media portrayals (n = 234, 29%), patient prefers use of natural products vs pharmaceuticals (n = 170, 21%), cannabis use was recommended by healthcare provider or other trusted parties (n = 136, 17%), use of cannabis was a "last resort" (n = 127, 16%) given that prior treatments had failed, or use was primarily driven by curiosity or other factors (n = 127, 16%).

3.4.1 Medical conditions

Throughout their responses, 432 participants (53%) cited a number of medical conditions and symptoms that they or their loved ones were coping with, or that they expressed an interest in regarding the potential impact of medicinal cannabis. These comprised 124 unique codes referring to medical conditions or symptoms that were collated into 12 broad categories, including: pain and nerve-related conditions, seizures, psychiatric, cancer, autism spectrum disorders, neurological and headache, sleep, autoimmune, poor QOL, gastrointestinal issues, movement disorders, and dermatological conditions. Most commonly cited among these were seizures (n = 171, 21%), pain (n = 157, 19%), and psychiatric symptoms (n = 99, 12%). These were generally mentioned in the context of reasons for seeking treatment or perceived benefits, e.g., "took away or lessened my symptoms from Lyme, and also my anxiety, and gastro issues" (ppt. 763).

3.4.2 Traditional treatments ineffective or intolerable

A major reason participants reported initiating medicinal cannabis use was because they found traditional treatments ineffective or intolerable (n = 415, 51%). Lack of efficacy of prior treatments was explicitly cited by 235 (29%) respondents, who provided statements such as, "No medications were controlling my [osteoarthritis] symptoms and my health was declining to the point I was afraid I would be soon disabled" (ppt. 884), and "15 failed seizure meds in 10 years" (ppt. 1050). As a result, many participants (n = 174, 22%) described medicinal cannabis as a welcome alternative to treatments they considered suboptimal, or as a means of avoiding unwanted treatments. For instance, ppt. 79, the mother of a 15-year-old with epilepsy and ASD wrote,

> The medications prescribed by doctors were not working and they had wanted to do surgery to decrease the seizures. As her mother I wanted anything to take these seizures away but surgery was not in the plan. So we chose to start her on these [CBD] oils hearing great things about them. In the beginning they did decrease a little so with the increase of the dosage and monitoring with her doctor she has been seizure free for 55 days and looking forward to being seizure free forever. It has also helped with her autism as well by this I mean she has been using words in sentences and communicating a lot more... Our goal for the future is to totally wean her off of her seizure medications.

Adverse side effects from traditional medications were another commonly mentioned reason for initiating medicinal cannabis (n = 163, 20%), e.g., "Seizure medications made her angry, anxious, not eat, would not do school work, fight with brother and sister, night terrors, leg cramps, constipated, and severe eczema" (ppt. 712). The parents of an 11-year-old with obsessive compulsive disorder (OCD) responded,

> He tried two different SSRI [selective serotonin reuptake inhibitor] medications. One made him have severe suicidal ideations. The other one increased his OCD compulsions and the distress became unbearable. When the psychiatrist gave us another prescription for a 3rd SSRI, we, the parents, decided that we could not put him (and us) through that again (ppt. 1233).

Likewise, ppt. 201, a 63-year-old woman struggling with post-traumatic stress disorder, depression, and anxiety reported, "Paradoxical effects to meds used in past, increased suicidal ideation, depression, anxiety, SSRI discontinuation syndrome. Haven't had great success w/pharmaceutical treatment."

3.4.3 Positive scientific or media portrayals

A number of respondents (n = 234, 29%) said they decided to try medicinal cannabis after doing their own research, examining online resources, popular media, and scientific literature. For example, "I began researching mj [marijuana] after the Sanjay Gupta airing on Charlotte's Web. After much reading on the Internet, I decided it was worth a try and had very little downside, except legal implications" (ppt. 874). Participant 1180 attributed their interest in medicinal cannabis to,

> The Harvard Conference on Addictions and Psychiatric researcher, Kevin Hill MD. The information about how CBD interacts in the brain producing calming and the studies that show it improving mood acting as an anti-psychotic. The information that attracted me the most was the positive literature on the studies for chronic pain and neuropathic pain. When I was offered a free sample of CBD I jumped at the chance.

These types of accounts were often viewed as attractive due to purported benefits, e.g., "I've read that it can help kill cancer"

(ppt. 90). Additionally, some responded they felt compelled to do their own research due to mistrust in the healthcare system and/or pharmaceutical companies. For instance, ppt. 1343 wrote, "The modern medical system (AMA) [American Medical Association] is exacerbating illnesses in humans or masking symptoms rather than treating in a fully comprehensive way." Similarly, participant 1186 remarked, "The word needs to get out that cannabis is not harmful and can and does help people. Prescription meds cause more harm and are no more than profit for big pharmaceutical companies and the politicians that they pay off."

3.4.4 Prefer natural products

In line with concerns about medications' side effects and misgivings about pharmaceutical industry motives, some participants (n = 170, 21%) expressed a preference for medicinal cannabis as a natural alternative that they viewed as safer and more effective than conventional treatments:

> I am a firm believer that there are many benefits to using holistic, natural treatments from plants (not synthetics). Many conditions are treated with medication that cause terrible side effects that end up complicating life more for the patient and can also damage their system (like robinul messing up my autonomic nervous system). (ppt. 179).

Echoing these sentiments, ppt. 110 said, "It is natural and my body seems to respond better to it. I don't want to use non-natural products. I don't trust most pharmaceutical companies and doctors to ensure what I am ingesting is the best for my body and me."

3.4.5 Recommended by healthcare provider or others

Some respondents (n = 136, 17%) said they initiated medicinal cannabis use as recommended by a healthcare provider or other trusted individual, e.g., "My family doctor suggested I try the CBD oil" (ppt. 1083). Healthcare providers were explicitly cited as suggesting or supporting medicinal cannabis use in 56 cases (7%), and family or friends were attributed for recommendations in 43 cases (5%). In other cases, respondents were encouraged by accounts from people managing similar medical conditions, for instance, "A friend who has Fibromyalgia had been using it and said it helped her so I tried it" (ppt. 1034).

3.4.6 Last resort

For some participants (n = 127, 16%), medicinal cannabis use was seen as a "last resort" after all other treatment options had been exhausted. For example, ppt. 1183, a 30-year-old chronic pain patient with PTSD, described his medicinal cannabis use as a, "Last resort after pain killers, anti-depressants, and anti-psychotics failed and only caused more suicidal ideations." Participant 892 said she tried medicinal cannabis, "Because everything failed and I was desperate for something that might work," for her Chiari malformation.

3.4.7 Curiosity and other factors

In addition to the reasons described above, respondents cited several other factors contributing to their medicinal cannabis use including hope for improved QOL (n = 50, 6%), curiosity (n = 42, 5%), and increasing availability and acceptance (n = 26, 3%). Participant 737 said she initiated CBD treatment for osteoarthritis and torn rotator cuffs, "with the hopes of improving overall quality of life." Participant 677 wrote, "I am curious to see the effect of CBD oil on my chronic pain." Regarding accessibility, ppt. 153 remarked, "North Dakota recently voted to allow therapeutic use of cannabinoids and we chose, as his parents, to begin treatment."

3.5 Good effects

The Good Effects theme consisted of perceived therapeutic benefits of medicinal cannabis use, which were reported by 624 (77%) participants (an additional 127 [16%] responded to this item that it was too early to say or they were unsure of benefits; and another 57 [7%] did not respond). Good Effects were classified into five subthemes including improvements in physical symptoms (n = 446, 55%), mental health (n = 232, 29%), and quality of life (QOL; n = 116, 14%), as well as reduced medication or healthcare utilization (n = 93, 12%), and too early to say or unsure of benefits (n = 127, 16%).

3.5.1 Physical symptom improvements

The most widely reported good effects or perceived benefits of medicinal cannabis use were broadly classified as physical symptom improvements. These were explicitly cited by 446 (55%) participants, and included the following categories: decreased pain (n = 227, 28%), reduced seizures (n = 146, 18%), improved sleep (n = 144, 18%), reduced movement symptoms (e.g., spasms, ticks; n = 73, 9%), gastrointestinal symptom relief (n = 66, 8%), reduced inflammation (n = 36, 4%), and headache or migraine relief (n = 36, 4%). In many cases (n = 267, 33%), participants reported numerous physical symptom improvements concurrently (Mean physical symptoms improved = 2.8, Range = 2-9). For example, ppt. 1058 reported, "Effective control of intractable seizures, improved sleep, appetite, relaxed muscle tone, digestive health, overall daily stability of all functions," in relation to their 20-year-old dependent daughter diagnosed with a lifelong seizure disorder.

3.5.2 Mental health improvements

Mental health improvements were reported by 232 (29%) respondents, referring generally to psychiatric symptom reduction or remission perceived to be associated with

medicinal cannabis use. The most common mental health improvement categories were reduced anxiety (n = 180, 22%), improved mood (n = 88, 11%), enhanced cognitive function (n = 61, 8%), improved communication (e.g., vocabulary, eye contact; n = 58, 7%), increased energy (n = 41, 5%), and reduced problem behavior (e.g., aggression, self-injury; n = 39, 5%). For example, ppt. 972 stated, "I've been dealing with acute depression and feel as if CBD is really helping." A 63-year-old male respondent with anxiety and depression (ppt. 1118) reported, "I can focus, remember tasks, organize better." Numerous participants (n = 158, 20%) cited multiple mental health benefits attributed to their medicinal cannabis use (Mean mental health symptoms improved = 2.8, Range = 2-8). For instance, ppt. 1076, a 45-year-old male with Generalized Anxiety Disorder said, "CBD appears to abate the majority of symptoms associated with anxiety and depression. While flare ups do occur, the severity is diminished compared to without cannabinoids." Similarly, the parent report (ppt. 1146) of a 5-year-old boy with Autism Spectrum Disorder (ASD) noted

> Self-harm stopped after first dose. Violent outbursts/meltdowns stopped with first dose. 1-2 h long meltdowns stopped with first dose. Was completely non-verbal but verbal skills are now emerging. Able to adjust to transitions throughout the day without panic attacks. Now able to follow verbal commands. Social awareness is drastically improving. Now smiles, laughs, and has clear and alert eyes.

3.5.3 Quality of life improvements

In addition to discrete physical and mental health symptom relief, 165 (20%) participants cited notable quality of life (QOL) improvements attributed to their medicinal cannabis use. These fell into two overarching categories including enhanced well-being (n = 71, 9%) and improved daily functioning (n = 56, 7%). Enhanced well-being included effects such as regaining a sense of hope, enjoying family life, and laughing more often. For instance, ppt. 134 said, "It helps with an overall happier more joyful countenance. Gives me energy to play and interact with my kids and my husband. Helps me get out of the house and do things with friends," and ppt. 1239 said, "CBD has transformed my son, has brought calmness to our home and has given us and our son a quality of life that we never thought possible." Improved daily function was defined by greater ability to engage in everyday activities such as exercise and work. A 52-year-old woman with Multiple Sclerosis (ppt. 826), commented that with CBD oil she "was able to sleep better and stretch/light exercise twice a day. It also made it easier to walk and go outside regularly." A 55-year-old woman with Lyme Disease (ppt. 885) said, "People such as myself are able to become productive and valued members of society again when we can have the quality of life improved so simply."

3.5.4 Reduced medication or healthcare utilization

One hundred (12%) respondents cited reductions in medication use or healthcare utilization as a benefit of using medicinal cannabis. These were often cited as nonspecific reductions in medication use, e.g., "I have been able to eliminate prescription pharmaceuticals, and sleep better while lowering my anxiety" (ppt. 1340). Although in some cases, particular medications or classes of medications were explicitly noted, with opioids (n = 13, 2%) and antiseizure medications (n = 8, 1%) being the most commonly mentioned. While some participants discontinued use of certain medications altogether, others described being able to use less. For example, ppt. 1039, a caregiver for a 46-year-old family member ("L") with post-traumatic Parkinsonism said, "Since using the CBD oil, *L* has been able to reduce her daily dosage of Dilaudid from 16 mg per day to 2 mg per day! The Dilaudid was toxic to her body, and she feels much better with a lower dosage. She would like to be able to completely eliminate the use of the Dilaudid. Also, she has been able to reduce her daily dosage of Baclofen in her intrathecal baclofen pump from over 1,200 mcg per day to 853 mcg per day. She has less spasticity and rigidity, and therefore, less pain." Additionally, small contingents of participants explained how their reduced medication use also helped provide relief from adverse side effects of those medications (n = 12, 1%): "Weaned off antiseizure med (Keppra) so there is less brain fog and moodiness" (ppt. 1270). A few people (n = 3, 0.4%) attributed reduced hospital visits to their medicinal cannabis use, "I used to suffer from migraines daily. I was in the ER/Urgent Care Weekly. My husband and I were discussing whether I should go on Full Time Medical Disability when I saw a documentary on CBD and started researching it high and low then I purchased a few tinctures and ever since my life has been "back to normal" (ppt. 845).

3.5.5 Too early or unsure of benefits

Some participants reported they had only recently initiated medicinal cannabis use and therefore it was too early to provide a conclusive evaluation of therapeutic benefits. For example, ppt. 1095 said, "Thus far I haven't noticed any difference; however, I have only been taking it for 17 days" Other respondents were simply unsure whether there had been any notable improvements from medicinal cannabis use, e.g., "I'm not sure it has helped at all. I have had three episodes of ovarian cancer. I just hope that the use of the hemp oil gives me more time between recurrences" (ppt. 678).

3.6 Issues or problems

A majority of participants responded they had not encountered notable harms or problems related to their medicinal cannabis use (n = 478, 59%), e.g., "No problems

TABLE 3 Self-reported cannabis side effects classified by body system and prevalence.

Side Effects	(*n*, %)
drowsiness/tiredness (N)	25, 3%
high (N)	13, 2%
brain fog (N)	10, 1%
anxiety (N); interfere with function (G); dizziness (G)	8, 1%
headache (N)	7, 1%
stomach upset (D); overdose (NA); nausea (D)	5, 1%
overeating / increased appetite (D); paranoia (N)	4, 0.5%
confusion (N); constipation (EX); insomnia (N); loss of motivation (N); memory problems (N); palpitations (C); general psychotropic effects (N)	3, 0.4%
diarrhea (EX); medication interference (G); agitation (N); bad dreams (N); hypersomnia (N); hypersensitivity (N); irritability (N); itchiness (IN); sore throat (RS); urinary incontinence (EX); weight gain (G); withdrawn (N); cannabis/medication combined side effects (NA)	2, 0.2%
cannabinoid withdrawal (N); psychotic episode (N); chapped lips/picking lips (IN); depression (N); dronabinol affects liver enzymes (C); dry eyes (N); dry mouth (D); ears ringing (N); elevated blood pressure (C); eye/vision problems (N); eye pain (N); hangover (G); hyperactivity (N); loss of appetite (D); mind racing (N); night sweat (G); desire for sweets (D); pain (G); possibly affecting menstrual cycle (RP); restlessness (N); sleep disruption (N); spasms (M); visual disturbances (N); vivid dreams (N); alters other medication absorption (EN); nerve problems/pain in combo w/meds (N); swelling in combo w/meds (G)	1, 0.1%

Note: Body systems cited as follows. Skeletal = S; muscular = M; circulatory = C; immune = IM; digestive = D; endocrine = EN; nervous = N; respiratory = RS; excretory = EX; reproductive = RP; integumentary = IN; general/other = G; not applicable = NA.

whatsoever" (ppt. 30). However, 330 (41%) reported a range of potential issues or problems related to medicinal cannabis use, or social ramifications and impacts surrounding their use. These encompassed nine subthemes including side effects (n = 130, 16%), lack of information or support (n = 127, 16%), high cost (n = 97; 12%), too early or unsure (n = 96, 12%), legal concerns (n = 81, 10%), difficult to access (n = 54, 7%), not fully effective (n = 46, 6%), social stigma (n = 31, 4%), and other concerns (n = 29, 4%).

3.6.1 Side effects

Roughly 16% of the sample (n = 130) reported side effects from medicinal cannabis use, or from a combination of medicinal cannabis with other treatments. These included 62 adverse effects that were classified according to bodily system in Table 3 (Wadhwa et al., 2018). Most common among these were drowsiness/tiredness (n = 24, 3%), high (n = 13, 2%), brain fog (n = 10, 1%), anxiety (n = 8, 1%), interferes with daily function (n = 8, 1%), dizziness (n = 8, 1%), headache (n = 7, 0.9%), upset stomach (n = 5, 0.6%), nausea (n = 5, 0.6%), dose higher than intended (n = 5, 0.6%), increased appetite (n = 4, 0.5%), and paranoia (n = 4, 0.5%). For example, ppt. 49 noted side effects such as, "lethargy, diarrhea, occasional nausea, and in general sleepiness." Additionally, some participants (n = 14, 2%) reported their side effects resolved over time or upon finding optimal dosing. Most side effects appeared mild to moderate in severity. However, a 38-year-old male with multiple sclerosis who was prescribed medical cannabis for trigeminal neuralgia (ppt. 1157) reported a psychotic episode associated as follows, "THC therapy for 9 months caused couch lock and when stopped caused a psychotic episode leading to behavioral hospitalization. I am also now left with less energy and gumption."

3.6.2 Lack of information or support

A major issue cited by participants (n = 127, 16%) consisted of a general lack of information or medical support in implementing their medicinal cannabis use. This included uncertainty around correct dosage (n = 72, 9%), appropriate products to use (n = 28, 3%), and deficient knowledge or support from healthcare providers (n = 38, 5%). For instance, ppt. 889 said, "It is difficult finding providers who know how to dose, what strains might work for specific problems, and which methods might work well. It is not easy to find complete ingredients in medicinal cannabis products or if and who has tested the product." Similarly, regarding their 13-year-old son with Lennox-Gastaut syndrome, ppt. 1106 remarked

> It is difficult to get support from doctors to manage dosing and related issues, such as how to handle surgeries or testing like MRI or dental when sedation is needed. Drs. even neurologist/epileptologists are not encouraging and some are even skeptical. As a parent, it is really trial and error, and knowing your child well, keeping accurate documentation to see how the CBD is working. Unfortunately, when at crossroads, there is no direction.

3.6.3 High cost

Another notable cause for concern among participants was the high cost of their medicinal cannabis products. This was expressed by 97 (12%) of participants, e.g.

> If it were not for the cost prohibitive nature of the oil (we are currently at $250/every 20 days), we would be going full forward with this treatment. My son has responded only favorably to the treatment and if it were less costly we would continue to pursue it and perhaps eliminate some of the more negative drugs we are using. (ppt. 1135)

Lack of insurance coverage was also explicitly mentioned by 19 (2%) respondents. For example, "The only problem that cannabis has caused for me is a financial burden. If I had access that could somehow be covered through insurance, let alone going without worry of legal consequences, my quality of life would be much better" (ppt. 122).

3.6.4 Too early or unsure of problems

As with perceived benefits, some participants (n = 96, 12%) thought it was too soon for them to provide a definitive opinion regarding problems or issues related to medicinal cannabis use, e.g., "Unsure, no problems yet" (ppt. 1121).

3.6.5 Legal concerns

Eighty-one respondents (10%) mentioned legal concerns as a problem surrounding their medicinal cannabis use. For example, ppt. 1062 wrote, "The stigma and continued illegality of cannabis products in our state causes undue stress and unnecessary effort to help our family." Similarly, ppt. 1133 stated, "I am so grateful for what this oil has done for my son. I am however nervous of the uncertainty of the legality of it. It needs to be fixed at the federal level not just State," highlighting conflicts between local and Federal regulations. Employment issues, such as potential drug tests and job loss were cited by 19 individuals (2%), e.g., "I risk being terminated from my job due to random drug testing or being arrested for illegal cannabis" (ppt. 1186). Others (n = 10, 1%) described worry surrounding travel with medicinal cannabis:

> Travelling is difficult as I cannot function without my CBD oil. I can now purchase it in Europe, but cannot fly with it from the United States, which makes it extremely expensive and a worry to have to source it overseas, as well as restricting where I can travel.

3.6.6 Difficult to access

Limited accessibility of medicinal cannabis was identified as problematic by 54 participants (7%) e.g., "I have tried oil. Both CBD and THC. CBD was better for work. Both helped with pain. Very hard for me to get" (ppt. 1313). Living in areas without legal provision for medicinal cannabis use was cited as an obstacle for treatment accessibility (n = 33, 4%). Participant 1108, a parent of a 14-year-old suffering from epilepsy, remarked,

> If we lived in a legal state, and had safe and legal access to all cannabis strains, we firmly believe that we could help our son achieve better seizure control. In my opinion, it is highly unethical for some children to legally be given this medical option, and not other children simply because of their zip code.

3.6.7 Not fully effective

Forty-six respondents (6%) described limited efficacy of medicinal cannabis. Some (n = 16, 2%) reported no observable differences related to medicinal cannabis use. Participant 1144 wrote, "We have hopes that this medicine can help our son, despite not seeing any benefit after 8 months of daily use we feel a little alone with it all." Others found only partial efficacy (n = 14, 2%), stating for example that, "This product has had some effect to somewhat dull my pain, but at times when pain is so severe it will hardly do so! I don't believe it is a silver bullet, but it does help and has not created more problems!" (ppt. 1124). In some cases (n = 7, 1%), participants described worsening of symptoms such as seizures or problem behaviors related to medicinal cannabis use: "We did see 2 days (mostly at school) of unusual destructive behaviors and aggression" (ppt. 137).

3.6.8 Social stigma

Social stigma around medicinal cannabis use was cited as a problem by 31 participants (4%). This was associated with difficulty discussing medicinal cannabis with healthcare providers and others, and feelings of isolation. For example, ppt. 1030 stated, "[I'm] not sure how people I know would react to my using this type of therapy. I do not feel like it would be accepted or understood." For some, this presented a barrier to initiating medicinal cannabis use. Participant 1216, a caregiver for their 74-year-old spouse with metastatic prostate cancer, remarked, "Took quite a bit of time, over a year, to decide to try this modality. Reluctant due to social stigma and legality concerns." For others, such stigma was seen as a potential difficulty in their professional careers or in their role as a parent, e.g., "I hate that I have to hide my interest in hemp and alternative therapy because I'm a nurse and fear this could negatively affect my career or employment" (ppt. 947). Similarly, as ppt. 895, a 41-year-old chronic pain patient noted, "Being a mom with two young children, people see using cannabinoids as a bad thing. The stigma makes it harder as a parent".

3.6.9 Other concerns

Twenty-nine respondents (4%) mentioned other concerns not explicitly falling into the subthemes described above. These primarily involved problems with medicinal cannabis formulations (n = 18, 2%), or considerations around discontinuing medicinal cannabis use (n = 11, 1%). Regarding formulation, six people reported that smoking was not their preferred route of administration, e.g., "Smoking flower gave me a sore throat" (ppt. 719). Four others noted that products were not always consistent: "Trying to find local products resulted in inconsistent products and an increase in seizures" (ppt. 75). Individuals considered discontinuation due to several of the

TABLE 4 Product related (CBD vs other) differences in medical cannabinoid outcomes.

	CBD users (n = 466)	Other products (n = 342)	Fisher's exact test
Major themes/subthemes	**n, %**	**n, %**	***p* (OR,CI)**
Good effects	353, 76%	271, 79%	0.27 (1.2, 0.9–1.7)
Physical symptom improvements	**241, 52%**	**205, 60%**	**0.02 (1.4, 1.1–1.9)**
Mental health improvements	134, 29%	98, 29%	>0.9 (1.0, 0.7–1.4)
QOL improvements	89, 19%	76, 22%	0.29 (1.2, 0.9–1.7)
Reduced medications/healthcare utilization	58, 12%	42, 12%	>0.9 (1.0, 0.7–1.5)
Issues or Problems	182, 39%	148, 44%	0.25 (1.2, 0.9–1.6)
Side effects	**61, 13%**	**69, 20%**	**0.009 (1.7, 1.2–2.4)**
Not fully effective	30, 6%	16, 5%	0.37 (0.7, 0.4–1.3)

Note: Prevalence data are presented as (n, %) participants who positively endorsed a particular outcome. Statistical results show two-tailed Fisher's exact tests including p value, odds ratio (OR), and 95% confidence interval (CI). Significant differences are highlighted in bold at $p< 0.05$. QOL, quality of life.

issues described above, including high cost, lack of information or support, inaccessibility, and ineffectiveness, e.g., "Temporarily stopped hoping to get guidance on dosage to optimize its use for my conditions" (ppt. 714).

3.7 Differences between users of CBD vs. other products

Participant responses were examined quantitatively for differences in prevalence of major themes and subthemes of interest between users of primarily CBD-based products (n = 466, 58%) compared with users of other (e.g., THC-containing) products (n = 342, 42%). These focused specifically on dichotomous (i.e., classified as yes or no) themes and subthemes regarding medicinal cannabinoid efficacy or adverse effects: Good Effects, physical symptom improvements, mental health improvements, QOL improvements, reduced medication or healthcare utilization, Issues or Problems, side effects, and not fully effective. Results found significant differences between product type subgroups on two subthemes, with respondents who used primarily CBD-based medical cannabinoid products reporting lower rates of both physical symptom improvements and lower rates of side effects (Table 4).

4 Discussion

Analysis of open-ended response data from a large-scale online study identified a number of key themes providing important insights into the experience and motivations of medicinal cannabis users and caregivers of medicinal cannabis users (Table 2). Findings indicate a majority of the current sample experienced notable physical, mental, and quality of life benefits attributed to medicinal cannabis use. Benefits were multifaceted, but consistently reported across this sample, who used a variety of cannabis products (primarily CBD dominant) for diverse medical conditions. These data are consistent with use of purified CBD formulations (Epidiolex) and synthetic THC (dronabinol) as FDA approved medications for seizures (CBD) and chemotherapy induced nausea or AIDS related weight-loss (THC), respectively (Levinsohn and Hill, 2020). Furthermore, international approval of novel combined CBD/THC formulations such as nabiximols (Sativex) and participant responses regarding specific cannabinoids such as THC-A highlight that there is still significant research yet to be conducted to fully assess myriad therapeutic indications of interest for cannabinoids.

Like benefits, issues or problems surrounding medicinal cannabis use were also multidimensional, including not only drug related adverse effects that were reported by a subset (16%) of respondents (Table 3), but also legal, social, and provider challenges. Participants lamented the lack of reliable information and medical support available for those seeking to initiate medicinal cannabis use or to integrate it within their treatment regimen. Healthcare providers were often seen as unknowledgeable or unsupportive regarding medicinal cannabis use, and unanswered questions around optimal products and dosing for particular conditions were commonplace. These responses highlight the urgent need for expanded research to produce high-quality data necessary to definitively answer such questions, as well as focused education for medical professionals regarding cannabis to improve healthcare support and integration. Respondents also voiced concerns about the high cost and lack of insurance coverage for medicinal cannabis, which were cited as barriers to

implementing and maintaining treatment. Other interrelated issues surrounding legal concerns, accessibility, and stigma highlight the complex regulatory and social landscape that patients must navigate in order to obtain and use cannabis as medicine, while managing potential legal penalties, employment challenges, difficulty traveling, or even the risk of being ostracized by health care providers or those in their social network.

Participants cited a number of factors driving their medicinal cannabis use, chief among these were that standard medications for their respective conditions were ineffective or had intolerable side effects. This is not surprising considering the high prevalence of neurological, pain, and mental health conditions among participants, and the limitations of current pharmacotherapies in these domains. For instance, epidemiological data suggest 20%–40% of patients diagnosed with epilepsy may be refractory to available treatments (French, 2007), and some 20% of patients with major depression do not respond to existing medications (Gaynes, 2009). Furthermore, adverse side effects of antiepileptic (Perucca and Gilliam, 2012), antidepressant (Ferguson, 2001), and opioid medications are well-established (Benyamin et al., 2008). Thus, participants described frustration with traditional treatment approaches leading them to seek feasible alternatives, and often doing their own research drawing on Internet-based resources, documentaries, and scientific literature. Such efforts, in an era of growing accessibility and diversity of medicinal cannabis products, have seemingly combined to contribute to the rising interest in and adoption of medicinal cannabis use (Vandrey, 2018; Spindle et al., 2019). Similarly, respondents expressed misgivings about the safety and efficacy of pharmaceutical treatments, mistrust towards medical practitioners, and a preference for products that were perceived as 'natural' and safer than pharmaceutical medications. On the one hand, this can result in health benefits and reduced medication and healthcare utilization as described above, when patients are successful in finding medicinal cannabis regimens that work for them. On the other, it highlights a concerning trend towards "do-it-yourself" healthcare approaches that may discount validated clinical expertise and treatments, and undermine honest and open communication with healthcare providers. In the broader landscape of patients seeking alternative treatments for intractable health conditions, this raises concerns about perceptions of natural products as being safer than prescription medicines considering many available supplements are unregulated and lacking in sufficient quality control or clinical data to establish safety and efficacy.

Quantitative analyses of code prevalence found participants using CBD dominant products exhibited lower rates of both physical symptom improvements and cannabinoid related side effects (Table 4). The latter is consistent with the pronounced intoxicating effects of THC (Heishman et al., 1990), which is likely present in non-CBD dominant products such as cannabis flower or oil. However, the observed relationship between non-CBD cannabinoid constituents and transdiagnostic physical symptom improvements necessitates further study, and could plausibly be related to hypothesized entourage effects between numerous cannabinoids and terpenoids present in whole plant cannabis (ElSohly and Slade, 2005; Radwan et al., 2009; Russo, 2011).

These findings should be considered in light of a number of limitations of the present study, design, and dataset. The heterogeneous convenience sample discussed here ranged from infancy to older adulthood and cut across a wide variety of medical conditions and cannabinoid products, but was fairly narrow with respect to ethnic diversity. Thus, it can be difficult to draw generalizable conclusions regarding specific subsamples, indications, and products based on the present data. Furthermore, this self-selected sample may not be representative of the wider population of medicinal cannabis users, and may be biased in favor of those with more positive experience with medicinal cannabis and/or higher socioeconomic status users who are able to afford medicinal cannabis, and have access to computers, Internet, and sufficient time and literacy necessary to respond to the present survey. Because the current study collected data regarding both adult and minor patients using medicinal cannabis, it is difficult to infer if the present sample is typical of the general population of medicinal cannabis users based on nationally representative data that primarily queries adults. According to available literature, the sample in this study may be somewhat older, include more women, and be more highly educated than nationally representative samples of medicinal cannabis users (Lin et al., 2016; Compton et al., 2017), again suggesting these results may not fully generalize to broader populations of medicinal cannabis users. That said, this is a sizeable sample of individuals for inclusion in qualitative analysis of open-ended questions that likely captures many key individual user/caregiver perspectives related to the medicinal use of cannabis.

The cross-sectional design of the current analysis and lack of a placebo group makes it impossible to draw any causal inference about the association of self-reported health impacts and medicinal cannabis use. The results from the present study should be interpreted with caution, particularly regarding the content and prevalence of themes and sub-themes in this sample, which may not reflect the experience of the wider population of medicinal cannabis users. Other limitations inherent in Internet-based research include the unverifiable nature of participant responses, possible social desirability bias in responses, and potential errors regarding information on cannabis products and doses used, which cannot be conclusively confirmed. Furthermore, data rely entirely on respondents' perceptions and self-report, meaning clinical assessments of benefit or risk from healthcare providers are lacking, but present an important future direction for additional research that incorporates these perspectives. Finally, the interpretive nature of qualitative analysis means these findings and thematic categories are not necessarily definitive, but represent the understanding of the

authors in their attempt to present a cogent account of participant responses. However, a key strength of qualitative approaches is the ability to allow people to describe their experience in their own words, which can otherwise be difficult to extrapolate using strictly quantitative methods.

In conclusion, this study adds to the growing literature around medicinal cannabis use and therapeutic potentials. Findings suggest health benefits that extend to a large number of diverse medical and mental health conditions, and also encompass general quality of life improvements. These results underline the importance of further prospective clinical research toward validation and development of cannabis-based therapies, as well as regulatory policies that can facilitate such research. Issues regarding lack of information and medical support and frustrations surrounding inconsistent legal status of medicinal cannabis represent critical challenges that require careful and targeted actions. It is recommended that healthcare professionals and policymakers expand initiatives related to education, transparency, and regulation of medicinal cannabis products, with particular focus on improving quality control, expanding clinical research, and continued vigilance in limiting misinformation. At present, a growing number of individuals are seeking and using medicinal cannabis and product availability is expanding rapidly. As such, this is a pressing public health opportunity that warrants substantial resources and concentrated efforts for improving outcomes of medicinal cannabis use, and patients' voices should be a vital factor in informing these efforts.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Johns Hopkins University School of Medicine Institutional Review Board. Written informed consent to participate in this study was provided by the participants or the participants' legal guardian/next of kin.

Author contributions

AG-R made substantial contributions to the design of the study, the analysis and interpretation of the data, and the drafting of the manuscript. JE and RM made substantial contributions to the analysis and interpretation of the data, and the drafting of the manuscript. NS, EM, JS, MB-M, and HJ made substantial contributions to the conception and design of the study, participant recruitment, acquisition and interpretation of the data, and made critical revisions to the manuscript. RV made substantial contributions to the conception and design of the study, participant recruitment, acquisition and interpretation of the data, the drafting of the manuscript, and made critical revisions to the manuscript. All authors approved the final version of this -manuscript and agree to be accountable for all aspects of the work.

Funding

Funding for this research was provided by the Realm of Caring Foundation. Support for Strickland and Schlienz was provided in part by National Institutes of Health T32 DA07209. Support for Martin was provided by NIH/NIDA T32 DA007288.

Acknowledgments

The authors would like to thank study participants and the support staff at Realm of Caring and Johns Hopkins University School of Medicine.

Conflict of interest

AG-R is a scientific advisor to Etha Natural Botanicals, Innerwell, and NeonMind Biosciences. RV has received compensation as a consultant or scientific advisor from MyMD Pharmaceuticals, Mira1a Pharmaceuticals Inc. Canopy Health Innovations, Syqe Medical Ltd. WebMD, and Radicle Science Inc. MB-M is employed by Canopy Growth Corporation.

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.

Publisher's note

References

Bachhuber, M., Arnsten, J. H., and Wurm, G. (2019). Use of cannabis to relieve pain and promote sleep by customers at an adult use dispensary. *J. Psychoact. Drugs* 51 (5), 400–404. doi:10.1080/02791072.2019.1626953

Benyamin, R., Trescot, A. M., Datta, S., Buenaventura, R., Adlaka, R., Sehgal, N., et al. (2008). Opioid complications and side effects. *Pain Physician* 11, S105–S120. doi:10.36076/ppj.2008/11/s105

Black, N., Stockings, E., Campbell, G., Tran, L. T., Zagic, D., Hall, W. D., et al. (2019). Cannabinoids for the treatment of mental disorders and symptoms of mental disorders: a systematic review and meta-analysis. *Lancet. Psychiatry* 6 (12), 995–1010. doi:10.1016/S2215-0366(19)30401-8

Bonn-Miller, M. O., Pollack, C. V., Casarett, D., Dart, R., ElSohly, M., Good, L., et al. (2019). Priority considerations for medicinal cannabis-related research. *Cannabis Cannabinoid Res.* 4 (3), 139–157. doi:10.1089/can.2019.0045

Bonn-Miller, M. O., Brunstetter, M., Simonian, A., Loflin, M. J., Vandrey, R., Babson, K. A., et al. (2022). The long-term, prospective, therapeutic impact of cannabis on post-traumatic stress disorder. *Cannabis Cannabinoid Res.* 7 (2), 214–223. doi:10.1089/can.2020.0056

Braun, V., and Clarke, V. (2006). Using thematic analysis in psychology. *Qual. Res. Psychol.* 3 (2), 77–101. doi:10.1191/1478088706qp063oa

Compton, W. M., Han, B., Hughes, A., Jones, C. M., and Blanco, C. (2017). Use of marijuana for medical purposes among adults in the United States. *JAMA* 317 (2), 209–211. doi:10.1001/jama.2016.18900

De Vries, H., Elliott, M. N., Kanouse, D. E., and Teleki, S. S. (2008). Using pooled kappa to summarize interrater agreement across many items. *Field Methods* 20 (3), 272–282. doi:10.1177/1525822x08317166

ElSohly, M. A., and Slade, D. (2005). Chemical constituents of marijuana: The complex mixture of natural cannabinoids. *Life Sci.* 78 (5), 539–548. doi:10.1016/j.lfs.2005.09.011

Ferguson, J. M. (2001). SSRI antidepressant medications: adverse effects and tolerability. *Prim. Care Companion J. Clin. Psychiatry* 3 (1), 22–27. doi:10.4088/pcc.v03n0105

French, J. A. (2007). Refractory epilepsy: clinical overview. *Epilepsia* 48, 3–7. doi:10.1111/j.1528-1167.2007.00992.x

Gaynes, B. N. (2009). Identifying difficult-to-treat depression: differential diagnosis, subtypes, and comorbidities. *J. Clin. Psychiatry* 70 (6), 10–15. doi:10.4088/JCP.8133su1c.02

Hall, W., Stjepanović, D., Caulkins, J., Lynskey, M., Leung, J., Campbell, G., et al. (2019). Public health implications of legalising the production and sale of cannabis for medicinal and recreational use. *Lancet* 394 (10208), 1580–1590. doi:10.1016/S0140-6736(19)31789-1

Heishman, S. J., Huestis, M. A., Henningfield, J. E., and Cone, E. J. (1990). Acute and residual effects of marijuana: Profiles of plasma THC levels, physiological, subjective, and performance measures. *Pharmacol. Biochem. Behav.* 37 (3), 561–565. doi:10.1016/0091-3057(90)90028-g

Hussain, S. A., Zhou, R., Jacobson, C., Weng, J., Cheng, E., Lay, J., et al. (2015). Perceived efficacy of cannabidiol-enriched cannabis extracts for treatment of pediatric epilepsy: a potential role for infantile spasms and lennox-gastaut syndrome. *Epilepsy Behav.* 47, 138–141. doi:10.1016/j.yebeh.2015.04.009

Levinsohn, E. A., and Hill, K. P. (2020). Clinical uses of cannabis and cannabinoids in the United States. *J. Neurol. Sci.* 411, 116717. doi:10.1016/j.jns.2020.116717

Lin, L. A., Ilgen, M. A., Jannausch, M., and Bohnert, K. M. (2016). Comparing adults who use cannabis medically with those who use recreationally: Results from a national sample. *Addict. Behav.* 61, 99–103. doi:10.1016/j.addbeh.2016.05.015

Martin, E. L., Strickland, J. C., Schlienz, N. J., Munson, J., Jackson, H., Bonn-Miller, M. O., et al. (2021). Antidepressant and anxiolytic effects of medicinal cannabis use in an observational trial. *Front. Psychiatry* 12, 729800. doi:10.3389/fpsyt.2021.729800

McHugh, M. L. (2012). Interrater reliability: the kappa statistic. *Biochem. Med.* 22 (3), 276–282. doi:10.11613/bm.2012.031

Meiri, E., Jhangiani, H., Vredenburgh, J. J., Barbato, L. M., Carter, F. J., Yang, H.-M., et al. (2007). Efficacy of dronabinol alone and in combination with ondansetron versus ondansetron alone for delayed chemotherapy-induced nausea and vomiting. *Curr. Med. Res. Opin.* 23 (3), 533–543. doi:10.1185/030079907X167525

National Academies of Sciences, Engineering, and Medicine (2017). *The health effects of cannabis and cannabinoids: The current state of evidence and recommendations for research*. Washington, DC: The National Academies Press. doi:10.17226/24625

Perucca, P., and Gilliam, F. G. (2012). Adverse effects of antiepileptic drugs. *Lancet. Neurol.* 11 (9), 792–802. doi:10.1016/S1474-4422(12)70153-9

Pratt, M., Stevens, A., Thuku, M., Butler, C., Skidmore, B., Wieland, L. S., et al. (2019). Benefits and harms of medical cannabis: A scoping review of systematic reviews. *Syst. Rev.* 8 (1), 320. doi:10.1186/s13643-019-1243-x

Radwan, M. M., ElSohly, M. A., Slade, D., Ahmed, S. A., Khan, I. A., and Ross, S. A. (2009). Biologically active cannabinoids from high-potency Cannabis sativa. *J. Nat. Prod.* 72 (5), 906–911. doi:10.1021/np900067k

Russo, E. B. (2011). Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol.* 163 (7), 1344–1364. doi:10.1111/j.1476-5381.2011.01238.x

Schleider, L. B.-L., Mechoulam, R., Lederman, V., Hilou, M., Lencovsky, O., Betzalel, O., et al. (2018). Prospective analysis of safety and efficacy of medical cannabis in large unselected population of patients with cancer. *Eur. J. Intern. Med.* 49, 37–43. doi:10.1016/j.ejim.2018.01.023

Schlienz, N. J., Scalsky, R., Martin, E. L., Jackson, H., Munson, J., Strickland, J. C., et al. (2020). A cross-sectional and prospective comparison of medicinal cannabis users and controls on self-reported health. *Cannabis Cannabinoid Res.* 6, 548. doi:10.1089/can.2019.0096

Serpell, M., Ratcliffe, S., Hovorka, J., Schofield, M., Taylor, L., Lauder, H., et al. (2014). A double-blind, randomized, placebo-controlled, parallel group study of THC/CBD spray in peripheral neuropathic pain treatment. *Eur. J. Pain* 18 (7), 999–1012. doi:10.1002/j.1532-2149.2013.00445.x

Spindle, T. R., Bonn-Miller, M. O., and Vandrey, R. (2019). Changing landscape of cannabis: Novel products, formulations, and methods of administration. *Curr. Opin. Psychol.* 30, 98–102. doi:10.1016/j.copsyc.2019.04.002

Stockings, E., Campbell, G., Hall, W. D., Nielsen, S., Zagic, D., Rahman, R., et al. (2018). Cannabis and cannabinoids for the treatment of people with chronic noncancer pain conditions: a systematic review and meta-analysis of controlled and observational studies. *Pain* 159 (10), 1932–1954. doi:10.1097/j.pain.0000000000001293

Strickland, J. C., Jackson, H., Schlienz, N. J., Salpekar, J. A., Martin, E. L., Munson, J., et al. (2021). Cross-sectional and longitudinal evaluation of cannabidiol (CBD) product use and health among people with epilepsy. *Epilepsy Behav.* 122, 108205. doi:10.1016/j.yebeh.2021.108205

Vandrey, R. (2018). The cannabis conundrum: steering policy and medicine with insufficient data. *Int. Rev. Psychiatry* 30, 181. doi:10.1080/09540261.2018.1491107

Wadhwa, S., Gupta, A., Dokania, S., Kanji, R., and Bagler, G. (2018). A hierarchical anatomical classification schema for prediction of phenotypic side effects. *PloS One* 13 (3), e0193959. doi:10.1371/journal.pone.0193959

Wallace, M. S., Marcotte, T. D., Umlauf, A., Gouaux, B., and Atkinson, J. H. (2015). Efficacy of inhaled cannabis on painful diabetic neuropathy. *J. Pain* 16 (7), 616–627. doi:10.1016/j.jpain.2015.03.008

Whiting, P. F., Wolff, R. F., Deshpande, S., Di Nisio, M., Duffy, S., Hernandez, A. V., et al. (2015). Cannabinoids for medical use: a systematic review and meta-analysis. *JAMA* 313 (24), 2456–2473. doi:10.1001/jama.2015.6358

Wilsey, B. L., Deutsch, R., Samara, E., Marcotte, T. D., Barnes, A. J., Huestis, M. A., et al. (2016). A preliminary evaluation of the relationship of cannabinoid blood concentrations with the analgesic response to vaporized cannabis. *J. Pain Res.* 9, 587–598. doi:10.2147/JPR.S113138

Zajicek, J., Fox, P., Sanders, H., Wright, D., Vickery, J., Nunn, A., et al. (2003). Cannabinoids for treatment of spasticity and other symptoms related to multiple sclerosis (CAMS study): multicentre randomised placebo-controlled trial. *Lancet* 362 (9395), 1517–1526. doi:10.1016/S0140-6736(03)14738-1

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 19 October 2022
DOI 10.3389/fphar.2022.981817

OPEN ACCESS

EDITED BY
Uraiwan Panich,
Mahidol University, Thailand

REVIEWED BY
Darrell Pilling,
Texas A&M University, United States
Honggang Zhou,
Nankai University, China

*CORRESPONDENCE
Manuel Tena-Sempere,
fi1tesem@uco.es
Eduardo Muñoz,
fi1muble@uco.es

†PRESENT ADDRESS
Carmen del Río,
Instituto de Biomedicina de Sevilla (IBiS),
Campus del Hospital Universitario
Virgen del Rocío Edificio IBiS- Avda,
Antonio Maura Montaner, Sevilla

†These authors have contributed equally to this work and share senior authorship

SPECIALTY SECTION
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

RECEIVED 29 June 2022
ACCEPTED 05 October 2022
PUBLISHED 19 October 2022

CITATION
del Río C, Ruiz-Pino F, Prados ME, Fiebich BL, Tena-Sempere M and Muñoz E (2022), Cannabidiol markedly alleviates skin and liver fibrosis. *Front. Pharmacol.* 13:981817. doi: 10.3389/fphar.2022.981817

Cannabidiol markedly alleviates skin and liver fibrosis

Carmen del Río[1,2,3†‡], Francisco Ruiz-Pino[4†], María E. Prados[4], Bernd L. Fiebich[5], Manuel Tena-Sempere[1,2,3,6*] and Eduardo Muñoz[1,2,3*]

[1]Instituto Maimónides de Investigación Biomédica de Córdoba-IMIBIC, Cordoba, Spain, [2]Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Cordoba, Spain, [3]Hospital Universitario Reina Sofía, Cordoba, Spain, [4]VivaCell Biotechnology España, Cordoba, Spain, [5]VivaCell Biotechnology GmbH, Dezlingen, Germany, [6]CIBER Fisiopatologia de la Obesidad y Nutrición, Instituto de Salud Carlos III, Cordoba, Spain

Cannabidiol (CBD) has been suggested as a potential therapy for inflammatory and fibrotic diseases. Cannabidiol was demonstrated to reduce alcohol-induced liver inflammation and steatosis but its specific activity on the fibrotic process was not investigated. Herein, the antifibrotic effects of cannabidiol in the skin were analysed *in vitro* using NIH-3T3 fibroblasts and human dermal fibroblasts and *in vivo* using the bleomycin-induced model of skin fibrosis. In a second model, non-alcoholic liver fibrosis was induced in mice by CCl_4 exposure. Cannabidiol was administered daily, intraperitoneally in mice challenged with bleomycin and orally in CCl_4 mice, and skin and liver fibrosis and inflammation were assessed by immunochemistry. Cannabidiol inhibited collagen gene transcription and synthesis and prevented TGFβ-and IL-4 induced fibroblast migration. In the bleomycin model, cannabidiol prevented skin fibrosis and collagen accumulation around skin blood vessels, and in the CCl_4 model cannabidiol significantly attenuated liver fibrosis measured by picrosirius red and Tenascin C staining and reduced T cell and macrophage infiltration. Altogether, our data further support the rationale of the medicinal use of this cannabinoid, as well as cannabis preparations containing it, in the management of fibrotic diseases including Systemic Sclerosis and Non-Alcoholic Fatty Liver Disease.

KEYWORDS

cannabidiol, fibrosis, systemic sclerosis, non-alcoholic fatty liver disease, COL1A2

Introduction

Cannabidiol (CBD), the main non-psychotropic component of *Cannabis sativa* L. (Cannabaceae), has aroused much interest due to its broad range of therapeutic potential. CBD antiinflammatory and antifibrotic properties stem from multiple pharmacological mechanisms but the relative contribution of each pathway is not known. CBD shows a very low affinity to CB_1 and CB_2 receptors, and recent evidence raised the possibility that CBD can act as a negative allosteric modulator of CB_1 receptor (Laprairie et al., 2015). Despite CBD behaves as a low affinity agonist, several studies support that CBD effects can be partially attributed to its activity on CB_2 receptor (Martinez-Pinilla et al., 2017; Vilela

et al., 2017). CB_1 and CB_2 receptors have been shown to play opposite roles in experimental models of fibrosis. While CB_1 inactivation exerted antifibrotic effects by indirectly regulating leukocyte infiltration (Marquart et al., 2010) and the production of transforming growth factor beta 1 (TGFβ1) (Teixeira-Clerc et al., 2006), activation of CB_2 receptors was reported to reduce tissue fibrosis in various rodent models of organ fibrosis, including skin (Akhmetshina et al., 2009), liver (Munoz-Luque et al., 2008) and heart (Li et al., 2016).

Apart from canonical cannabinoid receptors, CBD acts on numerous biological targets able to downregulate proinflammatory and profibrotic cytokines, including the modulation of different transient receptor potential vanilloid (TRPV) channels and GPR55 receptor antagonism (Sunda and Arowolo, 2020). In addition, CBD stimulation of PPARγ may inhibit the transcription of proinflammatory NF-κB dependent genes (Scirpo et al., 2015) and abrogate collagen synthesis by interfering TGFβ signalling (Ghosh et al., 2009). CBD also enhances adenosine signaling to reduce inflammation (Carrier et al., 2006), which has been shown to alleviate bleomycin-induced lung fibrosis (Chen et al., 2017). Moreover, the antioxidant properties of CBD can also contribute to its effects by downregulating intracellular ROS generation and lipid peroxidation (Sunda and Arowolo, 2020). More recently, it has been shown that the antioxidant activity of CBD can be also mediated by targeting Bach1 and inducing the expression of HMOX-1 (Casares et al., 2020).

CBD administration has been described to reduce inflammation and fibrosis in different experimental disease models, such as allergic asthma (Vuolo et al., 2019), diabetic cardiomyopathy (Rajesh et al., 2010) and alcohol-related fibrosis in the liver (De Ternay et al., 2019). Recently, we demonstrated that two different CBD aminoquinone derivatives, acting as dual PPARγ/CB_2 ligand agonists, inhibit fibroblast differentiation and collagen deposition *in vitro* and alleviate inflammation and fibrogenesis in a mouse model of experimental systemic sclerosis, when delivered either intraperitoneally or orally (Del Rio et al., 2018; Del Rio et al., 2018; Garcia-Martin et al., 2018). Similarly, the (+)-enantiomer of CBD and its derivative (+)-CBD hydroxypentylester mitigated immune cell infiltration and renal fibrosis (Gonzalez-Mariscal et al., 2021). Randomized controlled trials have shown that CBD and CBD derivatives are well-tolerated in a wide range of disease conditions with limited side effects (Devinsky et al., 2018; Taylor et al., 2018; Taylor et al., 2019) and the FDA approval of a CBD oral solution (Epidiolex®) for certain types of epilepsy supports its medicinal use.

Fibrosis is a lifelong pathological condition characterized by the excessive collagen and extracellular matrix (ECM) accumulation. Fibrosis can be classified based on anatomical location in systemic or organ specific fibrosis, with increased frequency in the skin, liver, heart, kidneys, and lungs (Pryimak et al., 2021). The etiology of fibrotic disorders is heterogeneous and the causative mechanisms remain still elusive. Systemic sclerosis (SSc) is a highly heterogeneous immune-mediated rheumatic disease characterised by the presence of vasculopathy that precedes fibrosis in the skin and internal organs (Varga and Abraham, 2007). In addition, abnormal epigenetic modifications in fibroblasts, endothelial cells and immune cells also participate in pathogenic pathways of SSc (Tsou et al., 2021). Damaged endothelial cells release growth factors and cytokines that promote inflammatory infiltration and autoimmunity. The long-lasting synthesis of proinflammatory mediators by the infiltrated immune cells triggers fibroblast proliferation and differentiation. These cells become the primary source of extracellular matrix causing excessive remodelling and tissue dysfunction (Kendall and Feghali-Bostwick, 2014). In the same way, hepatic stellate cells activated upon injury are responsible of the scarring response of liver, which can result from toxic, metabolic, and viral insults. Liver disease progression by sustained inflammation and progressive fibrosis leads to cirrhosis, a major cause of morbidity and mortality worldwide (Hernandez-Gea and Friedman, 2011). At the moment, there are no effective antifibrotic treatments for human use. In this context, the strong antiinflammatory and antifibrotic potential of CBD represent a useful pharmacological approach for the treatment of fibrosis in both pathological conditions. Therefore, we have investigated the ability of CBD to ameliorate fibrosis in experimental models of SSc and non-alcoholic liver fibrosis.

Methods

Cell lines

NIH-3T3 and human dermal fibroblasts (NHDFs) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin. All cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere. 99.73% pure synthetic trans (−) Cannabidiol was obtained from Symrise AG, lot number 10300010 (Holzminden, Germany).

Col1a2 and CAGA transcriptional assay and collagen synthesis measurement

NIH-3T3 cells were seeded in 24-well plates (5×10^4 cells/well). After 24 h, cells were transiently transfected with Col1a2-luc (1 μg/well) using Roti©-Fect (Carl Roth, Karlsruhe, Germany) following manufacturer's specifications. 24 h after transfection, cells were pre-treated with CBD for 1 h at the indicated concentrations and stimulated with TGFβ1 (5 ng/ml) for the following 24 h. Then, cells were lysed in 100 μL of lysis buffer and luciferase activity was measured using the Dual-

Luciferase® reporter assay system (Promega; Madison, WI, United States). Human dermal fibroblasts were seeded in 24-well plates (6×10^4 cells/well). The following day, complete media was replaced by serum-free DMEM supplemented with 1% (v/v) penicillin/streptomycin. After 24 h, cells were pre-treated with CBD or RGZ for 1 h and stimulated with TGFβ1 (10 ng/ml) for 48 h. At the indicated time points, cell media was collected and assayed for soluble collagen using the Sircol Collagen Assay (Biocolor, County Antrim, United Kingdom) according to the manufacturer's protocol. NIH3T3 cells were seeded in 24-well plates and after 24 h they were transiently transfected with CAGA-luc plasmid using Roti©-Fect (Carl Roth, Karlsruhe, Germany) following manufacturer´s specifications. The CAGA-luc reporter plasmid contains multimerized Smad-binding elements that bind active Smad2/3 complexes. After stimulation, the luciferase activities were quantified using Dual-Luciferase Assay (Promega, Madison, WI, United States). To correct for transfection efficacy, 100 ng Renilla luciferase (pRL-CMV) was cotransfected.

Fibroblast scratch assay

Normal human dermal fibroblasts (NHDF) (2×10^4 cells/well) were seeded in 96-well plates (Essen Bioscience, Newark, United Kingdom). Once fibroblast reached confluence, a scratch was made using the 96-pin WoundMaker (Essen Bioscience). Then, cell media was replaced by fresh serum-free DMEM supplemented with 1% (v/v) penicillin/streptomycin. At that point, CBD treatments were added in combination with either TGFβ1 or rhIL-4 (10 ng/ml) to induce cell proliferation. Images were taken every 3 h for 48 h, and data analysed using IncuCyte HD software.

Western blots

NHDF cells were incubated in low serum conditions (1% FBS) for 24 h. Then cells were pretreated with CBD or Rosiglitazone (RGZ) for 1 h and stimulated with TGFβ1 (10 ng/ml) for 2 h. After treatments, the cells were washed with PBS and proteins extracted in 50 μL of lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol and 1% NP-40) supplemented with 10 mM NaF, 1 mM Na3VO4, 10 μ g/ml leupeptine, 1 μg/ml pepstatin and aprotinin, and 1 μL/ml saturated PMSF. Protein concentration was determined by the Bradford assay (Bio-Rad, CA, United States) and 30 μg of proteins were boiled at 95°C in Laemmli buffer and electrophoresed in 10% SDS/PAGE gels. Separated proteins were transferred to PVDF membranes (20 V for 30 min) and blocked in TBS solution containing 0.1% Tween 20 and 5% non-fat dry milk for 1 h at room temperature. Immunodetection of specific proteins was carried out by incubation with primary antibody against pSMAD2 (1: 500; #AB3849, Merck Millipore), SMAD2 (1:500; #5339, Cell Signaling, MA, United States) or β -actin (1:10.000; #A5316, Merk, St Louis, MO, United States) overnight at 4°C. After washing membranes, horseradish peroxidase-conjugated secondary antibody was added and detected by chemiluminescence system (GE Healthcare Europe GmbH).

Animals

Animal work was performed in compliance with the ARRIVE and European Union guidelines and procedures were approved by the Animal Research Ethic Committee of the University of Cordoba and the Andalusian Regional Committee for Animal Experimentation (07/04/2021/044 and 03/11/14/145). Mice were housed under constant conditions of light (12 h light/dark cycle), temperature (20 ± 2 °C) and relative humidity (40–50%), with free access to standard food and water. Handling of animals was performed in compliance with the guidelines of animal care set by the EU guidelines 86/609/EEC. Measures to improve welfare assistance and clinical status as well as endpoint criteria were established to minimize suffering and ensure animal welfare. CBD dosing was chosen based on previous experience using CBD derivatives for treating fibrosis (Del Rio et al., 2018) and literature reporting pharmacological effects of CBD *in vivo* (Rajesh et al., 2010; Cheng et al., 2014; Austrich-Olivares et al., 2022).

Mice model of skin fibrosis

BALB/c female mice aged 6–8 weeks (Envigo, Valencia, Spain) were housed in groups of nine animals and acclimatized to manipulators for a week before the experiment. Skin fibrosis was induced by daily subcutaneous (s.c.) administration of BLM (50 μg/mice; 100 μL; Mylan, Barcelona, Spain) into the back for 3 weeks. Treatments were administered for 3 weeks in parallel to fibrosis induction by daily i. p. injections of CBD (20 mg/kg; 100 μL) or vehicle (4% DMSO, 6.2% Tween 20 in saline; 100 μL). Control group received s. c. saline instead of BLM and i. p. vehicle. During the protocol, mice were evaluated daily, and weight was monitored weekly. No significant changes in weight or behaviour were observed. Mice were euthanized by cervical dislocation and the back skin was collected. Macroscopic evaluation of internal organs did not reveal any pathological changes.

Induction of CCl4-induced liver fibrosis

Six-week-old male C57BL6 mice (from Charles Rivers Laboratories; l'Arbresle, France) were acclimated for 2 weeks. When eight-week-old, hepatic fibrosis was induced by intraperitoneal (ip) injection of 1 ml/kg body weight (BW) carbon tetrachloride (CCl4) -vehicle corn oil 1:4-, twice

weekly for 2 weeks (Scholten et al., 2015). Pair aged mice received the corresponding vehicle injections. Concurrent with the induction of hepatic fibrosis, mice were daily administered *via* oral gavage with vehicle (sesame oil) or CBD (20 mg/kg) for 2 weeks. CCl4, corn oil and sesame oil were purchased from Sigma-Aldrich.

Histological evaluation

Mice were euthanized 24 h after the last BLM administration or 72 h after the last dose of CCl4. Tissue samples were collected and fixed for a period of at least 48 h in fresh 4% paraformaldehyde (PFA) in 0.1 M PBS for monitoring progression of inflammation and fibrosis by histochemical analysis. Tissues were processed for histological analysis by formalin fixation. Paraffin-embedded skin and liver sections (5 µm-thick) were stained with Masson's trichrome technique. Toluidine blue staining was used for the detection of mast cells in the skin. Liver paraffin-embedded tissue sections were stained with Picrosirius Red (PSR) staining following manufacturer's instructions (Sigma-Aldrich) in order to detected liver collagen. Tenascin C expression, T lymphocyte and macrophage infiltration were detected with anti-TNC (1:100) (MAB3138, R&D Systems, Minneapolis, MN, United States), rat anti-CD3 (1:100) (ab11089, Abcam, Cambridge, United Kingdom), or anti-F4/80 (1:50) (MCA497, Bio Rad, Hercules, CA, United States) primary antibodies overnight at 4°C, respectively. For blocking endogenous mouse IgG and non-specific background, rodent block M (RBM961, Biocare Medical, Concord, CA) was used prior anti-CD3 antibody. Then, the slides were incubated for 1 h at room temperature with the appropriate biotin-conjugated secondary antibodies; goat anti-mouse (21538, Merck-Millipore) for CD3 and goat anti-rat (BP-9400, Vector Laboratories, Burlingame, CA, United States) for TNC and F4/80. Reaction products were detected by avidin-biotin-peroxidase (Vector Laboratories), the color reaction was developed with DAB (3,3′Diaminobenzidine) chromogen (Dako, Santa Clara, CA, United States) and subsequent counterstained with hematoxylin. Samples were analysed with a Leica DM2000 microscope and pictures were taken with a Leica MC190 or Leica DFC420c cameras and analysed using ImageJ software (https://imagej.nih.gov/ij/) for quantification.

Real-time PCR

Total RNA extraction from mice skin was performed using Qiazol lysis reagent (Qiagen, Hilden, Germany) and purified with RNeasy Lipid Mini Kit (Qiagen). 1 µg of total RNA was retrotranscribed using iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using the iQTM SYBR Green Supermix (Bio-Rad) in a CFX96 Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized to GAPDH in each sample and expressed using the $2^{-\Delta\Delta Ct}$ method. The oligonucleotide primers sequence used are listed in Table 1.

TABLE 1 Primer sequence information.

Primer	Sequence
mIl6 Fw	GTATGAACAACGATGATGCACTTG
mIl6 Rv	GTATGAACAACGATGATGCACTTG
mIl1β Fw	CTCCACCTCAATGGACAGAA
mIl1β Rv	GCCGTCTTTCATTACACAGG
mTnc Fw	CCACCAAGTTTACCACAGACCT
mTnc Rv	TCCACAGATTCATAGACCAGGAG
mGapdh Fw	TGGCAAAGTGGAGATTGTTGCC
mGapdh Rv	AAGATGGTGATGGGCTTCCCG

Statistical analysis

Statistical analyses were performed using Prism software (GraphPad Prism version 8.00, GraphPad Software, La Jolla, California, United States, https://www.graphpad.com/). *In vivo* data are expressed as the mean ± SEM. Unpaired two-tailed student t test or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for parametric analysis or Kruskal–Wallis post-hoc test for non-parametric analysis were used to determine the statistical significance. The level of significance was set at $p < 0.05$. Statistical details of each experiment can be found in the figures and the respective figure legends.

Results

Cannabidiol limits the profibrotic response *in vitro*

Accumulating evidence suggest that CBD, the main non psychotropic cannabinoid present in *Cannabis sativa*, exerts a protective role in fibrotic conditions. We first investigated the *in vitro* effect of CBD against the profibrotic activity induced by TGFβ1, the primary factor driving fibrosis. NIH-3T3 cells transiently transfected with the Col1A2-luc plasmid were pretreated with CBD at different non-cytotoxic concentrations. CBD treatment resulted in a significant inhibition of TGFβ1 stimulation on Col1A2 transcription in a concentration-dependent manner (Figure 1A). However, no

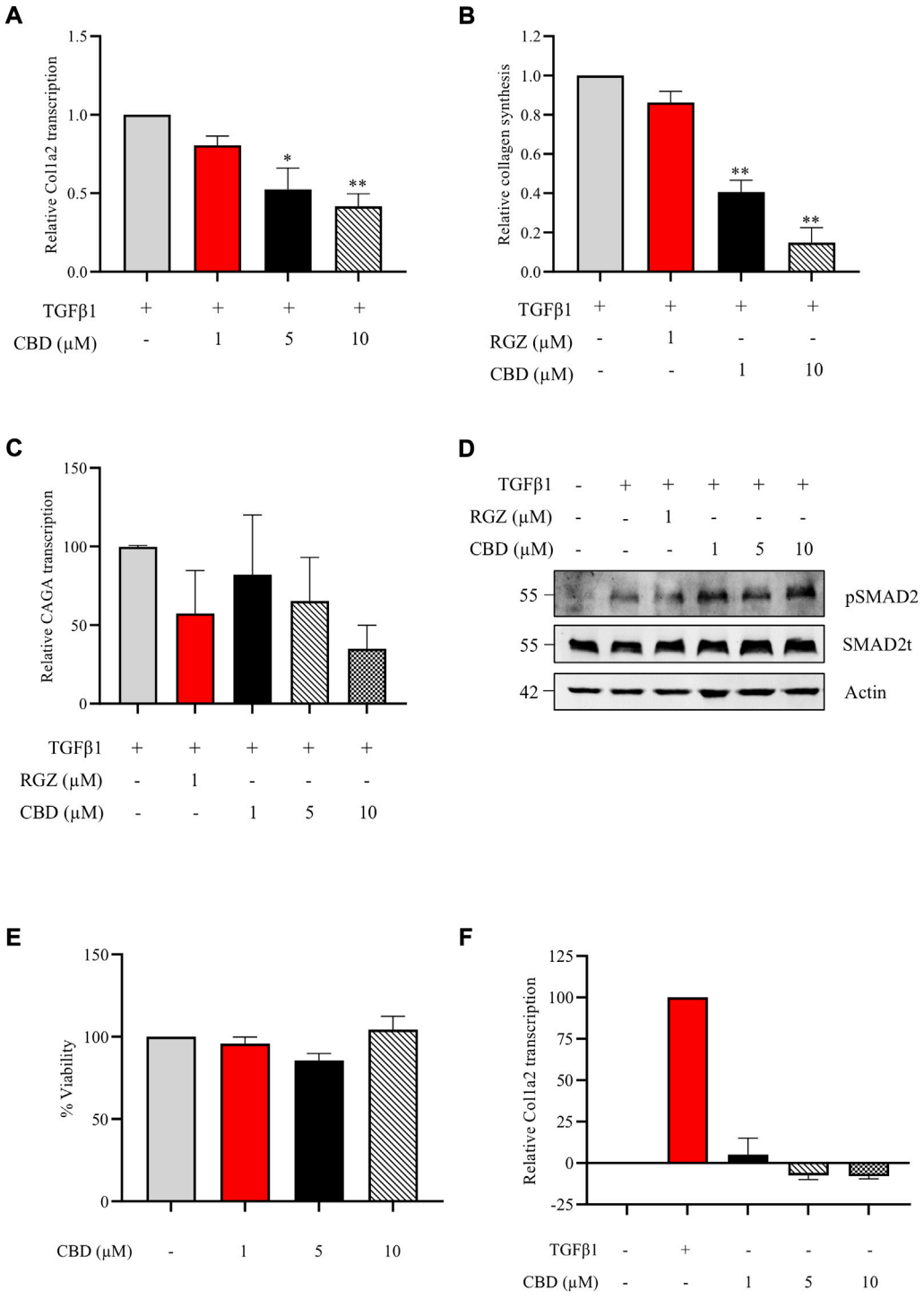

FIGURE 1
CBD inhibits collagen transcription and synthesis *in vitro* without altering SMAD2 pathway. **(A)** Col1a2 transcriptional activity in NIH-3T3 cells pretreated with CBD for 1 h and stimulated with TGFβ1 for the following 24 h (n = 4) **(B)** Effect of CBD pretreatment on soluble collagen release by human dermal fibroblast using the Sircol Assay. Fibroblasts were pretreated with CBD for 1 h and stimulated with TGFβ1 for 48 h and collagen was measured in the culture media (n = 3). **(C)** Effect of CBD on SMAD-dependent transcriptional activity. NIH-3T3 cells were transfected with the CAGA-Luc plasmid, preincubated with the indicated concentrations of CBD for 1 h and stimulated with TGFβ1 (10 ng/ml) for 6 h. Then, cells were lysed for luciferase activity (n = 3). Data are expressed as mean ± S.D. relative to control cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs TGFβ1-treated cells. **(D)** CBD effect on SMAD2 phosphorylation. NHDF cells were incubated in low serum conditions (1% FBS) for 24 h. Then cells were pretreated with CBD or Rosiglitazone (RGZ) for 1 h and stimulated with TGFβ 1 (10 ng/ml) for 2 h. **(E)** Viability of NIH3T3 cells treated with different concentrations of CBD after 24 h expressed as percentage taking control as 100% (n = 3). **(F)** Effect of CBD treatment on Col1a2 transcriptional activity in NIH-3T3 cells (n = 3).

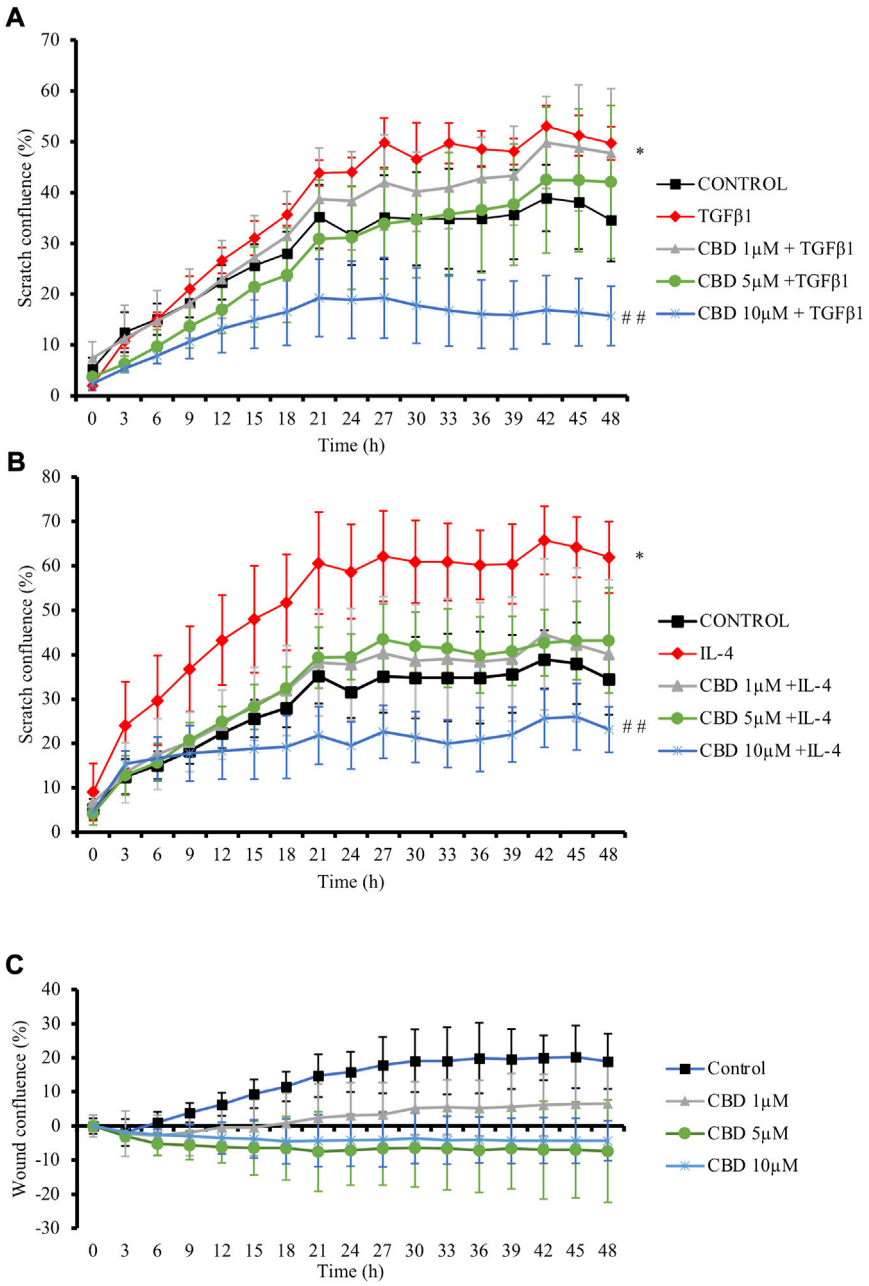

FIGURE 2
CBD reduces *in vitro* fibroblast migration. NHDF monolayers were scratched and treated with TGFβ1 **(A)** or IL-4 **(B)** in the presence of CBD or with CBD alone **(C)**. Results are expressed as percentage of closure (confluence) ± SD (n = 3). *$p < 0.05$ *versus* control; $^{\#\#}p < 0.01$ vs TGFβ1-treated cells.

effects were found when NIH3T3 cells were treated with CBD alone (Figure 1F). Next, we studied the resulting collagen synthesis using NHDFs stimulated with TGFβ1 for 48 h. NHDFs preincubated with increasing concentrations of CBD from 1 h prior to the addition of TGFβ1 showed a significant reduction in collagen release (Figure 1B). Also, we tested the capacity of CBD to interfere with upstream or downstream TGFβ signaling pathways in comparison with Rosiglitazone (RGZ), a PPARγ agonist. We found that both compounds were able to inhibit the transcriptional activity driven by SMAD proteins in CAGA-Luc transfected NIH-3T3 cells (Figure 1C). However, neither CBD nor RGZ inhibited TGFβ-induced SMAD2 phosphorylation (Figure 1D). CBD has been shown to activate PPARγ and our results are consistent with the view

10.3389/fphar.2022.981817

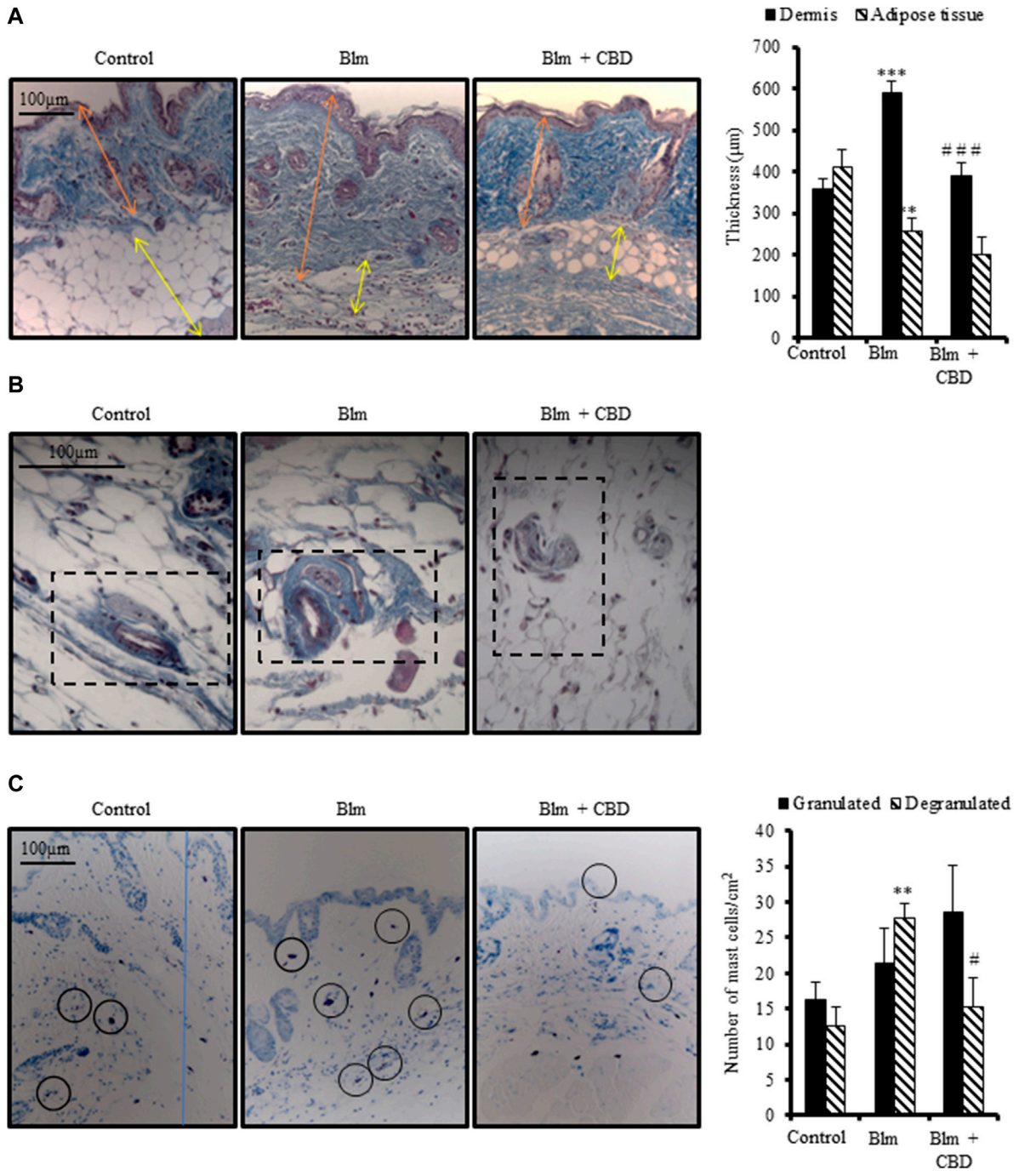

FIGURE 3
Treatment with CBD reduced skin fibrosis development in a mouse model of systemic sclerosis. Skin fibrosis was induced by daily subcutaneous administration of BLM for 3 weeks and mice were treated in parallel with CBD or vehicle i. p. injections (n = 9 mice per group). **(A)** Representative images of Masson's trichrome stained skin sections and quantification of dermal and subcutaneous adipose layers thickness. **(B)** Masson's trichrome staining showing collagen around skin blood vessels. **(C)** Representative images of toluidine blue stained skin sections showing mast cell degranulation and their corresponding quantification. Data represent the mean ± SEM. $^{*}p < 0.05$; $^{***}p < 0.001$ vs control mice. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs BLM-treated mice.

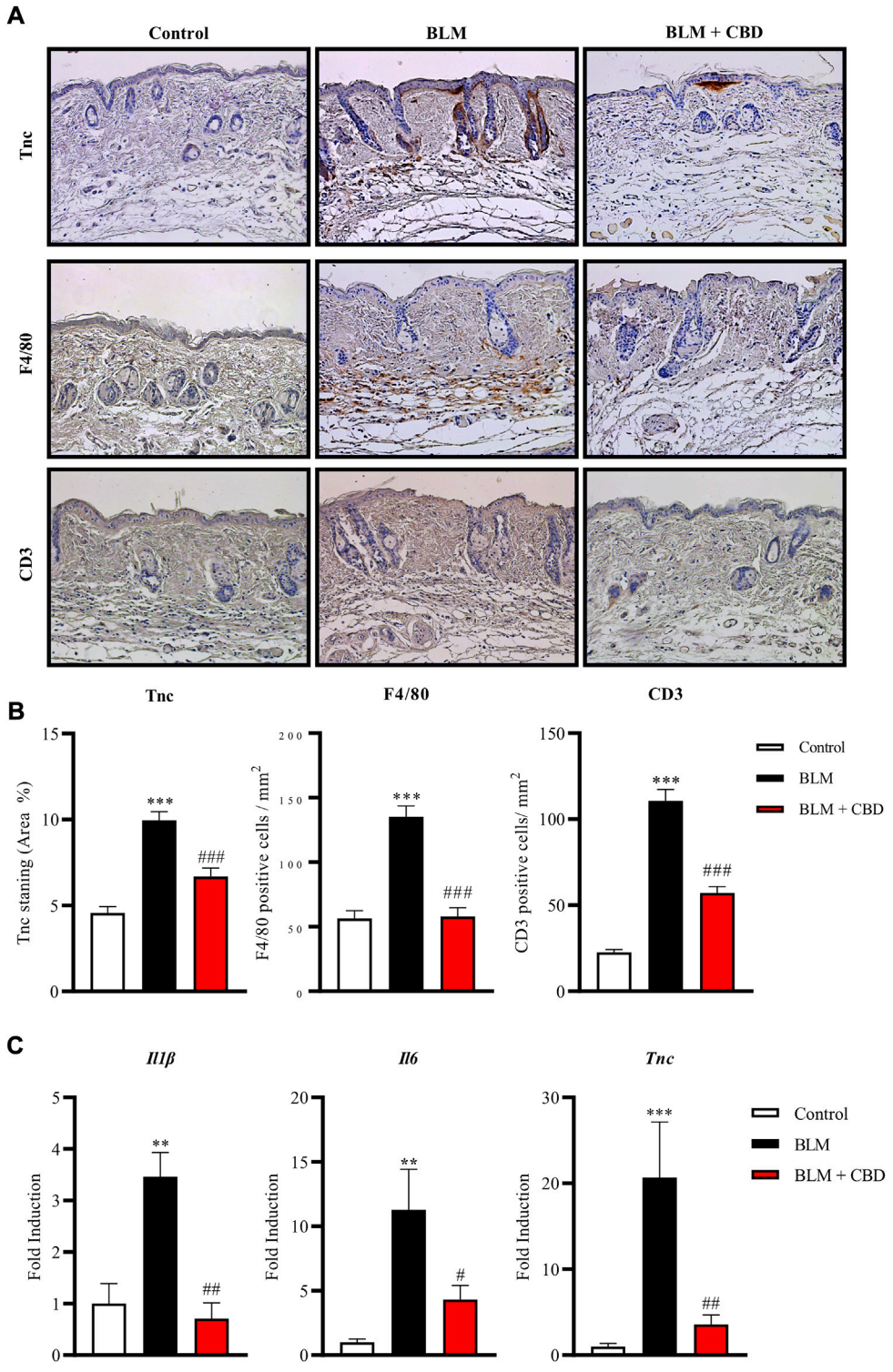

FIGURE 4
Treatment with CBD reduced skin fibrosis and inflammation in a mouse model of systemic sclerosis. **(A)** Representative images of Tnc, F4/80 and CD3 immunohistochemistry. **(B)** Quantifications of Tnc, F4/80 and CD3 expression were performed with Image J software. Values are expressed as mean ± SEM. ***$p < 0.001$ vs control mice. ### $p < 0.001$ vs BLM-treated mice. **(C)** Gene expression of inflammatory and fibrotic markers including *Il-6*, *Il-1β* and *Tnc* was significantly downregulated in CBD-treated mice compared with BLM mice. Data represent the mean ± SEM (n = four to six animals per group). **$p < 0.01$; ***$p < 0.001$ vs control mice. #$p < 0.05$; ## $p < 0.01$ vs BLM-treated mice.

that PPARγ agonists inhibit the expression of several TGFβ-activated genes by acting at the transcriptional level.

Increased speed of resident cells migration relative to the normal tissues is also promoted by profibrotic cytokines and plays a crucial role during fibrogenesis (Kai et al., 2016). Then, the effects of CBD on fibroblast migration were evaluated in NHDFs cells monolayer, which were scratched and treated with CBD in parallel to TGFβ1 or IL-4 for 48 h. TGFβ1 (Figure 2A) and IL-4 (Figure 2B) fostered cell confluence in the scratch while treatment with CBD at the highest non-cytotoxic concentration significantly reduced fibroblast migration by 35% in both conditions. Additionally, CBD alone did not exert any effect on scratch confluence (Figure 2C).

Effect of cannabidiol on skin fibrosis induced by BLM administration

Repeated subcutaneous administration of BLM is the most common model to study skin fibrosis. As expected, female BALB/c mice injected with BLM for 3 weeks developed skin thickening accompanied by a reduction of subcutaneous adipose layer. Treatment with CBD for 3 weeks during fibrosis induction significantly prevented dermal thickness secondary to collagen deposition (Figure 3A). However, CBD was not able to avoid significantly the reduction of subcutaneous adipose tissue. In addition, BLM induced vascular lesions manifested by the thickening of the vascular wall. CBD-treated mice showed reduced collagenic bundles around blood vessels to levels comparable to the control group (Figure 3B).

It is well known that the number of mast cells increase during the development of fibrosis in several tissues and early fibrogenesis associates with marked cell degranulation (Yamamoto and Nishioka, 2005). Accordingly, the lesioned skin of BLM treated mice presented a higher number of degranulated mast cells. CBD administration led to one-fold decrease in the number of degranulated mast cells in the skin (Figure 3C). Next, we studied the levels of Tenascin C (Tnc) in the skin. Tnc is involved in modulating the extracellular matrix (ECM) composition and is known to stimulate the profibrotic response and upregulate the expression of type I collagen by fibroblasts (Bhattacharyya et al., 2016). Local BLM administration in the skin elevated Tnc stained area while CBD treatment significantly diminished this Tnc upregulation (Figures 4A,B). In BLM-challenged mice a significant increase in the recruitment of inflammatory cells was observed. Mice treated with CBD exhibited a significant reduction of macrophages and T cells infiltration in the skin (Figures 4A,B). Consequently, the expression of cytokines associated with inflammation and fibrosis was analysed. As expected, BLM promoted Il1β, Il6 and Tnc expression in the skin, and intraperitoneal treatment with CBD prevented the proinflammatory boost induced by BLM administration (Figure 4C).

Effect of cannabidiol on CCl4-induced liver fibrosis

Next, we were also interested to study the antifibrotic and antiinflammatory effects of orally delivered CBD in another model of fibrosis. Eight-week-old C57Bl6J mice were randomized to healthy control group, CCl4-treated group in combination with vehicle (CCl4-vehicle) and CCl4-treated group in combination with 20 mg/kg of CBD. In addition, a group of mice treated with CBD alone was also included for reference purposes. As reported previously, PSR staining revealed that CCl4 treatment significantly induced accumulation of collagen in the liver compared to the healthy group, and CBD greatly reduced the fibrotic liver area compared to CCl4-vehicle mice, reaching levels close to the control group (Figure 5). Furthermore, CCl4 treatment also increased the protein level of Tnc, which is also an early fibrotic marker in liver (Kasprzycka et al., 2015), while CBD co-treatment led to a 2-fold reduction of protein levels of Tnc compared to CCl4-vehicle mice. Importantly, CBD in the absence of CCl4 did not induce fibrosis. In hepatocytes, cytochrome P450 proteins (CYP2E1) metabolize endogenous substrates as well as xenobiotic compounds, including carbon tetrachloride. In this sense, hepatic transformation of CCl4 produces trichloromethyl radicals, which trigger several free radical reactions and contribute to the induction of an inflammatory response. Immunostaining for CD3 showed that CCl4 induces a significant (3.7-fold) increase in $CD3^+$ T lymphocyte infiltration into the liver compared to control mice, which was reduced in animals treated with CBD (2.9 fold-increase; Figure 6). Similarly, liver sections in CCl4-vehicle mice had 2.8-fold higher infiltration of macrophages than healthy animals, as seen by F4/80 staining (Figure 6), and CBD induced a significant reduction (1.9-fold) in the magnitude of liver infiltration of F4/80 + cells compared to CCl4-vehicle group.

Discussion

Different reports have evidenced the antifibrotic effects of CBD *in vitro* and in various preclinical animal models of fibrotic diseases (Rajesh et al., 2010; Lee et al., 2016; De Ternay et al., 2019; Vuolo et al., 2019). To the best of our knowledge the antifibrotic effects of CBD on skin and liver fibrosis have not been studied previously. Herein, we have shown that treatment with CBD not only alleviated organ fibrosis but also attenuated inflammation, which is a major driving force for fibrosis development. We also found that CBD inhibits collagen gene transcription and synthesis and fibroblast migration *in vitro*.

The antifibrotic effects of CBD are still elusive and could be attributed to different signaling pathways. CBD weakly binds to the orthosteric binding sites of CB_1 (Ki values in the micromolar range) and CB_2 (Ki in the high nanomolar range) receptors

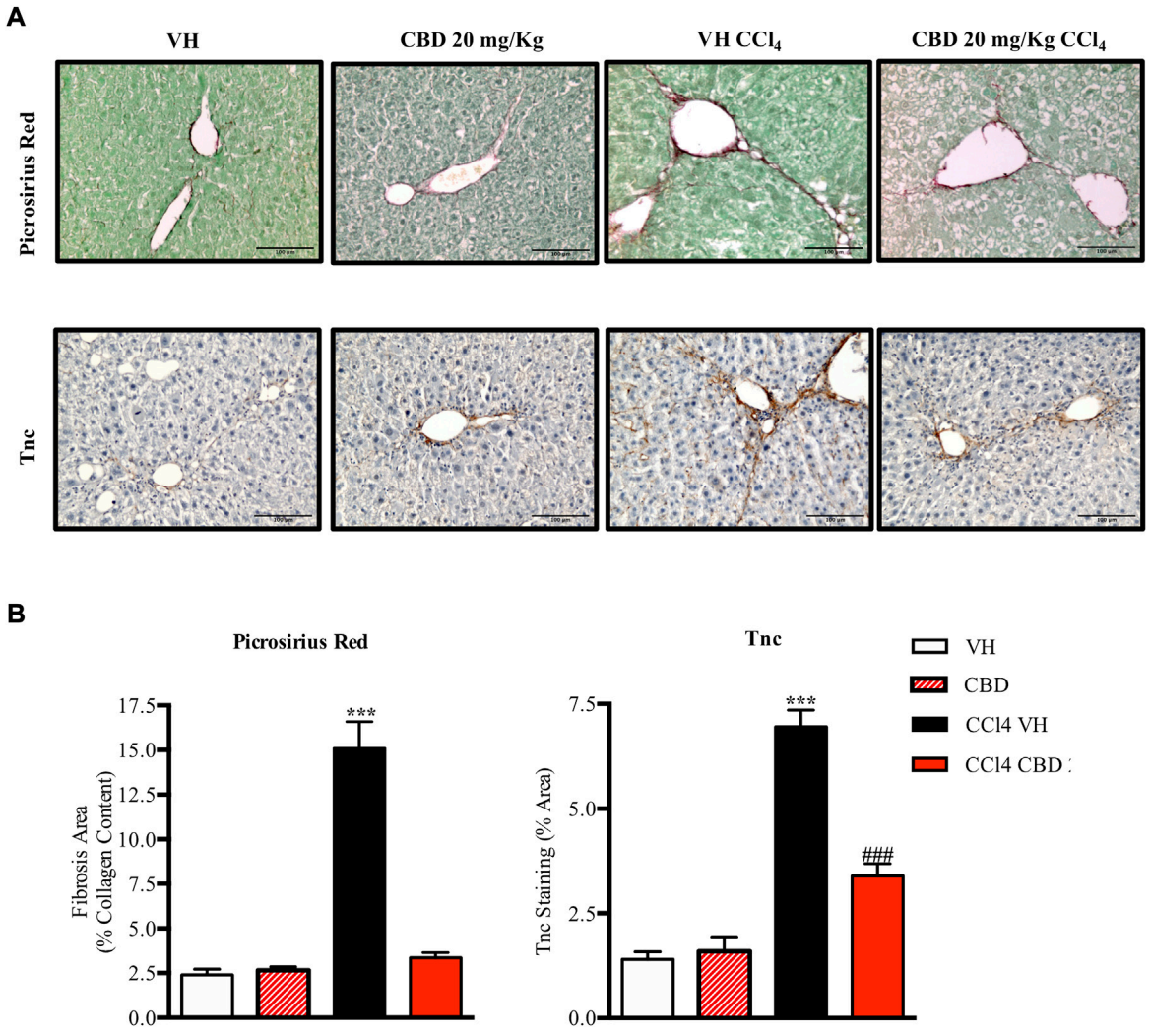

FIGURE 5
CBD reduces liver fibrosis induced by CCl4. **(A)** Representative images of collagen staining in the liver using picrosirius red dye (upper panel) or immunostaining with Tnc (bottom panel) from control, CBD, CCl4-vehicle, and CCl4 + CBD mice. Scale bars represent 100 and 50 μm, respectively. **(B)** Quantification of positive collagen content (left panel) and Tnc staining (right panel), expressed as a percentage of the total liver area. Values are expressed as mean ± SEM (n = 6 animals per group). ***$p < 0.001$ vs. control group; $^{###}p < 0.001$ vs. CCl4 group (ANOVA followed by Tukey's test).

(Rosenthaler et al., 2014). However, CBD is a negative allosteric modulator (NAM) for CB_1 receptor (Laprairie et al., 2015) and a CB_2 inverse agonist (Thomas et al., 2007; Martinez-Pinilla et al., 2017). There is evidence that CB_1 receptor activation is profibrotic in different fibrotic conditions (reviewed by (Rio et al., 2018). For instance, CB_1 deficient mice were protected against experimental fibrosis and the activation of CB_1 exacerbated mouse experimental fibrosis induced by BLM (Marquart et al., 2010). Therefore, CBD could exert its antifibrotic activity, at least in part, by acting as a NAM on CB_1 receptor.

CBD's multimodal pharmacologic profile further includes other receptors such as PPARγ that may explain its antifibrotic and antiinflammatory effects (O'Sullivan et al., 2009). PPARγ is a negative regulator of the inflammatory response, inhibits collagen synthesis and blunts fibrogenesis in a wide variety of organs (Dantas et al., 2015; Li et al., 2021). Skin fibrosis is associated with a progressive loss of PPARγ expression and activation of PPARγ using rosiglitazone reduced inflammation and dermal fibrosis (Wu et al., 2009). Activation of PPARγ by rosiglitazone also ameliorated bile duct ligation-induced liver fibrosis (Wei et al., 2019). Moreover, PPARγ viral overexpression has been reported to reduce CCl4-induced liver fibrosis in rats (Wang et al., 2011).

It is well known that BLM and CCl4 can induce cell damage and organ fibrosis through inflammatory processes, attributed in part to

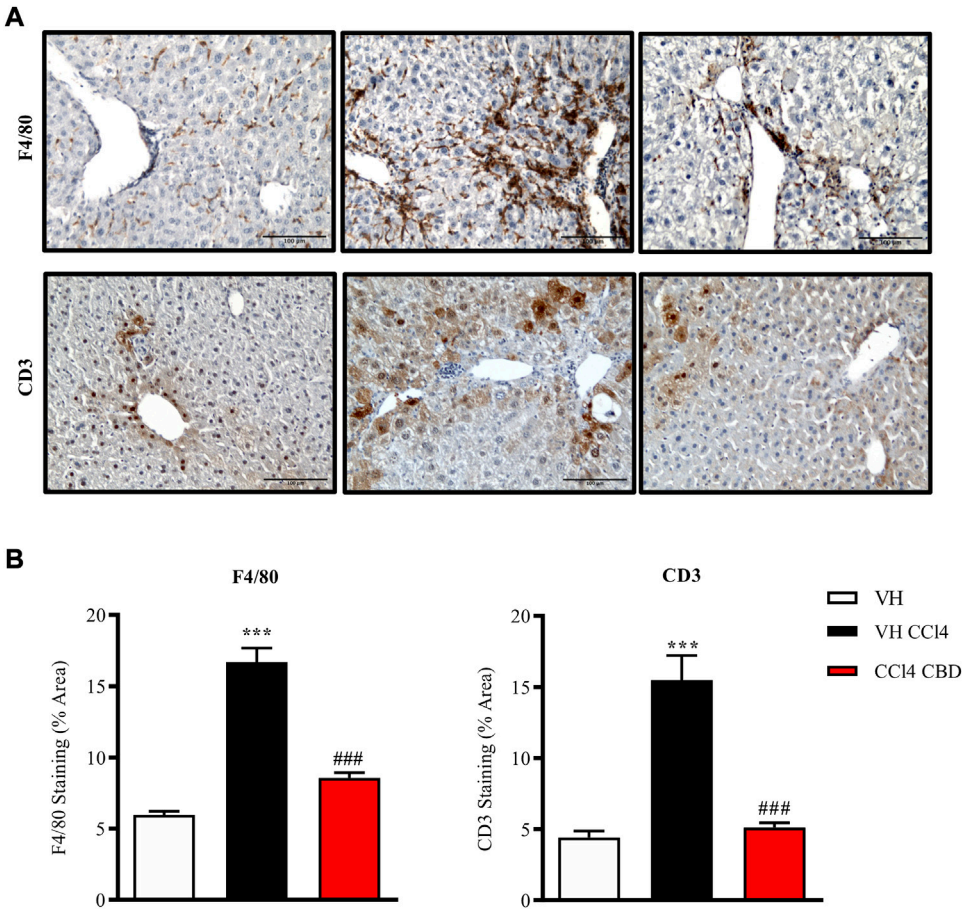

FIGURE 6
CBD reduces hepatic inflammation induced by CCl4. **(A)** Representative images of immunostaining for F4/80 (macrophage-specific marker; upper panel) and CD3 (lymphocyte-specific marker; bottom panel) from control, CCl4-vehicle, and CCl4 + CBD mice. Scale bars represent 100 μm. **(B)** Quantification of (percentage of total liver area) for F4/80 and CD3 (left and right panel respectively). Values are expressed as mean ± SEM (n = 6 animals per group). ***$p < 0.001$ vs. control group; ###$p < 0.001$ vs. CCl4 group (ANOVA followed by Tukey's test).

their free radical-promoting ability. CBD beneficial effects can be also explained in part by its potent antioxidant properties. It is plausible that the antioxidant activity of CBD can reduce the generation of the reactive oxygen species (ROS) in these mouse models and therefore limit the pathological manifestations. Accordinlgy, classical antioxidants, like phenolic compounds, were effective in preventing tissue inflammation and fibrosis (Pan et al., 2018; Shariati et al., 2019). Unlike other cannabinoids, CBD contains two phenolic groups. Direct antioxidant properties of CBD may be related to the location and surroundings of the hydroxyl groups in the phenolic ring (Atalay et al., 2019). Two main mechanisms can explain the protective role of CBD as an antioxidant: electron (the antioxidant compound give an electron to the radical) and hydrogen (the free radical removes a hydrogen atom from the antioxidant) abstraction (Silva, 2017). CBD also modulates antioxidant gene expression by inducing the activation of Nrf2, the master regulator of the antioxidant response (Singer et al., 2015). Recent report indicated that CBD is a weak inducer of Keap1/Nrf2 activation but a potent Bach1 inhibitor (Casares et al., 2020). Bach1 is a transcriptional repressor of Nrf2 and its inactivation by CBD mediated the expression of heme oxygenase 1 (HMOX1), an essential enzyme in oxidative degradation of heme group (Casares et al., 2020). Interestingly, HMOX-1 is thought to provide antinflammatory and antibrotic activity in different preclinical models (Barikbin et al., 2012; Wang et al., 2013).

We have studied the effect of intraperitoneal and oral CBD, administered since the initial induction of fibrosis, and therefore acting simultaneously to the development of the disease, which includes $CD3^+$ and $F4/80^+$ immune cell infiltration prior to collagen deposition. CBD antioxidant profile, together with its actions on PPARγ, Adenosine A_{2A} receptor and TRPV1 (Carrier et al., 2006) (Feng et al., 2017), are likely to be targeting the major pro-inflammatory and pro-oxidant signalling pathways involved in the initial stages of tissue injury leading to abnormal remodelling and, subsequently, fibrosis (reviewed by (Sunda and Arowolo, 2020)). The same was observed for Δ^9-THCA,

which only showed antifibrotic effect in the liver when the treatment started at the same time of CCl_4 challenge (Carmona-Hidalgo et al., 2021). However, although we have not tested the effect of CBD on preestablished fibrosis, a therapeutic effect can not be discarded given that CBD directly inhibited fibroblast migration and collagen transcription *in vitro*.

CBD metabolism has been demonstrated *in vitro* and *in vivo* (Martin et al., 1976; Brown, 1990). The route of administration considerably affects CBD pharmacokinetics. Oral bioavailability of CBD is low across species, known to be approximately 6% in humans (Nakano et al., 2019). We have demonstrated that both intraperitoneal and oral treatments are able to reduce inflammation and fibrosis. Oral administration usually delays serum peak concentration and sometimes show a second peak due to enterohepatic circulation (Hlozek et al., 2017). In the liver, CBD is metabolized by CYP2C19 and CYP3A4 isozymes and undergoes hydroxylation at multiple sites and further oxidations (Anderson and Chan, 2016). About a hundred CBD metabolites have been identified. Main CBD metabolites are hydroxylated 7-COOH derivatives of CBD but little is known about their pharmacological activity. *In vitro* biological activities of CBD metabolites include antiangiogenic, anticancer and antiinflammatory properties (Ujvary and Hanus, 2016). In the event of CBD metabolites reaching a pharmacologically relevant concentration, direct or indirect contribution to the observed therapeutic effect of CBD *in vivo* can not be excluded.

Altough we have found that CBD alone does not induce liver fibrosis, a major concern of CBD use is the risk of hepatotoxicity. Acute oral administration of a concentrated CBD-enriched extract was reported to produce hepatotoxicity indicated by marked increases in serum ALT, AST, and total bilirubin. However, the concentration used (2,460 mg/kg) is not therapeutically applicable (Ewing et al., 2019). In the same study, subacute 2-week administration of the extract revealed no measurable toxicological responses associated with liver injury in mice orally gavaged with CBD up to 184.5 mg/kg (Ewing et al., 2019). Several studies have reported that pure CBD can be hepatoprotective in mice (Magen et al., 2009; Avraham et al., 2011; Wang et al., 2017). The most common CBD therapeutic dose used for seizure disorders is 20 mg/kg/day. Human studies addressing potential CBD hepatotoxicity events of Epidiolex® included patients concomitantly taking other medications, such as valproic acid which is known for its hepatotoxicity. Therefore, whether CBD is endowed with adverse hepatic effects is unclear (Devinsky et al., 2016; Devinsky et al., 2017), and, overall, human studies indicate limited hepatic effects upon continued use of CBD (Stohs and Ray, 2020).

Conclusion

We have shown that both intraperitoneal and oral administration of CBD exerts potent anti-inflammatory and antifibrotic activities *in vivo*. Moreover, CBD blunted the effects of fibrogenic stimuli on cultured fibroblast. We have shown for the first time CBD efficacy in reducing BLM-induced dermal fibrosis and CCl4-induced hepatic fibrosis. Given the broad spectrum of CBD targets, *in vivo* effects might be mediated by a plethora of molecular mechanisms, directly or through its metabolites. Further studies are needed for dissecting the exact contribution of each mechanism involved.

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 Animal Research Ethic Committee of the University of Cordoba and the Andalusian Regional Committee for Animal Experimentation.

Author contributions

FR-P, CD, MT-S, BF and EM participated in research design. The experiments were performed by FR-P, CD and MP. Data were analysed by FR-P, CD, MT-S, BF and EM. CD, FR-P, BF, MT-S and EM wrote or contributed to the writing of the manuscript. In addition, CD, MT-S and EM reviewed the data and discussed the manuscript. All authors have read and approved the final version of the manuscript.

Funding

This work was partially supported by VivaCell Biotechnology España (Cordoba, Spain). This funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. All authors declare no other competing interests.

Conflict of interest

Authors FR-P and MP are employed by VivaCell Biotechnology España.

BF is employed by VivaCell Biotechnology GmbH.

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.

Publisher's note

References

Akhmetshina, A., Dees, C., Busch, N., Beer, J., Sarter, K., Zwerina, J., et al. (2009). The cannabinoid receptor CB2 exerts antifibrotic effects in experimental dermal fibrosis. *Arthritis Rheum.* 60 (4), 1129–1136. doi:10.1002/art.24395

Anderson, G. D., and Chan, L. N. (2016). Pharmacokinetic drug interactions with tobacco, cannabinoids and smoking cessation products. *Clin. Pharmacokinet.* 55 (11), 1353–1368. doi:10.1007/s40262-016-0400-9

Atalay, S., Jarocka-Karpowicz, I., and Skrzydlewska, E. (2019). Antioxidative and anti-inflammatory properties of cannabidiol. *Antioxidants (Basel)* 9 (1), E21. doi:10.3390/antiox9010021

Austrich-Olivares, A., Garcia-Gutierrez, M. S., Illescas, L., Gasparyan, A., and Manzanares, J. (2022). Cannabinoid CB1 receptor involvement in the actions of CBD on anxiety and coping behaviors in mice. *Pharm. (Basel)* 15 (4), 473. doi:10.3390/ph15040473

Avraham, Y., Grigoriadis, N., Poutahidis, T., Vorobiev, L., Magen, I., Ilan, Y., et al. (2011). Cannabidiol improves brain and liver function in a fulminant hepatic failure-induced model of hepatic encephalopathy in mice. *Br. J. Pharmacol.* 162 (7), 1650–1658. doi:10.1111/j.1476-5381.2010.01179.x

Barikbin, R., Neureiter, D., Wirth, J., Erhardt, A., Schwinge, D., Kluwe, J., et al. (2012). Induction of heme oxygenase 1 prevents progression of liver fibrosis in Mdr2 knockout mice. *Hepatology* 55 (2), 553–562. doi:10.1002/hep.24711

Bhattacharyya, S., Wang, W., Morales-Nebreda, L., Feng, G., Wu, M., Zhou, X., et al. (2016). Tenascin-C drives persistence of organ fibrosis. *Nat. Commun.* 7, 11703. doi:10.1038/ncomms11703

Brown, D. H. N. K. (1990). *In vitro* metabolism of cannabidiol in seven common laboratory mammals. *Res. Commun. Subst. abuse* 11, 27–37.

Carmona-Hidalgo, B., Gonzalez-Mariscal, I., Garcia-Martin, A., Prados, M. E., Ruiz-Pino, F., Appendino, G., et al. (2021). Δ9-Tetrahydrocannabinolic Acid markedly alleviates liver fibrosis and inflammation in mice. *Phytomedicine.* 81, 153426. doi:10.1016/j.phymed.2020.153426

Carrier, E. J., Auchampach, J. A., and Hillard, C. J. (2006). Inhibition of an equilibrative nucleoside transporter by cannabidiol: A mechanism of cannabinoid immunosuppression. *Proc. Natl. Acad. Sci. U. S. A.* 103 (20), 7895–7900. doi:10.1073/pnas.0511232103

Casares, L., Garcia, V., Garrido-Rodriguez, M., Millan, E., Collado, J. A., Garcia-Martin, A., et al. (2020). Cannabidiol induces antioxidant pathways in keratinocytes by targeting BACH1. *Redox Biol.* 28, 101321. doi:10.1016/j.redox.2019.101321

Chen, Y., Yu, X., He, Y., Zhang, L., Huang, X., Xu, X., et al. (2017). Activation of A2aR attenuates bleomycin-induced pulmonary fibrosis via the SDF-1/CXCR4 axis-related pathway. *Am. J. Transl. Res.* 9 (9), 4125–4136.

Cheng, D., Low, J. K., Logge, W., Garner, B., and Karl, T. (2014). Chronic cannabidiol treatment improves social and object recognition in double transgenic APPswe/PS1E9 mice. *Psychopharmacol. Berl.* 231 (15), 3009–3017. doi:10.1007/s00213-014-3478-5

Dantas, A. T., Pereira, M. C., de Melo Rego, M. J., da Rocha, L. F., Jr., Pitta Ida, R., Marques, C. D., et al. (2015). The role of PPAR gamma in systemic sclerosis. *PPAR Res.* 2015, 124624. doi:10.1155/2015/124624

De Ternay, J., Naassila, M., Nourredine, M., Louvet, A., Bailly, F., Sescousse, G., et al. (2019). Therapeutic prospects of cannabidiol for alcohol use disorder and alcohol-related damages on the liver and the brain. *Front. Pharmacol.* 10, 627. doi:10.3389/fphar.2019.00627

Del Rio, C., Cantarero, I., Palomares, B., Gomez-Canas, M., Fernandez-Ruiz, J., Pavicic, C., et al. (2018). VCE-004.3, a cannabidiol aminoquinone derivative, prevents bleomycin-induced skin fibrosis and inflammation through PPARgamma- and CB2 receptor-dependent pathwaysThe cannabinoid quinol VCE-004.8 alleviates bleomycin-induced scleroderma and exerts potent antifibrotic effects through peroxisome proliferator-activated receptor-gamma and CB2 pathways. *Sci. Rep.Sci Rep.* 1756 (19), 381321703–381323831. doi:10.1111/bph.1445010.1038/srep21703

Devinsky, O., Cross, J. H., and Wright, S. (2017). Trial of cannabidiol for drug-resistant seizures in the dravet syndrome. *N. Engl. J. Med.* 377 (7), 699–700. doi:10.1056/NEJMc1708349

Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., et al. (2016). Cannabidiol in patients with treatment-resistant epilepsy: An open-label interventional trial. *Lancet. Neurol.* 15 (3), 270–278. doi:10.1016/S1474-4422(15)00379-8

Devinsky, O., Patel, A. D., Thiele, E. A., Wong, M. H., Appleton, R., Harden, C. L., et al. (2018). Randomized, dose-ranging safety trial of cannabidiol in Dravet syndrome. *Neurology* 90 (14), e1204–e1211. doi:10.1212/WNL.0000000000005254

Ewing, L. E., Skinner, C. M., Quick, C. M., Kennon-McGill, S., McGill, M. R., Walker, L. A., et al. (2019). Hepatotoxicity of a cannabidiol-rich cannabis extract in the mouse model. *Molecules* 24 (9), E1694. doi:10.3390/molecules24091694

Feng, J., Yang, P., Mack, M. R., Dryn, D., Luo, J., Gong, X., et al. (2017). Sensory TRP channels contribute differentially to skin inflammation and persistent itch. *Nat. Commun.* 8 (1), 980. doi:10.1038/s41467-017-01056-8

Garcia-Martin, A., Garrido-Rodriguez, M., Navarrete, C., Del Rio, C., Bellido, M. L., Appendino, G., et al. (2018). EHP-101, an oral formulation of the cannabidiol aminoquinone VCE-004.8, alleviates bleomycin-induced skin and lung fibrosis. *Biochem. Pharmacol.* 157, 304–313. doi:10.1016/j.bcp.2018.07.047

Ghosh, A. K., Bhattacharyya, S., Wei, J., Kim, S., Barak, Y., Mori, Y., et al. (2009). Peroxisome proliferator-activated receptor-gamma abrogates Smad-dependent collagen stimulation by targeting the p300 transcriptional coactivator. *FASEB J.* 23 (9), 2968–2977. doi:10.1096/fj.08-128736

Gonzalez-Mariscal, I., Carmona-Hidalgo, B., Winkler, M., Unciti-Broceta, J. D., Escamilla, A., Gomez-Canas, M., et al. (2021). (+)-trans-Cannabidiol-2-hydroxy pentyl is a dual CB1R antagonist/CB2R agonist that prevents diabetic nephropathy in mice. *Pharmacol. Res.* 169, 105492. doi:10.1016/j.phrs.2021.105492

Hernandez-Gea, V., and Friedman, S. L. (2011). Pathogenesis of liver fibrosis. *Annu. Rev. Pathol.* 6 (1), 425–456. doi:10.1146/annurev-pathol-011110-130246

Hlozek, T., Uttl, L., Kaderabek, L., Balikova, M., Lhotkova, E., Horsley, R. R., et al. (2017). Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion *in vivo* of CBD to THC. *Eur. Neuropsychopharmacol.* 27 (12), 1223–1237. doi:10.1016/j.euroneuro.2017.10.037

Kai, F., Laklai, H., and Weaver, V. M. (2016). Force matters: Biomechanical regulation of cell invasion and migration in disease. *Trends Cell Biol.* 26 (7), 486–497. doi:10.1016/j.tcb.2016.03.007

Kasprzycka, M., Hammarstrom, C., and Haraldsen, G. (2015). Tenascins in fibrotic disorders-from bench to bedside. *Cell adh. Migr.* 9 (1-2), 83–89. doi:10.4161/19336918.2014.994901

Kendall, R. T., and Feghali-Bostwick, C. A. (2014). Fibroblasts in fibrosis: Novel roles and mediators. *Front. Pharmacol.* 5, 123. doi:10.3389/fphar.2014.00123

Laprairie, R. B., Bagher, A. M., Kelly, M. E., and Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br. J. Pharmacol.* 172 (20), 4790–4805. doi:10.1111/bph.13250

Lee, W. S., Erdelyi, K., Matyas, C., Mukhopadhyay, P., Varga, Z. V., Liaudet, L., et al. (2016). Cannabidiol limits T cell-mediated chronic autoimmune myocarditis: Implications to autoimmune disorders and organ transplantation. *Mol. Med.* 22, 136–146. doi:10.2119/molmed.2016.00007

Li, J., Guo, C., and Wu, J. (2021). The agonists of peroxisome proliferator-activated receptor-gamma for liver fibrosis. *Drug Des. devel. Ther.* 15, 2619–2628. doi:10.2147/DDDT.S310163

Li, X., Han, D., Tian, Z., Gao, B., Fan, M., Li, C., et al. (2016). Activation of cannabinoid receptor type II by AM1241 ameliorates myocardial fibrosis via nrf2-mediated inhibition of TGF-β1/smad3 pathway in myocardial infarction mice. *Cell. Physiol. biochem.* 39 (4), 1521–1536. doi:10.1159/000447855

Magen, I., Avraham, Y., Ackerman, Z., Vorobiev, L., Mechoulam, R., and Berry, E. M. (2009). Cannabidiol ameliorates cognitive and motor impairments in mice with bile duct ligation. *J. Hepatol.* 51 (3), 528–534. doi:10.1016/j.jhep.2009.04.021

Marquart, S., Zerr, P., Akhmetshina, A., Palumbo, K., Reich, N., Tomcik, M., et al. (2010). Inactivation of the cannabinoid receptor CB1 prevents leukocyte infiltration and experimental fibrosis. *Arthritis Rheum.* 62 (11), 3467–3476. doi:10.1002/art.27642

Martin, B., Nordqvist, M., Agurell, S., Lindgren, J. E., Leander, K., and Binder, M. (1976). Identification of monohydroxylated metabolites of cannabidiol formed by rat liver. *J. Pharm. Pharmacol.* 28 (4), 275–279. doi:10.1111/j.2042-7158.1976.tb04152.x

Martinez-Pinilla, E., Varani, K., Reyes-Resina, I., Angelats, E., Vincenzi, F., Ferreiro-Vera, C., et al. (2017). Binding and signaling studies disclose a potential allosteric site for cannabidiol in cannabinoid CB2 receptors. *Front. Pharmacol.* 8, 744. doi:10.3389/fphar.2017.00744

Munoz-Luque, J., Ros, J., Fernandez-Varo, G., Tugues, S., Morales-Ruiz, M., Alvarez, C. E., et al. (2008). Regression of fibrosis after chronic stimulation of cannabinoid CB2 receptor in cirrhotic rats. *J. Pharmacol. Exp. Ther.* 324 (2), 475–483. doi:10.1124/jpet.107.131896

Nakano, Y., Tajima, M., Sugiyama, E., Sato, V. H., and Sato, H. (2019). Development of a novel nano-emulsion formulation to improve intestinal absorption of cannabidiol. *Med. Cannabis Cannabinoids* 2 (1), 35–42. doi:10.1159/000497361

O'Sullivan, S. E., Sun, Y., Bennett, A. J., Randall, M. D., and Kendall, D. A. (2009). Time-dependent vascular actions of cannabidiol in the rat aorta. *Eur. J. Pharmacol.* 612 (1-3), 61–68. doi:10.1016/j.ejphar.2009.03.010

Pan, Y., Long, X., Yi, R., and Zhao, X. (2018). Polyphenols in liubao tea can prevent CCl_4-Induced hepatic damage in mice through its antioxidant capacities. *Nutrients* 10 (9), E1280. doi:10.3390/nu10091280

Pryimak, N., Zaiachuk, M., Kovalchuk, O., and Kovalchuk, I. (2021). The potential use of cannabis in tissue fibrosis. *Front. Cell Dev. Biol.* 9, 715380. doi:10.3389/fcell.2021.715380

Rajesh, M., Mukhopadhyay, P., Batkai, S., Patel, V., Saito, K., Matsumoto, S., et al. (2010). Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy. *J. Am. Coll. Cardiol.* 56 (25), 2115–2125. doi:10.1016/j.jacc.2010.07.033

Rio, C. D., Millan, E., Garcia, V., Appendino, G., DeMesa, J., and Munoz, E. (2018). The endocannabinoid system of the skin. A potential approach for the treatment of skin disorders. *Biochem. Pharmacol.* 157, 122–133. doi:10.1016/j.bcp.2018.08.022

Rosenthaler, S., Pohn, B., Kolmanz, C., Huu, C. N., Krewenka, C., Huber, A., et al. (2014). Differences in receptor binding affinity of several phytocannabinoids do not explain their effects on neural cell cultures. *Neurotoxicol. Teratol.* 46, 49–56. doi:10.1016/j.ntt.2014.09.003

Scholten, D., Trebicka, J., Liedtke, C., and Weiskirchen, R. (2015). The carbon tetrachloride model in mice. *Lab. Anim.* 49, 4–11. doi:10.1177/0023677215571192

Scirpo, R., Fiorotto, R., Villani, A., Amenduni, M., Spirli, C., and Strazzabosco, M. (2015). Stimulation of nuclear receptor peroxisome proliferator-activated receptor-γ limits NF-κB-dependent inflammation in mouse cystic fibrosis biliary epithelium. *Hepatology* 62 (5), 1551–1562. doi:10.1002/hep.28000

Shariati, S., Kalantar, H., Pashmforoosh, M., Mansouri, E., and Khodayar, M. J. (2019). Epicatechin protective effects on bleomycin-induced pulmonary oxidative stress and fibrosis in mice. *Biomed. Pharmacother.* 114, 108776. doi:10.1016/j.biopha.2019.108776

Silva, R. S. B. A. B. F. d. (2017). "Cannabidiol as an antioxidant," in *Cannabis and related pathologies: Biology, Pharmacology, diagnosis, and treatment*. Editor V. R. Preedy.

Singer, E., Judkins, J., Salomonis, N., Matlaf, L., Soteropoulos, P., McAllister, S., et al. (2015). Reactive oxygen species-mediated therapeutic response and resistance in glioblastoma. *Cell Death Dis.* 6, e1601. doi:10.1038/cddis.2014.566

Stohs, S., and Ray, S. (2020). Is cannabidiol hepatotoxic or hepatoprotective: A review. *Toxicol. Res. Appl.* 4, 239784732092294. doi:10.1177/2397847320922944

Sunda, F., and Arowolo, A. (2020). A molecular basis for the anti-inflammatory and anti-fibrosis properties of cannabidiol. *FASEB J.* 34 (11), 14083–14092. doi:10.1096/fj.202000975R

Taylor, L., Crockett, J., Tayo, B., and Morrison, G. (2019). A phase 1, open-label, parallel-group, single-dose trial of the pharmacokinetics and safety of cannabidiol (CBD) in subjects with mild to severe hepatic impairment. *J. Clin. Pharmacol.* 59 (8), 1110–1119. doi:10.1002/jcph.1412

Taylor, L., Gidal, B., Blakey, G., Tayo, B., and Morrison, G. (2018). A phase I, randomized, double-blind, placebo-controlled, single ascending dose, multiple dose, and food effect trial of the safety, tolerability and pharmacokinetics of highly purified cannabidiol in healthy subjects. *CNS Drugs* 32 (11), 1053–1067. doi:10.1007/s40263-018-0578-5

Teixeira-Clerc, F., Julien, B., Grenard, P., Tran Van Nhieu, J., Deveaux, V., Li, L., et al. (2006). CB1 cannabinoid receptor antagonism: A new strategy for the treatment of liver fibrosis. *Nat. Med.* 12 (6), 671–676. doi:10.1038/nm1421

Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., and Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists *in vitro*. *Br. J. Pharmacol.* 150 (5), 613–623. doi:10.1038/sj.bjp.0707133

Tsou, P. S., Varga, J., and O'Reilly, S. (2021). Advances in epigenetics in systemic sclerosis: Molecular mechanisms and therapeutic potential. *Nat. Rev. Rheumatol.* 17 (10), 596–607. doi:10.1038/s41584-021-00683-2

Ujvary, I., and Hanus, L. (2016). Human metabolites of cannabidiol: A review on their formation, biological activity, and relevance in therapy. *Cannabis Cannabinoid Res.* 1 (1), 90–101. doi:10.1089/can.2015.0012

Varga, J., and Abraham, D. (2007). Systemic sclerosis: A prototypic multisystem fibrotic disorder. *J. Clin. Invest.* 117 (3), 557–567. doi:10.1172/JCI31139

Vilela, L. R., Lima, I. V., Kunsch, E. B., Pinto, H. P. P., de Miranda, A. S., Vieira, E. L. M., et al. (2017). Anticonvulsant effect of cannabidiol in the pentylenetetrazole model: Pharmacological mechanisms, electroencephalographic profile, and brain cytokine levels. *Epilepsy Behav.* 75, 29–35. doi:10.1016/j.yebeh.2017.07.014

Vuolo, F., Abreu, S. C., Michels, M., Xisto, D. G., Blanco, N. G., Hallak, J. E., et al. (2019). Cannabidiol reduces airway inflammation and fibrosis in experimental allergic asthma. *Eur. J. Pharmacol.* 843, 251–259. doi:10.1016/j.ejphar.2018.11.029

Wang, Q. M., Du, J. L., Duan, Z. J., Guo, S. B., Sun, X. Y., and Liu, Z. (2013). Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation. *World J. Gastroenterol.* 19 (19), 2921–2934. doi:10.3748/wjg.v19.i19.2921

Wang, Y., Mukhopadhyay, P., Cao, Z., Wang, H., Feng, D., Hasko, G., et al. (2017). Cannabidiol attenuates alcohol-induced liver steatosis, metabolic dysregulation, inflammation and neutrophil-mediated injury. *Sci. Rep.* 7 (1), 12064. doi:10.1038/s41598-017-10924-8

Wang, Z., Xu, J. P., Zheng, Y. C., Chen, W., Sun, Y. W., Wu, Z. Y., et al. (2011). Peroxisome proliferator-activated receptor gamma inhibits hepatic fibrosis in rats. *Hepatobiliary Pancreat. Dis. Int.* 10 (1), 64–71. doi:10.1016/s1499-3872(11)60009-x

Wei, Z., Zhao, D., Zhang, Y., Chen, Y., Zhang, S., Li, Q., et al. (2019). Rosiglitazone ameliorates bile duct ligation-induced liver fibrosis by down-regulating NF-κB-TNF-α signaling pathway in a PPARγ-dependent manner. *Biochem. Biophys. Res. Commun.* 519 (4), 854–860. doi:10.1016/j.bbrc.2019.09.084

Wu, M., Melichian, D. S., Chang, E., Warner-Blankenship, M., Ghosh, A. K., and Varga, J. (2009). Rosiglitazone abrogates bleomycin-induced scleroderma and blocks profibrotic responses through peroxisome proliferator-activated receptor-gamma. *Am. J. Pathol.* 174 (2), 519–533. doi:10.2353/ajpath.2009.080574

Yamamoto, T., and Nishioka, K. (2005). Cellular and molecular mechanisms of bleomycin-induced murine scleroderma: Current update and future perspective. *Exp. Dermatol.* 14 (2), 81–95. doi:10.1111/j.0906-6705.2005.00280.x

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 24 October 2022
DOI 10.3389/fphar.2022.1038754

OPEN ACCESS

EDITED BY
Francesca Baratta,
University of Turin, Italy

REVIEWED BY
Abuzar Kabir,
Florida International University, United States
Carmina Sirignano,
University of Naples Federico II, Italy

*CORRESPONDENCE
Sebastiano Barco,
sebastianobarco@gaslini.org

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

RECEIVED 07 September 2022
ACCEPTED 10 October 2022
PUBLISHED 24 October 2022

CITATION
Pigliasco F, Malaca S, Lo Faro AF, Tini A, Cangemi G, Cafaro A, Barco S, Riva A, Pisati A, Amadori E, Striano P, Tagliabracci A, Huestis MA and Busardò FP (2022), Cannabidiol, Δ^9-tetrahydrocannabinol, and metabolites in human blood by volumetric absorptive microsampling and LC-MS/MS following controlled administration in epilepsy patients.
Front. Pharmacol. 13:1038754.
doi: 10.3389/fphar.2022.1038754

Cannabidiol, Δ^9-tetrahydrocannabinol, and metabolites in human blood by volumetric absorptive microsampling and LC-MS/MS following controlled administration in epilepsy patients

Federica Pigliasco[1†], Sara Malaca[2†], Alfredo Fabrizio Lo Faro[2], Anastasio Tini[2], Giuliana Cangemi[3], Alessia Cafaro[3,4], Sebastiano Barco[3*], Antonella Riva[1], Angelica Pisati[1], Elisabetta Amadori[1], Pasquale Striano[1,5], Adriano Tagliabracci[2], Marilyn Ann Huestis[6] and Francesco Paolo Busardò[2]

[1]Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health (DINOGMI), University of Genoa, Genoa, Italy, [2]Department of Excellence-Biomedical Sciences and Public Health, Università Politecnica delle Marche, Ancona, Italy, [3]Chromatography and Mass Spectrometry Section, Central Laboratory of Analyses, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [4]Department of Internal Medicine, Pharmacology & Toxicology Unit, University of Genoa, Genoa, Italy, [5]Paediatric Neurology and Muscular Disease Unit, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [6]Institute of Emerging Health Professions, Thomas Jefferson University, Philadelphia, PA, United States

Cannabidiol (CBD) exhibits anti-inflammatory, anxiolytic, antiseizure, and neuroprotective proprieties without addictive or psychotropic side effects, as opposed to Δ^9-tetrahydrocannabinol (THC). While recreational cannabis contains higher THC and lower CBD concentrations, medical cannabis contains THC and CBD in different ratios, along with minor phytocannabinoids, terpenes, flavonoids and other chemicals. A volumetric absorptive microsampling (VAMS) method combined with ultra-high-performance liquid chromatography coupled with mass spectrometry in tandem for quantification of CBD, THC and their respective metabolites: cannabidiol-7-oic acid (7-COOH-CBD); 7-hydroxy-cannabidiol (7-OH-CBD); 6-alpha-hydroxy-cannabidiol (6-α-OH-CBD); and 6-beta-hydroxycannabidiol (6-β-OH-CBD); 11- Hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH). After overnight enzymatic glucuronide hydrolysis at 37°C, samples underwent acidic along with basic liquid-liquid extraction with hexane: ethyl acetate (9:1, v/v). Chromatographic separation was carried out on a C18 column, with the mass spectrometer operated in multiple reaction monitoring mode and negative electrospray ionization. Seven patients with intractable epilepsy were dosed with various CBD-containing formulations and blood collected

just before their daily morning administration. The method was validated following international guidelines in toxicology. Linear ranges were (ng/ml) 0.5–25 THC, 11-OH-THC, THCCOOH, 6-α-OH-CBD and 6-β-OH-CBD; 10–500 CBD and 7-OH-CBD; and 20–5000 7-COOH-CBD. 7-COOH-CBD was present in the highest concentrations, followed by 7-OH-CBD and CBD. This analytical method is useful for investigating CBD, THC and their major metabolites in epilepsy patients treated with CBD preparations employing a minimally invasive microsampling technique requiring only 30 µL blood.

KEYWORDS

cannabinoids, medical cannabis, serum, CBD metabolites, UHPLC-MS/MS

1 Introduction

The most researched phytocannabinoids are cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) (Degenhardt et al., 2017; Barco et al., 2018). CBD exhibits anti-inflammatory, antiseizure, anxiolytic, and neuroprotective proprieties without addictive or psychotropic effects, as opposed to THC (Pigliasco et al., 2020). While recreational cannabis generally contains high THC concentrations, medical cannabis contains THC and CBD in varying amounts, along with minor terpenes, flavonoids, phytocannabinoids, and other chemicals (Malaca et al., 2021).

There is growing interest in cannabis-based therapies and clinical applications (Lattanzi et al., 2019, 2020; Arzimanoglou et al., 2020). Different CBD products are available on the market with effects varying based on purity, formulation, and concentration. The European Medicines Agency has authorized Epidyolex®, a pure CBD oral solution. As an add-on therapy for drug-resistant epilepsies including Dravet syndrome, tuberous sclerosis complex and Lennox-Gastaut syndrome (Dubois et al., 2020).

Therapeutic drug monitoring (TDM) of a variety of antiseizure medications (ASMs) is critical in therapeutic management of patients with epilepsy. TDM is particularly useful in emerging clinical practice for cannabis-based therapies to identify the dose associated with an optimal response (Patsalos et al., 2018; Striano et al., 2008; Brandt, 2019). Although TDM is often performed on plasma or serum samples, a major challenge is a need for repeated venipunctures, which is stressful, especially for children. Dried blood spots and other microsampling techniques offer advantages including easy, rapid, and less invasive sample collection, low sample volumes of 10–50 µL, minimal sample preparation and safe sample handling with minimum risk of transmission of infectious diseases (Biagini et al., 2020).

Other analytical methods focused on CBD and metabolite identification and quantification in different biological matrices, but none included simultaneous quantification with the volumetric absorptive microsampling (VAMS) method (Barco et al., 2018; Dubois et al., 2020; Pérez-Acevedo et al., 2020, 2021; Pichini et al., 2020, 2021; Pigliasco et al., 2020; Busardò et al., 2021; Malaca et al., 2021). This method quantifies CBD, THC, cannabidiol-7-oic acid (7-COOH-CBD); 7-hydroxy-cannabidiol (7-OH-CBD); 6-alpha-hydroxy-cannabidiol (6-α-OH-CBD); and 6-beta-hydroxycannabidiol (6-β-OH-CBD); 11-Hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in whole blood collected with VAMS and analyzed with ultra-high performance liquid chromatography coupled with mass spectrometry in tandem (UHPLC-MS/MS). In our previous research, we demonstrated that CBD plasma concentrations were comparable to those measured in venous or capillary blood with VAMS, allowing the use of this microsampling fingerpick device (Barco et al., 2017; D'Urso et al., 2019; Pigliasco et al., 2020). The validated method quantified cannabinoids in children's blood with drug-resistant epilepsy.

2 Materials and methods

2.1 Chemicals and reagents

Standards for CBD, 7-OH-CBD, 7-COOH-CBD, 6-α–OH–CBD and 6-β–OH–CBD, THC, 11-OH-THC, and THCCOOH were obtained from Dalton Research Molecules (Toronto, ON, Canada) and deuterated internal standards (ISTD) THC-d_3, 11-OH-THC-d_3, THCCOOH-d_3 and CBD-d_3 were purchased from Cayman Chemical (Ann Arbor, MI, United States) and stored at −20°C until use. LC-MS grade water, LC grade acetone, formic acid and acetonitrile acquired from Sigma-Aldrich® (Milano, Italy). Ammonium formate 5 mM was prepared with 97% pure ammonium formate ammonium salt (Sigma-Aldrich®) dissolved in LC-MS grade water. Beta-glucuronidase from *E. Coli* (>20.000 units mg/protein) was obtained from Sigma Aldrich® (Milano, Italy).

2.2 Instrumental conditions for UHPLC-MS/MS

A Waters® Xevo® TQ-S micro mass spectrometer (triple quadrupole), equipped with an electrospray ionization source operating in both negative and positive-ion mode (ESI), was used

TABLE 1 Mass spectrometry parameters for analytes and internal standards.

Compounds	Internal Standard	Cone voltage (eV)	Q1 mass (m/z)	Quantification transition		Confirmation transition		RT (min)
				Q3 mass (m/z)	CE (eV)	Q3 mass (m/z)	CE (eV)	
Standards								
7-COOH-CBD	11-OH-THC-d_3	40	343.1	179.2	20	231.2	26	5.57
6-α–OH–CBD	11-OH-THC-d_3	30	329.2	158.2	32	173.1	28	6.17
7-OH-CBD	11-OH-THC-d_3	25	329.1	261.2	20	268.1	24	6.42
6-β–OH–CBD	11-OH-THC-d_3	30	329.2	158.2	26	173.2	34	6.50
THCCOOH	THCCOOH-d3	40	345.1	193.1	24	299.2	24	7.61
11-OH-THC	11-OH-THC-d3	30	331.2	193.1	24	201.1	24	8.23
CBD	CBD-d_3	45	315.2	123	34	189.1	22	9.79
THC	THC-d3	45	315.2	123	34	193.1	22	11.27
Internal Standards								
THCCOOH-d_3	-	40	348.1	196.2	26	-	-	7.61
11-OH-THC-d_3	-	30	334.2	196.1	30	-	-	8.22
CBD-d_3	-	45	316.1	110.1	45	248.2	45	9.77
THC-d_3	-	45	318.2	123	34	196.1	22	11.27

CBD, Cannabidiol; 7-COOH-CBD, 7-Carboxy-cannabidiol; 7-OH-CBD, 7-Hydroxy-cannabidiol; 6-α–OH–CBD, 6-α-Hydroxy-cannabidiol; 6-β–OH–CBD, 6-β-Hydroxy-cannabidiol; THC, Δ^9-Tetrahydrocannabinol; 11-OH-THC, 11-Hydroxy-Δ^9-tetrahydrocannabinol; THCCOOH, 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol; CE, collision energy; RE, retention time.

to conduct the UHPLC-MS/MS analysis. The instrument was interfaced with an ACQUITY UPLC® I-Class (Waters®; Milan, Italy). Data were collected using the MassLynx® program version 4.1 (Waters®, Milano, Italy). A Waters® ACQUITYTM PREMIER UPLC® BEH C18 column (100 × 2.1 mm, 1.7 µm) was used for separation. Run time was 17 min with mobile phases (A) water with ammonium formate 5 mM pH 7.5 and (B) acetonitrile at a flow rate of 0.4 ml/min. The gradient program went from 5% B for 0.25 min to 30% B after 1 min, 80% B after 11.5 min and held for 0.5 min, 100% B after 11.55 min till 13.5 min, and then back to 5% B after 13.55 min and held for the remaining 17:00 min. Column oven and autosampler temperatures were 50°C and 10°C, respectively. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, with two transitions for each analyte and ISTD (see Table 1). By individually injecting neat standards into methanol and ramping cone voltage and collision energy, MS parameter settings were made to be fully optimized. (see Table 1). The ESI conditions were optimized to source temperature 150°C, capillary voltage −2.8 kV, cone gas flow rate 0.18 ml/min, desolvation temperature 650°C, and desolvation gas flow rate 1200 L/h. The scan speed (dwell time) was 0.023 s.

2.3 Preparation of quality control samples and calibration standards

Standard stock solutions with all five non-deuterated standards were prepared in methanol at 1 mg/ml, 100 µg/ml, 10 µg/ml, and 1 µg/ml. ISTD stock solution with THC-d_3, THCCOOH-d_3, 11-OH-THC-d_3 and CBD-d_3 was prepared in methanol at 1 µg/ml. Due to the unavailability of deuterated standards for the analytes, the deuterated 11-OH-THC standard was used for the CBD metabolites. Glass vials containing the stock solutions were kept at −20°C.

Pre-screened blood samples were donated by Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health (DI-NOGMI, University of Genoa, Genoa, Italy) as material discarded during current clinical practice. During method validation, samples were evaluated to rule out any potential sources of chromatographic interferences and then mixed to form a homogeneous pool of blank samples for the preparation of calibration standards and quality control samples.

Calibrator working solutions were prepared by diluting standard stock solutions with methanol (0.25, 0.5, 1, 2, 5 and 25 ng/ml for THC, 11-OH-THC, THCCOOH, 6-α–OH–CBD and 6-β–OH–CBD; 1.5, 25, 50, 100, 250 and 500 ng/ml for CBD and 7-OH-CBD; 3.5, 20, 100, 500, 1000 and 5000 ng/ml for 7-COOH-CBD). Low, medium and high-quality control (QC) working solutions were prepared daily from the standard stock solutions in methanol. THC, 11-OH-THC, THCCOOH, 6-α–OH–CBD and 6-β–OH–CBD QC concentrations were 0.75, 1.5 and 20 ng/ml, CBD and 7-OH-CBD QC concentrations were 5, 80 and 400 ng/ml and 7-COOH-CBD QC concentrations were 10, 350 and 4000 ng/ml.

2.4 Sample preparation

Since prior research and our preliminary experiments in real samples revealed that CBD metabolites were present as glucuronides, blood samples were extracted following enzymatic hydrolysis (Bergamaschi et al., 2013; Ujváry and Hanuš, 2016). Glucuronide hydrolysis was carried out by adding 2 µL ISTD solution (100 ng/ml), beta-glucuronidase (*E. Coli* >20,000 units mg/protein) was added to 30 ml of blood in a VAMS tip, followed by dilution in 800 ml of water and heated overnight at 37°C. After hydrolysis, acidic and basic extractions were performed to extract all metabolites based on their acid-base properties. For the basic extraction, 100 µL ammonium hydroxide (pH 9) was added to the hydrolyzed sample after the addition of 4 ml hexane: ethyl acetate (9:1). Samples underwent horizontal agitation for 30 min. Samples were centrifuged for 3 min at 3500 rpm, and the supernatant was then transferred to a clean tube. The samples were centrifuged for 3 minutes at 3500 rpm and the supernatant was then transferred to a clean tube. The remaining aqueous phase was then treated with 15 µL of formic acid (≥99.9%) (pH 3) for acid extraction. Four mL of hexane: ethyl acetate (9: 1) was added to the sample. The tubes were then capped, vortexed for 10 s, mixed for 10 min and centrifuged at 5000 g for 5 min . Both extractions' supernatants were combined in a glass tube (Safe-Lock Tube®, Eppendorf, Milano, Italy) and dried under nitrogen. Samples were resuspended with 100 µL water: methanol (1:1), transferred into autosampler glass vials, and 10 µL injected onto the chromatographic system.

2.5 Validation of the analytical method

2.5.1 Sensitivity

Sensitivity was determined by analyzing four replicates of negative samples and examination of the signal/noise ratio. The standard deviation (SD) of the mean noise level over the retention time window of analytes was used to determine the detection limit (LOD = 3 SD) and the quantification limit (LLOQ = 10 SD). The calculated LLOQ had to demonstrate precision and accuracy within the 20% relative SD and relative error, respectively, in order to be acceptable.

2.5.2 Selectivity and carryover

Selectivity evaluated the capacity of analytes to be identified in the presence of matrix elements. Blank blood samples were assayed to identify possible endogenous interferences. In addition, blank blood samples were assayed for possible exogenous drug interferences. For this, other commonly encountered analytes (such as common drugs or metabolites) encountered in routine work were analyzed with fortified matrix samples at high therapeutic or lethal concentrations. The acceptance criteria were no signal/noise ratio higher than 3 at ± 0.2 min of the retention time of the analytes (ranging from 5.57 to 11.27 min) in the quantitative and qualitative ions. To test for carryover, blank blood samples were analyzed immediately after the highest calibrator. Carryover was the highest fortified concentration at which no analyte carryover above the method's LOD was observed in the blank matrix.

2.5.3 Calibration curve

Six calibrators were assayed on five separate days to establish the calibration curve. The peak area ratio of each compound and its corresponding ISTD were plotted against each analyte's concentration. The minimally acceptable linearity requires a coefficient of correlation (r^2) ≥ 0.99 and each calibrator quantifying within ±20% of target concentration. Dilution integrity was checked for over-the-curve samples with concentrations 10 and 50 times higher than the highest calibrators, verifying precision and accuracy to be within 15%.

2.5.4 Imprecision and bias

Imprecision was expressed as the RSD (%), and bias was calculated as (determined concentration)/(nominal concentration)×100%. Acceptance criteria for intra- and inter-assay imprecision were CV ≤ 20% and bias ≤15%. To evaluate intra-assay imprecision, six blank blood samples each were fortified with the target analytes at three different concentrations (low, medium, and high QC) and analyzed on the same day. Evaluation of inter-assay imprecision and bias were performed over 5 days with a minimum of six concentrations.

2.5.5 Matrix effect and recovery

Matrix effect was determined by comparing peak areas of the extracted blank samples fortified with standards after the extraction procedure with the peak areas of pure diluted substances. Recovery was determined by comparing peak area of the extracted compounds fortified before extraction to the peak area obtained from samples fortified post-extraction (representing 100% recovery). The ISTD mixture was added to samples after extraction.

2.5.6 Stability

Compounds' stability in blood was evaluated through repeated analysis (n = 5) of QC samples after three freeze-thaw cycles (storage at −20°C) on the compounds stability in blood was evaluated by repeated analysis (n = 5) of QC samples. In addition, short term (24 and 48 h) and mid-term (1 month) stability were assessed using five different aliquots of QC stored at −20°C. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes in QC.

2.6 Application to patients' samples

The analytical method's applicability was demonstrated using real clinical samples from patients taking different CBD

TABLE 2 Patients' demographic, clinical, and treatment data.

Patient ID	Age (y), gender	Weight (Kg)	Epilepsy disorder	CBD formulation, dose (mg/Kg/day), treatment duration (d)	Concomitant drugs
1	9, ♂	24	Dravet Syndrome	Epidyolex oral solution, 20 820	STP, VPA
2	8, ♀	23.6	Aicardi Syndrome	Galenic CBD oil, 3.9, 1125	LTG
3	15, ♀	29	Noonan Syndrome	Galenic CBD oil 24%, 7.2, 240	VPA, LCM, LZP
4	12, ♂	28.4	Focal non-lesional epilepsy	CBD crystals & Bedrolite, 26.4 and 10.6, 2130	LEV, CLB
5	20, ♀	40	Focal non-lesional epilepsy	CBD crystals, 7.5, 1030	VPA, FBM, NTZ
6	3, ♂	11	Infantile spasms/West Syndrome	Epidyolex oral solution, 16.3, 30	VPA, PB, CLB
7	8, ♂	35	Focal non-lesional epilepsy	Galenic CBD oil, 10.8, 850	PB

CLB, clobazam; FBM, felbamate; LEV, levetiracetam; LCM, lacosamide; LTG, lamotrigine; LZP, lorazepam; NTZ, nitrazepam; PB, phenobarbital; STP, stiripentol; VPA, valproate.

formulations. (Epidyolex®, CBD oil, CBD oil Enecta and CBD crystal) for the treatment of drug-resistant epilepsy of different etiologies (see Table 2). Blood samples were collected in the morning before daily dose administration using the 30 µL VAMS devices (MITRA®, Neoteryx, 105 Torrance, CA, United States) for capillary blood collection. Capillary VAMS were obtained in accordance with the manufacturer's recommendations: before pricking the patient's finger with a micro-needle, the area was disinfected and after the first drop of blood was removed, the VAMS tip was placed in contact with the surface of the second drop to absorb the matrix. The study was approved by the Regional Ethical Committee (CER Liguria: 056/057/058/059-2019) and written informed consent was signed by patients or caregivers.

3 Results

Previous analytical methods determined CBD, THC and metabolites (Figure 1) by UHPLC-MS/MS methods with VAMS collection (Dubois et al., 2020; Pigliasco et al., 2020) but no assay is currently available to simultaneously quantify all of these analytes in whole blood.

3.1 Validation of the analytical method

The method was validated over 5 days in blood samples following the most recent criteria for bioanalytical method development and validation (Peters et al., 2018; Wille et al., 2018), Linearity, sensitivity [limits of detection (LOD) and quantification (LOQ)], selectivity, accuracy, imprecision and carryover were calculated using five daily replicates of calibrators (six for each calibration curve) and five replicates of the three QC samples. Method validation results, presented in Tables 3, 4 were following the internationally established criteria (Peters et al., 2018; Wille et al., 2018). No relevant degradation was observed after any of the three freeze/thaw cycles, with differences in the initial concentration less than 15% for all the compounds under investigation. Similar results (differences from the initial concentration always lower than 15%) were obtained for the case of short-term and mid-term stability tests, confirming the validity of stored samples for analysis.

3.2 Analysis of patients' samples

For proof of concept, the VAMS collection and analytical method were applied to seven samples from seven patients receiving various CBD formulations each at different therapeutic dosages. Four males (ages 3–12 years; weight: 11–28.4 kg) and three females (age range: 8–20 years; weight: 23.6–40 kg) were treated at the Giannina Gaslini Children's Hospital provided samples. The study was approved by the Regional Ethical Committee (CER Liguria: 056/057/058/059-2019) and written informed consent was provided by patients or caregivers. Table 5 summarizes the patients' results.

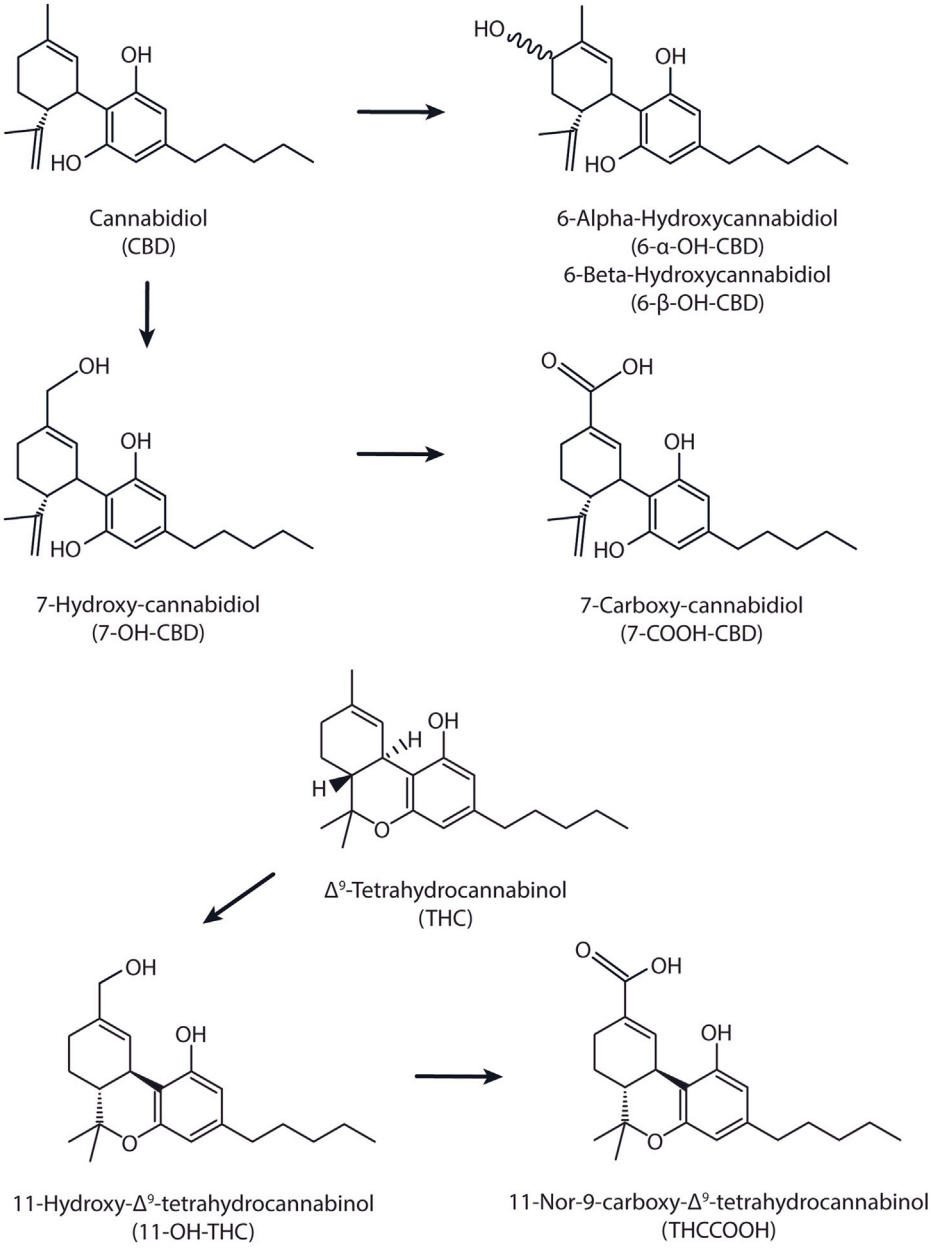

FIGURE 1
Chemical structures for CBD, THC, and their respective metabolites.

4 Discussion

An analytical method was validated for the determination of CBD, THC, and their respective metabolites and later applied to clinical samples. Blood samples were collected from seven patients under treatment with CBD formulations. Blood CBD concentrations were higher for the patients treated with Galenic CBD oil (patients #2 and #7) compared to patients treated with other CBD preparations. 7-COOH-CBD, the inactive metabolite, was present in the highest concentrations, followed by 7-OH-CBD, and CBD. 6-α–OH–CBD and 6-β–OH–CBD concentrations were always lower than concentrations of the other CBD metabolites, but for the first time were detected in all patients' samples, with the highest concentrations in patient #2. In previous studies, these two analytes were undetectable in

TABLE 3 Linearity, limits of detection (LOD) and limits of quantification (LOQ) for analytes under investigation in blood samples.

Analytes	Determination coefficient r^2	LOD ng/mL	LOQ ng/mL
CBD	0.996 ± 0.003	0.50	1.50
7-OH-CBD	0.999 ± 0.002	0.50	1.50
7-COOH-CBD	0.999 ± 0.002	1.10	3.50
6-α–OH–CBD	0.996 ± 0.004	0.10	0.25
6-β–OH–CBD	0.994 ± 0.003	0.10	0.25
THC	0.997 ± 0.002	0.10	0.25
11-OH-THC	0.998 ± 0.002	0.10	0.25
THCCOOH	0.998 ± 0.001	0.10	0.25

CBD, Cannabidiol; 7-COOH-CBD, 7-Carboxy-cannabidiol; 7-OH-CBD, 7-Hydroxy-cannabidiol; 6-α–OH–CBD, 6-α-Hydroxy-cannabidiol; 6-β–OH–CBD, 6-β-Hydroxy-cannabidiol; THC, Δ^9-Tetrahydrocannabinol; 11-OH-THC, 11- Hydroxy-Δ^9-tetrahydrocannabinol; THCCOOH, 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol; LOD, limit of detection; LOQ, limit of quantification.

TABLE 4 Validation parameters for cannabinoid analytes under investigation in blood samples.

Analytes	Intra-assay accuracy %CV			Inter-assay accuracy %CV			Intra-assay imprecision %CV			Inter-assay imprecision %CV			Recovery (%)		
	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
7-COOH-CBD	5.1	2.4	5.7	7.9	2.5	4.1	6.9	2.4	4.7	7.0	2.6	4.8	95.7	95.9	97.9
6-α–OH–CBD	7.9	9.1	2.8	7.8	8.4	3.3	3.1	3.5	2.9	7.9	3.0	3.7	96.5	84.3	78.2
7-OH-CBD	7.8	7.5	5.2	6.9	7.7	4.7	8.8	4.5	3.6	6.8	3.1	2.9	93.7	92.2	98.1
6-β–OH–CBD	7.9	5.8	7.8	9.6	8.6	5.2	3.9	2.8	1.5	9.4	6.2	6.5	81.7	87.9	89.9
THC-COOH	4.2	8.3	5.0	58	8.6	4.8	5.8	3.9	2.2	5.8	5.8	6.5	83.5	94.0	93.2
11-OH-THC	9.9	10.4	9.1	9.5	8.5	5.5	2.9	3.7	2.7	4.2	3.8	7.0	77.8	86.4	86.4
CBD	10.2	4.1	2.3	7.8	5.8	3.8	9.2	4.8	1.1	6.8	6.4	5.1	69.0	63.2	64.5
THC	7.1	4.9	7.0	6.9	7.4	6.9	5.8	4.0	2.6	6.1	6.7	2.8	80.1	85.6	88.9

CBD, Cannabidiol; 7-COOH-CBD, 7-Carboxy-cannabidiol; 7-OH-CBD, 7-Hydroxy-cannabidiol; 6-α–OH–CBD, 6-α-Hydroxy-cannabidiol; 6-β–OH–CBD, 6-β-Hydroxy-cannabidiol; THC, Δ^9-Tetrahydrocannabinol; 11-OH-THC, 11-Hydroxy-Δ^9-tetrahydrocannabinol; THCCOOH, 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol; CV, coefficient of variation; L, low; M, medium; H, high.

TABLE 5 Formulations, doses, and concentrations of CBD, THC, and their respective metabolites in patients' blood samples collected with the VAMS technique after CBD therapy.

ID	Formulation	CBD mg/kg/day	Concentration ng/mL							
			6-α-OH-CBD	6-β-OH-CBD	7-OH-CBD	CBD-COOH	CBD	11-OH-THC	THC-COOH	THC
1	Epidyolex oral solution	20	16.8	17.4	399	7335	109	N.D.	N.D.	N.D.
2	Galenic CBD Oil	3.9	28.9	38.8	883	6677	2503	N.D.	N.D.	N.D.
3	CBD Oil 24%	3.9	0.9	1.0	53.5	357	19.1	N.D.	N.D.	N.D.
4	CBD crystal & Bedrolite	250	8.2	5.8	498	15,371	333	N.D.	N.D.	N.D.
5	CBD crystal	100	2.0	4.0	77.3	211	39.0	N.D.	N.D.	N.D.
6	Epidyolex oral solution	0.9	2.6	6.2	266	1120	88.7	N.D.	N.D.	N.D.
7	Galenic CBD oil	18 drops	1.9	1.9	59.4	423	1878	N.D.	N.D.	N.D.

CBD, Cannabidiol; 7-COOH-CBD, 7-Carboxy-cannabidiol; 7-OH-CBD, 7-Hydroxycannabidiol; 6-α–OH–CBD, 6-α-Hydroxycannabidiol; 6-β–OH–CBD, 6-β-Hydroxycannabidiol; 11-OH-THC, 11-Hydroxy- Δ^9-tetrahydrocannabinol; THCCOOH, 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol THC, Δ^9-Tetrahydrocannabinol; N.D., not detected.

some patients' samples [4]. THC and its metabolites, THCCOOH and 11-OH-THC, were not detected, indicating that the CBD formulations contained little if any THC.

In conclusion, this simple and rapid UHPLC-MS/MS method enabled robust and sensitive quantification of CBD, THC, and their respective metabolites, with good precision, accuracy, and efficiency. Also, blood sample collection by a microsampling technique (VAMS) is a major advantage when dealing with patients, especially children to avoid invasive procedures during repeated venipunctures.

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.

Ethics statement

The studies involving human participants were reviewed and approved by Regional Ethical Committee (CER Liguria: 056/057/058/059-2019). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Conceptualization, FB, GC, and PS; Validation, SM, AL, AT, and FP; Formal Analysis, AC; Investigation, SM, AL, and AT; Resources, AR, AP, and EA; Writing – Original Draft Preparation, SM; Writing – Review & Editing, SM, AL, SB, FB, GC, and PS; Supervision, MH, FB, and PS; Funding Acquisition, GC, ADT, and PS.

Funding

This study was funded by the Italian Ministry of Health, RC 2022. We gratefully thank all Italian citizens who allocated the 5 × 1000 share of their tax payment in support of health research.

Acknowledgments

The authors would like to thank Simonetta di Carlo, Antonella Bacosi, Michele Sciotti, Josuè Gottardi, Chiara Fraioli and Laura Martucci for technical assistance.

Conflict of interest

The 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.

Publisher's note

References

Arzimanoglou, A., Brandl, U., Cross, J. H., Gil-Nagel, A., Lagae, L., Landmark, C. J., et al. (2020). Epilepsy and cannabidiol: A guide to treatment. *Epileptic Disord.* 22, 1–14. doi:10.1684/epd.2020.1141

Barco, S., Castagnola, E., Moscatelli, A., Rudge, J., Tripodi, G., and Cangemi, G. (2017). Volumetric adsorptive microsampling-liquid chromatography tandem mass spectrometry assay for the simultaneous quantification of four antibiotics in human blood: Method development validation and comparison with dried blood spot. *J. Pharm. Biomed. Anal.* 145, 704–710. doi:10.1016/j.jpba.2017.07.033

Barco, S., Fucile, C., Manfredini, L., Grandis, E. D., Gherzi, M., Martelli, A., et al. (2018). A UHPLC-MS/MS method for the quantification of Δ9-tetrahydrocannabinol and cannabidiol in decoctions and in plasma samples for therapeutic monitoring of medical cannabis. *Bioanalysis* 10, 2003–2014. doi:10.4155/bio-2018-0184

Bergamaschi, M. M., Barnes, A., Queiroz, R. H. C., Hurd, Y. L., and Huestis, M. A. (2013). Impact of enzymatic and alkaline hydrolysis on CBD concentration in urine. *Anal. Bioanal. Chem.* 405, 4679–4689. doi:10.1007/s00216-013-6837-x

Biagini, D., Antoni, S., Lomonaco, T., Ghimenti, S., Salvo, P., Bellagambi, F. G., et al. (2020). Micro-extraction by packed sorbent combined with UHPLC-ESI-MS/MS for the determination of prostanoids and isoprostanoids in dried blood spots. *Talanta* 206, 120236. doi:10.1016/j.talanta.2019.120236

Brandt, C. (2019). Pharmacodynamic monitoring of antiepileptic drug therapy. *Ther. Drug Monit.* 41, 168–173. doi:10.1097/FTD.0000000000000623

Busardò, F. P., Pérez-Acevedo, A. P., Pacifici, R., Mannocchi, G., Gottardi, M., Papaseit, E., et al. (2021). Disposition of phytocannabinoids, their acidic precursors and their metabolites in biological matrices of healthy individuals treated with vaporized medical cannabis. *Pharmaceuticals* 14, 59. doi:10.3390/ph14010059

Degenhardt, F., Stehle, F., and Kayser, O. (2017). "Handbook of cannabis and related pathologies biology, pharmacology, diagnosis, and treatment," in *The biosynthesis of cannabinoids*, 13–23. doi:10.1016/B978-0-12-800756-3.00002-8

Dubois, S., Marchese, F., Pigliasco, F., Barco, S., Tripodi, G., Lomonaco, T., et al. (2020). A volumetric absorptive microsampling technique to monitor cannabidiol levels in epilepsy patients. *Front. Pharmacol.* 11, 582286. doi:10.3389/fphar.2020.582286

D'Urso, A., Rudge, J., Patsalos, P. N., and De Grazia, U. (2019). Volumetric absorptive microsampling: A new sampling tool for therapeutic drug monitoring of antiepileptic drugs. *Ther. Drug Monit.* 41, 681–692. doi:10.1097/FTD.0000000000000652

Lattanzi, S., Brigo, F., Trinka, E., Zaccara, G., Striano, P., Del Giovane, C., et al. (2020). Adjunctive cannabidiol in patients with Dravet syndrome: A systematic Review and meta-analysis of efficacy and safety. *CNS Drugs* 343 34, 229–241. doi:10.1007/S40263-020-00708-6

Lattanzi, S., Trinka, E., Russo, E., Striano, P., Citraro, R., Silvestrini, M., et al. (2019). Cannabidiol as adjunctive treatment of seizures associated with Lennox-

Gastaut syndrome and Dravet syndrome. *Drugs Today* 55, 177–196. doi:10.1358/DOT.2019.55.3.2909248

Malaca, S., Gottardi, M., Pigliasco, F., Barco, S., Cafaro, A., Amadori, E., et al. (2021). UHPLC-MS/MS analysis of cannabidiol and its metabolites in serum of patients with resistant epilepsy treated with CBD formulations. *Pharmaceuticals* 14, 630. doi:10.3390/ph14070630

Patsalos, P. N., Spencer, E. P., and Berry, D. J. (2018). Therapeutic drug monitoring of antiepileptic drugs in epilepsy: A 2018 update. *Ther. Drug Monit.* 40, 526–548. doi:10.1097/FTD.0000000000000546

Pérez-Acevedo, A. P., Busardò, F. P., Pacifici, R., Mannocchi, G., Gottardi, M., Poyatos, L., et al. (2020). Disposition of cannabidiol metabolites in serum and urine from healthy individuals treated with pharmaceutical preparations of medical cannabis. *Pharmaceuticals* 13, E459. doi:10.3390/ph13120459

Pérez-Acevedo, A. P., Pacifici, R., Mannocchi, G., Gottardi, M., Poyatos, L., Papaseit, E., et al. (2021). Disposition of cannabinoids and their metabolites in serum, oral fluid, sweat patch and urine from healthy individuals treated with pharmaceutical preparations of medical cannabis. *Phytother. Res.* 35, 1646–1657. doi:10.1002/ptr.6931

Peters, F. T., Wissenbach, D. K., Busardo, F. P., Marchei, E., and Pichini, S. (2018). Method development in forensic toxicology. *Curr. Pharm. Des.* 23, 5455–5467. doi:10.2174/1381612823666170622113331

Pichini, S., Malaca, S., Gottardi, M., Pérez-Acevedo, A. P., Papaseit, E., Perez-Maña, C., et al. (2021). UHPLC-MS/MS analysis of cannabidiol metabolites in serum and urine samples. Application to an individual treated with medical cannabis. *Talanta* 223, 121772. doi:10.1016/j.talanta.2020.121772

Pichini, S., Mannocchi, G., Gottardi, M., Pérez-Acevedo, A. P., Poyatos, L., Papaseit, E., et al. (2020). Fast and sensitive UHPLC-MS/MS analysis of cannabinoids and their acid precursors in pharmaceutical preparations of medical cannabis and their metabolites in conventional and non-conventional biological matrices of treated individual. *Talanta* 209, 120537. doi:10.1016/j.talanta.2019.120537

Pigliasco, F., Barco, S., Dubois, S., Marchese, F., Striano, P., Lomonaco, T., et al. (2020). Cannabidiol determination on peripheral capillary blood using a microsampling method and ultra-high-performance liquid chromatography tandem mass spectrometry with on-line sample preparation. *Molecules* 25, E3608. doi:10.3390/molecules25163608

Striano, S., Striano, P., Capone, D., and Pisani, F. (2008). Limited place for plasma monitoring of new antiepileptic drugs in clinical practice. *Med. Sci. Monit.* 14, RA173–RA178.

Ujváry, I., and Hanuš, L. (2016). Human metabolites of cannabidiol: A Review on their formation, biological activity, and relevance in therapy. *Cannabis Cannabinoid Res.* 1, 90–101. doi:10.1089/can.2015.0012

Wille, S. M. R., Coucke, W., De Baere, T., and Peters, F. T. (2018). Update of standard practices for new method validation in forensic toxicology. *Curr. Pharm. Des.* 23, 5442–5454. doi:10.2174/1381612823666170714154444

frontiers | Frontiers in Pharmacology

TYPE Methods
PUBLISHED 27 March 2023
DOI 10.3389/fphar.2023.1143365

OPEN ACCESS

EDITED BY
Paola Brusa,
University of Turin, Italy

REVIEWED BY
Marsida Kallupi,
University of California, San Diego, United States
Benjamin B Land,
University of Washington, United States

*CORRESPONDENCE
Rafael Maldonado,
rafael.maldonado@upf.edu
Elena Martín-García,
elena.martin@upf.edu

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

RECEIVED 12 January 2023
ACCEPTED 14 March 2023
PUBLISHED 27 March 2023

CITATION
Cajiao-Manrique MdM, Maldonado R and Martín-García E (2023), A male mouse model of WIN 55,212–2 self-administration to study cannabinoid addiction.
Front. Pharmacol. 14:1143365.
doi: 10.3389/fphar.2023.1143365

A male mouse model of WIN 55,212–2 self-administration to study cannabinoid addiction

María del Mar Cajiao-Manrique[1], Rafael Maldonado[1,2*†] and Elena Martín-García[1,2,3*†]

[1]Laboratory of Neuropharmacology-Neurophar, Department of Medicine and Life Sciences, Universitat Pompeu Fabra (UPF), Barcelona, Spain, [2]Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain, [3]Departament de Psicobiologia i Metodologia de les Ciències de la Salut, Universitat Autònoma de Barcelona (UAB), Barcelona, Spain

We have established for the first time a mouse model of cannabinoid addiction using WIN 55,212–2 intravenous self-administration (0.0125 mg/kg/infusion) in C57Bl/6J mice. This model allows to evaluate the addiction criteria by grouping them into 1) persistence of response during a period of non-availability of the drug, 2) motivation for WIN 55,212–2 with a progressive ratio, and 3) compulsivity when the reward is associated with a punishment such as an electric foot-shock, in agreement with the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5). This model also allows to measure two parameters that have been related with the DSM-5 diagnostic criteria of craving, resistance to extinction and reinstatement, and two phenotypic traits suggested as predisposing factors, impulsivity and sensitivity to reward. We found that 35.6% of mice developed the criteria of cannabinoid addiction, allowing to differentiate between resilient and vulnerable mice. Therefore, we have established a novel and reliable model to study the neurobiological correlates underlying the resilience or vulnerability to develop cannabinoid addiction. This model included the chemogenetic inhibition of neuronal activity in the medial prefrontal cortex to the nucleus accumbens pathway to assess the neurobiological substrate of cannabinoid addiction. This model will shed light on the neurobiological substrate underlying cannabinoid addiction.

KEYWORDS

cannabinoid addiction, mouse model, WIN 55,212-2 self-administration, persistence of response, motivation, compulsive-like behavior

1 Introduction

Cannabis sativa derivatives are the most used illicit drugs worldwide, with an increased consumption over the recent years. In Europe, cannabis use has enhanced from 5.7% in 2015 to 7.7% in 2022 in adults aged from 15 to 64 (European Monitoring Centre for Drugs and Drug Addiction, 2015; European Monitoring Centre for Drugs and Drug Addiction, 2022). However, attitudes toward cannabis use have softened since there is a growing social perception that cannabis is relatively harmless (Weiss and Volkow, 2022). This lack of risk perception has led to an increase in the prevalence of cannabis use disorder, previously defined as cannabis dependence (Zehra et al., 2018).

Cannabis use disorder is defined as a chronically relapsing neuropsychiatric disorder diagnosed by applying the criteria defined in the 5th edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5). In this edition, the term addiction is synonymous with

severe substance-use disorder and requires the accomplishment of six out of eleven diagnostic criteria (Koob and Volkow, 2016).

Cannabis addiction results from the interaction between gene networks, epigenetics, and socio-environmental factors (Piazza and Deroche-Gamonet, 2013; Hamilton and Nestler, 2019; Lüscher et al., 2020; Maldonado et al., 2021). Not all individuals repeatedly exposed to the drug make this transition to addiction (Piazza and Deroche-Gamonet, 2013), raising the question of why some vulnerable individuals become addicted while others are resilient. Although multiple neuroadaptations induced by cannabinoids administration have been described, the precise neurobiological mechanisms underlying cannabinoid addiction in vulnerable individuals remain largely unknown. At the present moment, animal models of cannabinoid addiction have not been yet developed, and there is an urgent need of an accurate animal model to disentangle the neurobiological correlates of addiction to cannabinoids.

Animal models of drug exposure allow to investigate brain long-lasting changes produced by drugs of abuse. Non-contingent drug administration animal models were firstly developed to evaluate short and long-lasting changes after exposure to a drug (Panlilio et al., 1998). In contrast, contingent operant self-administration models allow to directly evaluate a drug's reinforcing property with a high predictive value to model voluntary drug consumption in humans. The current model provides for the first time an animal model of cannabinoid addiction that recapitulates the diagnostic criteria used in DSM-5 to define this human disorder (Deroche-Gamonet et al., 2004; Belin and Everitt, 2008; Maldonado et al., 2021). Indeed, our animal model has been generated based on the three main behavioral hallmarks of addiction that englobe DSM-5 addiction criteria (Supplementary Figure S1): 1) persistence of response (criteria 6 and 7 of DSM-5), 2) motivation for the drug (criteria 9 and 10 of DSM-5), 3) compulsive-like behavior defined as a disruption of inhibitory control despite negative consequences (criterion 11 of DSM-5). Our model also measures extinction reactiveness and reinstatement, parameters closely related with craving, another addiction diagnostic criteria of the DSM-5, as well as other phenotypic traits of predictive value for the development of cannabinoid addiction.

In this study, we used the synthetic cannabinoid agonist WIN 55,212–2, which is a potent full agonist of cannabinoid receptor 1 (CB1R) (Compton et al., 1992), to generate a model of cannabinoid addiction (Maldonado et al., 2011). Previous studies tried to obtain an operant intravenous (iv) self-administration of delta9-tetrahydrocannabinol (THC), which is the primary psychoactive component of the *cannabis sativa* plant. However, few studies were able to maintain a model with persistent, dose-related behavior regarding THC iv self-administration (Justinova et al., 2003; Spencer et al., 2018), as well as vapor self-administration (Freels et al., 2020), showing the necessity of alternative methods. In contrast to THC, mice achieved a reliable operant iv self-administration with the synthetic cannabinoid agonist WIN 55,212–2 (Martellotta et al., 1998; Mendizábal et al., 2006), although this cannabinoid has not yet been used to validate a model of cannabinoid addiction. This study aimed to validate for the first time a reliable mouse model of cannabinoid addiction by using WIN 55,212–2 iv operant self-administration.

This behavioral protocol can be combined with multiple neurochemical, electrophysiological, optogenetic, and chemogenetic manipulations to decipher the neurobiological mechanisms involved in cannabinoid addiction. For this purpose, we chemogenetically silenced the prelimbic (PL) -nucleus accumbens (NAc) pathway in our mouse model through a Designer Receptors Exclusively Activated by Designer Drugs (DREADD) approach, a pathway closely involved in the development of addictive behavior (Domingo-Rodríguez et al., 2004; Compton et al., 2022).

2 Materials and equipment

2.1 Animals

Eight weeks old male C57BL/6J mice (n = 30) (Charles River, France) were housed individually with food and water available *ad libitum* in controlled laboratory conditions (21oC ± 1°C, 55% ± 10%). Mice were tested during the first hours of the dark phase of a reversed light/dark cycle (lights off at 8:00 a.m. and on at 20:00 p.m.). Body weight and food intake were monitored throughout the entire experiment. All animal procedures were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB, agreement N°9687) and conducted in strict conformity with the guidelines of the European Communities Council Directive (2010/63/EU) regulating animal experimentation, in the animal facility at Universitat Pompeu Fabra-Barcelona Biomedical Research Park (UPF-PRBB; Barcelona, Spain). All the experiments were performed under blind and randomized conditions. The male sex was chosen accordingly with the previous literature that has validated the operant WIN 55,212–2 self-administration model only in males (Martellotta et al., 1998; Mendizábal et al., 2006; Mancino et al., 2015; Domingo-Rodriguez et al., 2020; Martín-García et al., 2020; García-Blanco et al., 2022).

2.2 Drugs

WIN 55,212–2 [(R)-(+)-WIN 55,212–2 mesylate salt, Sigma-Aldrich, U.S.A.] was dissolved in one drop of Tween 80 (TWEEN 80, Sigma-Aldrich, U.S.A.) and then diluted in heparinized (1%) sterile saline solution and made available at two different doses: 0.1 mg/kg for intraperitoneal (ip) injection 24 h before the first operant session and 12.5 μg/kg/infusion for the self-administered iv infusions. The preparation was covered from the light and stored at room temperature. After each self-administration session, 0.05 mL of sodic heparin (Hospira 5%, Hospira, Pfizer) was applied through the iv catheter to avoid coagulation and obstruction of the latter. Thiopental sodium (5 mg/mL, Braun Medical S.A.) was dissolved in distilled water and injected in a volume of 0.05 mL through the iv catheter to evaluate catheter patency.

2.3 Operant self-administration apparatus

Experiments were performed in mouse operant chambers (model ENV-307A-CT, Med Associates Inc., Georgia, VT, U.S.A.) equipped with two nose-pokes, one randomly selected as

the active hole and the other as the inactive hole. A house light was located on the chamber's ceiling, and two stimuli lights (cues) were placed one inside the active hole and the other above it. Nose-poking on the active hole resulted in the delivery of one WIN 55,212–2 infusion (under the associated schedule) paired with the activation of the stimulus light located above the active hole, while nose-poking on the inactive hole had no consequences. The chambers were made of aluminum and acrylic and placed inside sound- and light-attenuated boxes equipped with fans providing ventilation and white noise. The chamber's floor was a grid made with metal bars that could conduct electrical current when performing the shock test. WIN 55,212–2 (12.5 µL/kg/infusion) was delivered in a volume of 23.5 µL over 2 s *via* a syringe firmly attached to a micro infusion pump (PHM-100A, Med-Associates, Georgia, VT, U.S.A.) and connected with flexible polymer tubing (0.96 mm outer diameter, Portex Fine Bore Polythene Tubing, Portex Limited, Kent, England) to a single channel liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, U.S.A.) and the mouse iv catheter.

3 Methods

3.1 WIN 55,212–2 self-administration

3.1.1 Jugular vein catheterization

Mice (n = 30) were anesthetized by ip injection (0.2 mL/10 g of body weight) of ketamine hydrochloride (75 mg/kg of body weight, Ketamidor, Richterpharma ag, Austria) and medetomidine hydrochloride (1 mg/kg of body weight, Domtor, Esteve, Spain) dissolved in 0.9% sterile physiological saline and then implanted with indwelling iv silastic catheters in the right jugular vein, as previously described (Martín-García et al., 2009). Briefly, a 6 cm long silicone tubing (0.3 mm inner diameter, 0.6 mm outer diameter; Silastic, Dow Corning, Houdeng-Goegnies, Belgium) was adapted to a 22-gauge steel cannula (Semat, Herts, England) curved at a right angle and embedded in a dental cement disk (Dentalon Plus, Heraeus Kulzer, Germany) with a nylon mesh underneath. The catheter tubing was inserted 1.1 cm into the right jugular vein and attached with a suture. The remaining tubing was inserted subcutaneously (sc) to the cannula, exiting at the midscapular region. All incisions were sutured and coated with a local analgesic (Blastoestimulina, Almirall, Spain). Post-surgery procedure consisting of an ip injection of antibiotic (1 mg/kg of body weight, Gentamicine, Genta-Gobens, Laboratorios Normon, Spain), a sc injection of analgesic (mixture of glucose serum (GlucosaVet, B. Braun Vet Care, Spain) and meloxicam (2 mg/kg of body weight, Metacam, Boehringer Ingelheim, Rhein) and a sc injection of an anesthesia reversor, atipamezole hydrochloride (2.5 mg/kg of body weight, Revertor, Virbac, Spain), was applied all dissolved in 0.9% sterile physiological saline. Mice were allowed to recover for 3 days, with follow-up analgesics, prior to the initiation of the self-administration sessions. The patency of iv catheters was assessed by a thiopental sodium test at the end of the self-administration experimental sequence. The mouse was removed from the experiment if prominent signs of anesthesia were not observed immediately after injection (n = 1 in this study).

3.1.2 WIN 55,212–2 self-administration training

The operant model was applied accordingly to previous drug self-administration paradigms (Mendizábal et al., 2006; Vallée et al., 2014; Martín-García et al., 2016). To avoid the aversive effects of the drug's first administration, mice received an ip injection of WIN 55,212–2 (0.1 mg/kg) only 24 h before the first self-administration session (Valjent and Maldonado, 2000; Mendizábal et al., 2006; Vallée et al., 2014). Subsequently, mice (n = 29) were trained to acquire an operant self-administration conditioning maintained by iv infusions of WIN 55,212–2. The schedule was a fixed ratio (FR) 1 schedule of reinforcement during 5 consecutive sessions, followed by a progression to FR2 for another 5 sessions. All sessions were performed at the same time and scheduled every day. Each daily self-administration session was started with a priming injection of the drug (0.0125 mg/kg/infusion) automatically delivered iv through the catheter when the session was initiated (Mendizábal et al., 2006; Martín-García et al., 2016; Flores et al., 2020), followed by two 55 min active periods separated by a 15 min drug-free period for a total duration of 125 min. The initiation of each session was signaled by turning on the house light only during the first 3 s. The cue lights, together with the noise of the infusion pump, acted as environmental cues signaling the drug infusion. A 10 s time-out period was fixed after each drug delivery, during which the cue light was off, and no reward was provided after responding to the active nose-poke. Responses to the active and inactive holes and all responses executed during the time-out were recorded. During the drug-free period, no reinforcer nor cue was delivered, signaled by the activation of the house light. The session was concluded after 50 reinforcers were delivered or after 125 min, whichever occurred first. The acquisition of the self-administration behavior was achieved when the three following conditions were met: 1) mice maintained 80% of stability in three consecutive training sessions, meaning that the variance during these 3 days was 20% or less, 2) at least 75% responding on the active hole, and 3) a minimum of five reinforcers per session. After each session, mice were brought back to their home cages (Figure 1).

3.1.3 Three addiction criteria

The development of addictive-like behaviors was evaluated at the end of the training sessions based on three addiction-like criteria that summarize the addiction hallmarks according to the DSM-5 (Deroche-Gamonet et al., 2004; Piazza and Deroche-Gamonet, 2013; Domingo-Rodríguez et al., 2022). The addiction score developed was then attributed based on the results of these three criteria, each determined by the respective behavioral test:

3.1.3.1 Persistence to response

The number of non-reinforced active responses during the 15 min drug-free period was measured as persistence of drug-seeking behavior. Mice were scored on the three consecutive days before the progressive ratio (PR).

3.1.3.2 Motivation

The PR schedule of reinforcement evaluated the motivation towards the reinforcer. The responses required to receive one drug infusion escalated following this series: 1, 5, 12, 21, 33, 51, 75, 90, 120, 155, 180, 225, 260, 300, 350, 410, 465, 540, 630, 730, 850, 1000,

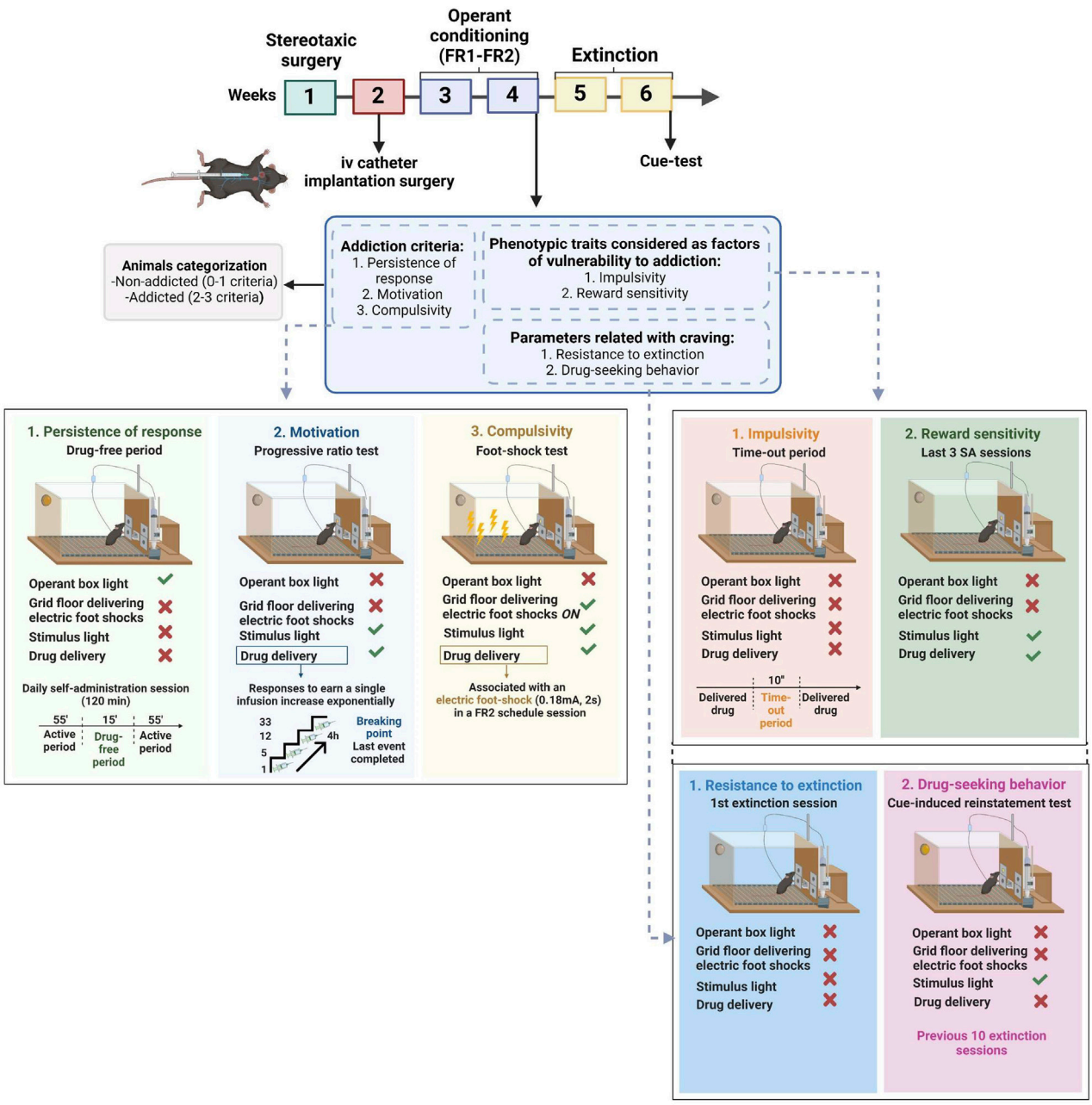

FIGURE 1
Complete experimental protocol of our mouse model of operant WIN 55,212–2 intravenous self-administration.

1200, 1500, 1800, 2100, 2400, 2700, 3000, 3400, 3800, 4200, 4600, 5000, and 5500. The breaking point, the maximal number of responses mice perform to obtain one infusion defined as the motivation value, corresponds to the last ratio completed. The duration of the PR session was maximum 4 h or until mice stopped responding to any nose-poke within 1 h.

3.1.3.3 Compulsivity

Resistance to punishment, now defined as compulsive-like behavior, corresponded to the maintenance of active responding behavior despite its association with a negative consequence. It was measured by the total number of shocks obtained in a 50 min shock test, during which each drug delivered was associated with a foot-shock-induced punishment. This shock session was performed after a stabilizing FR2 self-administration session following the PR test. Mice were placed in a different operant box than the one regularly used for the operant sessions. Then mice underwent an FR2 self-administration schedule of reinforcement for 50 min with two scheduled changes: after one active response, mice received an electric foot-shock (0.18 mA, 2 s), while after the second response, the electric foot-shock was paired with the drug delivery and the associated cue light. In parallel, if the second response was not completed within a min after completing the first response, the sequence was reinitiated.

3.1.4 Establishment of mice subpopulations

After the three behavioral tests were performed, mice were categorized into addicted and non-addicted animals based on the number of positive criteria achieved. A mouse was considered to be positive for an addiction-like criterion when the score of the behavioral test was equal to or beyond the 75th percentile of the normal distribution of the saline group. Mice that achieved 2 or 3 criteria were considered addicted and categorized as vulnerable, whereas those reaching 0 or 1 criterion were considered non-addicted and categorized as resilient.

3.1.5 Extinction and parameters related with craving

Only mice with patent catheters that reached all acquisition criteria continued to the extinction phase. After thiopental testing, mice were allowed to rest for 1 day, during which they underwent a 2-h locomotion test in individual locomotor activity boxes (10.8 × 20.3 × 18.6 cm, Imetronic, Pessac, France) equipped with infrared sensors to detect locomotor activity and an infrared plane to detect rearings.

During the extinction period, neither WIN 55,212–2 infusions, priming infusions, nor the associated environmental cues were delivered after nose-poking on the active hole. Mice were exposed to 2-h daily sessions for 10 consecutive days in the same operant chamber as the self-administration sessions. During this period, mice reached the extinction criterion when responses to the active nose-poke were < 35% of the mean responses obtained during the last 3 days of WIN 55,212–2 self-administration across three consecutive extinction sessions. Only mice that achieved the extinction criterion were evaluated for the following. Two parameters related with craving were evaluated before and after this extinction period:

3.1.5.1 Resistance to extinction

Number of active responses in 2 h during the first extinction session. Animals with significant sensitivity to drug withdrawal will increase their resistance to extinction by increasing the number of active nose-pokes to seek the drug when access is prevented.

3.1.5.2 Drug-seeking behavior

The day after achieving the extinction criterion, we performed a single cue-induced reinstatement session in the same operant chamber, in order to test reinstatement of drug-seeking behavior upon exposure to the environmental stimuli after a period of abstinence. The cue test was conducted under the same conditions used in the acquisition phase, except that active responding was not reinforced by the drug. This meant that mice were subject to a 90-min FR2 session, where the first 60 min were similar to an extinction session but in the last 30 min, nose-poking on the active hole resulted in the presentation of all the environmental cues associated (cue light, pump noise, and priming injection light) but not the delivery of WIN 55,212–2 (Martín-García et al., 2016; García-Blanco et al., 2022).

3.1.6 Behavioral tests to evaluate addiction-like phenotypic traits

Two additional phenotypic traits were also evaluated as factors of vulnerability to addiction:

3.1.6.1 Impulsivity

The number of non-reinforced active responses during the time-out periods (10 s) after each WIN 55,212-2 delivery was measured as impulsivity-like behavior, which indicated the inability to stop a response once it is initiated. The three consecutive days before the PR test were considered for this criterion.

3.1.6.2 Sensitivity to reward

The number of reinforcers obtained in 2-h sessions during the last three consecutive FR2 operant conditioning sessions maintained by WIN 55,212–2. Animals with higher levels of sensitivity to reward will obtain a higher number of reinforcers.

3.2 DREADD approach: Surgery and viral vector microinjection

The adeno-associated viral (AAV) vectors used were: AAV-hM4Di-DREADD (AAV8-hSyn-DIO-hM4D(Gi)-mCherry, 1.21E+13 gc/mL) and AAV-retrograde-Cre-EBFP (AAV pmSyn1-EBFP-Cre; 8.2E+12 gc/mL) (Viral Vector Production Unit, Universitat Autònoma de Barcelona).

Mice were anesthetized by ip injection (0.2 mL/10 g of body weight) of ketamine hydrochloride (75 mg/kg of body weight, Ketamidor, Richterpharma ag, Austria) and medetomidine hydrochloride (1 mg/kg of body weight, Domtor, Esteve, Spain) dissolved in 0.9% sterile physiological saline and located into a stereotaxic apparatus to receive the intracranial AAV injections. All injections were performed through a bilateral injection cannula (33-gauge internal cannula, Plastics One, United Kingdom) connected *via* a polyethylene tubing (PE-20, Plastics One, United Kingdom) to a 10 µL microsyringe (Model 1701 N SYR, Cemented NDL, 26 s GA, 2 in point style 3, Hamilton company, NV). The displacement of an air bubble along the tubing connecting the syringe to the injection needle was utilized to monitor the microinjections. For the precise inhibition of the PL-NAc pathway, two bilateral injections were performed, one targeting the PL and the other the NAc core. Mice were injected with 0.2 µL per site of the AAV-hM4Di-DREADD into PL (rate infusion of 0.05 µL/min) and 0.4 µL per site of the AAV-retrograde-Cre-EBFP into the NAc core (rate infusion of 0.10 µL/min). After infusion, the injection cannula was left untouched for an additional 10 min to permit the fluid to diffuse and prevent reflux, and then slowly withdrawn. A heating pad was used to preserve the body temperature at 35°C. The coordinates used followed the Paxinos and Franklin atlas (Franklin and Paxinos, 1997): (PL) AP +2.10 mm, L ±0.3 mm, DV -2.3 mm; (NAc core) AP +1.94 mm, L ±1 mm, DV -4.6 mm.

Clozapine-N-oxide (CNO, Enzo Life Sciences, NY), a behaviorally inert drug, was administered *via* Alzet osmotic minipumps (Model 2004; Alzet, Cupertino, CA) previously filled with either CNO (diluted in 0.9% sterile saline; 5 mg/mL) or physiological saline solution. Minipumps were sc implanted in the lower back of each mouse during the jugular vein catheterization surgery. The osmotic minipumps delivered CNO by osmosis at a constant sc flow rate of 0.25 µL/h for 15 days (Domingo-Rodríguez et al., 2020; Martín-García et al., 2020).

3.3 Statistical analysis

3.3.1 Statistical analysis of behavioral data

The number of animals (n) in each experimental condition is indicated in the figure legends. All statistical comparisons were performed with SPSS (IBM, version 25). Comparisons between two groups were performed by Student's t-test or U Mann-Whitney test according to the distribution defined by the Kolmogorov-Smirnov normality test. ANOVA with repeated measures was used when necessary to test the evolution over time, followed by *post hoc* analysis (Fisher LSD) for multiple group comparison. The Pearson correlation coefficient was performed to analyze the relationships between values in each addiction-like criterion and the final criteria achieved. The chi-square analysis were used to compare the percentage of addicted and non-addicted mice. Results were expressed as individual values with the median and the interquartile range or with the mean ± S.E.M, which is specified in the figure legend. A p-value <0.05 was applied to determine statistical significance.

The sample size was calculated based on the power analysis. The significance criterion (alpha) was set at 0.050, and the statistical test utilized was a two-sample t-test. With the sample size of 13–16 mice per group, our studies achieved a power superior to 80%. Supplementary tables (Supplementary Tables S1–S2) provide a complete report of the statistical results for the data described in the figures.

3.3.2 Principal component analysis

The principal component analysis (PCA) was performed to evaluate the multidimensional behavioral data by reducing it to fewer dimensions in order to observe trends, clusters, and outliers. PCA and varimax rotation were conducted using the three addiction-like criteria, the two parameters related to craving and the two phenotypic traits considered as vulnerability factors of addiction to dimensionality reduce them to the minimum number of components that best explain and maximize the variance present in the data set. An eigenvalue greater than 1 was set as selecting components criterion (Field, 2018).

4 Results

4.1 WIN 55,212–2 self-administration led to the development of an addictive-like phenotype in mice

We have developed for the first time a mouse model of cannabinoid addiction by using WIN 55,212–2 self-administration. In addition, we have evaluated in this model the possible involvement of the PL-NAc pathway in the development of this addictive behavior (Figure 1). Saline and CNO-treated mice were trained to acquire an operant self-administration sustained by iv infusions of WIN 55,212–2 (Figure 2A). The percentage of animals that achieved acquisition criteria of stability, discrimination, and number of reinforcers was 44.83% in the saline group and 55.17% in the CNO group (chi-square, C-S = 0.69, *n.s.*), with a progressive increase in the number of active nose-pokes across sessions (Repeated Measures ANOVA, [$F_{(1,54)}$ = 27.76 in FR1, $F_{(1,54)}$ = 0.01 in FR2, $p < 0.001$, DMS Actives vs. Inactives: $p < .001$]). No significant differences were found in active and inactive nose-pokes between CNO- and saline-treated mice in both FR1 and FR2 schedules, suggesting similar levels of operant conditioning maintained by WIN 55,212–2 (DMS Actives/Inactives CNO vs. Actives/Inactives Saline: *n.s.*).

The addiction score was calculated after the operant training using the three addiction-like criteria, as explained above. Extreme subpopulations of mice that present a high persistence of response, motivation and compulsivity, were revealed in both saline and CNO groups. Specifically, 26.1% (23.08% saline, 18.75% CNO), 51.7% (61.54% saline, 43.75% CNO) and 24.1% (30.77% saline, 18.75% CNO) of mice surpassed each criterion's threshold, suggesting the potential development of addictive-like behaviors after the chronic operant training. No significant differences were found between CNO- and saline-treated mice in persistence of response, motivation or compulsive-like behavior (Figures 2B–D). In the saline group, 23.08% (3/13) were considered addicted whereas 12.50% (2/16) were considered addicted in the CNO group (chi-square = 3.77, *n.s.*, Figure 2E). Addicted mice showed a strong tendency for higher persistence of response compared to non-addicted in both saline and CNO groups and only for the saline-treated animals in the motivation (Figures 2F,G), whereas a significantly higher compulsive-like behavior was observed for addicted mice compared to non-addicted mice regardless of the treatment (U Mann-Whitney test, U = 3,000 for NA vs. A saline and U = 1,500 for NA vs. A CNO, $p < 0.05$, Figure 2H), Moreover, positive correlations were found between the number of criteria achieved and the severity of each criterion in both CNO- and saline-treated mice for all addictive-like criteria except for motivation in CNO-treated mice (Pearson correlations, $p < 0.05$, Figures 2I–K).

After FR1 and FR2 training, mice underwent 10 sessions of extinction (Figure 3A). Both groups extinguished the self-administration behavior similarly (Repeated Measures ANOVA, [Active lever presses: $F_{(1,27)}$ = 0.15, *n.s.*, Inactive lever presses: $F_{(1,27)}$ = 0.38, *n.s.*]), despite a higher number of reinforcers obtained during the first session in saline-treated mice compared to the CNO group (U Mann-Whitney test, U = 42,000, $p < 0.01$, Figure 3B). Responses to the active nose-poke declined over time until reaching 58% and 43.7% decrease of the active nose-poke in the last session compared to the last operant session for the saline and CNO groups, respectively.

Animals that responded < 35% of the mean responses performed during the last 3 days of WIN 55,212–2 self-administration across three consecutive extinction sessions acquired the extinction criteria (30.77% of saline and 12.50% of CNO mice, chi-square = 1.92, *n.s.*). Resistance to extinction, measured on the first day of extinction, was significantly lower in the CNO group compared to saline-treated mice (U Mann-Whitney test, U = 42.000, $p < 0.01$, Figure 3B). In contrast, no significant differences were obtained between groups for the cue-induced reinstatement of drug-seeking behavior (Figure 3C). Non-addicted mice in the saline group showed higher levels of response in the first extinction session compared to non-addicted mice in the CNO group (U Mann-Whitney test, U = 32.000, $p < 0.05$,

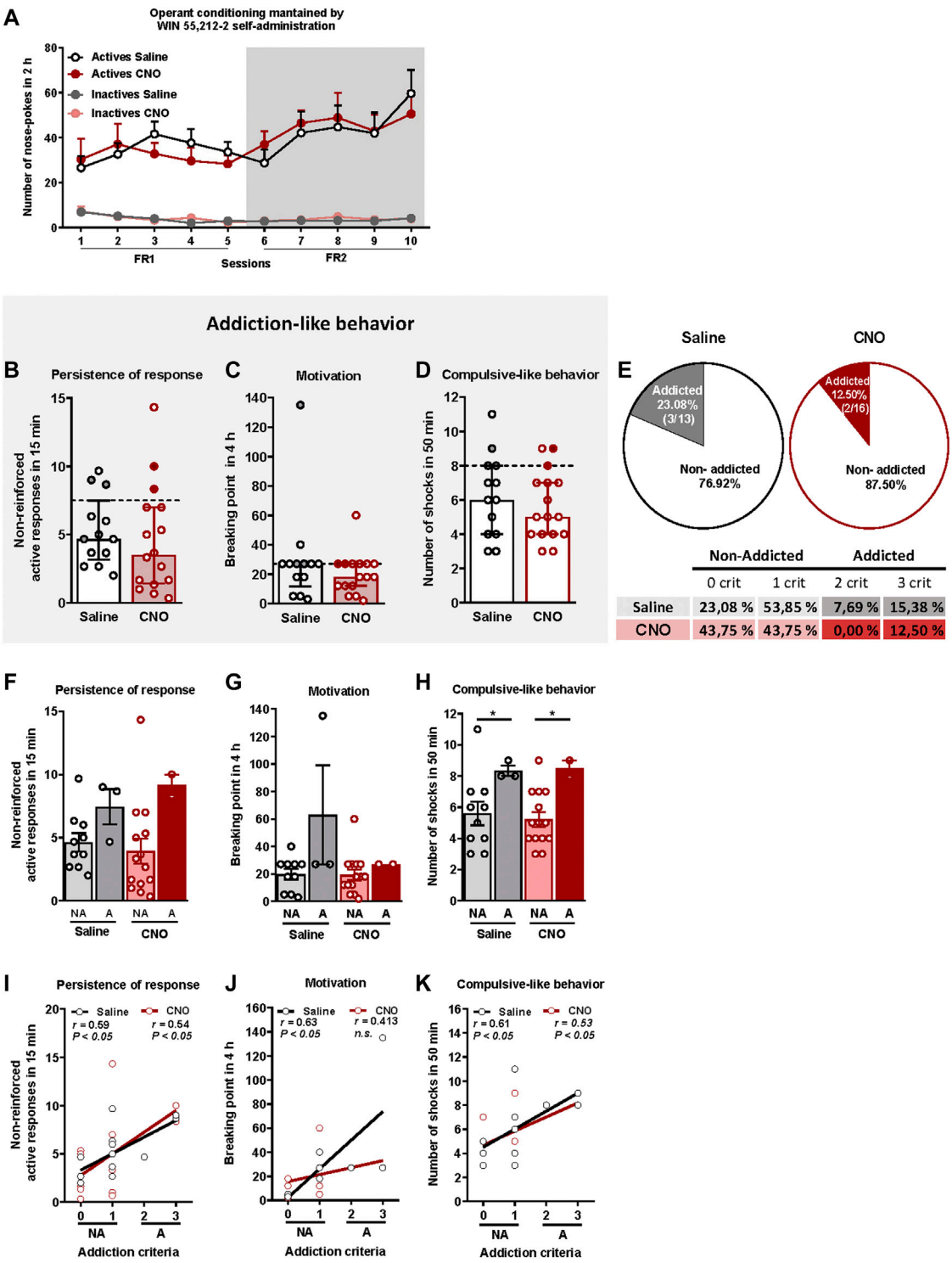

FIGURE 2
WIN 55,212–2 operant self-administration led to the development of an addictive-like phenotype in mice. **(A)** Similar number of total active and inactive nose-pokes performed by CNO- and saline-treated groups during 2 h of operant self-administration maintained by intravenous infusions of WIN 55,212–2 in both FR1 and FR2 schedules of reinforcement (mean ± S.E.M., repeated measures ANOVA). **(B–D)** Mice present similar responses in the three addiction-like criteria tests (individual data with median and interquartile range): **(B)** Persistence to response: Number of responses to the active nose-poke during the 15 min drug-free period (Student's t-test). **(C)** Motivation: Breaking point determined during a 4 h progressive schedule of reinforcement represents the maximal number of responses that an animal is able to do to obtain one drug infusion (U Mann-Whitney). **(D)** Compulsivity: Number of shocks received following the schedule described in the *Materials and Methods* section, reflecting the compulsivity level of each group (Student's t-test). The dashed horizontal line indicates the 75th percentile of the distribution of the group, used as the threshold to consider a mouse positive for one criterion. Addicted mice are represented in grey- and red-filled circles. **(E)** Percentage of mice categorized as addicted (Chi square). **(F–H)** Behavioral tests of the three addiction-like criteria showing increased compulsivity in addicted mice compared to non-addicted but similar persistence to response and motivation (individual data with mean ± S.E.M., U Mann-Whitney, *$p < 0.05$). **(I–K)** Pearson correlations between individual values of addiction-like criteria and **(I)** non-reinforced active responses in 15 min, **(J)** breaking point in 4 h and **(K)** number of shocks in 50 min (Saline-treated mice: n = 13; CNO-treated mice: n = 16; statistical details are included in Supplementary Table S1).

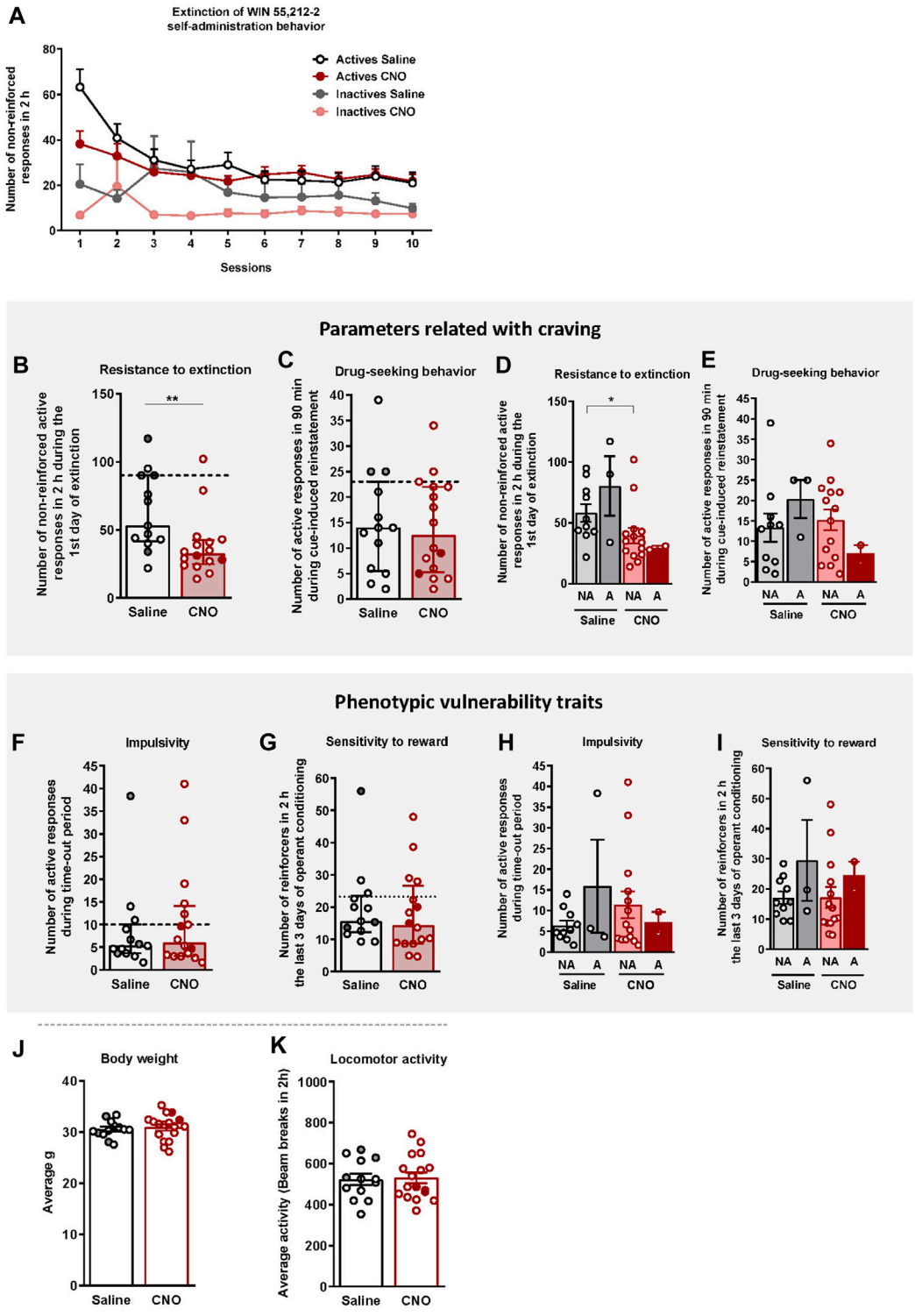

FIGURE 3
WIN 55,212–2 operant self-administration effects on two parameters related with craving and two phenotypic vulnerability traits to addiction-like behavior in mice. **(A)** Extinction pattern of the WIN 55,212–2 operant self-administration behavior (mean ± S.E.M., repeated measures ANOVA). **(B–C)** Behavioral tests of the two parameters related with craving (individual data with median and interquartile range): **(B)** Resistance to extinction: Number of responses to the active nose-poke during the first 2-h extinction session is significantly higher in saline-compared to CNO-treated mice (U Mann-Whitney, $^{**}p < 0.01$). **(C)** Drug-seeking behavior measured by the cue-induced reinstatement after abstinence: Number of active responses performed during the 90 min cue-induced drug-seeking test performed after extinction (Student's t-test). The dashed horizontal line indicates the 75th percentile of distribution of the group, used as the threshold to consider a mouse positive for one criterion. Addicted mice are represented in grey- and red-filled circles. **(D–E)** Behavioral tests of the parameters related with craving showing similar responses in cue-induced reinstatement between addicted and non-addicted mice (U Mann-Whitney), whereas a difference is observed between non-addicted mice in the 1st day of extinction (U Mann-Whitney, $^{*}p < 0.05$) (individual data with mean ± S.E.M.). **(F–G)** Behavioral tests used to evaluate the two phenotypic traits considered to be factors of vulnerability to

(*Continued*)

FIGURE 3 (Continued)
addiction-like behavior (individual data with median and interquartile range): **(F)** Impulsivity: Number of responses to the active nose-poke during the 10 s time-out period (U Mann-Whitney) **(G)** Sensitivity to reward: Number of reinforcers performed to the active nose-poke during the 2 h of the last three sessions of self-administration (Student's t-test). The dashed horizontal line indicates the 75th percentile of distribution of the group, used as the threshold to consider a mouse positive for one criterion. Addicted mice are represented in grey- and red-filled circles. **(H–I)** Behavioral tests of the phenotypic traits showing similar responses in impulsivity and behavior during the last 3 days of operant training between addicted and non-addicted mice (U Mann-Whitney) (individual data with mean ± S.E.M.). **(J)** Body weight: Body weight was measured every week during the self-administration protocol (mean ± S.E.M.; Student's t-test). **(K)** Locomotor activity: Activity was measured by the number of beam breaks during a 2 h test (mean ± S.E.M.; Student's t-test). (Saline-treated mice: n = 13; CNO-treated mice: n = 16; statistical details are included in Supplementary Table S2).

A

Variables	Factor loadings: Principal component 1 (45.0 %)	Factor loadings: Principal component 2 (20.2%)
Persistence of response	0,59	0,22
Motivation	0,80	0,31
Compulsivity	-0,12	0,83
Impulsivity	0,91	-0,18
Sensitivity to reward	0,94	-0,01
Resistance to extinction	0,53	0,67
Drug-seeking behavior	0,40	0,31

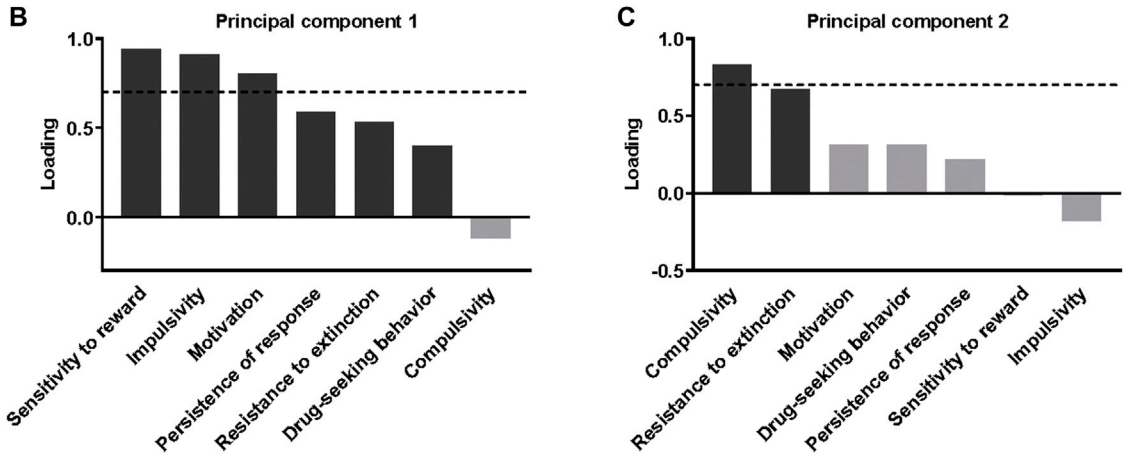

FIGURE 4
Principal component analysis of cannabinoid addiction through WIN 55,212–2 operant self-administration. **(A)** Factor loadings of the principal component 1 (PC1) and principal component 2 (PC2) in all variables studied. **(B–C)** Order of factor loading of the different variables in PC1 and PC2. The dashed horizontal line marks loading greater than 0.7, mainly contributing to the component. In regards to the addiction criteria, a dissociation between motivation and persistence of response, mainly contributing to PC1, and compulsivity, mainly contributing to PC2, can be observed. Regarding parameters related to craving, drug-seeking behavior weighted more in the PC1, while resistance to extinction weighted more in the PC2, even though its influence is also present in the PC1. For the phenotypic traits, both weighted more in the PC1.

Figure 3D), while no significant differences were obtained for the drug-seeking behavior (Figure 3E).

4.2 WIN 55,212–2 self-administration effects on phenotypic vulnerability traits to addiction-like behavior in mice

Two phenotypic traits considered as vulnerability factors to addiction-like behavior, impulsivity and sensitivity to reward, were also evaluated. No significant differences were found between CNO (mean ± S.E.M.: 10.85 ± 2.85; 18.08 ± 3.11) and saline-treated mice (mean ± S.E.M.: 8.56 ± 2.66; 19.90 ± 3.42), neither in impulsivity nor reward sensitivity respectively (Figures 3F,G). No significant differences between groups were neither observed when the population of saline and CNO groups was divided into addicted and non-addicted (Figures 3H,I).

To confirm that CNO treatment did not produce any effect that could bias our self-administration results (Roth, 2016), the body weight, locomotor activity, and food intake of mice were monitored

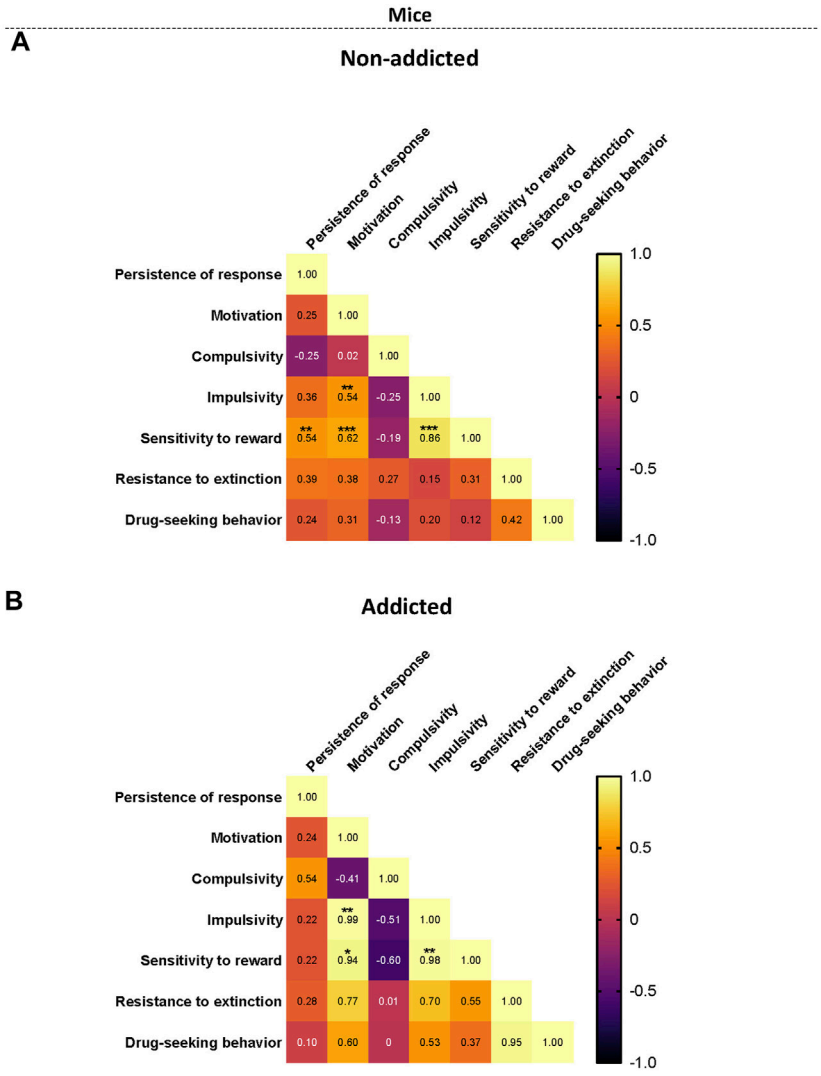

FIGURE 5
Correlation heatmaps of the variables of cannabinoid addiction criteria, parameters related to craving, and phenotypic vulnerability traits. **(A–B)** Pearson correlations between the three addiction-like criteria, the two parameters related with craving and the two phenotypic traits in both **(A)** non-addicted and **(B)** addicted groups. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

throughout the experiment. No significant differences were observed between saline- and CNO-treated mice in terms of body weight (Figure 3J), and food intake (*data not shown*) across the entire experiment. Moreover, no significant differences were observed between groups in the locomotor activity (Figure 3K), sustaining the absence of side effects of CNO treatment.

4.3 Principal component analysis of cannabinoid addiction through WIN 55,212–2 self-administration

A principal component analysis was used to determine whether the behavioral outcomes previously described could be reduced to fewer dimensions that might display individual differences in cannabinoid addiction. All addiction criteria, parameters related with craving and phenotypic traits were taken into account. Principal component 1, which accounts for 45.0% of the variance (Figure 4A), has strong loadings (>0.7) from all behavioral variables except compulsivity. These traits are associated to the development of cannabinoid addiction and, therefore, they contribute to this development. The second principal component, which is orthogonal to component 1 and accounts for 20.2% of the variance, is comprised of two variables, the criteria of compulsivity and resistance to extinction. Interestingly, impulsivity participates more in the first component, while compulsivity is more critical in the second component (Figures 4B,C), resembling the sequential feature of the transition from impulsivity to compulsivity described in addiction. Finally, most of the phenotypic traits of vulnerability are in the same component suggesting similar neurological correlates.

4.4 Correlation heatmap of the variables of cannabinoid addiction criteria, parameters related with craving and vulnerability phenotypic traits

When representing the addiction-like criteria, the parameters related with craving and the phenotypic traits into a heat map, non-addicted animals revealed significant correlations between persistence to response and sensitivity to reward (r = 0.54, $p < 0.01$), motivation and sensitivity to reward (r = 0.62, $p < 0.001$), impulsivity and sensitivity to reward (r = 0.86, $p < 0.001$), and motivation and impulsivity were significant (r = 0.54, $p < 0.01$) (Figure 5A). In addicted animals, the significant correlations between motivation and impulsivity (r = 0.99, $p < 0.01$), motivation and sensitivity to reward (r = 0.95, $p < 0.05$), and impulsivity and sensitivity to reward (r = 0.98, $p < 0.01$) were maintained (Figure 5B). Theses results were in agreement with the results obtained in the PCA. Interestingly, compulsivity and impulsivity showed a negative correlation (non-significant) in coherence with the differential load of each variable in the PCA.

5 Discussion

Cannabis addiction, defined in the 5th edition of the DSM as cannabis use disorder, is a major concern worldwide, and its neurobiological substrate is still largely unknown. The absence of animal models that recapitulate the hallmarks of cannabinoid addiction has impaired the animal research focused on this disorder. In this study, we developed for the first time an animal model of cannabinoid addiction that recapitulates the main diagnosis criteria of this disorder defined on the DSM-5 based on an operant paradigm of WIN 55,212–2 iv self-administration.

Drug self-administration paradigms based on their positive reinforcement effects can be used to model certain aspects of the human addictive behavior (Markou et al., 1999). However, previous studies have demonstrated the complexity in modeling cannabis self-administration compared to other drugs due to the difficulty to obtain a reliable self-administration of cannabinoids in animals (Panlilio and Justinova, 2018). Operant self-administration of THC is difficult to be reliably maintained in animals (Tanda and Goldberg, 2003). Only a few number of studies in squirrel monkeys (Justinova et al., 2003) and rats (Spencer et al., 2018; Freels et al., 2020) have been able to show that THC can maintain self-administration (intravenous or vapor) without a previous history of exposure to other drugs. The failure of THC to act as reinforcer in animal studies has been related to its delayed onset of pleasurable pharmacological effects, the long duration of its pharmacological and behavioral effects, and its partial agonist profile (Tanda and Goldberg, 2003). Due to these difficulties, most of the operant self-administration studies have used synthetic cannabinoids. WIN 55,212–2 is a potent synthetic cannabinoid with a shorter half-life than THC and a full agonist of the CB1R (Pertwee et al., 2010). These characteristics may explain the difference in reinforcing properties and the improving characteristics for operant self-administration models in comparison to THC (Maldonado et al., 2011). Several studies have achieved reliable operant paradigms to self-administer WIN 55,212–2 in mice and rats (Martellotta et al., 1998; Fattore et al., 2001; Mendizábal et al., 2006; Lefever et al., 2014). However, none of these self-administration studies has been used to generate a model of cannabinoid addiction.

In our study, we have established a model in which vulnerable mice present persistence to self-administer WIN 55,212–2, extremely high motivation to obtain WIN 55,212–2 infusions, and compulsivity to WIN 55,212-2-seeking and self-administer despite adverse consequences, which confirms the development of a cannabinoid addiction-like model based on iv infusions of WIN 55,212–2. These criteria allowed to separate two populations of mice with vulnerable and resilient phenotypes to develop cannabinoid addiction. Therefore, the establishment of this cannabinoid addiction mouse model using WIN 55,512–2 iv self-administration represents a pivotal tool for future research allowing to elucidate the neurobiological correlates underlying resilience and vulnerability to develop this disorder. These addiction-like hallmarks used to establish this cannabinoid addiction model have been extracted from an established rat model of cocaine addiction (Deroche-Gamonet et al., 2004), and have been repeatedly used as a reference to establish mice models of drug and food addiction (Martín-García et al., 2016; Domingo-Rodríguez et al., 2020).

A main problem in the treatment of addiction is the high rates of relapse to drug use after periods of abstinence (Venniro et al., 2016; Fredriksson et al., 2021). An important advantage of our cannabinoid addiction model is the possibility to evaluate two parameters closely related with craving and relapse, resistance to extinction and cue-induced reinstatement. Resistance to extinction measures an 'extinction burst' behavior typically seen in rodents during the first day of extinction (Cooper et al., 1987) that has been revealed with different drugs of abuse (Peltier et al., 2001; Shalev et al., 2002; Soria et al., 2008). The resistance to extinguish the operant behavior revealed in the present study suggests that mice had developed a reliable and persistent operant WIN 55,212–2 self-administration behavior, and this behavior has been reported to reflect a 'craving-like' state at the beginning of the extinction training (Fredriksson et al., 2021). Craving during abstinence has been suggested to be directly involved with the vulnerability to relapse (Venniro et al., 2016; Fredriksson et al., 2021). Reinstatement of drug seeking is typically assessed by the extinction-reinstatement model (Shaham et al., 2003; Weiss, 2010) and non-reinforced responding to the previously learned active nose-poke is the measure of drug seeking (Stewart and de Wit, 1987). In our model, we performed a cue-induced reinstatement procedure after extinction, in which re-exposure to conditioned cues when responding to the active nose-poke, cues that had been contingently paired with drug delivery during acquisition, reinstated drug seeking (Davis and Smith, 1976). Exposure to drug-associated cues can elicit drug desire and drug seeking, effects implicated both in the maintenance of ongoing drug use and inducing drug seeking after abstinence, which shows important resistance to extinction (Weiss and Volkow, 2022). However, some studies argue that human abstinence is often either forced or voluntary (self-imposed) (Venniro et al., 2016; Fredriksson et al., 2021). In fact, drug relapse and craving are commonly triggered not only by drug-associated cues, but also by acute exposure to the self-administered drug, stress and short-term or protracted withdrawal symptoms (Venniro et al., 2016; Fredriksson et al., 2021). Alternative animal

models have been developed in which abstinence is not obtained by extinction training but through forced abstinence, mainly assessed by the incubation of drug craving model (Grimm et al., 2001) or by voluntary abstinence, achieved either by the introduction of negative consequences to ongoing drug-administration (Panlilio et al., 2003; Cooper et al., 2007), or of alternative non-drug reinforcers (Ahmed et al., 2013).

This model also evaluates two phenotypic traits related to addiction vulnerability factors. Impulsivity is a complex construct composed of motor impulsivity and choice impulsivity (Belcher et al., 2014). In our model, we have considered the non-reinforced active nose-pokes during the time-out periods to evaluate the motor impulsivity defined as motor disinhibition, as previously described (Domingo-Rodriguez et al., 2022), The impulsivity trait has been associated with drug addiction since it predicts the transition to compulsive drug intake (Belin and Deroche-Gamonet, 2012; Weafer et al., 2014), and neurobiological correlates underlying this phenotypic trait could reveal potential biomarkers and/or therapeutic targets for cannabinoid addiction. Reward sensitivity is associated with an increased probability of responding with a positive hedonic component involving pathways that have a crucial role in the rewarding properties of drugs of abuse (Koob and Volkow, 2016).

We have used this cannabinoid addiction model to evaluate the possible involvement of the PL- NAc glutamatergic pathway, which plays a crucial role in food addiction (Domingo-Rodríguez et al., 2020). This pathway is modulated by the endocannabinoid system and deletion of the CB_1R of these glutamatergic neurons leads to a resilient phenotype to develop food addiction (Domingo-Rodríguez et al., 2020). CB1Rs are the main cannabinoid receptors involved in the development of cannabinoid addiction (Maldonado et al., 2011) and the inhibition of the activity of the PL-NAc pathway may modify the development of cannabinoid addiction. However, CNO activation of the inhibitory DREADDs expressed in the PL-NAc pathway did not alter the addictive-like behavior regarding the addictive criteria and phenotypic vulnerability traits. However, the resistance to extinction was decreased in CNO-treated animals, suggesting a protective effect of this manipulation on the craving-like state. It is important to underly that the patency of the catheters limited the protocol to 2 weeks. Thus, the time between when the minipumps filled with CNO were implanted and the performance of the addictive-like behavioral tests was merely 15 days. Hence, the CNO had only this short period of time to be released from the minipump and act on the DREADD to inhibit the pathway. We hypothesize this to be the reason for the absent effect of this pathway's inhibition, as the long-term action of the CNO and the hM4Di DREADDs in inhibiting a neuronal pathway has been confirmed in many studies (Domingo-Rodríguez et al., 2020; Martín-García et al., 2020).

The influence of the environment is key in the onset of consumption as well as the maintenance of cannabinoid addiction. Indeed, environments with high levels of social stressors, lack of opportunities, easy accessibility to drugs, and lack of alternative reinforcers, lead to an elevated risk for addiction development (Volkow et al., 2019). Consequently, the biggest shortcoming of this model is the absence of the environmental aspect of the disorder, which we cannot mimic in a mouse model. Moreover, the male sex was chosen considering the previous literature on drug addiction models (Martellotta et al., 1998; Mendizábal et al., 2006; Flores et al., 2020). In spite of all these studies previously performed in male rodents, further studies will be necessary to validate these models in female mice and rats.

We have established for the first time a novel and reliable mouse model of cannabinoid addiction using WIN 55,212–2 iv operant self-administration that allows to evaluate three addiction criteria, persistence of response, motivation, and compulsivity, based on the addiction hallmarks defined in the DSM-5. This model also allows to measure two parameters related craving, resistance to extinction and reinstatement, and two phenotypic traits related to cannabinoid addiction, impulsivity and sensitivity to reward. This model represents a pivotal tool to elucidate the neurobiological substrates of cannabinoid addiction and guide future research toward therapeutic strategies to address this disorder.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB, agreement N°9687.

Author contributions

EM-G and RM created and conceived the animal model and behavioral methodologies associated. MMC-M conducted the behavioral experiments with the supervision of EM-G and RM. MMC-M and EM-G performed catheterization surgeries. MMC-M performed the statistical and data analyses, generated the associated graphs, and interpretation of the results under the supervision of EM-G and RM. MMC-M wrote the manuscript and prepared the figures with the supervision of EM-G and RM. EM-G and RM revised the manuscript.

Funding

This work was supported by the Spanish "Ministerio de Ciencia e Innovación (MICIN), Agencia Estatal de Investigación (AEI)" (PID2020- 120029GB-I00/MICIN/AEI/10.13039/501100011033, RD21/0009/0019, to MR; the "Generalitat de Catalunya, AGAUR" (2017 SGR-669, to MR; the "ICREA-Acadèmia" (2020) to MR; the "European Commission-DG Research" (PainFact, H2020-SC1-2019-2-RTD-848099, QSPain Relief, H2020-SC1-2019-2-RTD-848068) to MR; the Spanish "la Caixa" Foundation under the project code LCF/PR/HR22/52420017 to MR, the Spanish "Instituto de Salud Carlos III, RETICS-RTA" (RD16/0017/0020) to MR; the Spanish "Ministerio de Sanidad, Servicios Sociales e Igualdad, Plan Nacional Sobre Drogas" (PNSD- 2021I076, to MR;

PNSD- 2019I006, to ME) and Ministerio de Ciencia e Innovación (ERA-NET) PCI2021-122073-2A to ME.

Acknowledgments

We thank R. Martín, M. Linares, D. Real, and F. Porrón for their technical support.

Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1143365/full#supplementary-material

References

Ahmed, S. H., Lenoir, M., and Guillem, K. (2013). Neurobiology of addiction versus drug use driven by lack of choice. *Curr. Opin. Neurobiol.* 23 (4), 581–587. doi:10.1016/j.conb.2013.01.028

Belcher, A. M., Volkow, N. D., Moeller, F. G., and Ferré, S. (2014). Personality traits and vulnerability or resilience to substance use disorders. *Trends Cogn. Sci.* 18 (4), 211–217. doi:10.1016/j.tics.2014.01.010

Belin, D., and Deroche-Gamonet, V. (2012) Responses to novelty and vulnerability to cocaine addiction: Contribution of a multi-symptomatic animal model. *Cold Spring Harb. Perspect. Med.*, 2, a011940, doi:10.1101/cshperspect.a011940

Belin, D., and Everitt, B. J. (2008). Cocaine seeking habits depend upon dopamine-dependent serial connectivity linking the ventral with the dorsal striatum. *Neuron* 3 (57), 432–441. doi:10.1016/j.neuron.2007.12.019

Compton, D. R., Gold, L. H., Ward, S. J., Balster, R. L., and Martin, B. R. (1992). Aminoalkylindole analogs: Cannabimimetic activity of a class of compounds structurally distinct from delta 9-tetrahydrocannabinol. *J. Pharmacol. Exp. Ther.* 263 (3), 1118–1126. PMID: 1335057.

Compton, W. M., Wargo, E. M., and Volkow, N. D. (2022). Neuropsychiatric model of addiction simplified. *Psychiatr. Clin. North Am.* 45 (3), 321–334. doi:10.1016/j.psc.2022.05.001

Cooper, A., Barnea-Ygael, N., Levy, D., Shaham, Y., and Zangen, A. (2007) A conflict rat model of cue-induced relapse to cocaine seeking. *Psychopharmacology*, 194, 117–125. doi:10.1007/s00213-007-0827-7

Cooper, J., Heron, T., and Heward, W. (1987). *Applied behavior analysis*. New York: Macmillan.

Davis, W. M., and Smith, S. G. (1976). Role of conditioned reinforcers in the initiation, maintenance and extinction of drug-seeking behavior. Pavlov. J. Biol. *Sci.* 11 (4), 222–236. doi:10.1007/BF03000316

Deroche-Gamonet, V., Belin, D., and Piazza, P. V. (2004) Evidence for addiction-like behavior in the rat. *Science*, 305, 1014–1017. doi:10.1126/science.1099020

Domingo-Rodriguez, L., Cabana-Domínguez, J., Fernàndez-Castillo, N., Cormand, B., Martín-García, E., and Maldonado, R. (2022). Differential expression of miR-1249-3p and miR-34b-5p between vulnerable and resilient phenotypes of cocaine addiction. *Addict. Biol.* 27 (5), e13201. doi:10.1111/adb.13201

Domingo-Rodriguez, L., Ruiz de Azua, I., Dominguez, E., Senabre, E., Serra, I., Kummer, S., et al. (2020). A specific prelimbic-nucleus accumbens pathway controls resilience versus vulnerability to food addiction. *Nat. Commun.* 11 (1), 782. doi:10.1038/s41467-020-14458-y

European Monitoring Centre for Drugs and Drug Addiction (2022). European drug report. *Trends Dev.* 22, 75644. doi:10.2810/75644

European Monitoring Centre for Drugs and Drug Addiction (2015). *European drug report; trends and developments*. Lisbon: EMCDDA

Fattore, L., Cossu, G., Martellotta, C. M., and Fratta, W. (2001). Intravenous self-administration of the cannabinoid CB1 receptor agonist WIN 55,212-2 in rats. *Psychopharmacology* 156 (4), 410–416. doi:10.1007/s002130100734

Field, A. (2018). *Discovering statistics using IBM SPSS statistics*. Los Angeles: Sage.

Flores, Á., Maldonado, R., and Berrendero, F. (2020). THC exposure during adolescence does not modify nicotine reinforcing effects and relapse in adult male mice. *Psychopharmacology* 237 (3), 801–809. doi:10.1007/s00213-019-05416-8

Franklin, K., and Paxinos, G. (1997). *The mouse brain in stereotaxic coordinates*. Academic Press. Massachusetts, United States.

Fredriksson, I., Venniro, M., Reiner, D. J., Chow, J. J., Bossert, J. M., and Shaham, Y. (2021). Animal models of drug relapse and craving after voluntary abstinence: A review. *Pharmacol. Rev.* 73 (3), 1050–1083. doi:10.1124/pharmrev.120.000191

Freels, T. G., Baxter-Potter, L. N., Lugo, J. M., Glodosky, N. C., Wright, H. R., Baglot, S. L., et al. (2020). Vaporized cannabis extracts have reinforcing properties and support conditioned drug-seeking behavior in rats. *J. Neurosci.* 40 (9), 1897–1908. doi:10.1523/JNEUROSCI.2416-19.2020

García-Blanco, A., Domingo-Rodriguez, L., Cabana-Domínguez, J., Fernández-Castillo, N., Pineda-Cirera, L., Mayneris-Perxachs, J., et al. (2022) miRNA signatures associated with vulnerability to food addiction in mice and humans. *J. Clin. Invest.*, 132 e156281, doi:10.1172/JCI156281

Grimm, J. W., Hope, B. T., Wise, R. A., and Shaham, Y. (2001) Neuroadaptation. Incubation of cocaine craving after withdrawal. *Nature*, 412, 141–142. doi:10.1038/35084134

Hamilton, P. J., and Nestler, E. J. (2019). Epigenetics and addiction. *Curr. Opin. Neurobiol.* 59, 128–136. doi:10.1016/j.conb.2019.05.005

Justinova, Z., Tanda, G., Redhi, G. H., and Goldberg, S. R. (2003). Self-administration of delta9-tetrahydrocannabinol (THC) by drug naïve squirrel monkeys. *Psychopharmacology* 169 (2), 135–140. doi:10.1007/s00213-003-1484-0

Koob, G., and Volkow, N. D. (2016). Neurobiology of addiction: A neurocircuitry analysis. *Lancet Psychiatry* 3 (8), 760–773. doi:10.1016/S2215-0366(16)00104-8

Lefever, T. W., Marusich, J. A., Antonazzo, K. R., and Wiley, J. L. (2014). Evaluation of WIN 55,212-2 self-administration in rats as a potential cannabinoid abuse liability model. *Pharmacol. Biochem. Behav.* 118, 30–35. doi:10.1016/j.pbb.2014.01.002

Lüscher, C., Robbins, T. W., and Everitt, B. J. (2020). The transition to compulsion in addiction. *Nat. Rev. Neurosci.* 21 (5), 247–263. doi:10.1038/s41583-020-0289-z

Maldonado, R., Berrendero, F., Ozaita, A., and Robledo, P. (2011). Neurochemical basis of cannabis addiction. *Neuroscience* 181, 1–17. doi:10.1016/j.neuroscience.2011.02.035

Maldonado, R., Calvé, P., García-Blanco, A., Domingo-Rodríguez, L., Senabre, E., and Martín-García, E. (2021). Vulnerability to addiction. *Neuropharmacology* 186, 108466. doi:10.1016/j.neuropharm.2021.108466

Mancino, S., Burokas, A., Gutiérrez-Cuesta, J., Gutiérrez-Martos, M., Martín-García, E., Pucci, M., et al. (2015). Epigenetic and proteomic expression changes promoted by eating addictive-like behavior. *Neuropsychopharmacology* 40 (12), 2788–2800. doi:10.1038/npp.2015.129

Markou, A., Arroyo, M., and Everitt, B. J. (1999) Effects of contingent and non-contingent cocaine on drug-seeking behavior measured using a second-order schedule of cocaine reinforcement in rats. *Neuropsychopharmacology*, 20, 542–555. doi:10.1016/S0893-133X(98)00080-3

Martellotta, M. C., Cossu, G., Fattore, L., Gessa, G. L., and Fratta, W. (1998). Self-administration of the cannabinoid receptor agonist WIN 55,212-2 in drug-naïve mice. *Neuroscience* 85 (2), 327–330. doi:10.1016/s0306-4522(98)00052-9

Martín-García, E., Barbano, M. F., Galeote, L., and Maldonado, R. (2009). New operant model of nicotine-seeking behaviour in mice. *Int. J. Neuropsychopharmacol.* 12 (3), 343–356. doi:10.1017/S1461145708009279

Martín-García, E., Bourgoin, L., Cathala, A., Kasanetz, F., Mondesir, M., Gutiérrez-Rodriguez, A., et al. (2016) Differential control of cocaine self-administration by GABAergic and glutamatergic CB1 cannabinoid receptors. *Neuropsychopharmacology*, 9, 2192–2205. doi:10.1038/npp.2015.351

Martín-García, E., Domingo-Rodriguez, L., and Maldonado, R. (2020). An operant conditioning model combined with a chemogenetic approach to study the neurobiology of food addiction in mice. *Bio Protoc.* 10, e3777. doi:10.21769/BioProtoc.3777

Mendizábal, V., Zimmer, A., and Maldonado, R. (2006) Involvement of kappa/dynorphin system in WIN 55,212-2 self-administration in mice. *Neuropsychopharmacology*, 31, 1957–1966. doi doi:10.1038/sj.npp.1300957

Panlilio, L. V., Goldberg, S. R., Gilman, J. P., Jufer, R., Cone, E. J., and Schindler, C. W. (1998). Effects of delivery rate and non-contingent infusion of cocaine on cocaine self-administration in rhesus monkeys. *Psychopharmacology* 137 (3), 253–258. doi:10.1007/s002130050618

Panlilio, L. V., and Justinova, Z. (2018). Preclinical studies of cannabinoid reward, treatments for cannabis use disorder, and addiction-related effects of cannabinoid exposure. *Neuropsychopharmacology* 43 (1), 116–141. doi:10.1038/npp.2017.193

Panlilio, L. V., Thorndike, E. B., and Schindler, C. W. (2003). Reinstatement of punishment suppressed opioid self-administration in rats: An alternative model of relapse to drug abuse. *Psychopharmacology* 168 (1-2), 229–235. doi:10.1007/s00213-002-1193-0

Peltier, R. L., Guerin, G. F., Dorairaj, N., and Goeders, N. E. (2001). Effects of saline substitution on responding and plasma corticosterone in rats trained to self-administer different doses of cocaine. *J. Pharmacol. Exp. Ther.* 299 (1), 114–120.

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P. H., Di Marzo, V., Elphick, M. R., et al. (2010). International union of basic and clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: Beyond CB_1 and CB_2. *Pharmacol. Rev.* 62 (4), 588–631. doi:10.1124/pr.110.003004

Piazza, P. V., and Deroche-Gamonet, V. (2013). A multistep general theory of transition to addiction. *Psychopharmacology* 229 (3), 387–413. doi:10.1007/s00213-013-3224-4

Roth, B. L. (2016). DREADDs for neuroscientists. *Neuron* 89 (4), 683–694. doi:10.1016/j.neuron.2016.01.040

Shaham, Y., Shalev, U., Lu, L., de Wit, H., and Stewart, J. (2003). The reinstatement model of drug relapse: History, methodology and major findings. *Psychopharmacology* 168, 3–20. doi:10.1007/s00213-002-1224-x

Shalev, U., Grimm, J. W., and Shaham, Y. (2002). Neurobiology of relapse to heroin and cocaine seeking: A review. *Pharmacol. Rev.* 54 (1), 1–42. doi:10.1124/pr.54.1.1

Soria, G., Barbano, M. F., Maldonado, R., and Valverde, O. (2008) A reliable method to study cue-priming-and stress-induced reinstatement of cocaine self-administration in mice. *Psychopharmacology*, 199:4, 593–603. doi:10.1007/s00213-008-1184-x

Spencer, S., Neuhofer, D., Chioma, V. C., Garcia-Keller, C., Schwartz, D. J., Allen, N., et al. (2018). A model of d9-tetrahydrocannabinol self-administration and reinstatement that alters synaptic plasticity in nucleus accumbens. *Biol. Psychiatry* 84 (8), 601–610. doi:10.1016/j.biopsych.2018.04.016

Stewart, J., and de Wit, H. (1987). "Reinstatement of drug-taking behavior as a method of assessing incentive motivational properties of drugs," in *Methods of assessing the reinforcing properties of abused drugs* (New York: Springer-Verlag).

Tanda, G., and Goldberg, S. R. (2003). Cannabinoids: Reward, dependence, and underlying neurochemical mechanisms - a review of recent preclinical data. *Psychopharmacology* 169 (2), 115–134. doi:10.1007/s00213-003-1485-z

Valjent, E., and Maldonado, R. (2000). A behavioural model to reveal place preference to delta 9-tetrahydrocannabinol in mice. *Psychopharmacology* 147 (4), 436–438. doi:10.1007/s002130050013

Vallée, M., Vitiello, S., Bellocchio, L., Hébert-Chatelain, E., Monlezun, S., Martín-García, E., et al. (2014) Pregnenolone can protect the brain from cannabis intoxication. *Science*: 343, 94–98. doi:10.1126/science.1243985

Venniro, M., Caprioli, D., and Shaham, Y. (2016). Animal models of drug relapse and craving: From drug-priming induced reinstatement to incubation of craving after voluntary abstinence. *Prog. Brain Res.* 224, 25–52. doi:10.1016/bs.pbr.2015.08.004

Volkow, N. D., Michaelides, M., and Baler, R. (2019). The neuroscience of drug reward and addiction. *Physiol. Rev.* 99 (4), 2115–2140. doi:10.1152/physrev.00014.2018

Weafer, J., Mitchell, S. H., and Wit, H. (2014). Recent translational findings on impulsivity in relation to drug abuse. *Curr. Addict. Rep.* 1 (4), 289–300. doi:10.1007/s40429-014-0035-6

Weiss, F. (2010). "Advances in animal models of relapse for addiction research," in *Advances in the neuroscience of addiction* (Boca Raton (FL: CRC Press). Chapter 1, PMID: 21656976.

Weiss, S. R. B., and Volkow, N. D. (2022). Coordinating cannabis data collection globally: Policy implications. *Addiction* 117 (6), 1520–1521. doi:10.1111/add.15751

Zehra, A., Burns, J., Liu, C. K., Manza, P., Wiers, C. E., Volkow, N. D., et al. (2018). Cannabis addiction and the brain: A review. *J. Neuroimmune Pharmacol.* 13 (4), 438–452. doi:10.1007/s11481-018-9782-9

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 26 April 2023
DOI 10.3389/fphar.2023.1145666

OPEN ACCESS

EDITED BY
Francesca Baratta,
University of Turin, Italy

REVIEWED BY
Astha Bhatia,
Guru Nanak Dev University, India
Sutapa Mukherjee,
Chittaranjan National Cancer Institute (CNCI), India

*CORRESPONDENCE
Nezaket Türkel,
nezaket.turkel@gmail.com

RECEIVED 16 January 2023
ACCEPTED 12 April 2023
PUBLISHED 26 April 2023

CITATION
Yüksel B, Hızlı Deniz AA, Şahin F, Sahin K and Türkel N (2023), Cannabinoid compounds in combination with curcumin and piperine display an anti-tumorigenic effect against colon cancer cells.
Front. Pharmacol. 14:1145666.
doi: 10.3389/fphar.2023.1145666

Cannabinoid compounds in combination with curcumin and piperine display an anti-tumorigenic effect against colon cancer cells

Büşra Yüksel[1], Ayşen Aslı Hızlı Deniz[1], Fikrettin Şahin[1], Kazim Sahin[2] and Nezaket Türkel[1]*

[1]Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Istanbul, Türkiye, [2]Department of Animal Nutrition, Faculty of Veterinary Medicine, Firat University, Elazig, Türkiye

Currently, use of cannabinoids is limited to improve adverse effects of chemotherapy and their palliative administration during treatment is curiously concomitant with improved prognosis and regressed progression in patients with different tumor types. Although, non-psychoactive cannabidiol (CBD) and cannabigerol (CBG) display antineoplastic effects by repressing tumor growth and angiogenesis both in cell line and animal models, their use as chemotherapeutic agents is awaiting further investigation. Both clinical and epidemiological evidence supported by experimental findings suggest that micronutrients such as curcumin and piperine may present a safer strategy in preventing tumorigenesis and its recurrence. Recent studies demonstrated that piperine potentiates curcumin's inhibitory effect on tumor progression via enhancing its delivery and therapeutic activity. In this study, we investigated a plausible therapeutic synergism of a triple combination of CBD/CBG, curcumin, and piperine in the colon adenocarcinoma using HCT116 and HT29 cell lines. Potential synergistic effects of various combinations including these compounds were tested by measuring cancer cell proliferation and apoptosis. Our findings revealed that different genetic backgrounds of HCT116 and HT29 cell lines resulted in divergent responses to the combination treatments. Triple treatment showed synergism in terms of exhibiting anti-tumorigenic effects by activating the Hippo YAP signaling pathway in the HCT116 cell line.

KEYWORDS

cannabidiol, cannabigerol, curcumin, piperine, colon cancer

1 Introduction

Colorectal cancer (CRC), which is a heterogeneous disease, involves the uncontrolled growth of cells in the rectum, colon, or gastrointestinal tract appendix (Fleming et al., 2012). Mostly because CRC patients receive their diagnosis at the later stages of the disease, CRC accounts for one of the highest mortality rate, corresponding to 883,200 deaths worldwide (WCRF/AICR, 2018). In addition to a higher mortality rate than other types of cancer, with 1.84 million cases recorded in 2018, CRC ranked as the second most commonly diagnosed cancer in females and the third in males (Bray et al., 2018). According to the stage of cancer and the degree of the complication, current treatment methods rely on the success of

chemotherapy either alone or combined with surgical resection or radiation therapy. Although therapy options remain limited, there are life style factors known to reduce the risk of CRC. For example, maintaining proper dietary habits is an important component of promoting disease prevention. A recent study reported a lower incidence of CRC associated with vegetarian diets when compared to carnivore diets underscoring the importance of the diet as a risk factor in the occurrence of the disease (Orlich et al., 2015). Another epidemiological study revealed that it is possible to reduce CRC death rate by as much as 90% through inclusion of naturally existing bio-compounds with the anti-cancer and anti-oxidant characteristics such as curcumin that is shown to exert distinctive anti-tumorigenic properties in various models (Goel et al., 2001).

Curcumin, chemically known as diferuloylmethane, is a hydrophobic polyphenol naturally present in the rhizome of the plant *Curcuma longa* (turmeric) (Nelson et al., 2017). It is suggested that curcumin can selectively kill tumor cells through its multifaceted metabolic effects, that culminate in its anti-oxidant and anti-inflammatory activities (Hewlings and Kalman, 2017). Several clinical trials classify curcumin as a potential chemo-preventive and chemotherapeutic agent (Doello et al., 2018).

Mechanistically, numerous factors operating in several signaling pathways are implicated in mediating the anti-oxidant effect of curcumin such as induction of the Nuclear factor erythroid 2-related factor 2 (Nrf2) as a protective response against oxidative damage induced by ferric nitrilotriacetate (Fe-NTA) (BALOGUN et al., 2003). While its anti-inflammatory action involves inhibtion cyclooxygenase-2 (COX-2), lipoxygenase (LOX), inducible nitric oxide synthase (iNOS), and downregulation of Janus kinase (JAK) signal transducer and activator of transcription signaling pathways, all of which are essential for inflammatory processes (Shanmugam et al., 2015). In exerting its anti-tumorigenic effects, curcumin blocks angiogenesis, and negatively regulates cancer cell cycle progression as well as metastatic activity (Bhandarkar and Arbiser, 2007).

In several combinatorial therapy approaches, where a secondary active drug agent or drug candidate is co-administered with curcumin, an increase in the therapeutic benefit from curcumin has been reported in diverse cancer models (Baldi et al., 1839), (Bolat et al., 2020), (Schmidt et al., 2020). Strikingly, the second agent turns out to enhance curcumin-dependent anti-cancer activity in a synergistic fashion in certain cases.

Among the proposed secondary agents, piperine, a dietary polyphenol isolated from black and long peppers, distinguished with its intrinsic features, improves -not only-curcumin's existing anti-cancer activity, but also its extremely poor bioavailability (Tang et al., 2017) (Tang et al., 2017) As a single agent, piperine alone also displays anti-mutagenic and anti-tumor activities (Chinta et al., 2015). For example, this agent can inhibit the proliferation of colon cancer cell lines via induction of a cell cycle arrest in the G1 phase, while it triggers apoptosis in prostate cancer models (Ouyang et al., 2013), (Yaffe et al., 2015).

Similar to curcumin and piperine, cannabinoids constitute another group of compounds that have been discovered as novel agents offering a promising anti-tumorigenic potential in multiple cancer types during their clinical use as palliative agents (Borrelli et al., 2014). Originally, cannabinoids were used to ameliorate the debilitating side effects of cytotoxic anti-cancer drugs such as anorexia, pain and emesis for a long time. Among more than 60 variants of cannabinoids, *Cannabis sativa*, and THC (delta-9-tetrahydrocannabinol), cannabidiol (CBD), cannabinol (CBN), cannabichromene (CBC), and cannabigerol (CBG), have been heavily studied (Sreevalsan et al., 2011; McAllister et al., 2015). For example, CBD inhibits the progression of many types of cancer, including glioblastoma multiforme (GBM), breast, lung, prostate and colon cancer (Orrego-González et al., 2020). Likewise, Cannabigerol (CBG) promotes apoptosis, stimulates reactive oxygen species production, and reduces cell growth in CRC cells. Moreover, CBG inhibits the progression of the chemically induced colon carcinogenesis and xenograft tumors *in vivo* (Borrelli et al., 2014). Remarkably, *Cannabis*-derived compounds display reduced cytotoxic behavior on normal colon cells, despite their well-established cytotoxic activity on colon carcinoma cells (Ben-Ami Shor et al., 2022).

Therefore, we chose CBD and CBG to pursue enhancing their demonstrated therapeutic potential in colon carcinoma through their supplementation with curcumin and piperine. Using human HT29 and HCT116 colon cancer cell lines, we made the first attempt to uncover the efficacy of a triple combination, where cannabinoid compounds CBD or CBG are added to a curcumin plus piperine dual cocktail (curcumin/piperine), both of which are considered inextricable in terms of providing most optimal therapeutic outcome possible. Our findings indicate that in the triple combinatory approach, these natural compounds exhibited enhanced anti-carcinogenic effects in colon cancer cells by inducing apoptosis and blocking cell proliferation. Finally, we demonstrate that the improved therapeutic potential of the triple combination entails the activation of the Hippo YAP signaling pathway.

2 Materials and methods

2.1 Cell lines and cell culture conditions

HT-29 (HTB-38, human colorectal adenocarcinoma cell lines), HCT-116 (CRL-247, human colorectal adenocarcinoma cell lines) were originally purchased from American Type Culture Collection (ATCC, Rockville, MD). HT-29 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI, #11875093, Invitrogen, Gibco, UK). The HCT-116 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, #41966-029, Invitrogen, Gibco, UK). Each medium was supplemented with 1% Penicillin/Streptomycin/Amphotericin (PSA, Invitrogen, Gibco, UK) and 10% fetal bovine serum (FBS, #10500-064, Invitrogen, Gibco, UK). Cells were maintained at 37°C and 5% CO2 in a humidified incubator.

2.2 Cytotoxicity assay

Effects of curcumin (Sigma-Aldrich, Germany), piperine (Sigma-Aldrich, Germany), cannabidiol (CBD), cannabigerol (CBG) on cell viability of HCT-116 and HT-29 cells were tested. Stock solutions of 1 μM curcumin, 1 μM piperine, 500 μg/ml CBD, and 500 μg/ml CBG molecules were dissolved in DMEM (for HCT116) or RPMI (for HT29) containing DMSO in 1:100 ratio.

HCT116 and HT29 cells were cultured in 96-well plates at a density of 5,000 cells/well. The following day, cells were treated with curcumin (doses ranging from 100 μM to 10 μM), piperine (doses ranging from 80 μM to 1 μM), CBD (doses ranging from 100 μg/ml to 10 μg/ml), CBG (doses ranging from 100 μg/ml to 10 μg/ml), and curcumin piperine CBD combinations (doses ranging from 50 μM/10 μM/15 μg/ml to 10 μM/2 μM/15 μg/ml) curcumin piperine CBG combinations (doses ranging from 50 μM/10 μM/25 μg/ml to 10 μM/2 μM/25 μg/ml. After administering the cells with different concentrations of the compounds for 72 h as described in similar studies (Bolat et al., 2020), cell viability was assessed via MTS assay 3-(4,5-dimethyl-thiazol-2)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolim salt (MTS) (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) following the procedure used in the same study (Bolat et al., 2020). Treatment containing medium was removed, and an MTS solution (PBS solution included 10% MTS and 4.5 g/L D-glucose solution) was added followed by 90 min of incubation at 37°C. Then, their absorbance was measured at 490 nm by using an ELISA plate reader (Biotek, Winooski, VT). IC50 values were calculated by the GraphPad prism software.

2.3 Annexin V assay

Determination of IC50 values was followed by the investigation of these concentrations of the compounds on apoptosis by Annexin V assay. HCT-116 and HT-29 cells were seeded into T25 flasks at a density of 50 × 103. The following day, media was aspirated, and cells were treated with CBD (25 μg/ml for HT-29 and 15 μg/ml for HCT116), CBG 50 μg/ml for HT-29 and 25 μg/ml for HCT116), curcumin (25 μM for both cell lines), piperine (5 μM for both cell lines) and their combinations. After 72 h of treatment cells, Annexin V assay was performed according to manufacturer's protocol (#sc-4252AK, Santacruz Biotechnology, United States). Cells were harvested and washed with ice-cold PBS. Then they were resuspended in Annexin V binding buffer and separated into four groups (Annexin V, propidium iodide (PI), Annexin V + PI, and NC). Cells were incubated for 15 min at room temperature for annexin V and PI staining (Kamiloglu et al., 2020) Data were analyzed by using FACSCalibur (BD biosciences) flow cytometry.

2.4 Cell cycle analysis

Cells were seeded into T25 flasks at a density of 50 × 103. The following day, treatments were applied, and cells were further incubated for 72 h at 37°C. Then, they were harvested and washed with PBS and fixed with 70% ice-cold ethanol for at least 2 hours at -20°C (Kim and Sederstrom, 2015). Cell pellets were permeabilized with 0.1% triton-X-100 (#85111, Thermo Scientific, United States) and incubated with 20 μg/ml RNase (#EN0531, Thermo Scientific, Lithuania) at room temperature for 30 min (Babes et al., 2018). Finally, cells were stained with PI (#sc-4252AK, Santacruz Biotechnology, United States) and immediately analyzed by a 488 nm single laser emitting device within 15 min.

2.5 Real-time PCR

Total RNA was isolated by using an RNA isolation kit (#740955.250, Macherey-NAGEL, Düren, Germany) according to the user's manual. After that, isolated total mRNAs were converted in cDNAs with QuantiTect Reverse Transcription Kit (#205313, QIAGEN, Hilden, Germany). RT-PCR was performed using SYBR Green (#4309155, Thermo Fisher, Waltham, ABD) and assayed in triplicate using the iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, United States). The expression levels were normalized with respect to RPL30 (Ribosomal Protein L30) gene (F: 5′-ACAGCATGCGGAAAATACTAC-3′ R: 5′-AAAGGAAAA TTTTGCAGGTTT-3′) levels. Genes and their corresponding primer sequences used in this study as follows; Tumor protein 53 (TP53) (F: 5′-GCCCAACAACACCAGCTCCT-3′ R: 5′-CCTGGG CATCCTTGAGTTCC-3′) Ataxia telangiectasia mutated (ATM) (F: 5′- TGTTCCAGGACACGAAGGGAGA - 3′ R: 5′- CAGGGTTCT CAGCACTATGGGA-3′), Ataxia telangiectasia and Rad3 related (ATR) (F: 5′-GGAGATTTCCTGAGCATGTTCGG-3′ R: 5′-GGC TTCTTTACTCCAGACCAATC-3′), Caspase7 (F: 5′-TCAGTG GATGCTAAGCCAGACC-3′ R: 5' –CGAACGCCCATACCT GTCAC-3′), Caspase8 (F: 5′-GCCACCCGGCTTCAGAATGGC-3′ R: 5′-TATGGGCCATCTGCTGTTGGCAGT-3′), baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5 or Survivin) (F: 5′-TCTTCACCGCTTTGCTTTC-3′ R: 5′- CGCACTTTCTCC GCAGTTTC-3′), Bcl-2-associated X protein (BAX) (F: 5′- TGC AGAGGATGATTGCCGCCG-3′ R: 5′-ACCCAACCACCCTGG TGTTGG-3′), Tyrosine-protein kinase (ABL-1) (F: 5′-TACCCG ATTGACCTGTC-3′ R: 5′-CGATTTCAGCAAACGACCCC-3′), proliferating cell nuclear antigen (PCNA) (F: 5′-CAAGTAATG TCGATAAAGAGGAGG-3′ R: 5′-GTGTCACCGTTGAAGAGA GTGG-3′), Kinetochore-associated protein-1 (KNTC-1) (F: 5′-ATAGTCAACCCAGAGTGGGCTGT-3′ R: 5′-TTTCACGTTTTT CGTGCTGCTGCG-3′), DNA replication licensing factor (MCM2) (F: 5′-TGCCACTGTCATCCTAGCCA-3′ R: 5′-GATCGAAGG AGCAA-3′), large tumor suprressor kinase 2 (LATS2) (F:5′-ACAAGATGGGCTTCATCCAC-3′ R: 5′-CTGACATGGCTC CCTTTCTG-3′) Yes1 associated transcriptional regulator (YAP) (F:5′-CACAGCATGTTCGAGCTCAT-3′ R:5′-GATGCTGAGCTG TGGGTGTA-3′) Salvador Family WW Domain Containing Protein 1 (SAV1) (F:5′-CCTGTGCTCCTAGTGTACCTC-3′ R:5′-GCGTAAACCTGAAGCCAGTC-3′) Neurofibromin 2 (Merlin) (F:5′-GACAGCTCTGGATATTCTGCAC-3′ R:5′-CTGCAAGGTG AGTTTGAGGG-3′). The fold changes for each sample were determined using the 2 [−Delta C(T)] method (Livak and Schmittgen, 2001).

2.6 Caspase activity assay

Caspase activity in HT29 and HCT116 cells were measured after treatment of cells for 72 h with each combination by using Caspase-Glo® 3/7, Caspase-Glo® 8, and Caspase-Glo® 9 assay systems (Promega, Madison, WI) according to manufacturer's instructions. Shortly, cells were cultured in white 96-well plates, and the following day they were treated with compounds for 72 h. Caspase levels were determined using a luminometer (Thermo Scientific- Varioskan Lux) after incubating with the kit's reagents for 30 and 60 min.

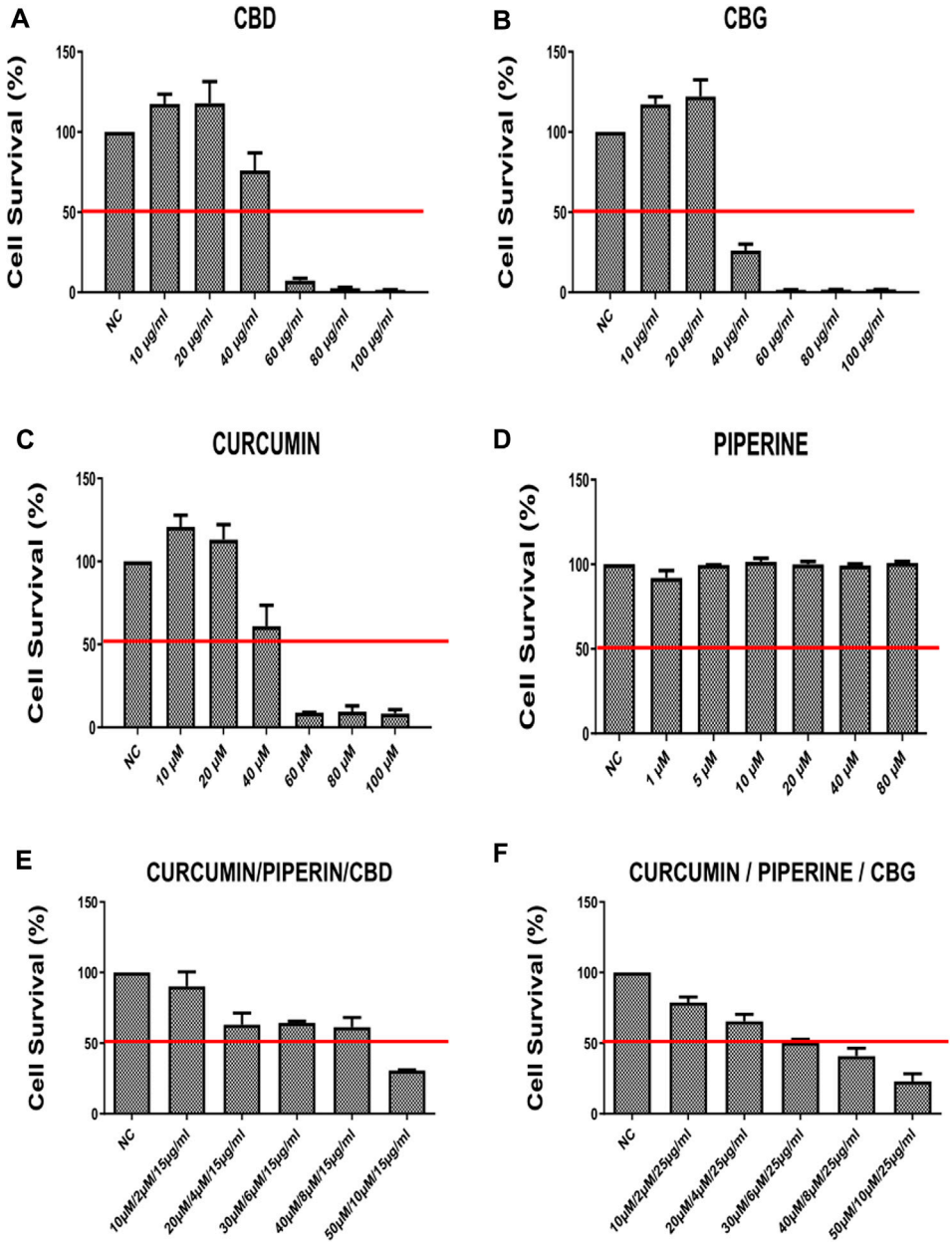

FIGURE 1
The cell survival rates (percent) of HCT-116 line at 72 h after treatment with **(A)** CBD (Cannabidiol) **(B)** CBD (Cannabigerol) **(C)** Curcumin **(D)** Piperline **(E)** combination of curcumin piperline and CBD **(F)** combination of curcumin piperline CBG. All groups were compared to their corresponding negative control.

2.7 Ethynyl-2′-deoxyuridine assay

EdU is a 5-ethynyl-2′-deoxyuridine analog that is absorbed into dividing cells during DNA synthesis. As a result, EdU inclusion is a marker for cell proliferation. As suggested by the manufacturer EdU Staining Proliferation Kit (iFluor 488) (Abcam, Cambridge, MA, United Kingdom; ab219801). HCT116 and HT-29 cells were seeded in 4 wells (Millicell® EZ Slide, 4-well), and after 72h, cells were treated with a culture medium containing 20 µM EdU reagent. Next, cells were incubated for 2 h and were fixed with paraformaldehyde. Nuclei were stained with DAPI. All image intensities were processed using Image J software through calculating the fluorescence intensity at the DAPI - and GFP-channels and taking their ratio as a quantified read-out for EdU-positivity.

2.8 Statistical analysis

All data are shown as the means ± standard errors. The statistical analysis of the results was performed with an unpaired *t*-test, and graphs were drawn using GraphPad Prism 5 software. Statistical significance was determined at $p < 0.05$.

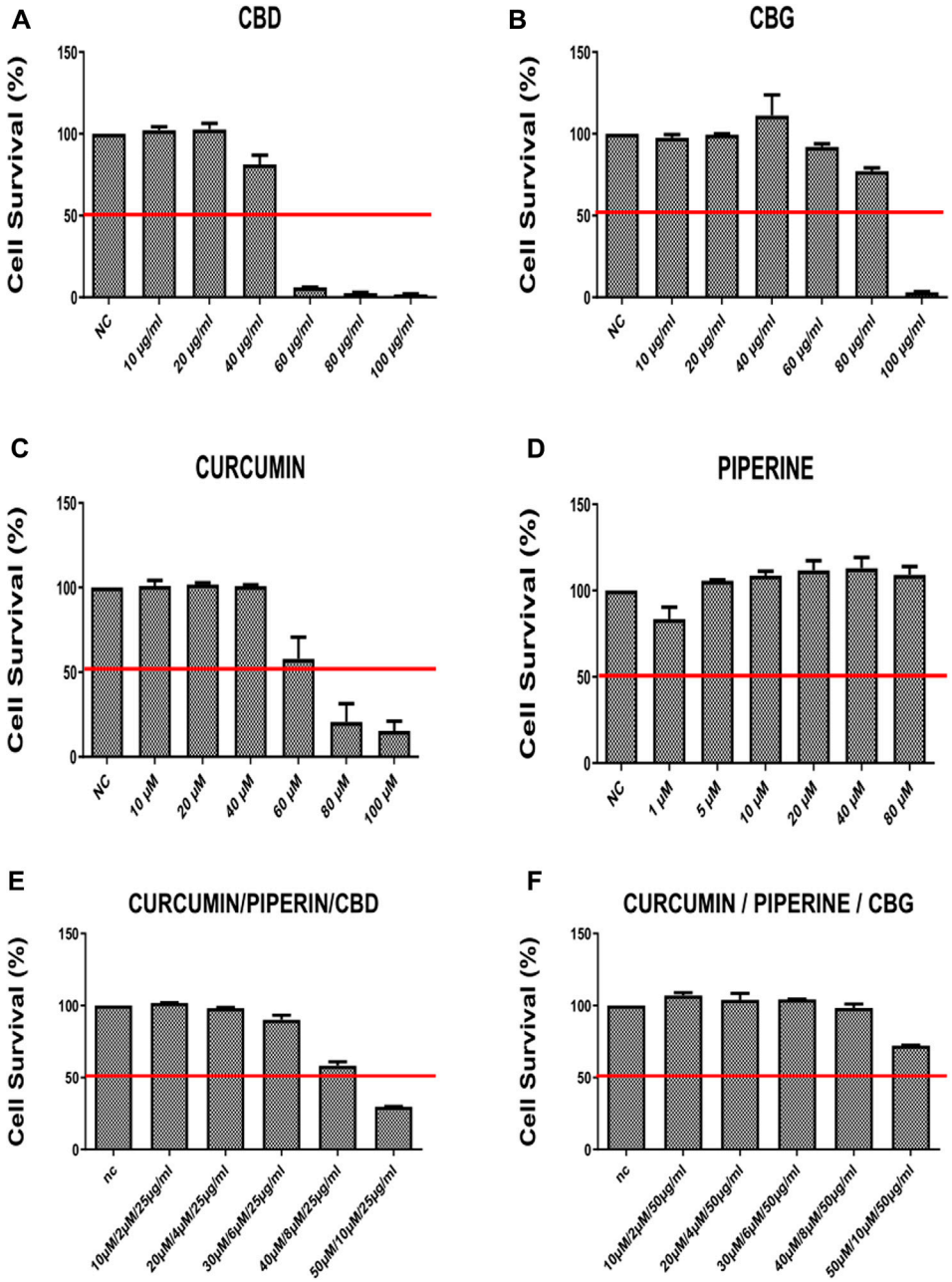

FIGURE 2
The cell survival rates (percent) of HT-29 cancer line 72 h after treatment with **(A)** CBG (Cannabidiol) **(B)** CBD (Cannabigerol) **(C)** Curcumin **(D)** Piperline **(E)** combination of curcumin piperline and CBD **(F)** combination of curcumin piperline CBG. All groups were compared to their corresponding negative control.

3 Results

3.1 Results

To evaluate a potential improvement in the therapeutic impact of triple combinations including CBD/CBG, curcumin, and piperine, an optimally dosed formulation was first determined based on the cell viability measurements. Next, anti-tumorigenic properties of the optimal combination were pursued in terms of inhibition of cancer cell proliferation and metastatic capacity, promoting cell death, alteration of cell cycle properties as well as the molecular pathways they engage to promote tumor suppression.

3.1.1 Effect of cannabinoid compounds together with curcumin and piperine on cell survival

HT29 and HCT116 colon cancer cell lines were treated with CBD, CBG, curcumin, piperine either alone or in triple combinations at a selected range of concentrations for 72 h (Figures 1, 2). 10–100 µg/ml of CBD and CBG, 10–100 µM of curcumin, and 10–80 µM of piperine were tested in both cell

TABLE 1 IC50 values of treatment for HCT116 and HT29 cell lines.

Treatment	IC50 values of HCT116	IC50 values of HT29
CBG	94.79 (μM)	284.37 (μM)
CBD	159.2 (μM)	143.3 (μM)
Cur/Pip	50/10 (μM)	50/10 (μM)
Cur/Pip/CBD	36/7.2/47.69 (μM)	37/7.4/79.6 (μM)
Cur/Pip/CBG	30/6/79 (μM)	ND

lines. In all of the treatments, DMSO content relative to total volume of cell medium was kept below 0.10% v/v to avoid excessive exposure of cells to DMSO. The half maximal inhibitory concentration (IC50) values at 72 h for all treatment groups were determined in both HT29 and HCT116 cell lines using an MTS-based cytotoxicity assay (Table 1).

According to our findings, curcumin, CBD and CBG alone (but not piperine) treatments reduce cell viability in a dose-dependent manner both in HCT-116 and HT-29 cells (Figures 1A–D, Figures 2A–D). Remarkably, while both cannabinoid variants were effective in HCT116 cell line (CBG being more potent) (Figures 1A, B), only CBD had a major effect on cell viability in the HT-29 cell line (Figure 2A). Interestingly, although piperine itself produces no significant change in cell viability in both cancer cell lines, its combination with CBD and CBG resulted in decreased cell viability in the HCT116 cell line compared to the negative control (Supplementary Figures S1C, E). Strikingly, although CBD (15 μg/ml) alone appeared to promote a mild increase in cell survival, combination in the triple cocktail of curcumin/piperine/CBD at the same doses promoted a cytotoxicity in the HCT116 cells displaying an additive effect based on Chou-Talalay Method (Figure 1E) (Chou, 2010). Furthermore, the doses in the triple combination of curcumin/piperine/CBG when compared to mono treatment doses displayed an antagonistic effect mildy in the HCT116 cells (Figure 1F) (Chou, 2010). On the other hand, triple combination of curcumin/piperine/CBD promoted a decrease in cell viability when combined with the non-toxic dose of CBD (25 μg/ml) and curcumin/piperine (Figures 2A, E; Supplementary Figure S2A), while curcumin/piperine/CBG did not display any cytotoxicity in the HT-29 cell line (Figures 2B, F; Supplementary Figure S2A). The obtained results indicate that the combination of CBD with either curcumin or curcumin/piperine has an additive effect in terms of decreasing cell viability in the HCT116 cell line (Figures 1A, E; Supplementary Figure S1), while combinations of cannabinoid compounds with either curcumin and curcumin/piperine showed *bona fide* antagonistic or no effect in cytotoxicity in HT-29 cells, ruling out synergism in this cell line (Figures 2E, F; Supplementary Figure S2) (Chou, 2010).

3.1.2 Cannabinoid compounds, together with curcumin and piperine induce apoptosis in colon cancer cell lines

The effect of individual or combination of the compounds on apoptosis was evaluated in HCT116 and HT29 cancer cells using the Annexin V staining protocol (Figures 3A, 4A). For each treatment group concentrations below the IC50 values were administered for 72 h. The lower left quadrant of the cytograms indicates live cells, while the right quadrant shows apoptotic cells (the lower right quadrant for early apoptotic cells and the upper right quadrant shows late apoptotic cells based on no PI inclusion). Finally, the upper left panel of the cytogram represents necrotic cells that are positive for PI. A histogram graph was drawn for further visual information (Figures 3A, 4A). Overall, treatment with CBD or CBG alone did not induce the apoptosis of HCT116 cells compared to the negative control (Figure 3A). However, application of the triple combination with either cannabinoid compounds resulted in extensive apoptotic effect (being more potent with CBD: 14.95% for late apoptosis and 11.11% for early apoptosis; CBG: 14.2% for late apoptosis and 4.88% for early apoptosis) (Figure 3A).

Mono-treatment of the HT29 cells with cannabinoid compounds increased necrosis compared to the negative control (NC by 1.68%; CBD by 22.98%; CBG by 15.35%) (Figure 4A). Furthermore, triple combination treatment with either cannabinoid compounds resulted in elevation of late apoptosis (with CBD by 37.67%; with CBG by 67.83%) and necroptosis (with CBD by 58.15%; with CBG by 26.53%), suggesting rapid initiation of programmed cell death and loss of cell membrane integrity (Figure 4A).

In order to further evaluate cell death in HCT116 and HT29 cells induced by the treatments at 72 h, caspase activity assay, where activities of caspase 3/7, caspase 8 and 9 were measured as a luminescence readout, was performed according to the manufacturer's instructions. Triple combinations of both cannabinoid compounds showed elevated caspase 3/7, 8, 9 levels at 72 h, measured and displayed at 0 min in the graphic for both cell lines (Figure 5). Caspase-dependent luminescence declined over time at the post-harvest timepoints of 60 and 90 min in both cell lines (Figure 5).

To understand the exact mechanism underlying the apoptotis triggered by the compounds in this study, expression levels of hallmark genes of programed cell death were analyzed. For example, Bax (Bcl-2 associated x) is a pro-apoptotic gene and a member of the Bcl-2 gene family (Kale et al., 2018). Its expression is regulated by tumour suppressor p53 (Miyashita et al., 1994). Expression levels of both Bax (≈2.28 fold) and p53 gene (≈0.71 fold) were elevated in response to triple combination with CBG compared to untreated HCT116 cells (Figure 3B). However, there were no significant changes in the levels of p53 when these cells were treated with the triple combination containing CBD and curcumin/piperine (Figure 3B). Consistent with the changes in Bax and p53 message levels as well as the results of Annexin V assay, curcumin/piperine/CBG treatment resulted in a significant increase in caspase 7 (≈4.42 fold) and caspase 8 (≈1.84 fold) levels (Figure 3B), indicating upregulation in programmed cell death (Figure 3A). ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) genes encode Serine/Threonine kinases that execute a key function in DNA damage response (DDR) and cell cycle checkpoint pathways. ATR gene expression levels were significantly increased upon all treatments compared to that negative control for HCT116 cells (Figure 3B). On the other hand,

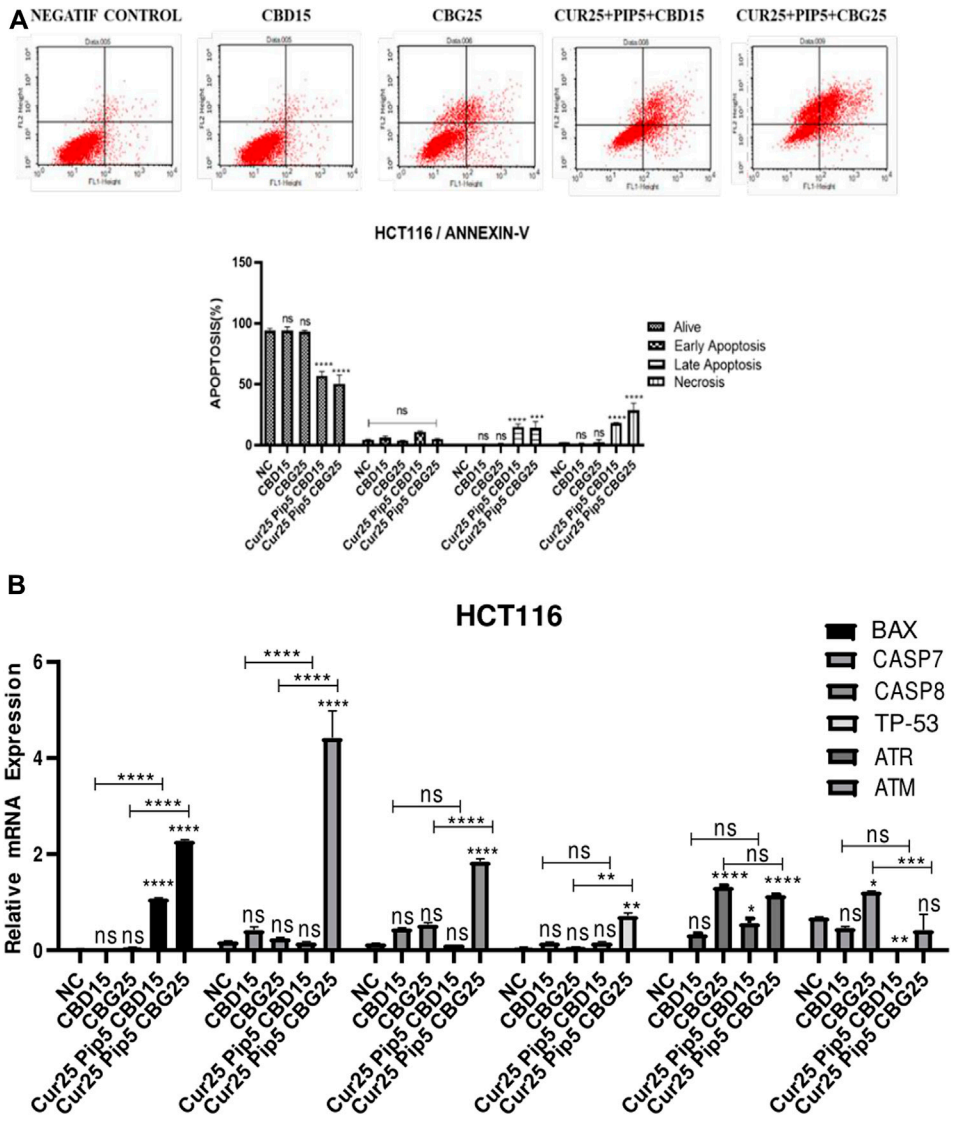

FIGURE 3
(A) Representative Annexin V-FITC/PI staining results for HCT116 cancer cells at 72 h quantitative analysis and their values are mean ± SD of three independent experiments **(B)** Representative graph apoptotic genes expression profiles of HCT116 cells after 72 h. (ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

while CBG mono-treatment resulted in significant increase in the levels of ATM gene, triple treatment comprising CBG restored ATM expression in untreated cells. Although CBD mono-treatment did not change the levels of ATM gene, CBD containing triple treatment lead to a dramatic decrease in the expression level of this gene (Figure 3B).

Correlating with Annexin V assay results, the mono or combination treatment schemes involving the three compounds did not alter the expression levels of caspase 7, 8, and p53 significantly in HT29 cells (Figure 4B). However, while CBG mono-treatment induced a significant increase in the expression levels of both ATR and ATM genes compared to the negative control, triple treatment with any cannabinoid compounds resulted in significant decrease in both ATR and ATM gene expression levels (Figure 4B).

3.1.3 Cannabinoid compounds, together with curcumin and piperine, induce cell cycle arrest in colon cancer cell lines

The observed adverse effect of the compounds of interest on cell viability can either be due to increased cell death (cytotoxic) or slowing down in the cell proliferation (cytostatic) through an arrest in the cell cycle progression. Therefore, cell cycle profiles of both HCT116 and HT29 cell lines were obtained for the same treatment conditions used in the apoptosis assays. In the mono-treatment of HCT116 cells with cannabinoid compounds an increase in the number of cells in G0-G1 phase accompanied with a decrease in the number of cells in the G2 phases compared to negative control were evident (Figure 6A). Furthermore, the triple combination, particularly the one comprising CBG, resulted in a significant increase in the G0-G1 phase population accompanied by a

10.3389/fphar.2023.1145666

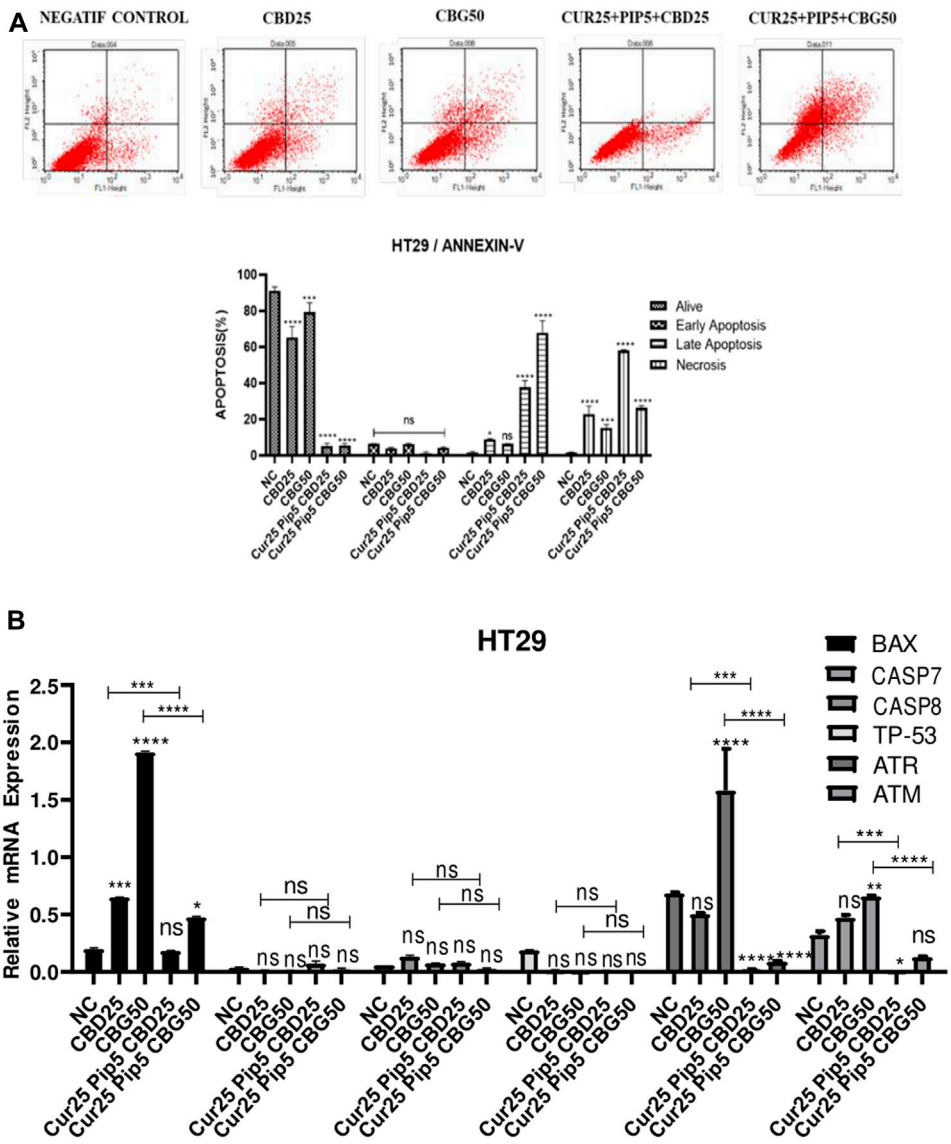

FIGURE 4
(A) Representative Annexin V-FITC/PI staining results for HT29 cancer cells at 72 h quantitative analysis and their values are mean ± SD of three independent experiments **(B)** Representative graph anti-apoptotic genes expression profiles of HT29 cells after 72 h. (ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

significant decrease in G2/M population. Non-etheless, a triple combination with CBD promoted the piling of cells in G0-G1, restoring the decrease induced in the mono-treatment in the G2/M phase (Figure 6A).

In HT29 cells, except for the mild increase in G1 cells induced by CBG alone, there was a no significant alterations in the cell cycle profiles obtained with any of the treatments (Figure 7A).

Non-etheless, changes in the expression of a panel of genes, including ABL-1 (Tyrosine-protein kinase), BIRC-5 (Baculoviral IAP Repeat Containing 5), PCNA (Proliferating cell nuclear antigen), KNTC-1 (Kinetochoreassociated protein 1) and MCM2 (Minichromosome Maintenance Complex Component 2), which are also implicated in the regulation of cell cycle, were examined both in HCT116 and HT29 cell lines under identical treatment conditions (Figures 6B, 7B). In HCT116 cells, mono-treatment with cannabinoid compounds resulted in significant decrease in ABL-1 gene expression, while triple treatments restored the levels of ABL-1 (Figure 6B). No significant change was observed for BIRC-5, PCNA, KNTC-1 and MCM2 gene expression levels in HCT116 cells. However, in HT29 cells, while expression levels of ABL-1 and PCNA were not altered with any of the treatments, BIRC-5 gene expression levels were downregulated upon all treatments compared to control cells. This decreasing trend was more pronounced in the triple treatment including CBD in comparison to that seen upon CBD mono-treatment (Figure 7B). Furthermore, all the treatments resulted in a decrease in the expression levels of KNTC-1 in HT29 cells (Figure 7B).

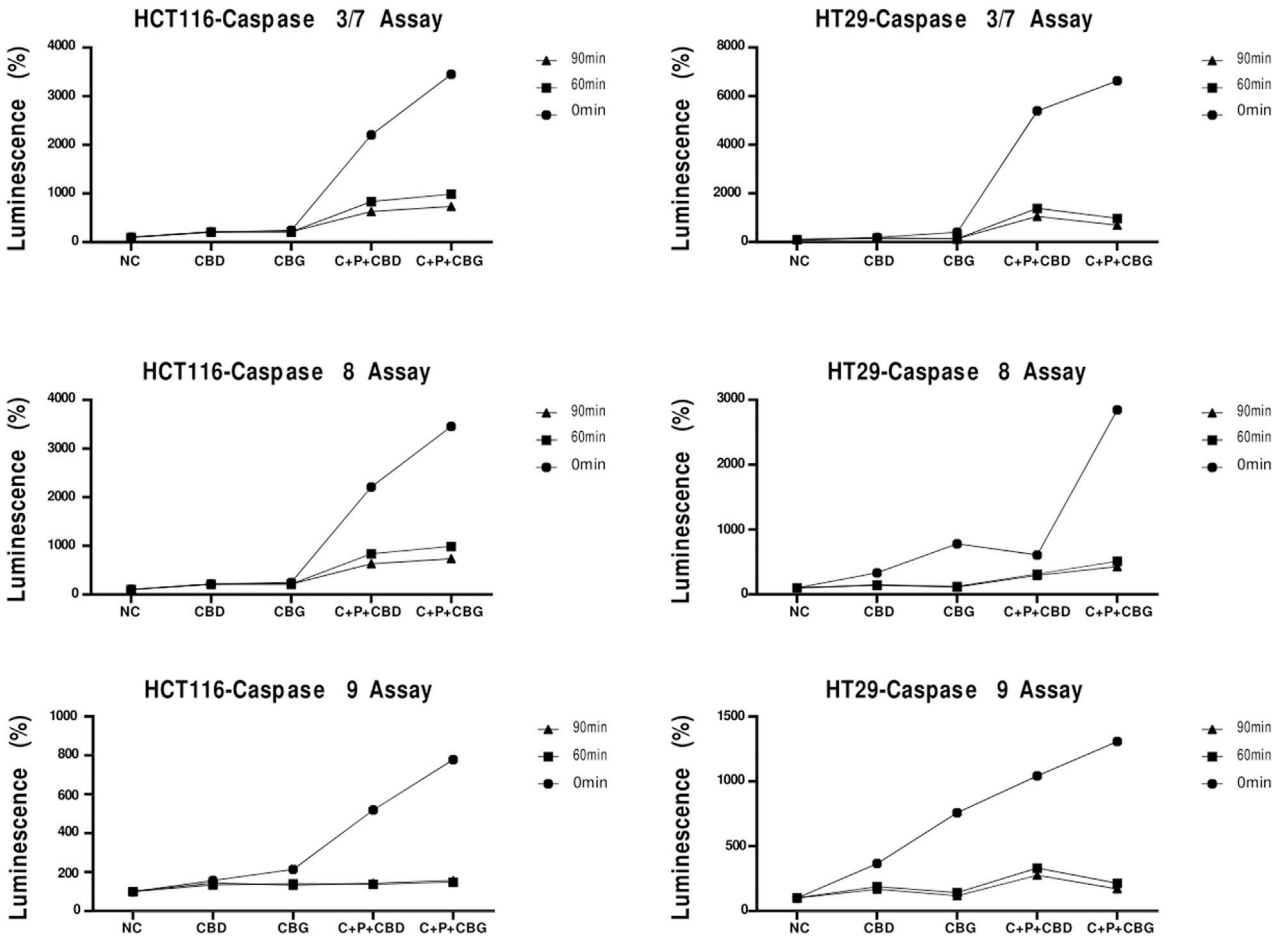

FIGURE 5
Caspase 3/7, Caspase 8 and Caspase 9 activation detected by Casepase-Glo 3/7, Caspase 8, and Caspase 9 luminescence assay for 0 min, 60 min and 90 min in HCT116 and HT29 cells that received the indicated treatments CBD (Cannabigerol), CBG (Cannabigerol), C + P + CBD (Combination of curcumin piperine CBD), C + P + CBG (combination of curcumin piperine CBG) for 72 h.

The effect of cannabinoid compounds together with curcumin and piperine on the cell proliferation of HCT116 (Figure 8) and HT29 (Figure 9) cells was examined using a DNA staining-based assay. In the HCT116 cell line, cellular proliferation was significantly suppressed only upon the triple treatment comprising CBD compared to negative control (Figure 8). On the other hand, in the HT29 cells, cell proliferation was significantly decreased upon all treatment schemes (Figure 9).

3.1.4 Effect of cannabinoid compounds together with curcumin and piperine on Hippo pathway

The expression of YAP, LATS2, Merlin and SAV1 genes were examined in both colon carcinoma cell lines (Figure 10). The results indicated that YAP expression levels were significantly decreased upon all treatments compared to that negative control for both HCT116 (Figure 10A) and HT29 (Figure 10B), suggesting anti-carcinogenic effects of the drugs tested depends on blocking the YAP oncogenic pathway. On the other hand, expression of the Merlin gene was decreased both in HCT116 and HT29 (only mild decrease was observed for CBD mono-treatment) cell lines for all treatments (Figures 10A, B). Furthermore, SAV1 mRNA expression was upregulated in all treatments for the HCT116 cell line (Figure 10A), while there was no significant change in the expression of SAV1 in HT29 cells (Figure 10B). Interestingly, the trend in LATS2 expression levels was in contrast in the two cell lines, in the sense that treatments comprising CBD (both mono and triple) resulted in elevation of LATS expression in HCT116 cells (Figure 10A), while the triple treatments with any of the cannabinoid compounds lead to decrease in levels of LATS2 expression in HT29 cells (Figure 10B).

Likewise, signaling downstream to suppression of oncogenic YAP pathway appears to be divergent between HCT116 and HT29 cells. While changes seen in the HCT116 background are consistent with the consequential events of the canonical YAP suppression as indicated by the significant increase in the LATS2 expression (more pronounced effect seen in mono and the triple combination consisting of CBD) in all of the treatments, a non-canonical YAP suppression accounts for the loss of tumorigenesis in the HT29 cells as implied by the reduction of LATS2 levels in the same treatment scheme.

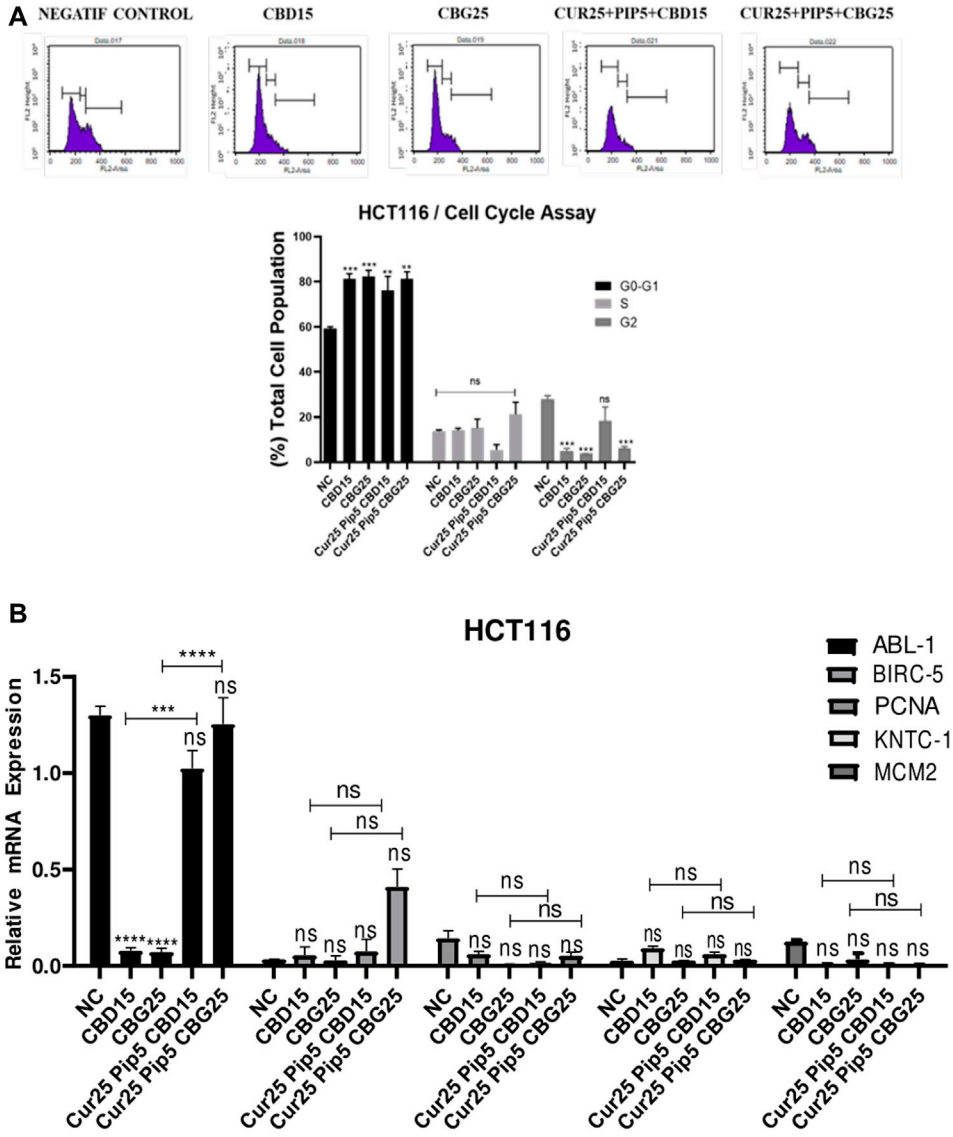

FIGURE 6
Distribution of cell cycle phases by flow cytometry for HCT116 cells **(A)** Distribution of cell cycle phases; the initial, middle, and last peaks show G0/G1, S and G2 respectively and the result of cell cycle analysis percentage of HCT116 cells in G0/G1, S and G2 phase compared with negative control. **(B)** Representative graph of cell cycle genes expression profiles of HCT116 cells after 72 h. (ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

4 Discussion

One of the major causes of cancer-related deaths worldwide is colon cancer, a disorder in which malignant tumors initially occur in the tissues of the colon and in the later stages of the disease, can metastasize to distal sites such as the liver, lung and ovaries. Although the standard of care for the treatment of colon carcinoma includes surgical resection, treatment with 5-FU (5-fluorouracil), and radiotherapy, all have adverse effects and unsatisfactory contributions to prognosis. Therefore, more effective and less toxic combination regimens are urgently needed in the clinic. To meet this pressing need, more and more plant-derived natural compounds targeting multiple molecular and cellular pathways in cancer cells are being investigated to bring novel therapeutic agents to the bedside (DU et al., 2013).

Among the numerous candidates tested so far, curcumin, piperine and certain types of cannabinoids performed promisingly well in colon carcinoma models as monotherapy agents. These results spurred an interest in the field to address the question whether the therapeutic potential of these agents can be boosted through combining them with other promising drug candidates or conventional chemotherapy agents. For example, Resveratrol, epigallocatechin gallate, sulforaphane and piperine are among the molecules studied for their contribution to the synergism seen in the anti-cancer behavior of their combination with curcumin (DU et al., 2013; Jin et al., 2017; Baspinar et al., 2018; Danafar et al., 2018). Meanwhile, chemo-potentiating effect of

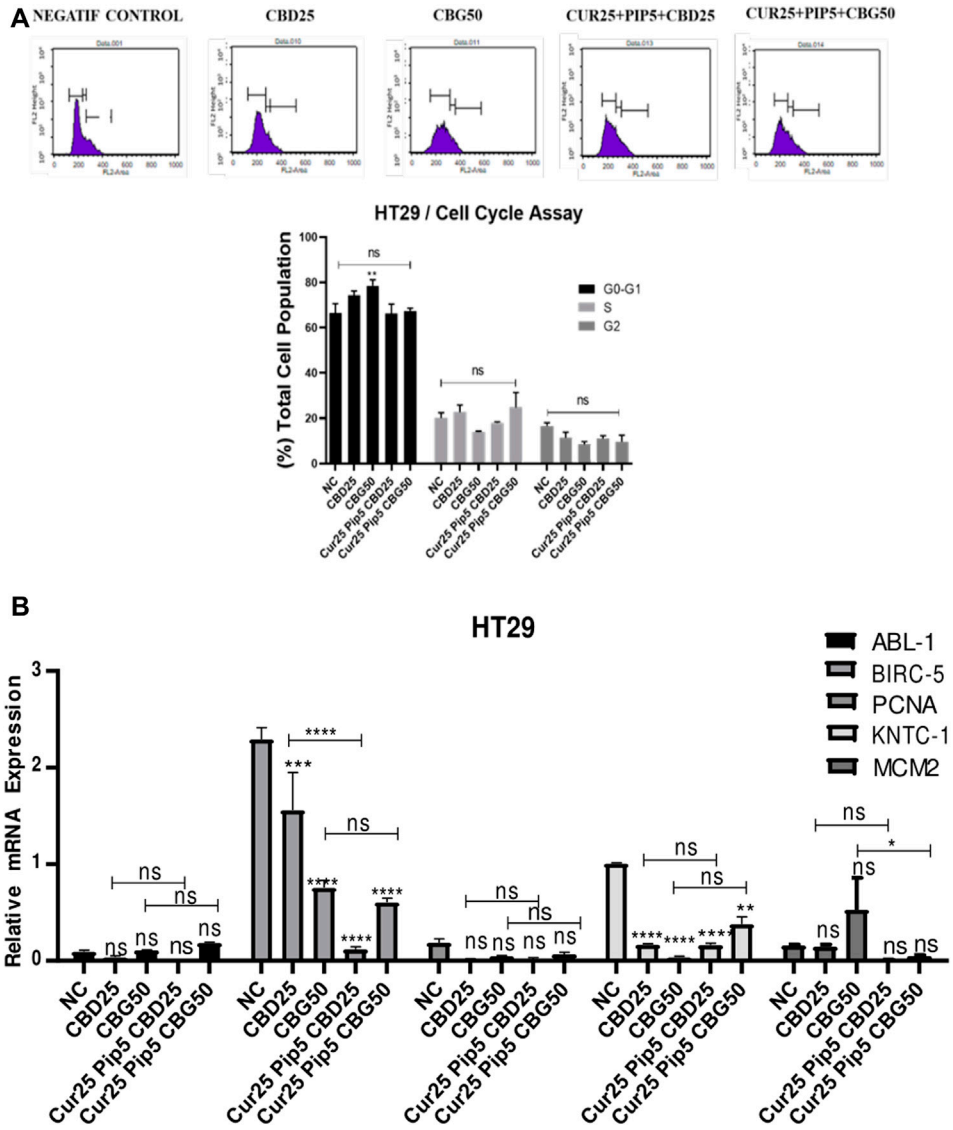

FIGURE 7
Distribution of cell cycle phases by flow cytometry for HT29 cells **(A)** Distribution of cell cycle phases; the initial, middle, and last peaks show G0/G1, S and G2 respectively and the result of cell cycle analysis percentage of HT29 cells in G0/G1, S and G2 phase compared with negative control. **(B)** Representative graph of cell cycle genes expression profiles of HT29 cells after 72 h. (ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

curcumin in combinations with conventional chemo-agents such as doxorubicin, docetaxel, gemcitabine, celebrex, paclitaxel, camptothecin and cisplatin is also reported in different cancer models (Kanai et al., 2011; Yan et al., 2016; Dash and Konkimalla, 2017; Abdallah et al., 2018); (Xiao et al., 2015; Li et al., 2017; Calaf et al., 2018).

However, despite the proven anti-tumor activities of curcumin, piperine and their combination, their low solubility and the poor chemical stability of the compounds in water largely limit their clinical applications. To overcome these challenges nanoparticle technology-based targeted and inducible drug delivery systems have been investigated as a prominent strategy to harness the full therapeutic potential these compounds offer (Wong et al., 2019). Polymeric nanoparticles, such as cyclodextrin nanoparticles, liposomes, copolymeric micelles, and solid lipid nanoparticles, are the most commonly applied in curcumin and piperine nanoformulations (Mahran et al., 2017).

The current study addresses the hypothesis whether a dose-wise optimally calibrated triple combination of curcumin, piperine, and cannabinoid compounds could offer a more effective therapeutic option in the treatment of colon carcinoma using established cell lines as a model system.

Here, we evaluated the cytotoxic and cytostatic effects of curcumin, piperine and cannabinoids alone and in combination in different concentration ranges on colon adenocarcinoma cell lines HCT116 and HT29. It was concluded that, although the same combination of compounds was used, each line responded differently to the treatment schemes tested. Overall,

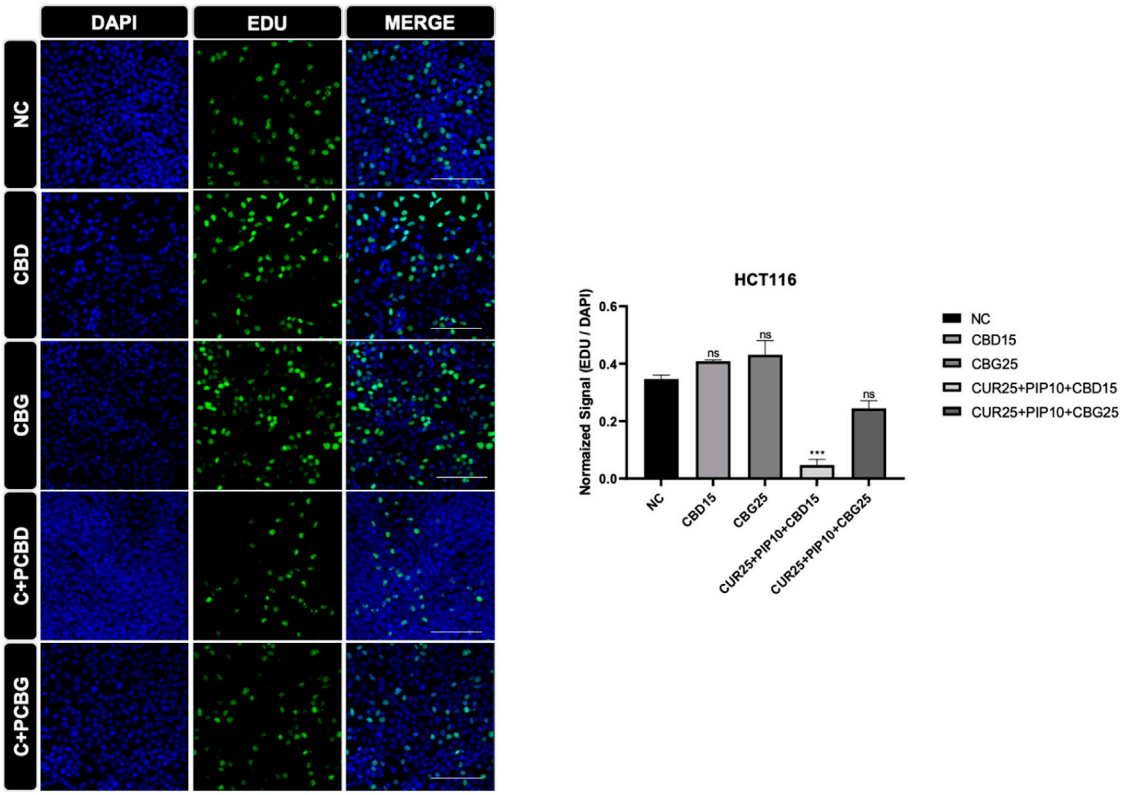

FIGURE 8
Representative results of the EDU assay are shown for HCT116 cell line. Dividing cell were labelled with EDU (green). DAPI staining was in blue in the nucleus. Histogram graph was indicated normalized signaling rate of EDU cells. (Scale bar is 100 μm) (ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

HCT116 cells displayed more sensitivity to CBD and CBG single or combination treatments compared to HT29 cells. Particularly, when HCT116 cells were treated with the triple combination including CBD induction of an additive therapeutic effect was noteworthy. Furthermore, combinations of curcumin, piperine and CBG showed more profound effect in HCT116 cells, whereas the same combinations were not effective in HT29 cells regarding cytotoxicity (Figures 1F, 2F).

One reason that could explain the differential response of the two cell lines to these agents either in single or triple form could be due to the differential expression of the target proteins that these agents interact with. For example, in their comprehensive review on cannabinoids and changes in the Endocannabinoid System (ESC) in intestinal inflammation and colorectal cancer, Cherkasova and colleagues point out that 1) in addition to CB1 and CB2, there are seven-transmembrane Gi/o-coupled receptors (GPCRs), most which are inhibitory, and respond to cannabinoids, including the most studied receptors are GPR119, GPR55, peroxisome proliferating activated receptor α (PPARα), and PPARγ, and 2) expression levels of the endocannabinoids fluctuate in response to satiety, diarrhea, emesis and inflammation, highlighting the scope of sophistication cannabinoids may elicit intracellularly (Cherkasova et al., 2021). In fact, this level of complexity becomes more advanced depending on the agonistic or antagonistic behavior of the ligand cannabinoid variant. For example, CBD-dependent induction of apoptosis via activation of p53-dependent apoptotic pathways is reported in the *in vitro* models glioblastoma multiforme (GBM), which present with high expression levels of CB1 and CB2 receptors. However, in their detailed study, Ivanov and colleagues demonstrate that as a poor ligand for CB1 and CB2 receptors, CBD-dependent signaling initiates independent of triggering of the receptors, but does engage in a downstream crosstalk with the CB1/CB2-mediated signaling in exertion of its pro-apoptotic effects (Ivanov et al., 2017).

Furthermore, differences in the genetic profile between the two cell lines, growth rate and mutations may also explain the divergent responses to these compounds. For example, HCT116 cells express wild-type forms of the BRAF and p53 genes, whereas both genes encode mutant protein forms with altered function in the HT29 cancer cell line. On the other hand, the HCT116 cell line bears mutations in the KRAS oncogene, while no mutations are reported for this gene in the HT29 cells. Although not dissected out in this study in detail, recent reports in the literature point to the importance of having a wild-type p53 status for the occurrence of a curcumin-dependent induction of apoptosis in breast and neuroblastoma cancer models (Heider et al., 2022), (Wang et al., 2022). Likewise, the detailed study by Raup-Konsavage et al. (2018) showed that among the 370 different cannabinoid compounds they tested on distinct molecular subtypes of CRC cell lines (SW480, SW620, HT-29, DLD-1, HCT-116, LS-174T, RKO), the extent of the therapeutic response was further influenced by the oncogenic

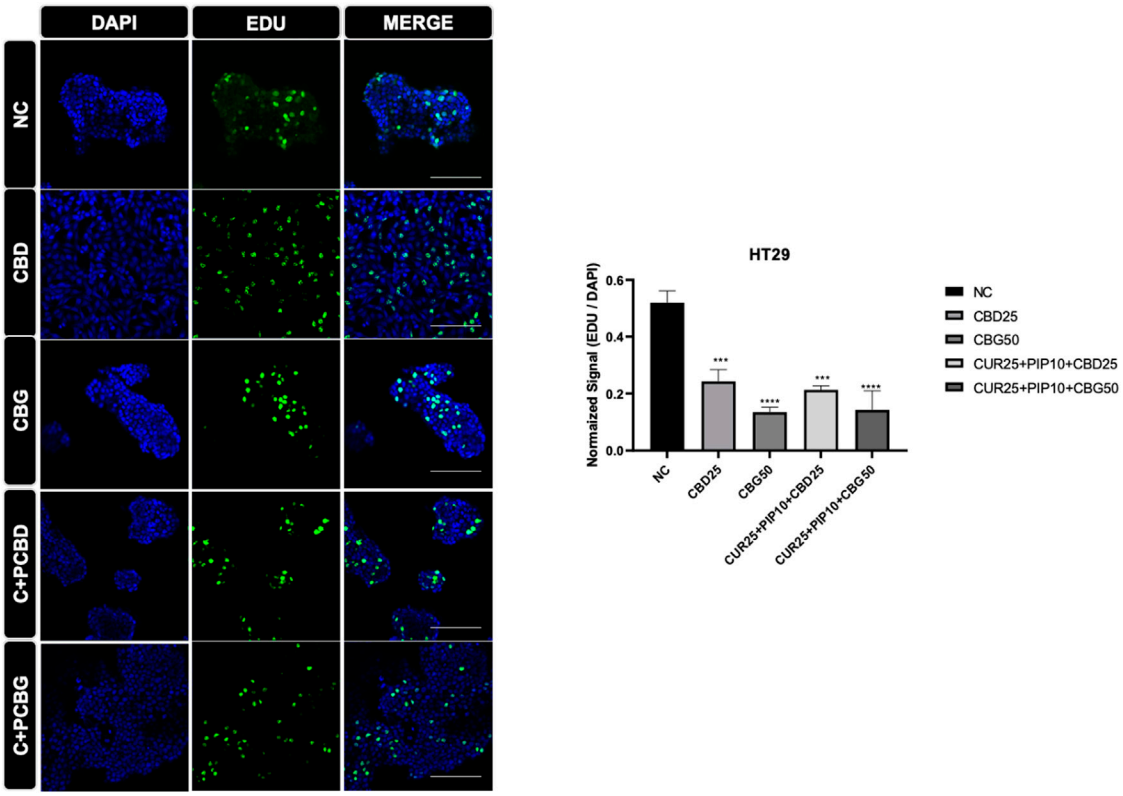

FIGURE 9
Representative results of the EDU assay are shown for HT29 cell line. Dividing cell were labelled with EDU (green). DAPI staining was in blue in the nucleus. Histogram graph was indicated normalized signaling rate of EDU cells.

mutations these cell lines carried (Raup-Konsavage et al., 2018). They also report that the cell lines with APC mutations (SW480, HT-29, DLD-1) were more sensitive to CBD than the cells mutated in the β-catenin pathway (HCT-116, LS-174T) (Raup-Konsavage et al., 2018).

Last but not least, potential direct interaction of CBD, CBG and curcumin/piperine with proteins of interest investigated in this study at the transcriptional level can be involved in eliciting differential therapeutic response in the two *in vitro* models of CRC. For example, curcumin has been shown to control the direct interaction between p53 and its binding partners in such a way that EGAP-p53 interaction becomes lost while NQO1-p53 interaction becomes promoted resulting in the profoundly increased stability of p53 protein (Patiño-Morales et al., 2020). In addition, effects of cannabinoid variant CBD in increasing transcript levels of p53 is reported in pancreatic carcinoma cells although whether this increase in p53 mRNA levels is a consequence of a direct interaction of CBD with factors that regulate p53 expression was not investigated (Luongo et al., 2020).

Another interesting study that addresses the differences between the effects of cannabinoids and curcumin as single agents and in dual combination reports an antagonistic impact of curcumin on cannabis-dependent intoxication via the Cannabinoid Receptor (Zhu et al., 2018). This antagonistic impact of curcumin has been shown in this detailed pharmacological study, where the authors base their claim on the results from binding capacity to the CB1R and other read outs such as inhibition of forskolin-stimulated cAMP accumulation, and β-arrestin2 recruitment in Chinese hamster ovary cells stably expressing human CB1R as well as different pharmacological assays (Zagzoog et al., 2022).

All these points suggest that mutational burden, possible changes in receptor expression for which the cannabinoid variant can be an agonist or antagonist, putative impact of curcumin and piperine on receptor levels, downstream crosstalk between signalizations in the cell lines used could contribute to the differential therapeutic response by HCT116 and HT29 cell lines used in this study. One powerful tool that can reduce this multifactorial rationale underlying the sensitivity versus irresponsiveness of the two cell lines involves the use of Structure Activity Relationship (SAR)-based studies prior to *in vitro* experimentation to obtain a preliminary opinion about the direct targets for these drugs.

In light of all these observations by other groups and the experimental evidence we collected in the colon carcinoma cells that are administered with these agents, anti-tumorigenic effects of cannabinoids as single agents can either be augmented or neutralized by curcumin and piperine depending on the cell line, levels of the target proteins expressed in those cells and the crosstalk between the downstream signalization these compounds trigger.

In terms of the candidate molecular mechanisms underlying anti-tumorigenic activity of the cannabinoids and their combinations with curcumin and piperine, putative

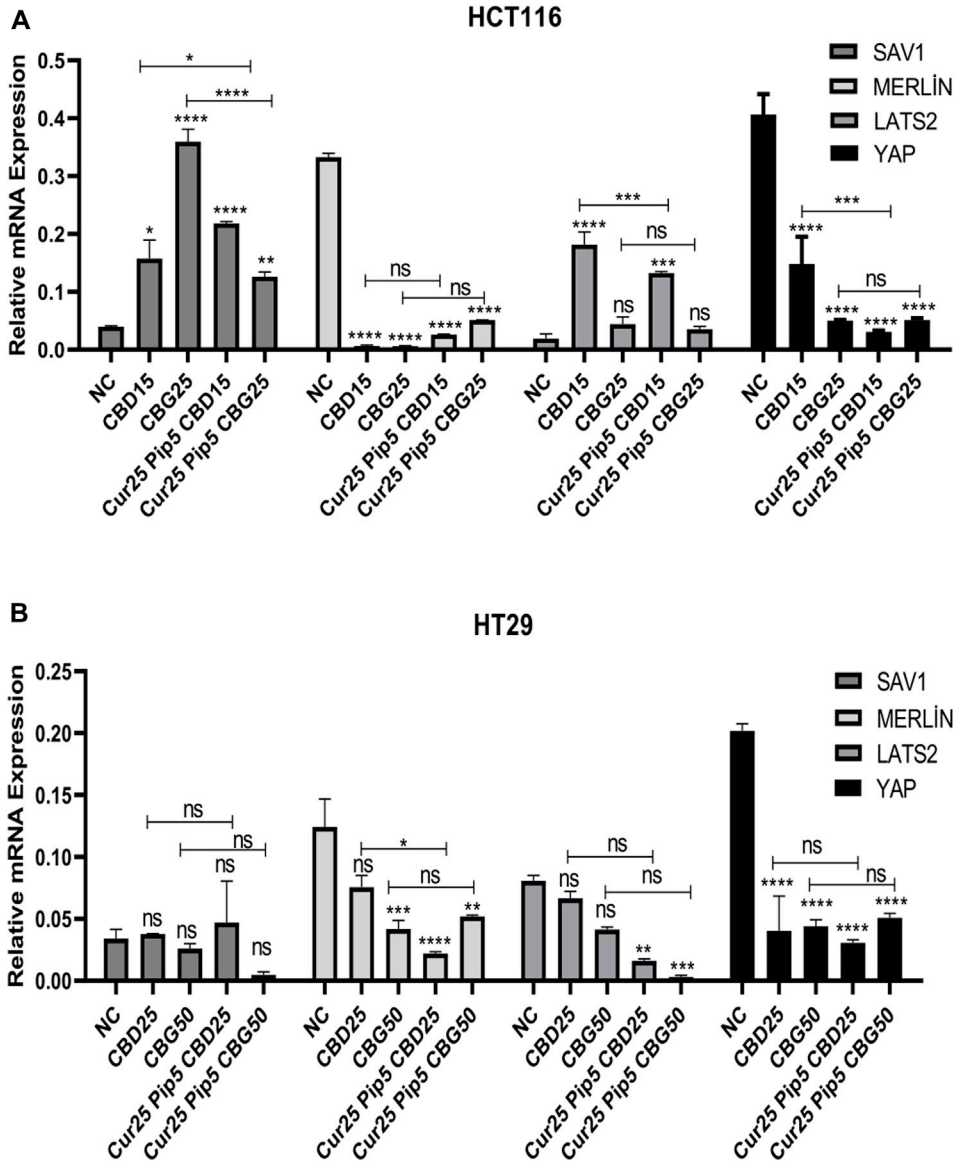

FIGURE 10
(A) Representative graph of Hippo pathway genes expression profiles of HCT116 cells after 72 h. **(B)** Hippo pathway genes expression profiles of HT29 cells after 72 h. (ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

involvement of YAP oncogenic pathway, which has been suggested as a biomarker for colon cancer, was investigated (Zhu et al., 2018). In this study, both mono and combination treatments promoted downregulation of YAP oncogene expression in HCT116 and HT29 cell lines, while expression levels of LATS2 and SAV1 tumor suppressor genes that are upstream players of the Hippo pathway were elevated significantly only in the HCT116 cell line. This may indicate that cannabinoid compounds, together with curcumin/piperine suppress proliferation of HCT116 cells through activating the Hippo signaling pathway, while suppression of proliferation by these compounds in the HT29 cell line was induced through a decrease in YAP expression level independently of Hippo signaling.

Since DNA synthesis is directly correlated with cell proliferation, elevated incorporation of 5-ethynyl-2′-deoxyuridine (EdU) stain enables visualization of newly synthesized DNA as a read-out for increased cell proliferation. Both types of triple cocktails of cannabinoid compounds (either with CBG or CBD) promoted a significant decrease in the rate of DNA synthesis in the HT29 cell line, whereas only the triple cocktail, including CBD but not CBG, resulted in a similarly significant decrease in DNA synthesis in HCT116 cells. Surprisingly, in the HT29 cell line all treatment regimes compared to control resulted in a significant decrease in the amount of newly synthesized DNA. It was concluded that the combination of distinct cannabinoids, such as the case of CBD in this study, with curcumin/piperine may elevate their antiproliferative effects in HCT116 cell line.

Cannabinoids have emerged as a promising novel class of anti-cancer agents that bind to cannabinoid receptors and activate multiple downstream pathways that induce suppression of cancer cell proliferation and trigger apoptosis. Their combination with curcumin/piperine resulted in a drastic induction of apoptosis based on the results of annexin V and caspase assays as well as increase in the levels of caspase 7, 8 and tumor suppressor gene Bax and TP53 in HCT116 cells. Furthermore, levels of those genes that function in the DNA repair pathways were elevated while levels of survival genes were reduced significantly upon the triple combination treatment of HCT116 cell line. On the contrary, despite the fact that mono-treatments of cannabinoids induced apoptosis and suppressed proliferation in the HT29 cell line, administration of neither cannabinoid variant together with curcumin/piperine did result in further elevation of caspase expression. Likewise, pronounced inductions in DNA repair gene levels in response to treatments of cannabinoids as single agents were lost in the triple combinations. In fact, others reported that curcumin can attenuate the intoxicating effects of *Cannabis* variants via indirect inhibition of Cannabinoid receptor 1 (CB1R) (Zagzoog et al., 2022). Therefore, we conclude that although cannabinoid compounds were effective as a single anti-cancer agents on HT29 cells, they are not suitable for combinatorial treatment with curcumin and piperine and, therefore, they have to be further assessed for their usage with other anti-cancer agents.

5 Conclusion

This study demonstrates that combination of curcumin, piperine and cannabinoid variants inhibit cell proliferation and induce apoptosis drastically in distinct models of colorectal cancer. Intriguingly, our findings point out that the compounds of interest, each of which are already known for their anti-tumorigenic and preventive role in colon cancer as single agents, displayed an augmented therapeutic effect in the cell lines tested. In the HT29 cell line, CBG significantly reduced cell proliferation and induced apoptosis as a monotherapy agent, whereas these anti-tumorigenic effects were overridden in the presence of curcumin/piperine. Therefore, findings from this study suggest a benefit in using cannabinoid compounds as single anti-cancer agents in the treatment of those colon carcinoma tumors that carry a genetic profile similar to that of the HT29 cell line. One major limitation of the current study was to reconcile these findings with the cannabinoid receptor 1 (CB1 receptor) and cannabinoid receptor 2 (CB2 receptor) expression profile of the cell lines used. Therefore, in future studies the link between the anti-tumorigenic effects of single cannabinoid compounds or their cocktails and the cannabinoid receptor expression should be interrogated to shed light on the differences in the responses of these cells to distinct cannabinoid-based regimens. In addition to the cannabinoid receptor status, role of other mutations in driver genes should be subject to more rigorous mechanistic studies to fully understand their role in determining the drug mechanism of action and the response to distinct treatment schemes involving cannabinoids as single agents their various combinations.

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

Conceptualization, FS, KS, and NT; methodology, BY, AH, FS, KS, and NT; validation, BY and NT; formal analysis, BY and NT; investigation, BY and NT; data curation, BY, AH, and NT; writing—original draft preparation, BY and NT; writing—review and editing, BY, AH, FS, KS, and NT. All authors have read and agreed to the published version of the manuscript. BY, FS, KS, and NT designed the study. BY and NT collected and analyzed the data. BY, AH, FS, KS, and NT contributed to results interpretation and the manuscript preparation.

Acknowledgments

We would like to thank Ayla Burçin Asutay and Murat Özpolat for their help during flow cytometry analysis.

Conflict of interest

The 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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1145666/full#supplementary-material

References

Abdallah, F. M., Helmy, M. W., Katary, M. A., and Ghoneim, A. I. (2018). Synergistic antiproliferative effects of curcumin and celecoxib in hepatocellular carcinoma HepG2 cells. *Naunyn Schmiedeb. Arch. Pharmacol.* 391 (12), 1399–1410. doi:10.1007/s00210-018-1557-6

Babes, R. M., Tofolean, I. T., Sandu, R. G., Baran, O. E., Cosoreanu, V., Ilie, M. T., et al. (2018). Simple discrimination of sub-cycling cells by propidium iodide flow cytometric assay in Jurkat cell samples with extensive DNA fragmentation. *Cell Cycle* 17 (6), 766–779. doi:10.1080/15384101.2018.1426415

Baldi, A., De Luca, A., Maiorano, P., D'Angelo, C., and Giordano, A. (1839). Curcumin as an anticancer agent in malignant mesothelioma: A review. *Int. J. Mol. Sci.* 21 (5), 1839. doi:10.3390/ijms21051839

Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C. J., Foresti, R., et al. (2003). Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem. J.* 371 (3), 887–895. doi:10.1042/bj20021619

Baspinar, Y., Üstündas, M., Bayraktar, O., and Sezgin, C. (2018). Curcumin and piperine loaded zein-chitosan nanoparticles: Development and *in-vitro* characterisation. *Saudi Pharm. J.* 26 (3), 323–334. doi:10.1016/j.jsps.2018.01.010

Ben-Ami Shor, D., Hochman, I., Gluck, N., Shibolet, O., and Scapa, E. (2022). The cytotoxic effect of isolated cannabinoid extracts on polypoid colorectal tissue. *Int. J. Mol. Sci.* 23 (19), 11366. doi:10.3390/ijms231911366

Bhandarkar, S. S., and Arbiser, J. L. (2007). "Curcumin as an inhibitor of angiogenesis," in *The molecular targets and therapeutic uses of curcumin in health and disease* (Boston, MA: Springer US), 185–195. doi:10.1007/978-0-387-46401-5_7

Bolat, Z. B., Islek, Z., Demir, B. N., Yilmaz, E. N., Sahin, F., and Ucisik, M. H. (2020). Curcumin- and piperine-loaded emulsomes as combinational treatment approach enhance the anticancer activity of curcumin on HCT116 colorectal cancer model. *Front. Bioeng. Biotechnol.* 8, 50. doi:10.3389/fbioe.2020.00050

Borrelli, F., Pagano, E., Romano, B., Panzera, S., Maiello, F., Coppola, D., et al. (2014). Colon carcinogenesis is inhibited by the TRPM8 antagonist cannabigerol, a Cannabis-derived non-psychotropic cannabinoid. *Carcinogenesis* 35 (12), 2787–2797. doi:10.1093/carcin/bgu205

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68 (6), 394–424. doi:10.3322/caac.21492

Calaf, G., Ponce-Cusi, R., and Carrin, F. (2018). Curcumin and paclitaxel induce cell death in breast cancer cell lines. *Oncol. Rep.* 40, 2381–2388. doi:10.3892/or.2018.6603

Cherkasova, V., Kovalchuk, O., and Kovalchuk, I. (2021). Cannabinoids and endocannabinoid system changes in intestinal inflammation and colorectal cancer. *Cancers (Basel)* 13 (17), 4353. doi:10.3390/cancers13174353

Chinta, G., Syed, S. B., Coumar, M. S., and Periyasamy, L. (2015). Piperine: A comprehensive review of pre-clinical and clinical investigations. *Curr. Bioact. Compd.* 11 (3), 156–169. doi:10.2174/1573407211666150915214425

Chou, T.-C. (2010). Drug combination studies and their synergy quantification using the chou-talalay method. *Cancer Res.* 70 (2), 440–446. doi:10.1158/0008-5472.CAN-09-1947

Danafar, H., Sharafi, A., Kheiri, S., and Kheiri Manjili, H. (2018). Co -delivery of sulforaphane and curcumin with PEGylated iron oxide-gold core shell nanoparticles for delivery to breast cancer cell line. *Iran. J. Pharm. Res.* 17 (2), 480–494.

Dash, T. K., and Konkimalla, V. B. (2017). Selection of P-glycoprotein inhibitor and formulation of combinational nanoformulation containing selected agent curcumin and DOX for reversal of resistance in K562 cells. *Pharm. Res.* 34 (8), 1741–1750. doi:10.1007/s11095-017-2182-7

Doello, K., Ortiz, R., Alvarez, P. J., Melguizo, C., Cabeza, L., and Prados, J. (2018). Latest *in vitro* and *in vivo* assay, clinical trials and patents in cancer treatment using curcumin: A literature review. *Nutr. Cancer* 70 (4), 569–578. doi:10.1080/01635581.2018.1464347

Du, Q., Hu, B., An, H. M., Shen, K. P., Xu, L., Deng, S., et al. (2013). Synergistic anticancer effects of curcumin and resveratrol in Hepa1-6 hepatocellular carcinoma cells. *Oncol. Rep.* 29 (5), 1851–1858. doi:10.3892/or.2013.2310

Fleming, M., Ravula, S., Tatishchev, S. F., and Wang, H. L. (2012). Colorectal carcinoma: Pathologic aspects. *J. Gastrointest. Oncol.* 3 (3), 153–173. doi:10.3978/j.issn.2078-6891.2012.030

Goel, A., Boland, C. R., and Chauhan, D. P. (2001). Specific inhibition of cyclooxygenase-2 (COX-2) expression by dietary curcumin in HT-29 human colon cancer cells. *Cancer Lett.* 172 (2), 111–118. doi:10.1016/S0304-3835(01)00655-3

Heider, C. G., Itenberg, S. A., Rao, J., Ma, H., and Wu, X. (2022). Mechanisms of cannabidiol (CBD) in cancer treatment: A review. *Biol. (Basel)* 11 (6), 817. doi:10.3390/biology11060817

Hewlings, S., and Kalman, D. (2017). Curcumin: A review of its effects on human health. *Foods* 6 (10), 92. doi:10.3390/foods6100092

Ivanov, V. N., Wu, J., and Hei, T. K. (2017). Regulation of human glioblastoma cell death by combined treatment of cannabidiol, γ-radiation and small molecule inhibitors of cell signaling pathways. *Oncotarget* 8 (43), 74068–74095. doi:10.18632/oncotarget.18240

Jin, G., Yang, Y., Liu, K., Zhao, J., Chen, X., Liu, H., et al. (2017). Combination curcumin and (−)-epigallocatechin-3-gallate inhibits colorectal carcinoma microenvironment-induced angiogenesis by JAK/STAT3/IL-8 pathway. *Oncogenesis* 6 (10), e384. doi:10.1038/oncsis.2017.84

Kale, J., Osterlund, E. J., and Andrews, D. W. (2018). BCL-2 family proteins: Changing partners in the dance towards death. *Cell Death Differ.* 25 (1), 65–80. doi:10.1038/cdd.2017.186

Kamiloglu, S., Sari, G., Ozdal, T., and Capanoglu, E. (2020). Guidelines for cell viability assays. *Food Front.* 1 (3), 332–349. doi:10.1002/fft2.44

Kanai, M., Yoshimura, K., Asada, M., Imaizumi, A., Suzuki, C., Matsumoto, S., et al. (2011). A phase I/II study of gemcitabine-based chemotherapy plus curcumin for patients with gemcitabine-resistant pancreatic cancer. *Cancer Chemother. Pharmacol.* 68 (1), 157–164. doi:10.1007/s00280-010-1470-2

Kim, K. H., and Sederstrom, J. M. (2015). Assaying cell cycle status using flow cytometry. *Curr. Protoc. Mol. Biol.* 111 (1), 1–28. doi:10.1002/0471142727.mb2806s111

Li, C., Ge, X., and Wang, L. (2017). Construction and comparison of different nanocarriers for co-delivery of cisplatin and curcumin: A synergistic combination nanotherapy for cervical cancer. *Biomed. Pharmacother.* 86, 628–636. doi:10.1016/j.biopha.2016.12.042

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25 (4), 402–408. doi:10.1006/meth.2001.1262

Luongo, M., Marinelli, O., Zeppa, L., Aguzzi, C., Morelli, M. B., Amantini, C., et al. (2020). Cannabidiol and oxygen-ozone combination induce cytotoxicity in human pancreatic ductal adenocarcinoma cell lines. *Cancers (Basel)* 12 (10), 2774. doi:10.3390/cancers12102774

Mahran, R. I., Hagras, M. M., Sun, D., and Brenner, D. E. (2017). Bringing curcumin to the clinic in cancer prevention: A review of strategies to enhance bioavailability and efficacy. *AAPS J.* 19 (1), 54–81. doi:10.1208/s12248-016-0003-2

McAllister, S. D., Soroceanu, L., and Desprez, P.-Y. (2015). The antitumor activity of plant-derived non-psychoactive cannabinoids. *J. Neuroimmune Pharmacol.* 10 (2), 255–267. doi:10.1007/s11481-015-9608-y

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., et al. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. *Oncogene* 9 (6), 1799–1805.

Nelson, K. M., Dahlin, J. L., Bisson, J., Graham, J., Pauli, G. F., and Walters, M. A. (2017). The essential medicinal chemistry of curcumin. *J. Med. Chem.* 60 (5), 1620–1637. doi:10.1021/acs.jmedchem.6b00975

Orlich, M. J., Singh, P. N., Sabaté, J., Fan, J., Sveen, L., Bennett, H., et al. (2015). Vegetarian dietary patterns and the risk of colorectal cancers. *JAMA Intern Med.* 175 (5), 767–776. doi:10.1001/jamainternmed.2015.59

Orrego-González, E., Londoño-Tobón, L., Ardila-González, J., Polania-Tovar, D., Valencia-Cárdenas, A., and Velez-Van Meerbeke, A. (2020). Cannabinoid effects on experimental colorectal cancer models reduce aberrant crypt foci (acf) and tumor volume: A systematic review. *Evidence-Based Complementary Altern. Med.* 2020, 2371527–2371613. doi:10.1155/2020/2371527

Ouyang, D., Zeng, L. h., Pan, H., Xu, L. h., Wang, Y., Liu, K. p., et al. (2013). Piperine inhibits the proliferation of human prostate cancer cells via induction of cell cycle arrest and autophagy. *Food Chem. Toxicol.* 60, 424–430. doi:10.1016/j.fct.2013.08.007

Patiño-Morales, C. C., Soto-Reyes, E., Arechaga-Ocampo, E., Ortiz-Sánchez, E., Antonio-Véjar, V., Pedraza-Chaverri, J., et al. 2020. Curcumin stabilizes p53 by interaction with NAD(P)H:quinone oxidoreductase 1 in tumor-derived cell lines. *Redox Biol.* 28, 101320. doi:10.1016/j.redox.2019.101320

Raup-Konsavage, W. M., Johnson, M., Legare, C. A., Yochum, G. S., Morgan, D. J., and Vrana, K. E. (2018). Synthetic cannabinoid activity against colorectal cancer cells. *Cannabis Cannabinoid Res.* 3 (1), 272–281. doi:10.1089/can.2018.0065

Schmidt, B., Ferreira, C., Alves Passos, C. L., Silva, J. L., and Fialho, E. (2020). Resveratrol, curcumin and piperine alter human glyoxalase 1 in MCF-7 breast cancer cells. *Int. J. Mol. Sci.* 21 (15), 5244. doi:10.3390/ijms21155244

Shanmugam, M., Rane, G., Kanchi, M. M., Arfuso, F., Chinnathambi, A., Zayed, M. E., et al. (2015). The multifaceted role of curcumin in cancer prevention and treatment. *Molecules* 20 (2), 2728–2769. doi:10.3390/molecules20022728

Sreevalsan, S., Joseph, S., Jutooru, I., Chadalapaka, G., and Safe, S. H. (2011). Induction of apoptosis by cannabinoids in prostate and colon cancer cells is phosphatase dependent. *Anticancer Res.* 31 (11), 3799–3807.

Tang, J., Ji, H., Ren, J., Li, M., Zheng, N., and Wu, L. (2017). Solid lipid nanoparticles with tpgs and brij 78: A co-delivery vehicle of curcumin and piperine for reversing P-glycoprotein-mediated multidrug resistance *in vitro*. *Oncol. Lett.* 13 (1), 389–395. doi:10.3892/ol.2016.5421

Wang, B., Li, D., Cherkasova, V., Gerasymchuk, M., Narendran, A., Kovalchuk, I., et al. (2022). Cannabinol inhibits cellular proliferation, invasion, and angiogenesis of

neuroblastoma via novel miR-34a/tRiMetF31/PFKFB3 Axis. *Cancers (Basel)* 14 (8), 1908. doi:10.3390/cancers14081908

WCRF/AICR (2018). *World cancer research fund/American Institute for cancer research (WCRF/AICR) continuous update project report: Diet, nutrition, physical activity and colorectal cancer 2016*. London, UK: World Cancer Research Fund International.

Wong, K. E., Ngai, S. C., Chan, K.-G., Lee, L.-H., Goh, B.-H., and Chuah, L.-H. (2019). Curcumin nanoformulations for colorectal cancer: A review. *Front. Pharmacol.* 10, 152. doi:10.3389/fphar.2019.00152

Xiao, B., Si, X., Han, M. K., Viennois, E., Zhang, M., and Merlin, D. (2015). Co-delivery of camptothecin and curcumin by cationic polymeric nanoparticles for synergistic colon cancer combination chemotherapy. *J. Mater Chem. B* 3 (39), 7724–7733. doi:10.1039/C5TB01245G

Yaffe, P. B., Power Coombs, M. R., Doucette, C. D., Walsh, M., and Hoskin, D. W. (2015). Piperine, an alkaloid from black pepper, inhibits growth of human colon cancer cells via G1 arrest and apoptosis triggered by endoplasmic reticulum stress. *Mol. Carcinog.* 54 (10), 1070–1085. doi:10.1002/mc.22176

Yan, J., Wang, Y., Zhang, X., Liu, S., Tian, C., and Wang, H. (2016). Targeted nanomedicine for prostate cancer therapy: Docetaxel and curcumin co-encapsulated lipid–polymer hybrid nanoparticles for the enhanced anti-tumor activity *in vitro* and *in vivo*. *Drug Deliv.* 23 (5), 1757–1762. doi:10.3109/10717544.2015.1069423

Zagzoog, A., Cabecinha, A., Abramovici, H., and Laprairie, R. B. 2022. Modulation of type 1 cannabinoid receptor activity by cannabinoid by-products from Cannabis sativa and non-cannabis phytomolecules," *Front. Pharmacol.* 13, 956030. doi:10.3389/fphar.2022.956030

Zhu, J., Zhao, B., Xiong, P., Wang, C., Zhang, J., Tian, X., et al. (2018). Curcumin induces autophagy via inhibition of yes-associated protein (YAP) in human colon cancer cells. *Med. Sci. Monit.* 24, 7035–7042. doi:10.12659/MSM.910650

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 24 May 2023
DOI 10.3389/fphar.2023.1135453

OPEN ACCESS

EDITED BY
Francesca Baratta,
University of Turin, Italy

REVIEWED BY
Richard C. Kevin,
The University of Sydney, Australia
Elena Martín-García,
Pompeu Fabra University, Spain

*CORRESPONDENCE
Jacob M. Vigil,
vigilj@unm.edu

RECEIVED 31 December 2022
ACCEPTED 09 May 2023
PUBLISHED 24 May 2023

CITATION
Stith SS, Li X, Brockelman F, Keeling K, Hall B and Vigil JM (2023), Understanding feeling "high" and its role in medical cannabis patient outcomes.
Front. Pharmacol. 14:1135453.
doi: 10.3389/fphar.2023.1135453

Understanding feeling "high" and its role in medical cannabis patient outcomes

Sarah S. Stith[1], Xiaoxue Li[1], Franco Brockelman[2], Keenan Keeling[2], Branden Hall[2] and Jacob M. Vigil[3]*

[1]Department of Economics, University of New Mexico, Albuquerque, NM, United States, [2]MoreBetter, Ltd., Hyattsville, MD, United States, [3]Department of Psychology, University of New Mexico, Albuquerque, NM, United States

Introduction: We measure for the first time the associations between subjective patient experiences of feeling "high" and treatment outcomes during real-time *Cannabis* flower consumption sessions.

Methods: Our study uses data from the mobile health app, Releaf App™, through which 1,882 people tracked the effects of *Cannabis* flower on a multitude of health conditions during 16,480 medical cannabis self-administration sessions recorded between 6/5/2016 and 3/11/2021. Session-level reported information included plant phenotypes, modes of administration, potencies, baseline and post-administration symptom intensity levels, total dose used, and real-time side effect experiences.

Results: Patients reported feeling high in 49% of cannabis treatment sessions. Using individual patient-level fixed effects regression models and controlling for plant phenotype, consumption mode, tetrahydrocannabinol (THC) and cannabidiol (CBD) potencies, dose, and starting symptom level, our results show that, as compared to sessions in which individuals did not report feeling high, reporting feeling high was associated with a 7.7% decrease in symptom severity from a mean reduction of −3.82 on a 0 to 10 analog scale (coefficient = −0.295, $p < 0.001$) with evidence of a 14.4 percentage point increase ($p < 0.001$) in negative side effect reporting and a 4.4 percentage point ($p < 0.01$) increase in positive side effect reporting. Tetrahydrocannabinol (THC) levels and dose were the strongest statistical predictors of reporting feeling high, while the use of a vaporizer was the strongest inhibitor of feeling high. In symptom-specific models, the association between feeling high and symptom relief remained for people treating pain ($p < 0.001$), anxiety ($p < 0.001$), depression ($p < 0.01$) and fatigue ($p < 0.01$), but was insignificant, though still negative, for people treating insomnia. Although gender and pre-app cannabis experience did not appear to affect the relationship between high and symptom relief, the relationship was larger in magnitude and more statistically significant among patients aged 40 or less.

Discussion: The study results suggest clinicians and policymakers should be aware that feeling high is associated with improved symptom relief but increased negative side effects, and factors such as mode of consumption, product potency, and dose can be used to adjust treatment outcomes for the individual patient.

KEYWORDS
high, cannabis, marijuana, intoxication, cannabidiol, tetrahydrocannabinol, inebriation

1 Introduction

Perhaps there is no more widely referenced, yet under-defined term involving the *Cannabis* plant than that of being or feeling "high" (National Academies of Sciences, Engineering, and Medicine, 2017). Merriam-Webster Dictionary (2022) defines high as "intoxicated by or as if by a drug or alcohol" with "intoxicated" defined both as being under the influence of drugs or alcohol to the point of physical or mental impairment or as "excited, elated, or exhilarated." In the scientific literature, high is almost always used to convey a pejorative concept associated with intoxication, steaming in part from strong evidence that cannabis intoxication can be associated with significant behavioral risks (Hartman and Huestis, 2013; Chihuri and Li, 2020; Preuss et al., 2021), and in part from the altered state of sensorimotor functioning that defines the very concept of feeling high. Healthcare providers, public health officials, and researchers regularly warn the public of dangers from getting high and the threats posed by people who are high or that get high. State and federal laws and regulations are operationalized based on whether or not the phenotypic expression of the *Cannabis* plant variant (e.g., "hemp") can get a person high, and recent marketing trends often use the phrasing that cannabidiol (CBD) products can offer medicinal benefits without the risk of producing "a high or any disorientation" (e.g., U.S. Pain Foundation, 2021). At the same time, most medical cannabis patients report enjoying the hyper-sensory experience of feeling high (Clem et al., 2020; Lake et al., 2020), often attributing its visceral euphoria to an enhanced state of peacefulness and relaxation (Stith et al., 2018).

To date, the scientific and medical communities have yet to clearly define the subjective experience of feeling high within the context of pharmaceutical applications of the *Cannabis* plant. Aside from general (millennia-old) descriptions of feeling an enhanced sense of euphoria and experiencing alterations in sensory perceptions, cognition, attention, and abstract thought processing (Russo, 2007; National Institute on Drug Abuse, 2021), there has been little scientific interest in how distinct psychological experiences may be correlated with the plant's ability to improve or impair the average medical patient's health outcomes. Phytocannabinoids, such as tetrahydrocannabinol (THC) and CBD, interact with numerous receptors (e.g., CB1) throughout the central nervous system, as well as receptors (e.g., CB2) found in the peripheral immune system, including in white blood cells and the spleen (Kendall and Yudowski, 2017; Bie et al., 2018). However, the precise mechanisms by which cannabis effects changes in psychological functioning and clinical outcomes remain elusive, as does the ability to clearly isolate potentially significant changes in psychological functioning from desired clinical outcomes.

Existing research clearly has shown that at least some of the exhilarating, intoxicating, and impairing characteristics of feeling high are driven by the plant's THC potency levels (Curran et al., 2020; Cuttler et al., 2021). Since the molecule's discovery by Raphael Mechoulam's lab in 1964 (Pertwee, 2006), THC has been identified within the scientific and medical communities as one of the primary determinants of feeling high (National Institute on Drug Abuse, 2021), and hence, THC is the singled-out molecule upon which current U.S. *Cannabis* plant legislation is based, with the 2018 Hemp Farming Act (The Farm Bill) arbitrarily defining legal hemp as *Cannabis* plant variants with less than 0.3% THC potency.

While cannabis with hemp-level THC potency has been shown to induce therapeutic benefits (Blessing et al., 2015; Fakhoury, 2016; Seltzer et al., 2020; Vigil et al., 2020), it is not clear that such treatments are optimal for all patients and conditions, especially health impairments whose very definitions are based on aversive percepts. Conditions such as chronic pain, depression, and anxiety are specific, aversive states of a person's consciousness (Vigil, 2009; Vigil and Stregnth, 2014; Durisko et al., 2016), and therefore, the ability for psychotropics and entheogens, such as the *Cannabis* plant, to alter the individual's visceral sensations, perceptions, and attention would seem to be integral to the treatment's ability to improve these types of health conditions. This thesis is supported by findings showing that THC potency levels are stronger predictors of patient symptom relief than are CBD levels (Stith et al., 2019). While the hope may be that cannabis treatments can offer relief without inducing a feeling of being high, an altered state of consciousness in association with symptom relief is not an unusual side effect among conventional medications such as opioids, benzodiazepines, muscle relaxants, and treatments for hyperactivity and attention deficit disorders.

The current study seeks to measure the associations among experiences of feeling high, patient symptom relief, and side effect outcomes during real-time cannabis administration sessions, accounting for plant phenotype, product potency, consumption mode, and dose and testing for differences across health conditions, sex, and age subgroups. We focus on flower products as the most widely used and homogenous product category with comparable percentage-based potency information across products (Stith et al., 2018). Our data come from the largest database of *Cannabis* flower administration sessions in the U.S., collected by the educational mobile software application, Releaf App™, which provides patients the ability to record the symptom relief and side effects generated by their cannabis usage in real time, across product types, consumption modes, doses, and symptoms. In order to better understand what it means for a patient to feel high, we assess pairwise correlations between feeling high and the other 46 side effects available for selection. We then use individual patient-level fixed effects regression models to assess real-time associations between feeling high and patients' symptom severity and categories of experienced side effects, both negative and positive. We further evaluate which product characteristics are associated with feeling high and analyze whether feeling high remains an independent predictor of symptom relief and side effect reporting even after controlling for plant phenotype, consumption mode, dose, and THC and CBD potencies. We run subgroup analyses by age, gender, pre-app cannabis experience, and for the five most frequently reported symptoms in the data: pain, anxiety, depression, fatigue, and insomnia.

2 Methods

2.1 Study design

We analyzed previously collected, de-identified data recorded through the educational mobile software app, Releaf App, which was

TABLE 1 Descriptive statistics.

	Observations	Patients	Mean	N or SD	Min	Max
Panel A: Outcome Variables						
Symptom Change	16,480	1,882	−3.82	(2.21)	−10	0
Starting Symptom Level	16,480	1,882	5.85	(2.09)	1	10
Minimum Symptom Level	16,480	1,882	2.03	(1.90)	0	10
Reporting Feeling "High"	7,904	1,882	49%	4,041	0	1
Any Negative Side Effect	7,904	1,882	64%	5,074	0	1
Any Positive Side Effect	7,904	1,882	95%	7,531	0	1
Any Context-Specific Side Effect	7,904	1,882	81%	6,439	0	1
Panel B: Treatment Variables						
Reporting Feeling "High"	16,480	1,882	51%	8,471	0	1
Dose	16,480	1,882	9.03	(9.33)	1	115
Plant Phenotype						
Hybrid	16,480	1,882	47%	7,820	0	1
C. indica	16,480	1,882	30%	5,015	0	1
C. sativa	16,480	1,882	22%	3,645	0	1
Combustion Method						
Joint	16,480	1,882	14%	2,355	0	1
Pipe	16,480	1,882	43%	7,091	0	1
Vape	16,480	1,882	43%	7,034	0	1
THC						
% THC	16,480	1,882	18.05	(6.75)	0	30
THC <10%	16,480	1,882	12%	2,031	0	1
THC 10%–20%	16,480	1,882	42%	6,927	0	1
THC 21%–30%	16,480	1,882	46%	7,522	0	1
CBD						
% CBD	16,480	1,882	5.32	(5.32)	0	30
CBD <1%	16,480	1,882	40%	6,668	0	1
CBD 1%–9%	16,480	1,882	34%	5,557	0	1
CBD 10%–30%	16,480	1,882	26%	4,255	0	1
Panel C: Subgroup Variables						
Common symptoms						
Pain	16,480	1,882	32%	5,307	0	1
Anxiety	16,480	1,882	27%	4,507	0	1
Depression	16,480	1,882	9%	1,436	0	1
Fatigue	16,480	1,882	5%	858	0	1
Insomnia	16,480	1,882	5%	856	0	1
Patient characteristics						
Male	12,478	1,139	49%	6,407	0	1

(Continued on following page)

TABLE 1 (*Continued*) Descriptive statistics.

	Observations	Patients	Mean	N or SD	Min	Max
Age<=40	12,478	1,139	57%	7,165	0	1
Experienced	13,075	1,210	57%	6,414	0	1

Notes: Observation counts are either treatment-level (16,480 observations in the main sample) or session-level (7,904 observations in the main sample.) Demographic characteristics are reported at the treatment level. Dichotomous variables are measured {0,1} and are reported in the tables as percentages ranging from 0 to 100, along with the number of sessions reporting "1." For non-dichotomous variables, standard deviations are reported in parentheses. In addition to "High," nineteen positive, seventeen negative, and ten context-specific side effects were available for selection.

designed to allow cannabis patients to track their symptom relief and side effect experiences over time across cannabis product characteristics, consumption modes, and symptom types. The de-identified, app-user-level data were provided to the research team by the owner of the Releaf App, MoreBetter, Ltd., under a data confidentiality agreement. (MoreBetter, Ltd. is owned by coauthors, Brockelman, Keeling, and Hall.). The patient-entered data were deemed exempt from IRB review by the Institutional Review Board at the University of New Mexico due to their preexistence and anonymous nature.

To use the Releaf App, patients voluntarily download the free app, available for both Android and iOS devices. Before starting a cannabis session, patients are instructed to record the product characteristics, including product type (flower, concentrate, edible, topical, pill, or tincture), and combustion method for "flower" and "concentrate" (joint, pipe, or vaporizer). Optional reporting includes plant phenotype (*C. sativa*, *C. indica*, or hybrid), which is widely used in marketing and consumer purchasing decisions and recorded by most app users, and THC and CBD potency levels, which are much less widely reported as they are typically available only for dispensary-sourced cannabis due to the cost of potency testing. Once the patient records product characteristics for the cannabis product, the product is saved in the app for future selection. To begin recording the effects of their cannabis treatment, patients are first prompted to select a symptom or set of symptoms for treatment and the cannabis product to be used in treatment before recording their starting symptom intensity level for each symptom on an analog scale from 0 to 10. (For flower and concentrate, combustion method must also be selected.) A session, in which one or more symptoms are tracked, begins when the patient records initial consumption and ends when the patient closes out the session and enters a final rating. While a session is active, patients can update their symptom-specific severity levels at any time, record additional doses (e.g., inhalations in the case of flower), and are able to track a variety of side effects, including seventeen negative side effects, nineteen positive side effects, and eleven side effects which are positive or negative depending on the context, e.g., feeling high or hungry. When setting up an account with the Releaf App, patients are encouraged to record basic demographic information, including age and gender.

The original sample consisted of 232,256 symptom-specific treatment events during 10,6801 sessions recorded by 13,539 app users between 6/5/2016 and 3/11/2021. Restricting the sample to those treatment events with positive starting symptom intensity levels, i.e., involving a health condition in need of treatment, reduced to sample to 228,835 treatment events, 103,825 sessions, and 12,910 app users. In addition, we required that a second symptom level was reported within 4 hours following the session inception to ensure an active cannabis treatment event and that assigned treatment effects are proximate to the timing of cannabis consumption–this restriction left a sample of 196,412 treatment events, 89,258 sessions and 12,908 app users. We further restrict the analysis to sessions using flower, which is the most common type of cannabis product in the data, leaving us with 120,023 treatment events in 57,884 sessions recorded by 9,045 app users. We further restrict the sample to sessions with any side effects reported, leaving us with 94,612 treatment events in 42,751 sessions recorded by 7,396 app users. Because not all variables included in our analyses are required reporting, we lose observations depending on the covariates in the analysis. Including plant phenotype reduces the sample to 84,011 treatment events in 37,991 sessions recorded by 6,862 app users and requiring THC and CBD potencies leaves 18,458 treatment events in 8,780 sessions recorded by 2,100 app users. Our most complete specification uses data on 16,480 treatment events in 7,904 sessions recorded by 1,882 app users. In addition to our main analyses, we conduct subgroup analyses by gender (male versus female), by age (40 or less versus over 40), for the five symptoms most prevalent in our data, and by pre-app cannabis experience ("none" or "a little" versus "a lot" or "expert."). Out of the total 1,882 users, 1,139 users reported demographic information and 1,210 report pre-app cannabis experience.

2.2 Variable construction

We focus on two primary sets of outcomes in this study: symptom relief and side effects. Symptom relief is a treatment event-level outcome and the goal of the patient's cannabis consumption, while side effects (including high) are reported at the session level and are optional reporting for the patient. Therefore, we use treatment events as our sample in our analyses of symptom relief and sessions as our sample in our analyses of side effects. To measure symptom relief, we calculate symptom change as the lowest symptom level reported within 4 hours from session inception minus the starting symptom level. As shown in Table 1 Panel A, the average symptom change was −3.82, with an average starting symptom level of 5.85 and an average minimum symptom level of 2.03. For side effects, we create three dummy variables for whether a patient reported feeling high (a side effect categorized as context-specific), any negative side effect (out of seventeen) and any positive side effect (out of nineteen) at any time during the session. Patients recorded feeling high in 49% of sessions. With respect to

TABLE 2 Side effect categorization, prevalence, and pairwise correlation with feeling "high."

Side effect	Category	% Sessions reporting	Pairwise correlation with "high"
Active	Positive	7	0.0615*
Chill	Positive	52	0.3109*
Clear	Positive	21	−0.0477
Comfy	Positive	36	0.0820*
Creative	Positive	9	−0.001
Dreamy	Positive	31	0.1258*
Energetic	Positive	11	0.0241
Focused	Positive	21	−0.0255
Frisky	Positive	9	0.0906*
Grateful	Positive	16	0.1394*
Great	Positive	16	0.1140*
Happy	Positive	25	0.1845*
Light	Positive	23	0.0833*
Optimistic	Positive	19	0.1100*
Peaceful	Positive	51	0.0246
Productive	Positive	14	0.0274
Reflective	Positive	20	−0.0014
Relaxed	Positive	60	0.0500*
Tuned	Positive	22	0.1047*
Anxious	Negative	7	−0.006
Clumsy	Negative	4	0.0970*
Confused	Negative	4	0.0753*
Coughing	Negative	16	0.1304*
Dizzy	Negative	8	0.0695*
Dry Mouth	Negative	30	0.2452*
Foggy	Negative	18	0.1325*
Forgetful	Negative	11	0.0714*
Headache	Negative	5	0.0274
Irritable	Negative	5	0.0234
Nausea	Negative	2	0.0249
Paranoid	Negative	3	0.0727*
Rapid Pulse	Negative	3	0.0603*
Red Eyes	Negative	14	0.2014*
Restless	Negative	14	0.1246*
Scattered	Negative	16	0.1941*
Unmotivated	Negative	12	0.0923*
Couchlocked	Context-Specific	15	0.1377*
Distracted	Context-Specific	13	0.1166*

(Continued on following page)

TABLE 2 (*Continued*) Side effect categorization, prevalence, and pairwise correlation with feeling "high."

Side effect	Category	% Sessions reporting	Pairwise correlation with "high"
High	Context-Specific	49	1.0000*
Hungry	Context-Specific	20	0.0964*
Silly	Context-Specific	8	0.1557*
Sleepy	Context-Specific	24	0.0914*
Talkative	Context-Specific	10	0.0578*
Thinky	Context-Specific	15	0.1069*
Thirsty	Context-Specific	29	0.2043*
Tingly	Context-Specific	25	0.2257*
Visuals	Context-Specific	9	0.1848*

Notes: Table includes data from 7,904 sessions. Side effects were categorized as positive, negative, or context-specific by the authors. We report Bonferroni-adjusted Pearson's correlation coefficients ρ with *'s indicating a statistical significance level of at least 0.01.

other side effects, patients reported one or more negative side effect in 64% of sessions, one or more positive side effect in 95% of sessions, and one or more context-specific side effects in 81% of sessions. Table 2 lists all the side effects, the category of side effect (negative, positive, or context-specific), and their prevalence in the data.

Panel B of Table 1 describes the treatment variables. In our analyses of symptom relief, our primary treatment outcome, we use the sample of symptom-specific treatment events and the associated variables are reported in Panel B, noting that we also include these variables in the side effect analyses and rates of prevalence may vary slightly between samples. For example, feeling high was reported in 49% of sessions in Panel A and is reported in 51% of treatment events in Panel B. In order to control for the quantity of cannabis flower consumed, we include the reported dose in our analyses with nine inhalations or "hits" consumed during the average treatment event. The rest of Panel B breaks out the categories of product and consumption characteristics for which high may proxy. Hybrid product (47%) was the most common plant phenotype and using a pipe (43%) was the most common combustion method. The average THC potency level was 18.05% while the average CBD level was 5.32%.

The variables used to define our subsamples are included in Panel C. In terms of symptom subgroups, 32% of treatment events were for Pain (including the specified symptoms: abdominal, arm or leg, back, cramping, gastrointestinal, headache, joint, menstrual, migraine, muscle, neck, nerve and other); 27% for Anxiety (including anxiety, stress, or agitation/irritation); 9% for Depression; 5% for Fatigue; and 5% for Insomnia. For demographics, among users who reported demographics, 49% of treatment events were recorded by males and 57% were recorded by patients 40 years old or younger. The majority of patients in our sample (57%) report being experienced with cannabis prior to starting use of the Releaf App.

2.3 Statistical analysis

This study seeks to examine the association between feeling high and symptom relief and side effects experienced, including the mediating roles of dose, plant phenotype, consumption mode, and THC and CBD potencies. Given the lack of a pre-existing clear definition of what it means to feel high, we first present Pearson correlation coefficients (with a Bonferroni adjustment) between reporting feeling high and the other side effects available in the Releaf App. We then proceed in evaluating whether feeling high is associated with changes in symptom relief and side effect reporting by reporting correlations between feeling high, symptom relief, and side effects, controlling for individual fixed effects for all outcomes and controlling for starting symptom level in our symptom relief regressions as it is naturally correlated with the potential extent of symptom relief. Including individual fixed effects allows us to approximate the within-user difference in symptom relief and side effects between sessions in which the patient reported feeling high and sessions in which they did not report feeling high. We next control for dose (the quantity of cannabis consumed) to ensure that feeling high is not merely capturing a greater quantity of cannabis consumed. As the distribution for dose has a long positive tail, we use a natural log transformation in our analysis to diminish the possibility that outliers are influencing the effect. To further explore what factors may be driving respondents feeling high, we regress high on plant phenotype, consumption method and product potency, controlling for individual fixed effects and the natural log of dose, before evaluating whether the effects of feeling high on symptom relief and side effects disappears after we control for plant phenotype, combustion method, and product potency. We also control throughout for starting symptom intensity levels in our symptom relief regressions, and in all our regressions including product characteristics, the natural log of total dose to capture the quantity of cannabis consumed.

In regressions analyzing the role of plant phenotype, consumption mode, and product potency, we measure *C. indica* and *C. sativa* relative to hybrid strains, joint and vape relative to pipe, THC 10%–20% and THC 21%–30% relative to THC less than 10%, and CBD 1%–9% and CBD 10%–30% relative to CBD of less than 1%. We use categorical variables for THC and CBD potency to capture how products are sold (low, medium, and high levels of THC and CBD) and to allow for non-linearities in the relationships. In order to evaluate the difference in coefficients across gender, age, and pre-app cannabis experience subgroups, we use Wald tests to

TABLE 3 Associations between feeling high and treatment outcomes.

	(1)	(2)	(3)	(4)	(5)	(6)
	Symptom change	Any negative	Any positive	Symptom change	Any negative	Any positive
High	−0.317***	0.136***	0.061***	−0.285***	0.132***	0.057***
	(0.023)	(0.009)	(0.007)	(0.023)	(0.009)	(0.007)
Starting Symptom Level	−0.674***			−0.671***		
	(0.007)			(0.007)		
Ln Dose				−0.306***	0.041***	0.034***
				(0.022)	(0.005)	(0.004)
Constant	0.195***	0.356***	0.819***	0.721***	0.284***	0.759***
	(0.047)	(0.004)	(0.003)	(0.062)	(0.011)	(0.008)
Observations	94,703	42,842	42,842	94,612	42,751	42,751
R-squared	0.378	0.018	0.005	0.385	0.020	0.008
N Users	7,419	7,419	7,419	7,396	7,396	7,396

Notes: All regressions are estimated using an individual-level fixed effects model. Standard errors, clustered at the individual patient level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

compare coefficients across regressions. As a robustness check, we also run regressions for our main outcomes controlling for the number of context-specific side effects reported (excluding high) in order to control for side effect reporting behavior, such as the same user recording their side effects in greater detail in some sessions, which might mechanistically increase the likelihood of recording a negative or positive side effect. In all regressions, we cluster the standard errors at the user level to control for heteroskedasticity and arbitrary correlation within users. Analyses were conducted using Stata 15.1.

3 Results

Table 2 shows reporting frequencies for the side effects available in the Releaf App along correlations between reporting feeling high and reporting of other individual side effects. "High," reported in 49% of sessions, is one of the most frequently reported side effects–only "Relaxed" (60% of sessions), "Chill" (52% of sessions), and "Peaceful" (51% of sessions) are reported more often. Among Bonferroni-adjusted correlations statistically significant at the 0.01 level ($p < 0.01$), feeling high has the largest statistically significant positive correlations with feeling "Chill," "Tingly," and "Thirsty" and experiencing "Dry Mouth" and "Red Eyes." "High" is negatively correlated with feeling "Clear," "Focused," "Anxious," "Reflective," and "Creative," but these correlations are not statistically significant at the 0.01 level. Connecting to the definitions of high discussed in the introduction, feeling high is statistically significantly correlated with impairment-related side effects such as "Clumsy," "Confused," "Dizzy," "Foggy," and "Paranoid," as well as euphoria or exhilaration-related effects like "Happy," "Grateful," "Great," and "Optimistic." The results in Table 2 flesh out what is meant by feeling high among patients in our sample and show that the sensations reported by patients in our sample relate directly to common definitions of feeling high.

We proceed in Table 3 by evaluating the association between feeling high and the treatment outcomes, symptom change, any negative side effect, and any positive side effect, controlling for starting symptom level in the symptom relief regressions and individual fixed effects in all the regressions. We add in the quantity of cannabis consumed, the natural log of the dose, in the second three columns of Table 3. Reporting feeling high is associated with 0.317 greater within-user symptom relief than experienced in treatment events in which the same user did not report feeling high ($p < 0.001$). This improved symptom relief is offset by a 13.6 percentage point increase in the likelihood of reporting a negative side effect, perhaps partially compensated for by a 6.1 percentage point increase in positive side effect reporting. Positive side effects, while not the treatment goal, may improve the overall patient experience, and therefore, medication compliance. A higher starting symptom level is, as expected, positively correlated with symptom relief. The results controlling for the natural log of dose suggest that the variable feeling high is partially capturing the consumption of larger quantities of cannabis, i.e., a larger dose, as the coefficients on the variable high are smaller with the inclusion of Ln Dose. Ln Dose is independently a strongly statistically significant predictor of greater symptom relief and a higher likelihood of reporting side effects, both negative and positive. The effect of a given percentage change in the natural log of the dose variable can be calculated by multiplying the coefficient on the natural log of dose variable by the natural log of one plus the increase, e.g., $\boldsymbol{\beta_{dose}}^{*}\mathbf{ln}(\mathbf{1.1})$ for a 10% increase or $\boldsymbol{\beta_{dose}}^{*}\mathbf{ln}(\mathbf{2})$ for the effect of doubling the dose. Doubling the dose is associated with a 0.212-point increase in symptom relief ($\mathbf{-0.306}^{*}\mathbf{ln}(\mathbf{2})$ and relatively small percentage point increases of 0.028 ($\mathbf{-0.041}^{*}\mathbf{ln}(\mathbf{2})$ and 0.024 ($\mathbf{-0.034}^{*}\mathbf{ln}(\mathbf{2})$ for negative and positive side effects, respectively.

10.3389/fphar.2023.1135453

TABLE 4 Associations between plant phenotype, combustion method, and potency and feeling high.

	(1)	(2)	(3)	(4)	(5)
	High	High	High	High	High
C. indica		0.006			−0.002
		(0.007)			(0.017)
C. sativa		−0.003			−0.022
		(0.008)			(0.018)
Joint			0.004		0.005
			(0.015)		(0.036)
Vape			−0.089***		−0.122***
			(0.014)		(0.026)
THC 10%–20%				0.124**	0.132**
				(0.045)	(0.046)
THC 21%–30%				0.158***	0.164***
				(0.046)	(0.047)
CBD 1%–9%				0.022	0.033
				(0.020)	(0.022)
CBD 10%–30%				−0.030	−0.030
				(0.022)	(0.023)
Ln Dose	0.061***	0.064***	0.068***	0.078***	0.093***
	(0.006)	(0.007)	(0.007)	(0.013)	(0.014)
Constant	0.374***	0.375***	0.393***	0.217***	0.246***
	(0.011)	(0.013)	(0.011)	(0.048)	(0.041)
Observations	42,751	37,991	40,638	8,780	7,904
R-squared	0.007	0.007	0.010	0.025	0.033
N Users	7,396	6,862	7,046	2,100	1,882

Notes: All regressions are estimated using an individual fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC, categories are relative to THC, between 0% and 10%, CBD, categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual patient level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Because feeling high may be correlated with specific product and consumption methods, particularly THC, the results in Table 3 may be only capturing a proxy relationship, in which it is not feeling high that is leading to increased symptom relief and negative and positive side effects, but rather a particular plant phenotype, consumption method, or product potency that is driving the effect. Table 4 reports the effects of plant phenotype, consumption method and product potency on the likelihood of reporting feeling high. Starting symptom level and the natural log of total dose are included in all specifications. Results are session-level as side effects are recorded at the session level rather than being specific to an individual symptom being tracked by the patient. In column 1, the likelihood of feeling high is regressed on the natural log of dose and in columns 2–4, we separately include plant phenotype, combustion method, and THC and CBD levels. All covariates are included in column 4. Plant phenotype is not correlated with feeling high. Relative to the use of pipe or joint, vaping is associated with a significantly lower probability of reporting high as a side effect. As expected, a high THC level is a strong predictor of reporting feeling high, while CBD is not associated with feeling high. The quantity of cannabis consumed (Ln Dose) is, not surprisingly, a strong predictor of feeling high.

Table 5 presents the association between feeling high and symptom relief, controlling for independent effects from plant phenotype in Column 1, consumption method in Column 2, THC and CBD in Column 3 and all factors jointly in Column 4. In Column 5, we omit high as a covariate to further explore how feeling high interacts with the covariates in Table 4. Throughout our results, plant phenotype is an insignificant predictor of symptom relief, while using a pipe is significantly predictive of greater symptom relief relative to smoking a joint or vaping. THC and CBD are generally insignificant with THC only becoming marginally significant once the variable for feeling high is excluded. This suggests that feeling high is not simply a proxy for consuming a higher THC product. In fact, none of the included product and consumption method covariates dramatically affects

10.3389/fphar.2023.1135453

TABLE 5 Effects of feeling high and product and combustion characteristics on symptom relief.

	(1)	(2)	(3)	(4)	(5)
	Symptom change	Symptom change	Symptom change	Symptom change	Symptom change
High	−0.291***	−0.277***	−0.296***	−0.295***	
	(0.024)	(0.023)	(0.037)	(0.040)	
C. indica	−0.000			0.007	−0.010
	(0.020)			(0.053)	(0.049)
C. sativa	0.028			0.018	0.018
	(0.023)			(0.054)	(0.053)
Joint		0.140**		0.283**	0.284***
		(0.047)		(0.088)	(0.084)
Vape		0.198***		0.298***	0.352***
		(0.055)		(0.069)	(0.066)
THC 10%–20%			−0.000	−0.067	−0.112
			(0.081)	(0.080)	(0.079)
THC 21%–30%			−0.053	−0.079	−0.164*
			(0.084)	(0.086)	(0.083)
CBD 1%–9%			−0.019	−0.018	−0.005
			(0.054)	(0.054)	(0.052)
CBD 10%–30%			0.040	0.037	0.019
			(0.059)	(0.061)	(0.061)
Ln Dose	−0.311***	−0.320***	−0.360***	−0.397***	−0.434***
	(0.024)	(0.024)	(0.037)	(0.041)	(0.040)
Starting Symptom Level	−0.675***	−0.671***	−0.668***	−0.672***	−0.656***
	(0.008)	(0.008)	(0.016)	(0.016)	(0.016)
Constant	0.731***	0.649***	0.928***	0.879***	0.767***
	(0.067)	(0.064)	(0.155)	(0.150)	(0.147)
Observations	84,011	89,701	18,458	16,480	19,411
R-squared	0.391	0.388	0.389	0.397	0.380
N Users	6,862	7,046	2,100	1,882	2,181

Notes: All regressions are estimated using an individual patient-level fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC categories are relative to THC between 0% and 10%, CBD categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual patient level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

the statistical significance or magnitude of the association between feeling high and symptom relief. In our most conservative model, with the full set of covariates included, sessions in which patients reported feeling high have symptom reductions of −0.295 points on average or a 7.7% improvement in symptom relief relative to the average symptom relief of −3.82.

Figure 1, 2 further demonstrates the relationship between symptom relief, feeling high, and THC level by showing the predicted covariate-adjusted symptom relief by THC levels and whether or not the patient reported feeling high. Consistent with the pattern in Table 4, sessions in which users reported feeling high were associated with greater symptom relief in every THC category.

Tables 6, 7 show the effects of reporting feeling high and product and consumption characteristics on negative and positive side effects. Findings suggest that feeling high is associated with higher probabilities of reporting negative and positive side effects. The estimated effects become larger for negative side effects but smaller for positive side effects with the inclusion of product and consumption mode characteristics. Higher THC values are associated with increased negative side effect reporting, but do not appear to be mediating the

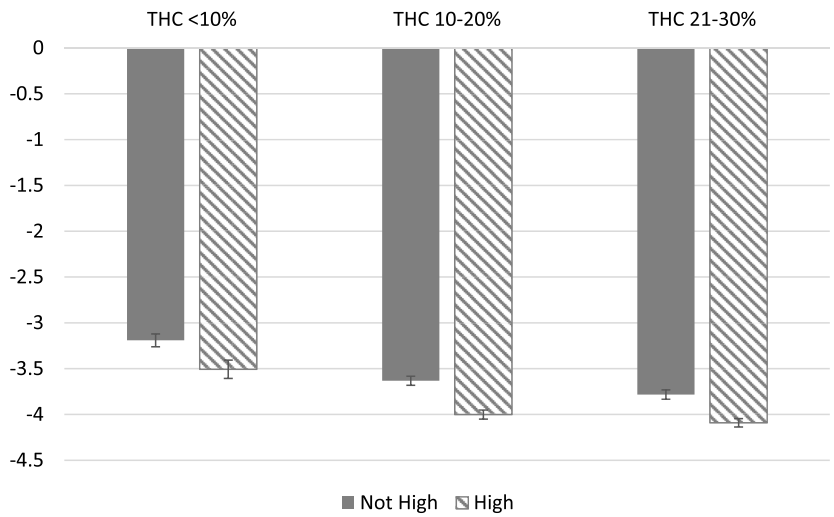

FIGURE 1
Symptom Relief by High Status and THC levels. Notes: Predicted, covariate-adjusted changes in symptom severity are presented with 95% confidence intervals reported. Covariate-adjusted symptom relief is obtained from an individual fixed effects model controlling for plant phenotype, combustion method, THC and CBD categories, natural log of dose, and starting symptom level.

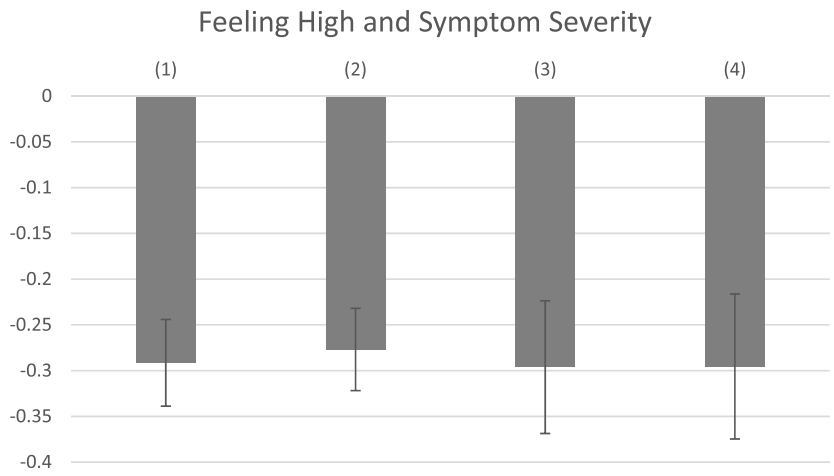

FIGURE 2
Symptom Relief by Covariate Controls. Note: Covariates-adjusted associations between feeling high and symptom severity are reported with 95% confidence intervals. All regressions control for the log of dosage and starting symptom level. In addition, (1) includes Plant Phenotype; (2) includes Combustion Method; (3) includes THC and CBD levels; (4) includes the full set of product characteristics.

relationship between feeling high and negative side effects–the coefficients on feeling high increase in magnitude with the inclusion of THC levels and the effects of THC levels on the likelihood of negative side effect reporting are largely unchanged by the inclusion of feeling high, as shown in Columns 4 and 5 of Table 6. We do not find evidence that feeling high is strongly proxying for any of our other covariates with respect to positive side effect reporting, although the negative effect of *C. sativa* and the positive effects of THC increase in magnitude and statistical significance when feeling high is omitted in Column 5 of Table 7.

Tables 8, 9 present our subgroup analyses. To conduct these analyses, we further restrict the analysis sample to patients who reported their age or gender in Table 8 and to patients who reported treating one of the five most common symptoms in our sample in Table 9. As shown in Columns 2 and 3 of Table 8, the association between feeling high on symptom relief is significant for both male and female patients. Male patients show a larger coefficient, but it is not statistically different from the coefficient of the female users (Wald test, $p = 0.71$). Greater variation exists by age group with those over 40 experiencing a smaller and less statistically significant relationship between feeling high and symptom relief (Wald test,

TABLE 6 Effects of feeling high and product and combustion characteristics on negative side effect reporting.

	(1)	(2)	(3)	(4)	(5)
	Any negative	Any negative	Any negative	Any negative	Any negative
High	0.128***	0.131***	0.147***	0.146***	
	(0.009)	(0.009)	(0.016)	(0.017)	
C. indica	0.010			−0.017	−0.021
	(0.007)			(0.016)	(0.015)
C. sativa	0.001			0.001	−0.009
	(0.007)			(0.017)	(0.016)
Joint		−0.010		0.008	0.023
		(0.013)		(0.039)	(0.037)
Vape		−0.036**		−0.065*	−0.039
		(0.012)		(0.026)	(0.024)
THC 10%–20%			0.048*	0.071**	0.075**
			(0.022)	(0.022)	(0.025)
THC 21%–30%			0.074**	0.100***	0.101**
			(0.028)	(0.028)	(0.031)
CBD 1%–9%			−0.011	0.000	0.000
			(0.017)	(0.017)	(0.018)
CBD 10%–30%			−0.021	0.003	−0.008
			(0.017)	(0.017)	(0.020)
Ln Dose	0.041***	0.048***	0.047***	0.055***	0.032**
	(0.006)	(0.005)	(0.011)	(0.012)	(0.012)
Constant	0.281***	0.285***	0.219***	0.205***	0.403***
	(0.012)	(0.012)	(0.034)	(0.036)	(0.034)
Observations	37,991	40,638	8,780	7,904	9,404
R-squared	0.020	0.021	0.030	0.034	0.005
N Users	6,862	7,046	2,100	1,882	2,181

Notes: All regressions are estimated using an individual patient-level fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC, categories are relative to THC, between 0% and 10%, CBD, categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual patient level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

$p < 0.001$). The other coefficients in the table suggest fundamental differences between younger and older patients, as combustion methods and THC levels are more relevant for symptom relief among older patients than among younger patients. In our second subgroup analysis in Table 9, we see that feeling high is associated with similar magnitudes of increased symptom relief for Pain, Anxiety, Depression, Fatigue, but not Insomnia, suggesting that feeling high may be too stimulating an experience for sleep induction.

Table 10 offers suggestive results with respect to the effect of experience on the relationship among symptom relief, product characteristics including THC, and feeling high. Feeling high has similar effects on symptom relief for both experienced and inexperienced users (Wald test, $p = 0.71$). However, the relationship between THC and feeling high does vary between the two groups with the effect of THC larger in magnitude and more statistically significant among inexperienced users than among experienced users and the negative impact of vaping on feeling high only evident among experienced users.

For a final robustness check on our results, we rerun our main analyses in Table 11, controlling for the total number of context-specific side effects in order to capture within-user, cross-session behavioral factors influencing the likelihood of reporting side effects. We find little effect on the overall pattern of results, but the number of context-specific side effects reported is correlated with an increased likelihood of reporting feeling high, symptom relief, and positive and negative side effect reporting, leading us to hypothesize that cannabis sessions that are overall more stimulating and encourage greater app engagement—e.g.,

10.3389/fphar.2023.1135453

TABLE 7 Effects of feeling high and product and combustion characteristics on positive side effect reporting.

	(1)	(2)	(3)	(4)	(5)
	Any positive	Any positive	Any positive	Any positive	Any positive
High	0.058***	0.056***	0.044**	0.044**	
	(0.007)	(0.007)	(0.016)	(0.016)	
C. indica	−0.007			−0.029	−0.025
	(0.006)			(0.017)	(0.015)
C. sativa	−0.003			−0.041*	−0.044**
	(0.006)			(0.017)	(0.016)
Joint		−0.006		−0.005	0.001
		(0.009)		(0.018)	(0.016)
Vape		−0.012		0.015	0.020
		(0.014)		(0.025)	(0.021)
THC 10%–20%			0.022	0.034	0.042*
			(0.017)	(0.020)	(0.018)
THC 21%–30%			0.001	0.012	0.019
			(0.020)	(0.023)	(0.020)
CBD 1%–9%			0.019	0.015	0.006
			(0.016)	(0.016)	(0.015)
CBD 10%–30%			0.020	0.018	0.013
			(0.018)	(0.019)	(0.017)
Ln Dose	0.034***	0.036***	0.049***	0.046***	0.036***
	(0.005)	(0.005)	(0.010)	(0.010)	(0.008)
Constant	0.763***	0.761***	0.724***	0.731***	0.789***
	(0.009)	(0.010)	(0.023)	(0.025)	(0.020)
Observations	37,991	40,638	8,780	7,904	9,404
R-squared	0.008	0.008	0.011	0.012	0.008
N Users	6,862	7,046	2,100	1,882	2,181

Notes: All regressions are estimated using an individual patient-level fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC, categories are relative to THC, between 0% and 10%, CBD, categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual user level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

as proxied by a greater likelihood of reporting side effects—are associated with greater symptom relief and increased side effect reporting.

4 Discussion

The current study extends the previous literature in multiple ways. First, we explore in detail how the experience of feeling high relates to other side effects reported during cannabis consumption and find that it is positively correlated with most side effects. This suggests that feeling high may be associated with increased engagement in the cannabis consumption and app use, as well as both feelings of impairment and feelings of exhilaration and elation. Second, we find that variation in whether or not a person reports feeling high is primarily driven by THC levels and whether a vaporizer was used for combustion. Associated with increased relief from health symptoms and side effects experienced during cannabis consumption. Third, we show that feeling high is associated with increased symptom relief and side effects reporting (both positive and negative) and these relationships remain statistically significant even after controlling for the quantity of cannabis consumed, the characteristics of the flower product (plant phenotype and THC and CBD potencies), and the mode of consumption (pipe, joint, vaporizer). Our results appear generalizable across genders and symptom types, although some heterogeneity exists in these relationships between older and younger patients and across symptom types. The results support

TABLE 8 Effects of feeling high and product and combustion characteristics on symptom relief, by gender and age.

	(1)	(2)	(3)	(4)	(5)
	All with demographics	Male	Female	<=40	40+
High	−0.302***	−0.312***	−0.276***	−0.377***	−0.123*
	(0.047)	(0.064)	(0.072)	(0.060)	(0.052)
C. indica	0.024	0.031	0.025	0.154	−0.073
	(0.062)	(0.087)	(0.071)	(0.089)	(0.064)
C. sativa	0.041	−0.080	0.158	0.056	0.021
	(0.066)	(0.082)	(0.104)	(0.097)	(0.084)
Joint	0.293**	0.293*	0.275*	0.235	0.555***
	(0.097)	(0.133)	(0.135)	(0.130)	(0.105)
Vape	0.307***	0.349***	0.235*	0.242*	0.405***
	(0.079)	(0.103)	(0.111)	(0.119)	(0.099)
THC 10%–20%	−0.099	0.055	−0.272	−0.020	−0.165
	(0.097)	(0.113)	(0.139)	(0.157)	(0.110)
THC 21%–30%	−0.131	−0.122	−0.178	0.064	−0.304*
	(0.101)	(0.120)	(0.168)	(0.153)	(0.120)
CBD 1%–9%	0.013	0.013	−0.026	0.039	−0.047
	(0.063)	(0.085)	(0.081)	(0.089)	(0.076)
CBD 10%–30%	0.021	0.083	−0.080	0.094	−0.051
	(0.072)	(0.093)	(0.107)	(0.104)	(0.104)
Ln Dose	−0.379***	−0.415***	−0.339***	−0.385***	−0.413***
	(0.044)	(0.073)	(0.056)	(0.070)	(0.051)
Starting Symptom Level	−0.680***	−0.691***	−0.672***	−0.685***	−0.683***
	(0.019)	(0.031)	(0.022)	(0.019)	(0.043)
Constant	0.900***	1.012***	0.860**	0.718**	1.104***
	(0.178)	(0.228)	(0.265)	(0.251)	(0.283)
Observations	12,478	6,071	6,407	7,558	4,958
R-squared	0.403	0.432	0.382	0.421	0.390
N Users	1,139	531	608	838	298

Notes: All regressions are estimated using an individual patient-level fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC, categories are relative to THC, between 0% and 10%, CBD, categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual patient level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

the thesis that changes in cognizance that characterize the distinct experience of feeling high may play a statistically and clinically significant role in the medicinal effects of the *Cannabis* plant for some patients.

Among the reported product characteristics, THC potency levels were the only independent predictors of an increased likelihood of reporting feeling high, while vaporizing was associated with a reduced likelihood of feeling high. As in prior work (Stith et al., 2019), THC predicted symptom relief and side effect reporting, but once feeling high was included, THC was no longer predictive of increased symptom relief, although it remained predictive of increased negative side effect reporting. It appears that for most patients in our sample, higher THC levels are only effective at increasing symptom relief if they induce feeling high. However, regardless of whether a patient reports feeling high, higher THC levels appear to be strongly associated with increased side effect reporting. These results suggest that ever-increasing THC levels are not the key to therapeutic benefits. Instead, the seeming drive in the cannabis industry towards ever-increasing THC levels may increase medication non-compliance due to the association between higher THC levels and negative side effect reporting.

TABLE 9 Effects of feeling high and product and combustion characteristics on symptom relief, by common symptom types.

	(1)	(2)	(3)	(4)	(5)
	Pain	Anxiety	Depression	Fatigue	Insomnia
High	−0.290***	−0.317***	−0.302**	−0.375**	−0.057
	(0.072)	(0.068)	(0.105)	(0.131)	(0.167)
C. indica	−0.147*	0.093	0.340**	0.049	0.258
	(0.064)	(0.080)	(0.118)	(0.174)	(0.138)
C. sativa	−0.032	0.040	0.035	−0.020	0.259
	(0.081)	(0.074)	(0.118)	(0.128)	(0.245)
Joint	0.156	0.150	0.301	0.477	0.343
	(0.159)	(0.149)	(0.194)	(0.292)	(0.321)
Vape	0.188*	0.161	0.222	0.188	0.192
	(0.088)	(0.121)	(0.201)	(0.273)	(0.237)
THC 10%–20%	−0.049	−0.018	−0.202	−0.047	−0.180
	(0.122)	(0.097)	(0.117)	(0.144)	(0.264)
THC 21%–30%	−0.044	−0.087	−0.232	−0.118	−0.220
	(0.133)	(0.110)	(0.164)	(0.181)	(0.298)
CBD 1%–9%	0.044	−0.041	−0.147	0.043	−0.085
	(0.084)	(0.089)	(0.127)	(0.196)	(0.137)
CBD 10%–30%	0.098	0.038	0.108	−0.041	0.201
	(0.086)	(0.091)	(0.139)	(0.151)	(0.211)
Ln Dose	−0.401***	−0.310***	−0.525***	−0.077	−0.268
	(0.064)	(0.057)	(0.100)	(0.061)	(0.155)
Starting Symptom Level	−0.629***	−0.743***	−0.643***	−0.660***	−0.696***
	(0.025)	(0.023)	(0.033)	(0.070)	(0.064)
Constant	1.157***	0.791***	1.003**	0.439	0.615
	(0.223)	(0.189)	(0.349)	(0.484)	(0.562)
Observations	5,307	4,507	1,436	858	856
R-squared	0.348	0.488	0.412	0.375	0.333
N Users	1,057	1,142	545	357	312

Notes: All regressions are estimated using an individual patient-level fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC, categories are relative to THC, between 0% and 10%, CBD, categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual user level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

These results complicate the common belief that the experience of feeling high is always a negative, tangential side effect of cannabis-based therapies, and instead, support the thesis that feeling high may be a fundamental factor for effective cannabis-based treatment for some patients, perhaps even more relevant than THC potency in determining symptom relief. Therefore, the experience of feeling high may highlight the cost-benefit tradeoffs of therapeutic cannabis use, i.e., the potential costs of increased risk of behavioral/cognitive impairments and the potential benefits of improved symptom management. For some people, the costs of impairment from feeling high may outweigh the perceived benefits, rendering cannabis treatments that make a person feel high a suboptimal choice for such individuals. For chronic health conditions that are not characterized by transient states of aversive percepts, such as metabolic or cellular diseases, feeling high may present indirect detriments (e.g., cognitive and behavioral impairments) or benefits sometimes recorded in the literature as positive side effects, such as increased reported quality of life, behavioral motivations, experienced creativity, ability to accomplish personally fulfilling tasks, and/or improved social relations (Schlienz et al., 2020; Aviram et al., 2021). Among other patients, feeling high may be a direct benefit from consuming cannabis. For health conditions such as chronic pain,

TABLE 10 Effects of feeling high and product and combustion characteristics on symptom relief, by pre-app cannabis experience.

	(1)	(2)	(3)	(4)
	Not experienced	Experienced	Not experienced	Experienced
High	−0.289***	−0.311***		
	(0.068)	(0.060)		
C. indica	0.005	0.064	0.022	0.025
	(0.066)	(0.105)	(0.025)	(0.029)
C. sativa	0.006	0.039	−0.022	0.002
	(0.086)	(0.093)	(0.032)	(0.032)
Joint	0.309*	0.272*	−0.005	−0.028
	(0.151)	(0.127)	(0.051)	(0.060)
Vape	0.336**	0.249*	−0.076	−0.213***
	(0.116)	(0.103)	(0.049)	(0.054)
THC 10%–20%	−0.177	0.038	0.157*	0.081
	(0.113)	(0.160)	(0.067)	(0.053)
THC 21%–30%	−0.140	−0.078	0.197**	0.132*
	(0.136)	(0.157)	(0.072)	(0.058)
CBD 1%–9%	−0.015	0.043	0.018	0.082*
	(0.075)	(0.105)	(0.028)	(0.036)
CBD 10%–30%	−0.003	0.076	−0.064*	0.013
	(0.094)	(0.118)	(0.031)	(0.050)
Log dosage	−0.338***	−0.444***	0.074***	0.111***
	(0.045)	(0.084)	(0.022)	(0.025)
Starting Symptom Level	−0.669***	−0.688***		
	(0.030)	(0.021)		
Constant	0.590*	1.219***	0.279***	0.291***
	(0.255)	(0.244)	(0.067)	(0.070)
Observations	6,661	6,414	6,661	6,414
R-squared	0.377	0.428	0.045	0.032
N Users	526	684	526	684

Notes: The outcome in Columns 1 and 2 is symptom relief; the outcome in Columns 3 and 4 is reporting feeling "high." All regressions are estimated using an individual patient-level fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC, categories are relative to THC, between 0% and 10%, CBD, categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual user level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

depression, and anxiety, experiencing euphoria is the very inverse of the forms of visceral sensations and cognitive percepts that characterize these disorders, meaning the primary goal of the treatments may be to achieve the euphoric state of feeling high and/or the behavioral changes that can results from feeling high (e.g., increased physical activity levels). At a mechanistic level, the therapeutic potential of feeling high may arise from interactions between heuristic feelings of euphoria and the tendency for cannabis to induce attentional distraction (Lundqvist, 2005; Hartman and Huestis, 2013), including the ability to alter the user's attention away from viscerally unpleasant sensations, thoughts, and memories, and habituation of the startle reflex (Kedzior and Martin-Iverson, 2006; Kedzior et al., 2016).

Given the widespread prevalence of clinical and subclinical medical conditions in the general population, one potential implication from the current results may be that some so-called "recreational" cannabis usage, based on the premise that the user is solely motivated to get high, may be offering medicinal benefits, whether the consumer is aware of such an outcome or not. Survey data shows a strong overlap between medicinal and recreational use among cannabis patients (Pacula et al., 2016), and for many individuals it may be impractical to operationalize the distinction between medical versus recreational cannabis use, as actual usage tends to result in essentially complementary outcomes that cannot easily be independently achieved.

TABLE 11 Associations between feeling high and treatment outcomes, controlling for all covariates and side effect reporting behavior.

	(1)	(2)	(3)	(4)
	High	Symptom change	Any negative	Any positive
High		−0.223***	0.103***	0.034*
		(0.040)	(0.014)	(0.017)
C. indica	−0.003	0.023	−0.025	−0.029
	(0.018)	(0.054)	(0.015)	(0.017)
C. sativa	−0.019	0.019	0.003	−0.042*
	(0.019)	(0.054)	(0.016)	(0.018)
Joint	0.018	0.273**	0.011	−0.002
	(0.039)	(0.088)	(0.036)	(0.018)
Vape	−0.096**	0.270***	−0.048	0.026
	(0.030)	(0.064)	(0.025)	(0.027)
THC 10%–20%	0.094*	−0.033	0.046*	0.029
	(0.043)	(0.078)	(0.023)	(0.020)
THC 21%–30%	0.135**	−0.044	0.075**	0.006
	(0.046)	(0.080)	(0.027)	(0.023)
CBD 1%–9%	0.018	−0.033	0.017	−0.020
	(0.024)	(0.056)	(0.018)	(0.015)
CBD 10%–30%	−0.035	0.023	0.015	−0.005
	(0.027)	(0.066)	(0.018)	(0.016)
N context specific side effects	0.057***	−0.122***	0.082***	0.019**
	(0.006)	(0.015)	(0.007)	(0.006)
Log dosage	0.076***	−0.370***	0.039***	0.042***
	(0.014)	(0.038)	(0.011)	(0.010)
Starting Symptom Level		−0.671***		
		(0.016)		
Constant	0.212***	1.007***	0.120***	0.731***
	(0.045)	(0.139)	(0.036)	(0.021)
Observations	16,480	16,480	7,904	7,904
R-squared	0.062	0.405	0.093	0.017
N Users	1,882	1,882	1,882	1,882

Notes: All regressions are estimated using an individual patient-level fixed effects model. *C. indica* and *C. sativa* are relative to Hybrid, THC, categories are relative to THC, between 0% and 10%, CBD, categories are relative to <1% CBD, and Joint and Vape are relative to Pipe. Standard errors, clustered at the individual user level, are shown in parentheses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

It is interesting that so many users reported feeling high, across THC potency levels, suggesting an important role for additional constituents, e.g., terpenes, in the psychological effects of cannabis consumption (McPartland and Russo, 2012; Fischedick and Elzinga, 2015). Phytochemicals, such as terpenes, have been shown to induce changes (e.g., anesthetic, anxiolytic, sedative) in mood but such studies have been limited by a lack of *in vitro* or *in vivo* data (Behr and Johnen, 2009), mice rather than human subjects (Ito and Ito, 2013); or much higher doses than found in the *Cannabis* plant (Surendran et al., 2021). Our own recent work tested how common combinations of THC, CBD and primary terpenes affected patient outcomes. We found differing effects, even across products with similar THC and CBD levels, suggesting an important role for terpenes and the possibility of a multitude of pharmacodynamics resulting from consuming different cannabis strains with varying phytochemical combinations, or chemovars (Vigil et al., 2023). Precisely how these "entourage" effects arise remains unknown with *in vitro*

studies indicating that terpenes do not directly affect cannabinoid receptors, e.g., CB1 and CB2 (Santiago et al., 2019; Finlay et al., 2020). Further supporting a role for cannabinoids beyond THC and CBD, our results throughout showed a strong association between the quantity of cannabis consumed and the effects experienced, regardless of whether the individual felt high. In regressions controlling for both feeling high and the natural log of the dose, doubling the dose of cannabis was associated with three-fourths of the effect of feeling high on symptom relief, with somewhat smaller relative impacts on side effect reporting. Controlling for product characteristics and ingestion methods, including THC, only strengthened the association between the quantity of cannabis consumed and patient outcomes. Adding additional nuance to the relationship, the results showing patients were less likely to report high when vaporizing cannabis or smoking cannabis through a joint, regardless of THC levels, might also support a role for additional constituents in the psychological effects of cannabis consumption beyond THC and CBD (McPartland and Russo, 2012; Fischedick and Elzinga, 2015) as different ingestion methods are associated with different levels of bioavailability for THC (Spindle et al., 2019), CBD, and phytochemicals, such as terpenes (Hädener et al., 2019). Further supporting the role of additional constituents beyond THC, regressions by pre-app cannabis experience suggest that while individuals appear to develop tolerance to THC, other factors, e.g., vaping, become more important determinants of feeling high as experience increases. Placebo effects, arguably more likely among less experienced users, could also explain the closer tie between THC and feeling high among less experienced users. Future research clearly should consider the role of phytochemicals beyond THC and CBD, tolerance, and placebo effects in patient outcomes. Likewise, more research is needed on naturally occurring ratios of major cannabinoids such as THC and CBD, which tend to be expressed asynchronously and can have antithetical pharmacodynamics effects. Because CBD can act as both an inverse agonist (CB_2 receptors) and as a non-competitive negative allosteric modulator (CB_1 receptors; Laprairie et al., 2015; McPartland et al., 2015; Pertwee, 2008; Tham et al., 2019; Thomas et al., 2007), it is unclear whether hybridized flower strains and/or synthetic formulates with extracted THC and CBD (e.g., 1-to-1 cannabis products) are aggregating, moderating, or perhaps, *de facto* canceling out each other's effects.

Despite these important implications, the current dataset has fundamental limitations, particularly due to the lack of randomization of treatment interventions or inclusion of controlled placebo conditions, and the self-selection into app use, both in terms of opting into app use and with respect to recording sessions. For example, our sample is more likely to consist of individuals who anticipate some benefit from cannabis consumption and our sample likely does not include every time an app user consumed cannabis during our sample period. Selection bias could be associated with the possibility of both underestimation and overestimation of the association between reporting feeling high and reported symptom relief. Individuals who tend to feel high and experience significant symptom relief might be satisfied with their cannabis experience and chose not to opt into app use as might those who feel high from cannabis but do not experience symptom relief. Other limitations of the study include the absence of information on the patients' medical histories, concurrent medication and substance use, and the contexts and settings of cannabis usage. Finally, studies have shown that THC and CBD potency levels reported on product labels can be inaccurate (Vandrey et al., 2015; Bonn-Miller et al., 2017), suggesting the need for improvements in testing and regulatory oversight within the recreational and medical cannabis industries. More comprehensive testing will also enable identification of varying plant chemovars, consisting of unique volumes and ratios of terpenes and even minor cannabinoids, which may facilitate eventual identification of plant variants with reliable psychotropic and clinical effects.

In conclusion, this study finds a novel, positive link between feeling high and symptom relief, even after controlling for THC. However, the benefits in terms of increased symptom relief must be weighed against a statistically and clinically significant increase in negative side effects. Our results suggest a complex relationship between the characteristics of a specific *Cannabis* plant, the consumption process, and therapeutic outcomes. Future studies would benefit from measurement of the mental and physical effects of consuming other, non-cannabinoid phytochemicals that commonly develop in the *Cannabis* plant, such as terpenes, as well as how heat exposure (e.g., through temperature-controlled vaping) and pressure affect their bioavailability and pharmacodynamics. Until we better understand these factors, the medical cannabis available to patients largely will be limited to plant variants developed by for-profit firms that may or may not be formulated for optimal symptom management. Prices remain highly correlated with THC levels, one of the primary factors driving the experience of feeling high, suggesting that the private sector is developing products that make people feel high. Both clinically and policy relevant, the results of this study imply that, for many patients, medical benefits may be optimized by achieving the sensation of feeling high at the minimum necessary THC level. Unfortunately, without further research into the role of other phytochemicals in the plant on symptom management, using commercially available cannabis products to target specific symptoms or develop customized treatments likely will remain elusive.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Data are available subject to a data confidentiality agreement and licensing fees. Requests to access these datasets should be directed to https://releafapp.com/research/.

Ethics statement

The studies involving human participants were reviewed and approved by University of New Mexico Institutional Review Board.

Author contributions

JV, SS, and XL conceived the study. FB, KK, and BH independently designed and developed the ReleafApp™ and server infrastructure. XL conducted the analyses. JV, SS, and XL drafted the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

We thank all the donors to the University of New Mexico Medical Cannabis Research Fund for helping to make this research possible.

Conflict of interest

FB, KK, and BH were employed by MoreBetter, Ltd.

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.

Publisher's note

References

Aviram, J., Lewitus, G. M., Vysotski, Y., Yellin, B., Berman, P., Shapira, A., et al. (2021). Prolonged medical cannabis treatment is associated with quality of life improvement and reduction of analgesic medication consumption in chronic pain patients. *Front. Pharmacol.* 12, 613805–613814. doi:10.3389/fphar.2021.613805

Behr, A., and Johnen, L. (2009). Myrcene as a natural base chemical in sustainable chemistry: A critical review. *ChemSusChem* 2 (12), 1072–1095. doi:10.1002/cssc.200900186

Bie, B., Wu, J., Foss, J. F., and Naguib, M. (2018). An overview of the cannabinoid type 2 receptor system and its therapeutic potential. *Curr. Opin. Anaesthesiol.* 31 (4), 407–414. doi:10.1097/ACO.0000000000000616

Blessing, E. M., Steenkamp, M. M., Manzanares, J., and Marmar, C. R. (2015). Cannabidiol as a potential treatment for anxiety disorders. *Neurotherapeutics* 12 (4), 825–836. doi:10.1007/s13311-015-0387-1

Bonn-Miller, M. O., Loflin, M. J. E., Thomas, B. F., Marcu, J. P., Hyke, T., and Vandrey, R. (2017). Labeling accuracy of cannabidiol extracts sold online. *J. Am. Med. Assoc.* 318, 1708–1709. doi:10.1001/jama.2017.11909

Chihuri, S., and Li, G. (2020). Direct and indirect effects of marijuana use on the risk of fatal 2-vehicle crash initiation. *Inj. Epidemiol.* 7 (1), 49–10. doi:10.1186/s40621-020-00276-9

Clem, S. N., Bigand, T. L., and Wilson, M. (2020). Cannabis use motivations among adults prescribed opioids for pain versus opioid addiction. *Pain Manag. Nurs.* 21 (1), 43–47. doi:10.1016/j.pmn.2019.06.009

Curran, T., Devillez, H., YorkWilliams, S. L., and Bidwell, L. C. (2020). Acute effects of naturalistic THC vs. CBD use on recognition memory: A preliminary study. *J. Cannabis Res.* 2 (1), 28. doi:10.1186/s42238-020-00034-0

Cuttler, C., Lafrance, E. M., and Stueber, A. (2021). Acute effects of high - potency cannabis flower and cannabis concentrates on everyday life memory and decision making. *Sci. Rep.* 1, 13784. doi:10.1038/s41598-021-93198-5

Durisko, Z., Mulsant, B. H., McKenzie, K., and Andrews, P. W. (2016). Using evolutionary theory to guide mental health research. *Can. J. Psychiatry* 61 (3), 159–165. doi:10.1177/0706743716632517

Fakhoury, M. (2016). Could cannabidiol be used as an alternative to antipsychotics? *J. Psychiatric Res.* 80, 14–21. doi:10.1016/j.jpsychires.2016.05.013

Finlay, D. B., Sircombe, K. J., Nimick, M., Jones, C., and Glass, M. (2020). Terpenoids from cannabis do not mediate an entourage effect by acting at cannabinoid receptors. *Front. Pharmacol.* 11, 359. doi:10.3389/fphar.2020.00359

Fischedick, J, and Elzinga, S (2015). Cannabinoids and terpenes as chemotaxonomic markers in cannabis. *Nat. Prod. Chem. Res.* 03. doi:10.4172/2329-6836.1000181

Hädener, M., Vieten, S., Weinmann, W., and Mahler, H. (2019). A preliminary investigation of lung availability of cannabinoids by smoking marijuana or dabbing BHO and decarboxylation rate of THC- and CBD-acids. *Forensic Sci. Int.* 295, 207–212. doi:10.1016/j.forsciint.2018.12.021

Hartman, R. L., and Huestis, M. A. (2013). Cannabis effects on driving skills. *Clin. Chem.* 59 (3), 478–492. doi:10.1373/clinchem.2012.194381

Ito, K., and Ito, M. (2013). The sedative effect of inhaled terpinolene in mice and its structure-activity relationships. *J. Nat. Med.* 67 (4), 833–837. doi:10.1007/s11418-012-0732-1

Kedzior, K. K., and Martin-Iverson, M. T. (2006). Chronic cannabis use is associated with attention-modulated reduction in prepulse inhibition of the startle reflex in healthy humans. *J. Psychopharmacol.* 20 (4), 471–484. doi:10.1177/0269881105057516

Kedzior, K. K., Wehmann, E., and Martin-Iverson, M. (2016). Habituation of the startle reflex depends on attention in cannabis users. *BMC Psychol.* 4 (1), 50–11. doi:10.1186/s40359-016-0158-8

Kendall, D. A., and Yudowski, G. A. (2017). Cannabinoid receptors in the central nervous system: Their signaling and roles in disease. *Front. Cell. Neurosci.* 10, 294–310. doi:10.3389/fncel.2016.00294

Lake, S., Nosova, E., Buxton, J., Walsh, Z., Socías, M. E., Hayashi, K., et al. (2020). Characterizing motivations for cannabis use in a cohort of people who use illicit drugs: A latent class analysis. *PLoS ONE* 15 (5), 02334633–e233518. doi:10.1371/journal.pone.0233463

Laprairie, R. B, Bagher, A. M, Kelly, M. E. M, and Denovan-Wright, E. M (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br. J. Pharmacol.* 172, 4790–4805. doi:10.1111/bph.13250

Lundqvist, T. (2005). Cognitive consequences of cannabis use: Comparison with abuse of stimulants and heroin with regard to attention, memory and executive functions. *Pharmacol. Biochem. Behav.* 81 (2), 319–330. doi:10.1016/j.pbb.2005.02.017

McPartland, J. M, Duncan, M, Di Marzo, V, and Pertwee, R. G (2015). Are cannabidiol and Δ9-tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. *Br. J. Pharmacol.* 172 (3), 737–753. doi:10.1111/bph.12944

McPartland, J. M., and Russo, E. B. (2012). "Cannabis and Cannabis extracts: Greater than the sum of their parts?," in *Cannabis therapeutics in HIV/AIDS*. doi:10.1300/J175v01n03_08

National Academies of Sciences, Engineering, and Medicine (2017). "The health effects of cannabis and cannabinoids: The current state of evidence and recommendations for research," in *The health effects of cannabis and cannabinoids*. doi:10.17226/24625

National Institute on Drug Abuse (2021). *Marijuana drug facts*. Retrieved from https://www.drugabuse.gov/publications/drugfacts/marijuana.

Pacula, R. L., Jacobson, M., and Maksabedian, E. J. (2016). In the weeds: A baseline view of cannabis use among legalizing states and their neighbours. *Addiction* 111 (6), 973–980. doi:10.1111/add.13282

Pertwee, R. G. (2006). Cannabinoid pharmacology: The first 66 years. *Br. J. Pharmacol.* 147 (Suppl. 1), S163–S171. doi:10.1038/sj.bjp.0706406

Pertwee, R. G. (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *Br. J. Pharmacol.* 153, 199, 215. doi:10.1038/sj.bjp.0707442

Preuss, U. W., Huestis, M. A., Schneider, M., Hermann, D., Lutz, B., Hasan, A., et al. (2021). Cannabis use and car crashes: A review. *Front. Psychiatry* 12, 643315–643411. doi:10.3389/fpsyt.2021.643315

Russo, E. B. (2007). History of cannabis and its preparations in saga, science, and sobriquet. *Chem. Biodivers.* 4, 1614–1648. doi:10.1002/cbdv.200790144

Santiago, M., Sachdev, S., Arnold, J. C., McGregor, I. S., and Connor, M. (2019). Absence of entourage: Terpenoids commonly found in*Cannabis sativa* do not modulate the functional activity of Δ^9-THC at human CB_1 and CB_2 receptors. *Cannabis cannabinoid Res.* 4 (3), 165–176. doi:10.1089/can.2019.0016

Schlienz, N. J., Scalsky, R., Martin, E. L., Jackson, H., Munson, J., Strickland, J. C., et al. (2020). A cross-sectional and prospective comparison of medicinal cannabis users and controls on self-reported health. *Cannabis Cannabinoid Res.* X (X), 548–558. doi:10.1089/can.2019.0096

Seltzer, E. S., Watters, A. K., Jr, D. M., and Granat, L. M. (2020). Cannabidiol (CBD) as a promising anti-cancer drug. *Cancers(Basel)* 12 (11), 3203. doi:10.3390/cancers12113203

Spindle, T. R., Cone, E. J., Schlienz, N. J., Mitchell, J. M., Bigelow, G. E., Flegel, R., et al. (2019). Acute pharmacokinetic profile of smoked and vaporized cannabis in human blood and oral fluid. *J. Anal. Toxicol.* 43 (4), 233–258. doi:10.1093/jat/bky104

Stith, S. S., Vigil, J. M., Brockelman, F., Keeling, K., and Hall, B. (2018). Patient-reported symptom relief following medical cannabis consumption. *Front. Pharmacol.* 9, 916. doi:10.3389/fphar.2018.00916

Stith, S. S., Vigil, J. M., Brockelman, F., Keeling, K., and Hall, B. (2019). The association between cannabis product characteristics and symptom relief. *Sci. Rep.* 9 (1), 2712–2718. doi:10.1038/s41598-019-39462-1

Surendran, S., Qassadi, F., Surendran, G., Lilley, D., and Heinrich, M. (2021). Myrcene—what are the potential health benefits of this flavouring and aroma agent? *Front. Nutr.* 8, 699666–699714. doi:10.3389/fnut.2021.699666

Tham, M, Yilmaz, O., Alaverdashvili, M., Kelly, M. E. M., Denovan-Wright, E. M., and Laprairie, R. B. (2019). Allosteric and orthosteric pharmacology of cannabidiol and cannabidiol-dimethylheptyl at the type 1 and type 2 cannabinoid receptors. *Br. J. Pharmacol.* 176, 1455–1469. doi:10.1111/bph.14440

Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., and Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB 1 and CB 2 receptor agonists *in vitro*. *Br. J. Pharmacol.* 150 (5), 613–623. doi:10.1038/sj.bjp.0707133

U. S. Pain Foundation (2021). Looking to try CBD? Read this first. Retrieved from: https://uspainfoundation.org/blog/looking-to-try-cbd-read-this-first/

Vandrey, R., Raber, J. C., Raber, M. E., Douglass, B., Miller, C., and Bonn-Miller, M. O. (2015). Cannabinoid dose and label accuracy in edible medical cannabis products. *JAMA - J. Am. Med. Assoc.* 313, 2491–2493. doi:10.1001/jama.2015.6613

Vigil, J. M. (2009). A socio-relational framework of sex differences in the expression of emotion. *Behav. Brain Sci.* 32, 375–390. doi:10.1017/S0140525X09991075

Vigil, J. M., Montera, M. A., Pentkowski, N. S., Diviant, J. P., Orozco, J., Ortiz, A. L., et al. (2020). The therapeutic effectiveness of full spectrum hemp oil using a chronic neuropathic pain model. *Life* 10, 69. doi:10.3390/life10050069

Vigil, J. M., Stith, S. S., Brockelman, F., Keeling, K., and Hall, B. (2023). Systematic combinations of major cannabinoid and terpene contents in cannabis flower and patient outcomes: A proof-of-concept assessment of the Vigil index of cannabis chemovars. *J. Cannabis Res.* 5 (1), 4. doi:10.1186/s42238-022-00170-9

Vigil, J. M., and Stregnth, C. (2014). No pain, no social gains: A social-signaling perspective of human pain behaviors. *World J. Anesthesiol.* 4 (3), 18–30. doi:10.5313/wja.v3.i1.18

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 06 June 2023
DOI 10.3389/fphar.2023.1142680

OPEN ACCESS

EDITED BY
Francesca Baratta,
University of Turin, Italy

REVIEWED BY
Patricia Di Ciano,
University of Toronto, Canada
Janet Schloss,
Southern Cross University, Australia
Chun Guang Li,
Western Sydney University, Australia

*CORRESPONDENCE
Iain S. McGregor,
iain.mcgregor@sydney.edu.au

RECEIVED 12 January 2023
ACCEPTED 09 May 2023
PUBLISHED 06 June 2023

CITATION
Cairns EA, Benson MJ, Bedoya-Pérez MA, Macphail SL, Mohan A, Cohen R, Sachdev PS and McGregor IS (2023), Medicinal cannabis for psychiatry-related conditions: an overview of current Australian prescribing.
Front. Pharmacol. 14:1142680.
doi: 10.3389/fphar.2023.1142680

Medicinal cannabis for psychiatry-related conditions: an overview of current Australian prescribing

Elizabeth A. Cairns[1,2,3], Melissa J. Benson[1,3,4], Miguel A. Bedoya-Pérez[1,3,4], Sara L. Macphail[1,3,4], Adith Mohan[5,6], Rhys Cohen[1], Perminder S. Sachdev[5,6] and Iain S. McGregor[1,3,4]*

[1]The Lambert Initiative for Cannabinoid Therapeutics, The University of Sydney, Sydney, NSW, Australia, [2]Faculty of Medicine and Health, School of Pharmacy, The University of Sydney, Sydney, NSW, Australia, [3]Brain and Mind Centre, The University of Sydney, Camperdown, NSW, Australia, [4]Faculty of Science, School of Psychology, The University of Sydney, Sydney, NSW, Australia, [5]Centre for Healthy Brain Ageing (CHeBA), Discipline of Psychiatry and Mental Health, Faculty of Medicine and Health, University of New South Wales, Sydney, NSW, Australia, [6]Neuropsychiatric Institute, The Prince of Wales Hospital, Randwick, NSW, Australia

Objective: Evidence is accumulating that components of the *Cannabis sativa* plant may have therapeutic potential in treating psychiatric disorders. Medicinal cannabis (MC) products are legally available for prescription in Australia, primarily through the Therapeutic Goods Administration (TGA) Special Access Scheme B (SAS-B). Here we investigated recent prescribing practices for psychiatric indications under SAS-B by Australian doctors.

Methods: The dataset, obtained from the TGA, included information on MC applications made by doctors through the SAS-B process between 1st November 2016 and 30th September 2022 inclusive. Details included the primary conditions treated, patient demographics, prescriber location, product type (e.g., oil, flower or capsule) and the general cannabinoid content of products. The conditions treated were categorized according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition, text revision (DSM-5-TR). Trends in prescribing for conditions over time were analyzed via polynomial regression, and relationships between categorical variables determined via correspondence analyses.

Results: Approximately 300,000 SAS-B approvals to prescribe MC had been issued in the time period under investigation. This included approvals for 38 different DSM-5-TR defined psychiatric conditions (33.9% of total approvals). The majority of approvals were for anxiety disorders (66.7% of psychiatry-related prescribing), sleep-wake disorders (18.2%), trauma- and stressor-related disorders (5.8%), and neurodevelopmental disorders (4.4%). Oil products were most prescribed (53.0%), followed by flower (31.2%) and other inhaled products (12.4%). CBD-dominant products comprised around 20% of total prescribing and were particularly prevalent in the treatment of autism spectrum disorder. The largest proportion of approvals was for patients aged 25–39 years (46.2% of approvals). Recent dramatic increases in prescribing for attention deficit hyperactivity disorder were identified.

Conclusion: A significant proportion of MC prescribing in Australia is for psychiatry-related indications. This prescribing often appears somewhat

"experimental", given it involves conditions (e.g., ADHD, depression) for which definitive clinical evidence of MC efficacy is lacking. The high prevalence of THC-containing products being prescribed is of possible concern given the psychiatric problems associated with this drug. Evidence-based clinical guidance around the use of MC products in psychiatry is lacking and would clearly be of benefit to prescribers.

KEYWORDS

medicinal cannabis, Australia, psychiatry, anxiety disorders, prescribing, medicinal cannabis use

Introduction

Cannabis is a drug that has had a somewhat troubled relationship with psychiatry. The classic writings of Moreau de Tours described how hashish can precipitate an acute psychotic state (Abel, 2005), and numerous subsequent studies have probed the complex relationship between chronic cannabis use and schizophrenia (Arseneault et al., 2002; D'Souza et al., 2022; Hill, 2015; National Academies of Sciences, 2017; Pasman et al., 2018). Recent analyses suggest that high frequency use of more potent cannabis may be a risk factor for schizophrenia, although the debate continues (Colizzi et al., 2020; D'Souza et al., 2022; Di Forti et al., 2019). A modest non-causal association between cannabis use and depression is also widely proposed (Gorfinkel et al., 2020; Hodgson et al., 2020; Onaemo et al., 2021). Delayed initiation of cannabis use by adolescents most likely benefits their mental health, although claims of cannabis use causing irreversible adolescent brain damage have been largely debunked, though some uncertainty remains (DeLisi, 2008; Weiland et al., 2015; Fischer et al., 2022).

Sitting somewhat uncomfortably against this backdrop is the growing access to cannabis for medicinal purposes across many jurisdictions. Medicinal cannabis (MC) in Australia became legally available to prescribe in November 2016, enabling patient access to quality standardized medicinal cannabis products, even though they fall outside the "Australian Register of Therapeutic Goods". Access is regulated by the Therapeutic Goods Administration (TGA), with the main access mechanism known as the Special Access Scheme B (SAS-B), whereby healthcare practitioners apply to the TGA to prescribe a specific type of product to an individual patient with a specific indication (reviewed in MacPhail et al., 2022).

Patient access to medical cannabis in Australia was initially very slow (MacPhail et al., 2022), due to a cumbersome application processes and high cost of products on offer (Lintzeris et al., 2022). An additional problem was that medical professionals felt relatively uneducated about medicinal cannabis products, regulatory frameworks, and therapeutic value despite ever-increasing patient interest (Karanges et al., 2018; Benson et al., 2020; Bawa et al., 2022). The past 2 years, however, has seen a dramatic rise in prescribing due to streamlined application processes, improved doctor education and a rise in cannabis-access clinics that specialize in MC prescribing (Karanges et al., 2018; Benson et al., 2020; Bawa et al., 2022). Accordingly, at the time of writing, the TGA has now issued more than 360,000 approvals for medicinal cannabis access in Australia through the SAS-B scheme. An increasing number of prescriptions are also now being made under the "Authorised Prescriber" scheme which provides a blanket approval for a healthcare practitioner to prescribe products to patients with a specific indication (Therapeutic Goods Administration, 2022a).

There are now more than 360 distinct medicinal cannabis products currently accessible to patients involving many different formulations, routes of administration, and cannabinoid profiles (Therapeutic Goods Administration, 2022b). The majority are oral formulations (oils, sprays, capsules) although there has been a recent surge in the use of plant cannabis products (also known as "flower" or "flos") (MacPhail et al., 2022). The TGA identifies five different categories of product according to their Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) content, with around one third of available products primarily containing CBD (Therapeutic Goods Administration, 2022b).

The optimal clinical use of different products and cannabinoid profiles across different conditions is still uncertain. Recent analyses lend some support to the use of THC in treating chronic pain, multiple sclerosis spasticity, anorexia/cachexia and Tourette syndrome (National Academies of Sciences, 2017; Therapeutic Goods Administration, 2019) while evidence supports CBD efficacy in the treatment of epilepsy (Devinsky et al., 2017; Devinsky et al., 2018). CBD may attenuate some of the intoxicating and other adverse psychological effects of THC, although the evidence for this is mixed (Arkell et al., 2019; Freeman et al., 2019; Englund et al., 2022; Hutten et al., 2022; Zamarripa et al., 2023).

CBD has generated some excitement in neurology and psychiatry with the proprietary oil-based CBD formulation "Epidyolex" now an FDA- and TGA-approved medicine for the treatment of specific intractable childhood epilepsies (Pauli et al., 2020). With antiepileptic drugs often successfully co-opted as psychiatric medications, it is perhaps not surprising to learn that CBD given either alone (Leweke et al., 2012) or as an adjunct to standard antipsychotic therapy (McGuire et al., 2018) shows some promise in the treatment of psychosis. Observational studies of patients, as well as open-label trials and small randomized-controlled trials (RCTs), indicate additional promise for CBD in the treatment of anxiety disorders (Masataka, 2019; Appiah-Kusi et al., 2020; Gulbransen et al., 2020; Berger, Amminger, et al., 2022). Preclinical evidence has suggested that CBD may curb addictions, with notable effects in animal models of methamphetamine and alcohol self-administration (Hay et al., 2018; Turna et al., 2019). These effects are currently being translated into clinical trials (e.g., NCT03248167, NCT03252756), with some recent findings suggesting beneficial utility in related substance abuse - cannabis-use disorder (Freeman et al., 2020).

Overall, despite some promise, the conclusions of recent systematic reviews are cautious around the use of cannabis-based medicines in psychiatric disorders citing the poor quality and patchy outcomes underpinning current evidence (Black et al., 2019; Bonaccorso et al., 2019; Hoch et al., 2019; Bahji et al., 2020; Botsford et al., 2020; Khan et al., 2020; Sarris et al., 2020; Kloiber et al., 2021; Stanciu et al., 2021). Establishing the therapeutic potential of cannabis-based medicines in psychiatry, therefore, remains a work in progress.

With this in mind, the current study involved analysis of the recent patterns of prescribing medicinal cannabis within Australia as it pertains to psychiatry-related conditions. Through information available through the TGA on SAS-B approvals, we examined the extent to which these products are being accessed via current schemes for psychiatry-related conditions, and relevant patient demographics and product characteristics.

Materials and methods

TGA approvals dataset

Anonymous de-identified data were obtained from the TGA through a Freedom of Information (FOI) request, informed by previous datasets (FOI 2013, 2250, 2274, 2370, 2419, 3653). Data were released from the TGA on 18th November 2022, and provided information around all SAS-B applications submitted by clinicians between 1st January 2016 and 30th September 2022 (n = 297,409). Applications "awaiting decision", "cancelled/withdrawn", or "rejected" were not included in the analyses (n = 4,662) or those with applications dated prior to November 2016 (n = 13).

Data preparation

Data were received in a Microsoft Excel file. As in our previous analysis of SAS-B prescribing trends (MacPhail et al., 2022), the indication noted by clinicians in their SAS-B applications were not systematic and so required recoding. Indications were first coded according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10; WHO, version 2019—English). Where required, ambiguous indications were assigned the nearest possible indication. They were then further categorized according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition, Text Revision (DSM-5-TR), and verified by two independent practicing psychiatrists (Supplementary Table S1).

Products were grouped into 11 types (capsules, extracts, crystal, flower, inhaled, lozenge, oil, spray, tablet, topical, wafer; Supplementary Table S2). The dataset contained little information that would allow an accurate analysis of the dose and/or specific medicinal cannabis product being used other than whether the product fell within Schedule 4 (≥98% CBD of total cannabinoid content) or Schedule 8 (containing ≥2% THC of total cannabinoid content) as specified by the Standard for the Uniform Scheduling of Medicines and Poisons (SUSMP). Prescriber specialty was not clear in the dataset: prior to November 2021 prescribers could volunteer their specialty as part of the application process but were not obliged to do so.

Data on patient ages were collected and were grouped for the analysis according to stratifications from the Australian Bureau of Statistics, with an additional separation of ages 10–24 to distinguish those below 18 years of age. Population data were also obtained from this source (Australian Bureau of Statistics, 2022).

Statistical analysis

Data were analyzed with general descriptive analyses and, where described, best fit using non-linear regression models and correspondence analyses, as previously reported (MacPhail et al., 2022). Prior to non-linear regression analyses, data were processed using "tidyverse" (Wickham et al., 2019), "padr" (Thoen, 2020) and "dplyr" (Wickham et al., 2021) packages. Non-linear regressions were performed using "MASS" (Venables & Ripley, 2002), plotted with "ggplot" (Wickham, 2016), "cowplot" (Wilke, 2020) and "ggpubr" (Kassambara, 2020). Appropriate error distribution for each regression fit (i.e., Poisson or Negative binomial) was determined via Residuals plots and Pearson's dispersion test. Fits of 1st, 2nd, 3rd, and 4th-degree polynomials were assessed via stepwise comparison of the Corrected Akaike Information Criterion (AICc) using "MuMIn" (Bartoń, 2020). Δm was calculated between models, excluding models with Δm >2 as having substantially less support (Burnham & Anderson, 2002). To estimate the goodness of fit, R^2 was calculated for each of the best-fitted regressions by the equation: 1—deviance/residual deviance, and classified according to Moore and Kirkland (2013). Averages are listed as means ± standard error unless otherwise specified.

Associations between variables were investigated by constructing a contingency table and performing a correspondence analysis using the "Factoshiny" package (Vaissie et al., 2021). Statistics that deviated from the expected values of independence were reported in the text. Chi-squared tests were used on nominal variables through the package "stats" (R Core Team, 2022), and asymptotic linear-by-linear association tests were used on ordinal variables (i.e., age groups) through the package "coin" (Hothorn et al., 2006). This analysis is used to provide insight into overall differentiation of variables (distance from origin), similarity between variables of the same type (e.g., between two different product types; proximity), and association between variables of different types (e.g., between an indication and product; angle between the vectors connecting variables to the origin).

Results

Overall trends

The TGA approved 297,409 SAS-B applications for medicinal cannabis between November 2019 and September 2022 (Table 1).

TABLE 1 Overview of SAS-B approvals for psychiatric and non-DSM indications by sex and age.

	Sex[a]			Age						
	Total (%[b])	Female (%[c])	Male (%[c])	0–9 (%[d])	10–17 (%[d])	18–24 (%[d])	25–39 (%[d])	40–54 (%[d])	55–74 (%[d])	>74 (%[d])
Non-DSM	196,743 (66.2)	88,828 (45.1)	107,336 (54.6)	733 (0.4)	924 (0.5)	6,501 (3.3)	49,200 (25.0)	57,942 (29.5)	58,537 (29.8)	22,896 (11.6)
Anxiety Disorders	67,133 (22.6)	23,911 (35.6)	42,964 (64)	165 (0.2)	741 (1.1)	8,123 (12.1)	33,116 (49.3)	17,063 (25.4)	6,782 (10.1)	1,141 (1.7)
Anxiety	67,095 (22.6)	23,894 (35.6)	42,943 (64.0)	165 (0.2)	741 (1.1)	8,117 (12.1)	33,101 (49.3)	17,051 (25.4)	6,778 (10.1)	1,140 (1.7)
GAD	19 (0.0)	10 (52.6)	9 (47.4)	0 (0)	0 (0)	3 (15.8)	6 (31.6)	6 (31.6)	4 (21.1)	0 (0)
Panic disorder	10 (0.0)	5 (50.0)	5 (50.0)	0 (0)	0 (0)	2 (20.0)	4 (40.0)	3 (30.0)	0 (0)	1 (10.0)
SAD	9 (0.0)	2 (22.2)	7 (77.8)	0 (0)	0 (0)	1 (11.1)	5 (55.6)	3 (33.3)	0 (0)	0 (0)
Sleep-Wake Disorders	18,321 (6.2)	5,905 (32.2)	12,379 (67.6)	27 (0.1)	86 (0.5)	1,528 (8.3)	7,429 (40.5)	5,481 (29.9)	3,275 (17.9)	491 (2.7)
Sleep disorder	11,202 (3.8)	3,495 (31.2)	7,698 (68.7)	14 (0.1)	47 (0.4)	1,031 (9.2)	4,814 (43.0)	3,263 (29.1)	1,790 (16.0)	240 (2.1)
Insomnia	6,877 (2.3)	2,308 (33.6)	4,544 (66.1)	13 (0.2)	37 (0.5)	491 (7.1)	2,591 (37.7)	2,166 (31.5)	1,365 (19.8)	213 (3.1)
RLS	239 (0.1)	101 (42.3)	135 (56.5)	0 (0)	2 (0.8)	6 (2.5)	23 (9.6)	51 (21.3)	119 (49.8)	38 (15.9)
Narcolepsy	1 (0.0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Hypersomnia	1 (0.0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Parasomnia	1 (0.0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Trauma- and Stressor-Related Disorders										
PTSD	5,799 (1.9)	2,216 (38.2)	3,546 (61.1)	0 (0)	18 (0.3)	419 (7.2)	2,372 (40.9)	2,141 (36.9)	799 (13.8)	50 (0.9)
Neurodevelopmental Disorders	4,450 (1.5)	961 (21.6)	3,455 (77.6)	550 (12.4)	1,138 (25.6)	807 (18.1)	1,550 (34.8)	330 (7.4)	67 (1.5)	7 (0.2)
ASD	2,206 (0.7)	522 (23.7)	1,667 (75.6)	480 (21.8)	931 (42.2)	389 (17.6)	330 (15.0)	62 (2.8)	13 (0.6)	1 (0.0)
ADHD	2,078 (0.7)	392 (18.9)	1,672 (80.5)	66 (3.2)	169 (8.1)	384 (18.5)	1,170 (56.3)	247 (11.9)	38 (1.8)	3 (0.1)
Tourette's syndrome	163 (0.1)	47 (28.8)	113 (69.3)	1 (0.6)	38 (23.3)	34 (20.9)	50 (30.7)	21 (12.9)	16 (9.8)	3 (1.8)
Intellectual impairment	3 (0.0)	0 (0)	3 (100)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Depressive Disorders	4,003 (1.3)	1,353 (33.8)	2,639 (65.9)	4 (0.1)	20 (0.5)	483 (12.1)	1,785 (44.6)	1,121 (28)	529 (13.2)	59 (1.5)
Depression	3,247 (1.1)	1,103 (34)	2,134 (65.7)	0 (0)	17 (0.5)	390 (12)	1,474 (45.4)	884 (27.2)	429 (13.2)	51 (1.6)
Mood disorder	736 (0.2)	243 (33)	492 (66.8)	4 (0.5)	3 (0.4)	90 (12.2)	307 (41.7)	230 (31.3)	94 (12.8)	8 (1.1)
Major depression	15 (0.0)	2 (13.3)	13 (86.7)	0 (0)	0 (0)	1 (6.7)	2 (13.3)	6 (40.0)	6 (40.0)	0 (0)
PDD	5 (0.0)	5 (100)	0 (0)	0 (0)	0 (0)	2 (40.0)	2 (40.0)	1 (20.0)	0 (0)	0 (0)
Neurocognitive Disorders	428 (0.1)	252 (58.9)	176 (41.1)	0 (0)	1 (0.2)	4 (0.9)	10 (2.3)	26 (6.1)	112 (26.2)	275 (64.3)
Alzheimer's disease	272 (0.1)	166 (61.0)	106 (39.0)	0 (0)	0 (0)	3 (1.1)	9 (3.3)	8 (2.9)	69 (25.4)	183 (67.3)
Unspecified dementia	132 (0.0)	75 (56.8)	57 (43.2)	0 (0)	1 (0.8)	1 (0.8)	0 (0)	4 (3.0)	36 (27.3)	90 (68.2)
Huntington chorea	22 (0)	9 (40.9)	13 (59.1)	0 (0)	0 (0)	0 (0)	1 (4.5)	14 (63.6)	6 (27.3)	1 (4.5)
Memory loss	1 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Cognitive decline	1 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Bipolar and Related Disorders										
Bipolar disorder	212 (0.1)	83 (39.2)	127 (59.9)	0 (0)	1 (0)	17 (8)	98 (46)	79 (37)	17 (8)	0 (0)
Disruptive, Impulse-Control, and Conduct Disorders	155 (0.1)	53 (34.2)	100 (64.5)	13 (8.0)	34 (22.0)	23 (15.0)	36 (23.0)	14 (9.0)	14 (9.0)	21 (14.0)
Behavior disorder	131 (0.0)	45 (34.4)	84 (64.1)	12 (9.0)	30 (23.0)	23 (18.0)	34 (26.0)	12 (9.0)	10 (8.0)	10 (8.0)

(Continued on following page)

TABLE 1 (*Continued*) Overview of SAS-B approvals for psychiatric and non-DSM indications by sex and age.

	Sex[a]			Age						
	Total (%[b])	Female (%[c])	Male (%[c])	0–9 (%[d])	10–17 (%[d])	18–24 (%[d])	25–39 (%[d])	40–54 (%[d])	55–74 (%[d])	>74 (%[d])
Aggressive behavior	13 (0.0)	5 (38.5)	8 (61.5)	1 (8.0)	4 (31.0)	0 (0)	0 (0)	0 (0)	3 (23.0)	5 (38.0)
Agitation	11 (0.0)	3 (27.3)	8 (72.7)	0 (0)	0 (0)	0 (0)	2 (18.0)	2 (18.0)	1 (9.0)	6 (55.0)
Substance-Related and Addictive Disorders	126 (0.0)	20 (15.9)	106 (84.1)	0 (0)	0 (0)	21 (17.0)	63 (50.0)	26 (21.0)	16 (13.0)	0 (0)
Cannabis use disorder	117 (0.0)	17 (14.5)	100 (85.5)	0 (0)	0 (0)	21 (18.0)	59 (50.0)	22 (19.0)	15 (13.0)	0 (0)
Unspecified addiction	6 (0.0)	3 (50.0)	3 (50.0)	0 (0)	0 (0)	0 (0)	3 (50.0)	3 (50.0)	0 (0)	0 (0)
Alcohol dependence	2 (0.0)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	1 (50.0)	1 (50.0)	0 (0)	0 (0)
Tobacco use disorder	1 (0.0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Schizophrenia Spectrum and Other Psychotic Disorders										
Schizophrenia	31 (0.0)	3 (9.7)	28 (90.3)	0 (0)	0 (0)	3 (10.0)	17 (55.0)	9 (29.0)	2 (6.0)	0 (0)
Obsessive-Compulsive and Related disorders										
OCD	4 (0.0)	1 (25.0)	3 (75.0)	0 (0)	0 (0)	1 (25.0)	1 (25.0)	1 (25.0)	1 (25.0)	0 (0)
Somatic Symptom and Related Disorders	2 (0.0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50.0)	1 (50.0)	0 (0)	0 (0)
Bruxism	1 (0.0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Psychogenic seizures	1 (0.0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Medication-Induced Movement Disorders and Other Adverse Effects of Medication	2 (0.0)	1 (50.0)	1 (50.0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
Extrapyramidal symptoms	1 (0.0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Tardive dyskinesia	1 (0.0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Total	**297,409 (100)**	**123,589 (42.0)**	**172,860 (58.1)**	**1,492 (0.5)**	**2,963 (1.0)**	**17,930 (6.0)**	**95,678 (32.2)**	**84,236 (28.3)**	**70,151 (23.6)**	**24,940 (8.4)**

[a]Sex indeterminate/intersex/unspecified data not shown (n = 960 or 0.4%; 381 for psychiatric indications).
[b]Percentage of prescribing over all indications (including non-DSM, indications).
[c]Percentage of prescribing in each indication by sex.
[d]Percentage prescribing in each indication by age group (age unknown not shown, n = 19). ADHD: attention deficit hyperactivity disorder; ASD: autism spectrum disorder; GAD: generalized anxiety disorder; OCD: Obsessive-compulsive disorder; PDD: premenstrual dysphoric disorder; PTSD: Post-traumatic stress disorder; RLS: restless legs syndrome; SAD: social anxiety disorder.

Psychiatric indications represented 33.9% (n = 100,666) of total approvals and included two out of the top three indications in the dataset [pain, n = 164,055 (55.2% of total prescribing); anxiety, n = 67,095 (22.6%); and sleep disorders, n = 11,202 (3.8%)].

Approvals for psychiatric indications covered thirteen general DSM-5-TR categories: anxiety disorders (n = 67,133; 22.6% of total prescribing); sleep-wake disorders (n = 18,321; 6.2%); trauma and stressor-related disorders (n = 5,799; 1.9%); neurodevelopmental disorders (n = 4,450; 1.5%); depressive disorders (n = 4,003; 1.3%); neurocognitive disorders (n = 428, 0.1%); bipolar and related disorders (n = 212; 0.1%); disruptive, impulse-control, and conduct disorders (n = 155; 0.1%); substance-related and addictive disorders (n = 126; <0.1%); schizophrenia spectrum and other psychotic disorders (n = 31; <0.1%); obsessive-compulsive and related disorders (n = 4; <0.1%); somatic symptom and related disorders (n = 2; <0.1%); and medication-induced movement disorders and other adverse effects of medication (n = 2; <0.1%).

Within the thirteen general DSM-5-TR categories, approvals for 38 specific psychiatric indications were identified, 12 of which accrued more than 200 approvals (Table 1).

Patient demographics

A larger proportion of approved applications for psychiatric indications were for males (65.1%) compared to females (34.5%; Table 1). A total of 381 applications (0.4%) that had no sex listed or were indeterminant or intersex. Approvals for psychiatric indications were

TABLE 2 SAS-B approvals by product schedule and indication. The number of approvals by SUSMP Schedule (S4 or S8) showing percentage split between S4 and S8 for each indication or indication group in brackets.

	S4 (%)	S8 (%)
Non-DSM	44,441 (22.6)	152,302 (77.4)
Anxiety Disorders	14,508 (21.6)	52,625 (78.4)
Anxiety	14,503 (21.6)	52,592 (78.4)
GAD	0 (0)	19 (100)
Panic Disorder	5 (50.0)	5 (50.0)
SAD	0 (0)	9 (100)
Sleep-Wake Disorders	2,630 (14.4)	15,691 (85.6)
Sleep disorder	1,610 (14.4)	9,592 (85.6)
Insomnia	923 (13.4)	5,954 (86.6)
RLS	95 (39.7)	144 (60.3)
Narcolepsy	0 (0)	1 (100)
Hypersomnia	1 (100)	0 (0)
Parasomnia	1 (100)	0 (0)
Trauma- and Stressor-Related Disorders		
PTSD	874 (15.1)	4,925 (84.9)
Neurodevelopmental Disorders	1,620 (36.4)	2,830 (63.6)
ASD	1,243 (56.3)	963 (43.7)
ADHD	330 (15.9)	1,748 (84.1)
Tourette's syndrome	44 (27.0)	119 (73.0)
Intellectual impairment	3 (100)	0 (0)
Depressive Disorders	509 (12.7)	3,494 (87.3)
Depression	447 (13.8)	2,800 (86.2)
Mood disorder	58 (7.9)	678 (92.1)
Major depression	0 (0)	15 (100)
PDD	4 (80.0)	1 (20)
Neurocognitive Disorders	119 (27.8)	309 (72.2)
Alzheimer's disease	83 (30.5)	189 (69.5)
Unspecified dementia	36 (27.3)	96 (72.7)
Huntington chorea	0 (0)	22 (100)
Memory loss	0 (0)	1 (100)
Cognitive decline	0 (0)	1 (100)
Bipolar and Related Disorders		
Bipolar disorder	35 (16.5)	177 (83.5)
Disruptive, Impulse-Control, and Conduct Disorders	59 (38.1)	96 (61.9)
Behavior disorder	56 (42.7)	75 (57.3)
Aggressive behavior	0 (0)	13 (100)
Agitation	3 (27.3)	8 (72.7)

(Continued on following page)

TABLE 2 (*Continued*) SAS-B approvals by product schedule and indication. The number of approvals by SUSMP Schedule (S4 or S8) showing percentage split between S4 and S8 for each indication or indication group in brackets.

	S4 (%)	S8 (%)
Substance-Related and Addictive Disorders	8 (6.3)	118 (93.7)
Cannabis use disorder	7 (6.0)	110 (94.0)
Unspecified addiction	1 (16.7)	5 (83.3)
Alcohol dependence	0 (0)	2 (100)
Tobacco use disorder	0 (0)	1 (100)
Schizophrenia Spectrum and Other Psychotic Disorders		
Schizophrenia	22 (71.0)	9 (29.0)
Obsessive-Compulsive and Related disorders		
Obsessive-compulsive disorder	1 (25.0)	3 (75.0)
Somatic Symptom and Related Disorders	1 (50.0)	1 (50.0)
Bruxism	1 (100)	0 (0)
Psychogenic seizures	0 (0)	1 (100)
Medication-Induced Movement Disorders and Other Adverse Effects of Medication	1 (50.0)	1 (50.0)
Extrapyramidal symptoms	0 (0)	1 (100)
Tardive dyskinesia	1 (100)	0 (0)
Total	**64,828 (21.8)**	**232,581 (78.2)**

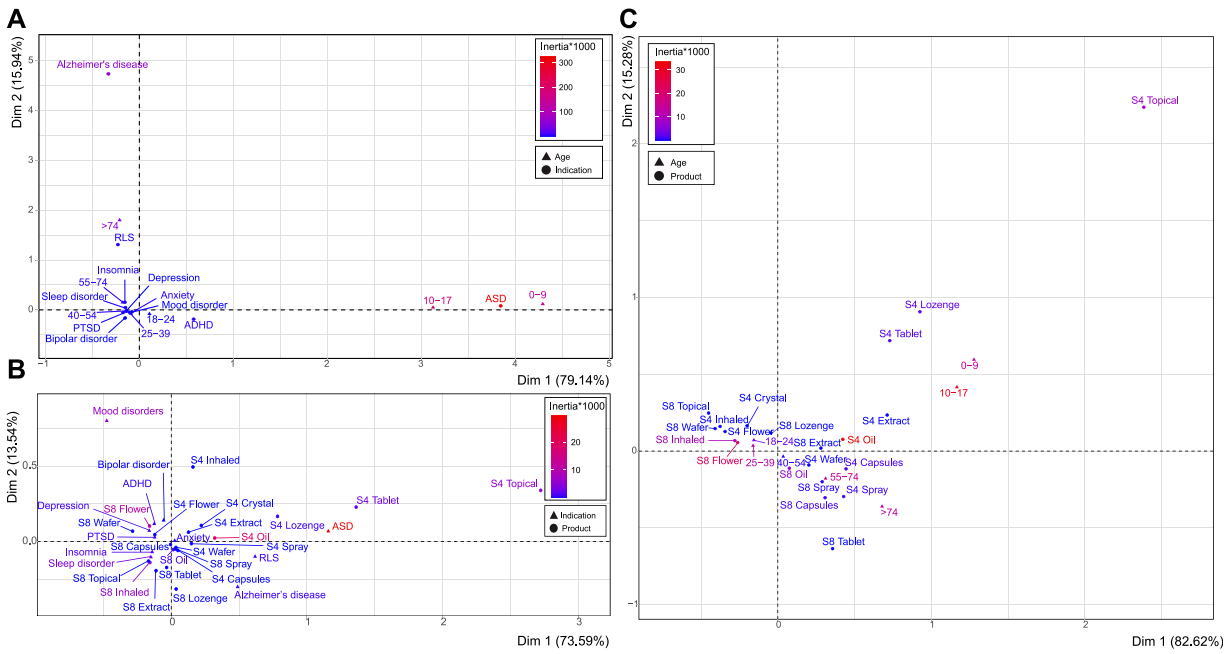

FIGURE 1
Associations between age, product schedule and type, and indication. Correspondence analyses between age and indication **(A)**, indication and product schedule and type **(B)**, and age and product schedule and type **(C)**. Deviation from independence described by the dimensions on each axis (Dim 1 and Dim 2), with the scaled contribution to the overall variance depicted by the inertia*1,000 (red to blue color gradient). See Supplementary Table S6 for related statistics.

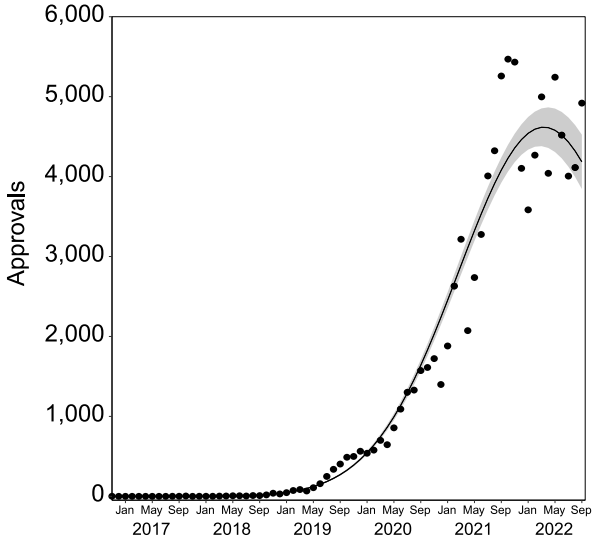

FIGURE 2
Number of SAS-B approvals per month for psychiatric indications from November 2016 to September 2022 (n = 100,666). The solid line represents the best fit, with shading depicting standard error of the mean (SEM).

primarily for younger patients, with approvals for patients <40 years representing 60.4% of total approvals. The largest proportion of approvals was for patients 25–39 years (46.2% of total psychiatry-related approvals). When normalized by overall population, this group also had the largest *per capita* prescribing for psychiatric indications (851 approvals per 100,000; Supplementary Table S3). By contrast, this age group represented only 25.0% of the total for non-psychiatric approvals. Prescribing for 25–39 year olds also represented the largest proportion of prescribing across different psychiatric categories, with a few exceptions (neurocognitive disorders; obsessive-compulsive and related disorders; and medication-induced movement disorders). Age was unknown for nine approvals for psychiatric indications.

Prescriber location

Queensland had the largest proportion of prescribing for psychiatric indications (54.9%), which was disproportionate to the population in this state (1,071 approvals per 100,000; Supplementary Table S4). Prescribing in the Northern Territory and South Australia were the lowest *per capita* (27 approvals per 100,000).

Products prescribed and cannabinoid content

The type of products being prescribed varied across psychiatric indications (Supplementary Table S5). The top three product types were oil (n = 53,347; 53.0%), flower (n = 31,518; 31.3%), and unspecified inhaled products ("inhaled"; n = 12,532; 12.4%). This varied greatly by indication: for example, 93.2% of prescribing for neurocognitive disorders was for oil products, while 44.8% of prescribing for depressive disorders was for flower products.

A greater proportion of prescribed products were S8 (>2% THC content; 79.7% of total) than S4 (>98% CBD content; 20.3% of total; Table 2). This proportion varied by psychiatric indication; for example, S4 products were more commonly used for disruptive, impulse-control, and conduct disorders (38.1%), neurodevelopmental disorders (36.4%), and neurocognitive disorders (27.8%). On the other hand, S4 approvals for depressive disorders were only 12.7% of the total, while sleep-wake-disorders had only 14.4% of approvals as S4.

Associations between patient profiles, indication, and products

Associations between indication, product type, and age group were investigated for psychiatric indications with >200 approvals. The contribution to variance (CoV) on each dimension and expected variances for all analyses are included in Supplementary Table S6.

There was a clear association between age group and indication ($Z = -26.815$, $p < 0.001$) (Figure 1A). Distinct conditions were ASD (CoV Dim 1 = 94.76%, inertia*1,000 = 325.731) and AD (CoV Dim 2 = 88.37%; inertia*1,000 = 62.71). AD corresponded to patients aged >74 (CoV Dim 2 = 90.56%; inertia*1,000 = 64.582), and ASD was associated with ages 0–9 (CoV Dim 1 = 39.72%; inertia*1,000 = 136.794) and 10–17 (CoV Dim 1 = 55.79%; inertia*1,000 = 191.633).

Similarly, there was an association between the condition treated and selected product format and schedule (e.g., S8 flower; χ^2_{119} = 3,719.300, $p < 0.001$) (Figure 1B). Patients with ASD displayed the most distinct product preference (CoV Dim 1 = 75.583%, inertia*1,000 = 33.406), and was associated with S4 oil (CoV Dim 1 = 47.339%; inertia*1,000 = 19.026), S4 tablet (CoV Dim 1 = 7.495%; inertia*1,000 = 3.317), and S4 topical preparations (CoV Dim 1 = 13.826%; inertia*1,000 = 6.766). S8 flower products also represented a distinct product choice (CoV Dim 2 = 45.518%; inertia*1,000 = 11.389),

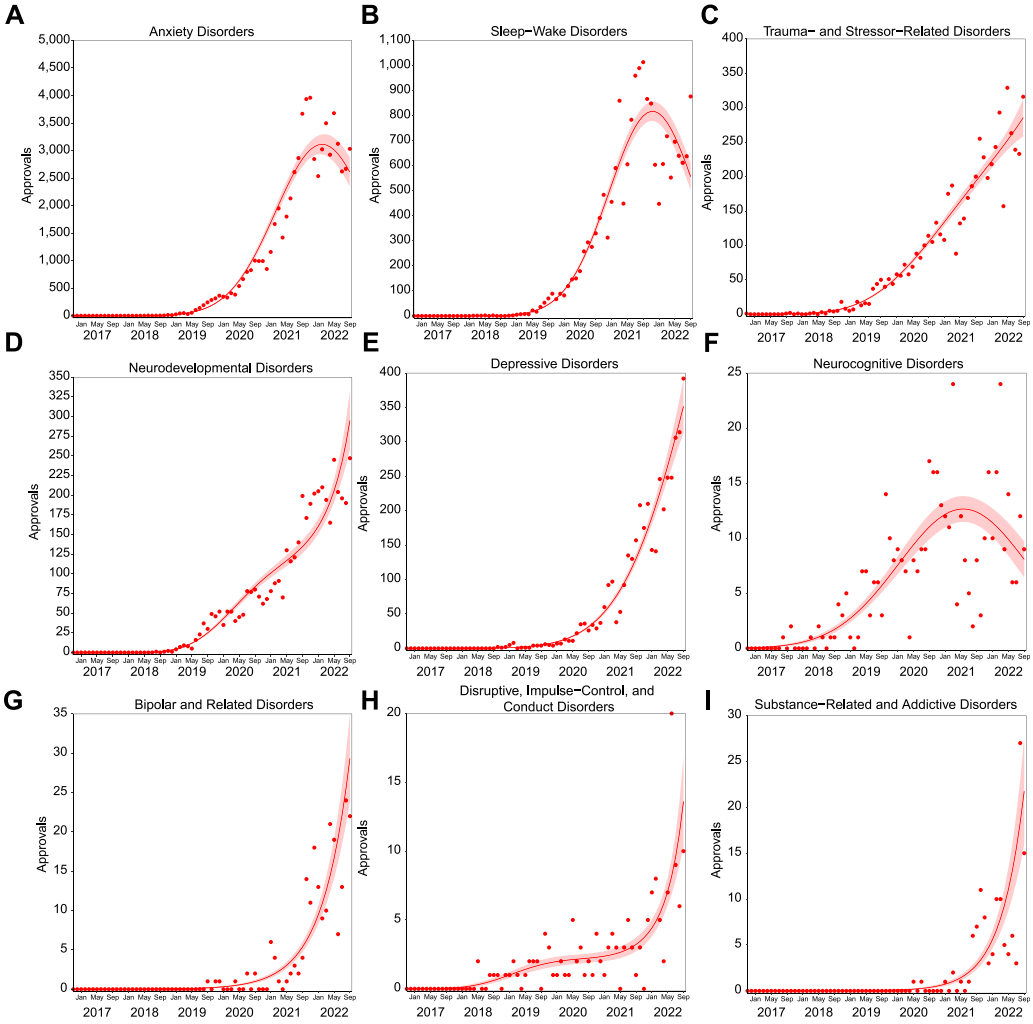

FIGURE 3
Approvals per month in psychiatric indication categories with >100 approvals followed different patterns of prescribing growth. Approvals over time for anxiety disorders [**(A)**, *n* = 67,133]; sleep wake disorders [**(B)**, *n* = 18,321], trauma- and stressor-related disorders [**(C)**, *n* = 5,799] neurodevelopmental disorders [**(D)**, *n* = 4,450]; depressive disorders [**(E)**, *n* = 4,003]; neurocognitive disorders [**(F)**, *n* = 428]; bipolar and related disorders [**(G)**, *n* = 212]; disruptive, impulse-control, and conduct disorders [**(H)**, *n* = 155]; and substance-related and addictive disorders [**(I)**, *n* = 126]. Solid lines represent the best fit, with shading depicting standard error of the mean (SEM).

which was associated with mood disorders (CoV Dim 2: 65.62%, inertia*1,000 = 6.575), amongst others. S8 inhaled formulations (CoV Dim 2 = 33.781%; inertia*1,000 = 6.493) were most associated with approvals for sleep disorders (CoV Dim 2 = 58.867%; inertia*1,000 = 8.561) and insomnia (CoV Dim 2 = 19.019%; inertia*1,000 = 4.609). A large proportion of approvals were for patients with anxiety, which represented the average profile across all product choices, as indicated by the proximity to the origin (coordinate for Dim 1 = 0.032 and Dim 2 = −0.006), as well as S8 capsules (Dim 1 = −0.006, Dim 2 = −0.020).

Product preference was also investigated in relation to patients' age group, in which there was a clear association (Z = −10.889, $p < 0.001$) (Figure 1C). Almost all age groups had distinct product preferences (see Supplementary Table S6C). S4 oil and S8 flower represented the most distinct subgroups (CoV Dim 1 = 43.184%, inertia*1,000 = 33.748; and CoV Dim 1 = 28.237%, inertia*1,000 = 22.387, respectively). S4 oil was most associated with ages 0–9 (CoV Dim 2 = 19.133%, inertia*1,000 = 15.634) and 10–17 (CoV Dim 1 = 36.494%, inertia*1,000 = 31.445), as was S4 topical (CoV Dim 2 = 26.038%, inertia*1,000 = 7.769). S8 flower was associated with patient ages 25–39 (CoV Dim 1 = 15.922%, inertia*1,000 = 12.69), as was S8 inhaled (CoV Dim 1 = 12.861%, inertia*1,000 = 10.496). The product choice of S8 oil (CoV Dim 2 = 30.974%, inertia*1,000 = 6.315) was more commonly selected for older age groups, particularly patients aged 55–74 (CoV Dim 2 = 26.312%, inertia*1,000 = 14.93) and >74 (CoV Dim 2 = 18.912%, inertia*1,000 = 12.169).

Trends over time

Prescribing for psychiatric conditions has grown rapidly from November 2019, as is the case for all SAS-B prescribing (MacPhail

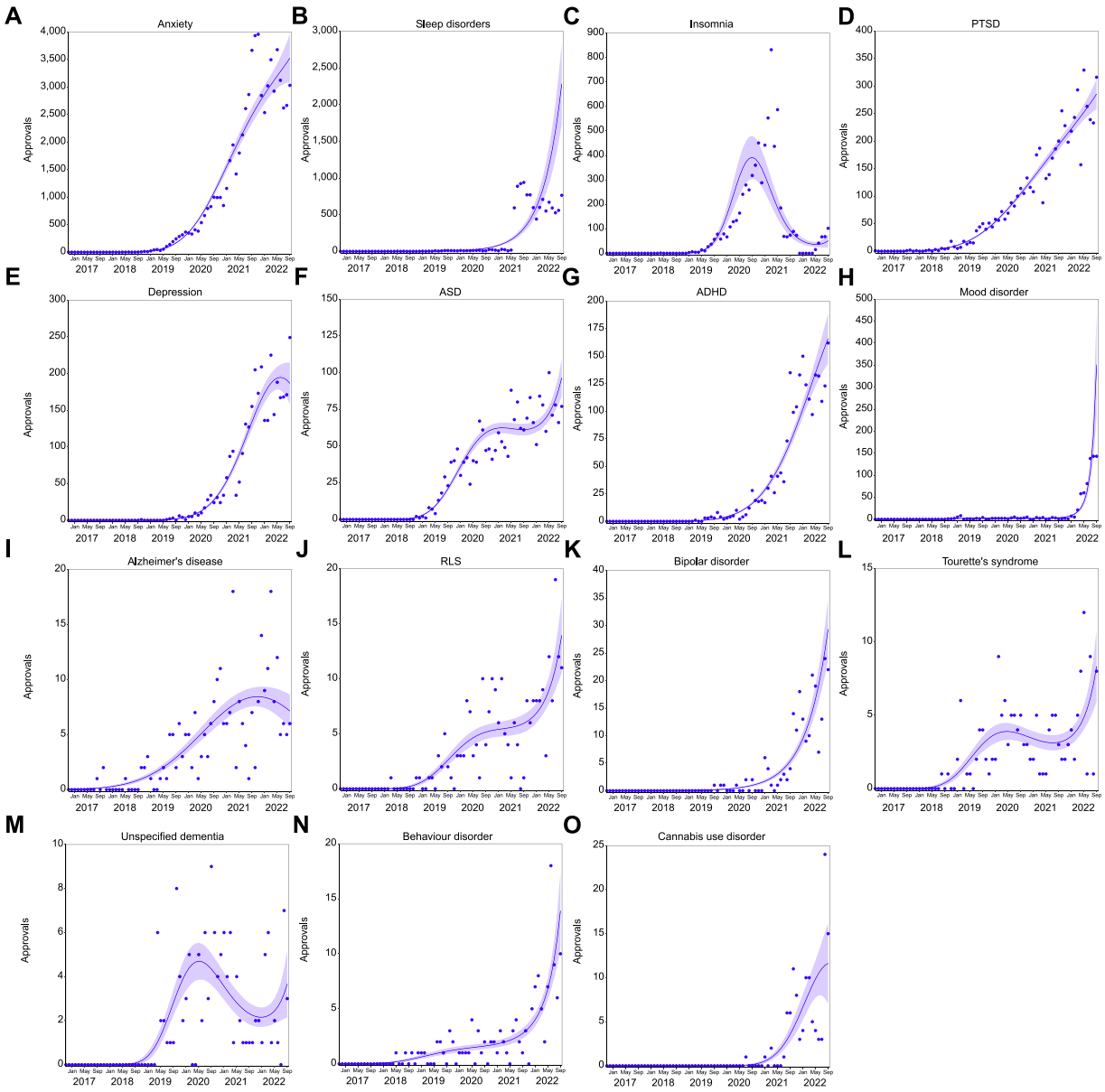

FIGURE 4
Approvals per month in psychiatric indications with >100 approvals. Approvals over time for anxiety [**(A)**, *n* = 67,095]; sleep disorder [**(B)**, *n* = 11,202)], insomnia [**(C)**, *n* = 6,877); post-traumatic stress disorder [PTSD; **(D)**, *n* = 5,799]; depression [**(E)**, *n* = 3,247]; autism spectrum disorder [ASD; **(F)**, *n* = 2,206]; attention deficit hyperactivity disorder [ADHD; **(G)**, *n* = 2,078]; mood disorder [**(H)**, *n* = 736]; Alzheimer's disease [**(I)**, *n* = 272]; restless leg syndrome [RLS; **(J)**, *n* = 239]; bipolar disorder [**(K)**, *n* = 212]; Tourette's syndrome [**(L)**, *n* = 163]; unspecified dementia [**(M)**, *n* = 132]; behavior disorder [**(N)**, *n* = 131]; cannabis use disorder [**(O)**, *n* = 117]. Solid lines represent the best fit, with shading depicting standard error of the mean (SEM).

et al., 2022). However, growth seems to be slowing or decreasing as of approximately November 2021 (2nd degree polynomial, R^2 = 0.988, Δm = 17,133.400; Figure 2).

Trends over time are not uniform across all psychiatric indications. Prescribing for neurodevelopmental disorders (3rd degree polynomial, R^2 = 0.975, Δm = 30.228); depressive disorders (2nd degree polynomial, R^2 = 0.973, Δm = 19.527); bipolar and related disorders (1st degree polynomial, R^2 = 0.873); disruptive and related disorders (3rd degree polynomial, R^2 = 0.728, Δm = 9.483); and substance-and related addictive disorders (1st degree polynomial, R^2 = 0.862) have shown dramatic increases in approvals (Figure 3). At the individual indication level (>100 approvals), prescribing also continues to increase for sleep disorders (2nd degree polynomial, R^2 = 0.726, Δm = 5.805), PTSD (3rd degree polynomial, R^2 = 0.972, Δm = 4.642), ADHD (2nd degree polynomial, R^2 = 0.966, Δm = 19.397), mood disorder (3rd degree polynomial, R^2 = 0.893, Δm = 39.410), bipolar disorder (1st degree polynomial, R^2 = 0.873), and behavior disorder (3rd degree polynomial, R^2 = 0.730, Δm = 6.371; Figure 4).

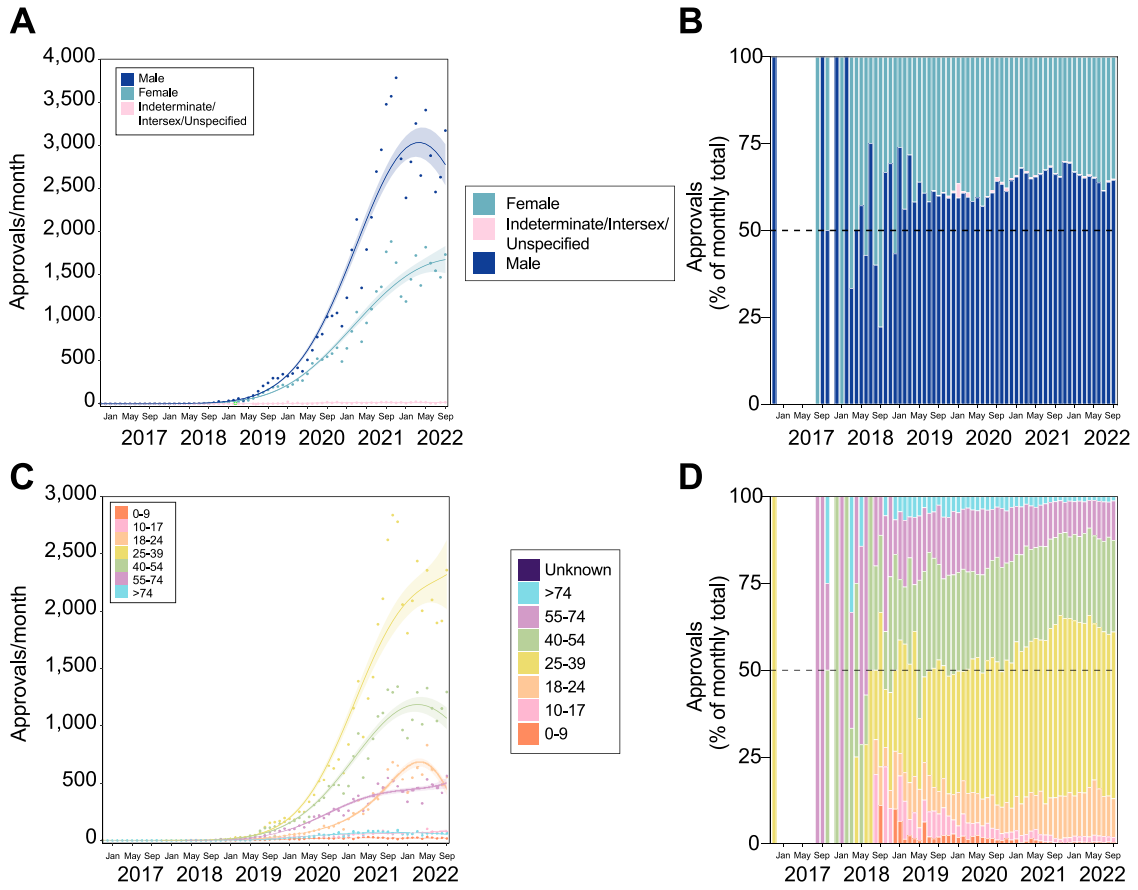

FIGURE 5
Patients receiving medicinal cannabis for psychiatric indications are predominantly younger and male. Trends in patient sex **(A,B)** and age **(C,D)**. Approval trends over time showing a recent decrease in the rate of approvals for males **(A)**, but continued growth in young patients, particularly aged 25–39 **(C)**. The proportion of these changes is also shown **(B,D)**, and suggests that while the number of male prescriptions may be decreasing, the relative proportion of prescribing remains relatively consistent. The lines of best fit in panels **(A,B)** are shown by the solid line with shaded area showing standard error of the mean. The gap in panels **(B,D)** indicates no applications submitted during this period.

Monthly numbers of SAS-B approvals for psychiatric indications in males grew significantly, but seems to be recently decreasing (2nd degree polynomial, R^2 = 0.986, Δm = 12,106.310; Figure 5A), while the rate of growth for females was less compared to males (3rd degree polynomial, R^2 = 0.989, Δm = 3.970). The proportion of prescribing for males has changed little over time following the initial prescription increase in November 2019 (Figure 5B).

The prescribing rate for patients aged 25–39 has grown sharply from November 2019, far outpacing any other group (4^{th} degree polynomial, R^2 = 0.988, Δm = 12.218; Figure 5C). Unsurprisingly, the proportion of approvals made up by this age group has increased over time, while the proportion of patients aged 55–74 and >74 has decreased (Figure 5D).

Prescribing of Schedule 4 products for psychiatric indications has grown slowly over time (3rd degree polynomial, R^2 = 0.979, Δm = 29.083; Figure 6A) compared with S8 (2nd degree polynomial, R^2 = 0.987, Δm = 14,260.970). Likewise, the proportion of S8 products approved for psychiatric indications has grown over time, though appears fairly uniform within the last few months (Figure 6B).

Approvals for oil products have rapidly increased over time, though has reduced in rate since the peak around September 2021 (2nd degree polynomial, R^2 = 0.984, Δm = 9,691.022; Figure 6C). Approvals for flower products followed a similar, but delayed trend, with peak around May 2022 (3rd degree polynomial, R^2 = 0.991, Δm = 10,422). Overall, the proportion of approvals for flower products has increased in recent years, and is now approaching that of oil products (Figure 6D).

Discussion

The prescribing of unregistered medicinal cannabis products is a relatively new development in Australia that appears to have strong community support (Australian Institute of Health and Welfare, 2020) and attracts significant patient curiosity (Karanges et al., 2018). The current study shows that prescribing medicinal cannabis for psychiatric indications has gained significant momentum after a slow start. Although the general profile of patients with approvals for psychiatric indications is similar in

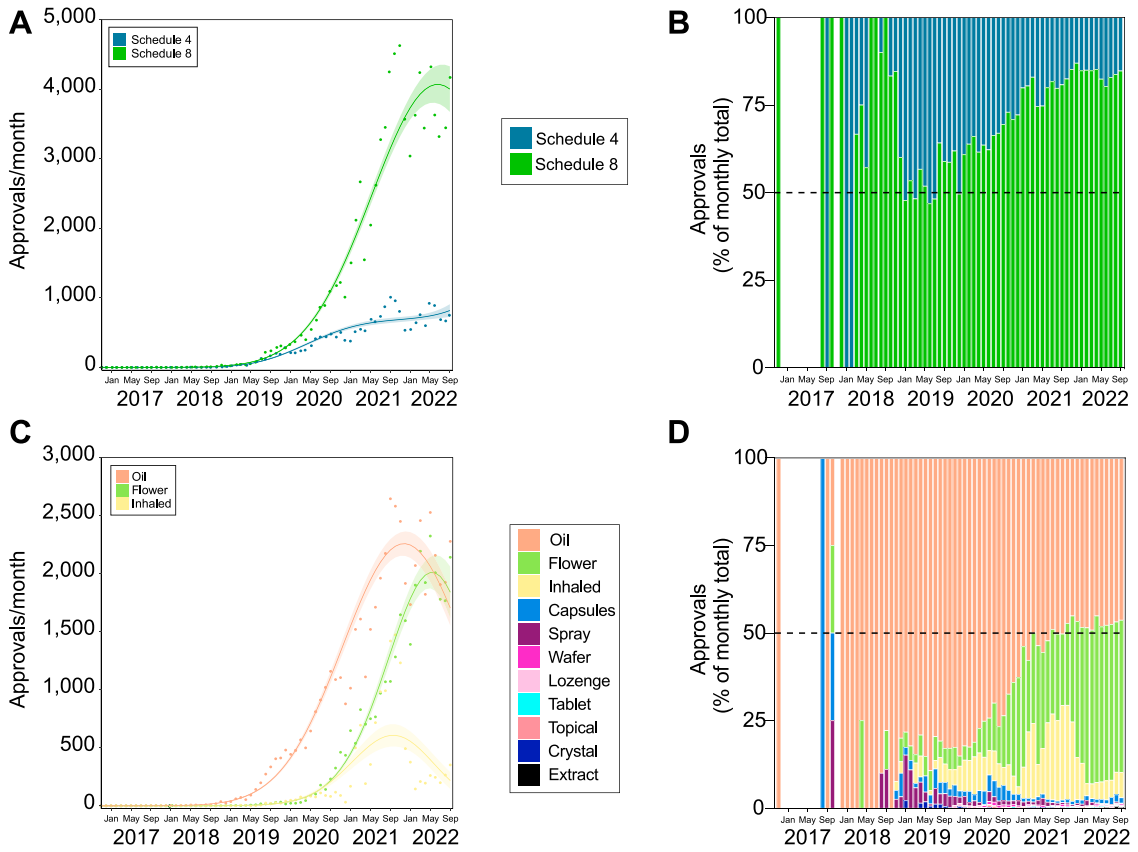

FIGURE 6
Approvals for medicinal cannabis products for the treatment of psychiatric indications are largely THC-containing oil or flower products. Trends in product schedule **(A,B)** and type **(C,D)**. Approval trends over time showing continued growth of S8 access **(A)**, which is reflected in the proportional access **(B)**. The rate of approvals for the top three product types (oil, flower, and inhaled) all appear to be decreasing **(C)**. However, oil and flower at least seem to have consistent proportional approvals **(D)**. The lines of best fit in panels **(A,B)** are shown by the solid line with shaded area showing standard error of the mean. The gap in panels **(B,D)** indicates no applications submitted during this period.

some ways to the overall SAS-B dataset (predominantly male patients obtaining S8 products; MacPhail et al., 2022), this analysis reveals several important distinctions and recent trends that were not previously captured. The majority of patients who have SAS-B approval for psychiatric indications are younger, and are more likely to obtain flower products. The proportion of SAS-B approvals for males is greater for psychiatric conditions than for non-psychiatric indications. This pattern does not seem to match with proportional estimates of mental health conditions in Australia (Australian Institute of Health and Welfare, 2022), though does align with overall cannabis usage patterns (Australian Institute of Health and Welfare, 2020). Approvals for several psychiatric indication groups, including developmental disorders and depressive disorders, have increased substantially in recent times. The minimal number of approvals for the major psychiatric indications of schizophrenia, bipolar disorder, and obsessive-compulsive disorders (Australian Institute of Health and Welfare, 2022) was also particularly notable.

As outlined in recent systematic reviews and meta-analyses, the quality of evidence supporting the use of medicinal cannabis in psychiatric indications is patchy (Black et al., 2019; Bonaccorso et al., 2019; Hoch et al., 2019; Bahji et al., 2020; Khan et al., 2020; Sarris et al., 2020; Kloiber et al., 2021; Stanciu et al., 2021; Berger, Amminger, et al., 2022). Large RCTs with a low risk of bias are few and far between, and most clinical evidence has been gained from observational or retrospective cohort studies, open-label pilot trials, or laboratory studies. Such evidence often falls short of the standards that would be required for the formal registration of a new pharmaceutical entity by regulatory agencies (Black et al., 2019).

However, cannabis is not a novel pharmaceutical entity, having been used for millennia for therapeutic purposes. To add complexity, "medicinal cannabis" covers a diverse variety of cannabinoids with varying routes of administration, doses, and formulations. Recent systematic reviews, therefore, attempt to synthesize data from trials involving multiple conditions treated by diverse pharmaceutical and artisanal products. Consider, for example, the difference between a patient vaporizing a high dose of THC-containing cannabis flower to treat PTSD (a feasible option under the current SAS-B scheme) and another patient orally ingesting a moderate dose of a CBD-containing oil to treat generalized anxiety (also feasible). Both are "medicinal cannabis products" used for "anxiety" under the SAS-B, but their use, route of administration, and psychoactive effects are dramatically different. In this context, the available systematic reviews are an imperfect guide to optimal prescribing of the currently available products.

Therefore, in contemplating whether the current prescribing of medicinal cannabis products in Australia for psychiatric conditions is rational and evidence-based, we must carefully dissect and interpret the evidence base, noting the limitations.

Anxiety disorders

By far the largest number of psychiatry-related approvals in the present study are for anxiety disorders. In some ways, this represents an interesting ongoing experiment, given the limited current evidence for the anxiolytic effects of cannabinoids. Two recent systematic reviews and meta-analyses (Black et al., 2019; Stanciu et al., 2021) concluded there may be some evidence supporting efficacy for cannabinoids in treating anxiety, but that evidence was of very low quality, with a third analysis showing no effect when studies were corrected for publication bias (Bahji et al., 2020).

However, research in this space is rapidly evolving. An observational study of patients receiving CBD prescriptions in New Zealand for various conditions found a significant overall reduction of anxiety and improved quality of life in those prescribed CBD (dose range = 40–300 mg/day) for mental health conditions and non-cancer pain (Gulbransen et al., 2020). Two recent open-label trials have also reported significant effects: the first found a significant reduction in anxiety and comorbid depressive symptoms with 200–800 mg/day CBD in patients aged 12–25 with refractory anxiety (Berger, Li, et al., 2022), and the second showed positive effects in adults (ages 22–64) with moderate to severe anxiety with a CBD sublingual solution (dose range = 23–46 mg/day) (Dahlgren et al., 2022). These complement previous experimental clinical studies showing anxiolytic effects of CBD in healthy volunteers (Linares et al., 2019) and social phobia patients (Bergamaschi et al., 2011; Crippa et al., 2011) and in patients at high risk of developing psychosis (Appiah-Kusi et al., 2020). It is hoped that future and current clinical trials can clarify optimal dosing, products, and anxiety subtypes that might best benefit. The role of expectancy effects with CBD administration should also be clarified: a small study of adults undergoing an acute stress test showed the importance of *a priori* beliefs about the anxiolytic properties of CBD in determining outcomes (Spinella et al., 2021).

On the other hand, the widespread use of THC-containing S8 products in treating anxiety (Table 2) gives some grounds for concern, given that THC can reliably induce anxiety and paranoia in higher doses (Martin-Santos et al., 2012; Freeman et al., 2015). However, THC-induced anxiety may be obviated by the gradual up-titration of doses in patients, and by using oral low-dose formulations (also containing CBD) rather than smoking or vaporizing herbal cannabis. Outcomes such as this may be probed further in large registry studies currently underway in the UK (Project Twenty21) and Australia with patients being prescribed medicinal cannabis, including those using it for treatment of anxiety and PTSD (Drug Science, 2022; Sakal et al., 2022; Vickery et al., 2022).

Trauma- and stressor-related disorders

Current evidence around the efficacy of cannabis and cannabinoid pharmaceuticals in PTSD has been reviewed recently in a focused fashion (Orsolini et al., 2019; Hindocha et al., 2020; Forsythe & Boileau, 2021; Rehman et al., 2021; Steardo et al., 2021; Sakal et al., 2022) and also included in the larger systematic reviews of psychiatric conditions published in the past 3 years (Black et al., 2019; Bonaccorso et al., 2019; Hoch et al., 2019; Botsford et al., 2020; Khan et al., 2020; Sarris et al., 2020; Kloiber et al., 2021; Stanciu et al., 2021). There are conspicuously high levels of self-medication with cannabis in patients with PTSD (Loflin et al., 2017), which is reported to provide symptomatic relief, particularly with respect to sleep and nightmares/flashbacks (Fraser, 2009; Passie et al., 2012); however, some studies suggest detrimental effects of cannabis use on PTSD symptom severity (Wilkinson et al., 2015). Studies of the pharmaceutical THC analogue nabilone have shown particular efficacy (Fraser, 2009; Cameron et al., 2014; Jetly et al., 2015), and some studies of THC/THC-predominant medicinal cannabis products have noted improved global functioning in PTSD (Mashiah, 2012; Roitman et al., 2014). The studies evaluating CBD only for PTSD symptom control are currently restricted to positive case reports (Shannon & Opila-Lehman, 2016; Elms et al., 2019), and a study on traumatic memory recall using a single administration of CBD (300 mg) showed little effect (Bolsoni et al., 2022). Again, we await larger well-controlled studies to validate current prescribing practice around CBD only products as well as those containing THC in PTSD, which appear to be ongoing (Telch et al., 2022).

Insomnia and other sleep disorders

The existing evidence base supporting cannabinoids for the treatment of insomnia is limited. Recent systematic reviews (Suraev et al., 2020; Lavender et al., 2022) highlighted the limited evidence supporting cannabinoids in treating clinician-diagnosed insomnia disorder (as opposed to patient self-reported insomnia, or "sleep problems"). Most published RCTs of "insomnia" are often secondary to other conditions, such as chronic pain, and are only conducted over acute timelines (e.g., single dose to a maximum of 4 weeks), making conclusions about the longevity of the self-reported effects of cannabinoids uncertain. Current SAS-B prescribing for insomnia is predominantly for THC-containing products (Table 2), which arguably aligns with available evidence, although there are exceptions. Two RCTs of nabilone in patients with sleep issues secondary to chronic pain showed modest efficacy in improving total sleep time and efficiency (Ware et al., 2010; Zalai, 2015). However, one such study concluded that nabilone was not an effective option as it concurrently increased sleep onset latency (Zalai, 2015). A third RCT of THC observed a reduction in sleep latency but only evaluated the effects of a single acute dose (Cousens & DiMascio, 1973), while early results from another trial with a single 200 mg CBD and 10 mg THC administration suggested a decrease in total sleep time (Suraev et al., 2022). Similarly, a study in healthy volunteers reported no effect of THC alone on sleep parameters, and when combined with CBD (in the form of nabiximols) actually increased wakefulness (Nicholson et al., 2004). Finally, a recent RCT in adults with chronic insomnia reported improvements over 2 weeks in

subjective sleep quality, sleep-onset latency, total sleep time, feeling of rest upon waking with nightly administration of used ZTL-101 (containing 10 mg THC, 1 mg cannabinol, and 0.5 mg CBD) (Walsh et al., 2021). The majority of support for THC prescribing in sleep disorders (including insomnia) seems to be from the community and patient self-report (Lintzeris et al., 2022) as opposed to a robust clinical evidence base (with the exception of one recent RCT), and this is reflected in the recommendations of the American Academy of Sleep Medicine (Ramar et al., 2018).

At present, there is no compelling rationale for prescribing CBD for chronic refractory insomnia. Evidence for the use of CBD in insomnia is limited to a retrospective case series (in patients with "poor sleep") (Shannon et al., 2019) and a single acute dose self-report RCT (Carlini & Cunha, 1981), neither of which makes a strong case for long term CBD efficacy to support prescribing. Again, it is anticipated that evidence from these larger, longer duration studies will shed better light on the therapeutic use of cannabinoids for insomnia and sleep disorders.

Neurodevelopmental disorders (ASD, ADHD)

Prescribing THC-containing products to children is controversial in any setting. Prescribing for psychiatric indications is particularly so, given the deleterious impacts of chronic THC exposure on the developing brain and adult behavioral phenotype that is routinely observed in animal models (Quinn et al., 2008; Trezza et al., 2008). Our analysis uncovers noteworthy prescribing of medicinal cannabis products to <18-year-olds largely divided between anxiety disorders and neurodevelopmental disorders (primarily ASD). While prescribing CBD in specific pediatric epilepsies is now evidence-based (Devinsky et al., 2017; Laux et al., 2019), the evidence for cannabinoid efficacy in conditions such as anxiety, ASD and ADHD is minimal. This is particularly true for ADHD–a single RCT in adults that concluded no significant effect of nabiximols treatment on cognitive performance and only suggestive effects on secondary hyperactivity measures (Cooper et al., 2017). There have been no studies evaluating CBD-only preparations in ADHD cohorts, despite this accounting for 15.9% of ADHD approvals under SAS-B (Table 2).

ASD attracts even more SAS-B prescribing than ADHD, yet the current evidence base consists of only a single RCT comparing a 20:1 CBD:THC whole extract, a purified isolate product at the same CBD:THC ratio, and placebo, in patients aged 5–21 years. Parent-reported measures of behavior did not reveal a significant effect over 12 weeks of either treatment compared with placebo, but clinical evaluation of disruptive behavior was improved with the extract product (Aran et al., 2021). Overall improvements in anxiety, sleep, and behavior remain inconsistent across case series and observational studies, with approximately one-third of children seemingly responding well (Efron, 2021; Fletcher et al., 2021). However, a significant caveat to these studies is their reliance on parental reports, which is notoriously variable and/or prone to bias. Notably, CBD-only prescribing accounts for 56.3% of ASD approvals, yet there are no published studies of CBD-only products in this patient cohort. Current CBD prescribing for ASD may be more reflective of caution around the use of THC products in children rather than being evidence-driven.

Schizophrenia, bipolar disorder and depressive disorders

A major finding of the current study is the minimal SAS-B approvals for the major psychiatric disorders of schizophrenia and bipolar disorder. The prescribing for depression is rising dramatically of late, though it still represents a small proportion of the overall prescribing. Presumably, the uncertainty that surrounds a causal association between cannabis use and psychosis (Colizzi et al., 2020; D'Souza et al., 2022; Di Forti et al., 2019; Hill, 2015; National Academies of Sciences, 2017; Pasman et al., 2018) explains the high degree of caution in prescribing medicinal cannabis products for schizophrenia and bipolar disorder, although it does not necessarily account for the limited S4 (CBD-dominant) prescribing (n = 22). A number of moderate-quality RCTs, with well-sized patient cohorts, evaluating CBD-only products for the treatment of schizophrenia have shown positive outcomes (Leweke et al., 2012; Boggs et al., 2018; McGuire et al., 2018). The limited approvals for schizophrenia highlight a notable gap between the existing evidence base and prescribing decisions. Similarly, the only published study involving CBD for treating bipolar disorder produced equivocal findings and concluded that CBD was ineffective in treating mania (Zuardi et al., 2010), and yet 16.5% of approvals have been for CBD-only-containing products. Psychiatric prescribing practices in these disorders are interesting examples that highlight a potential disconnect between current prescribing and awareness of the current evidence base.

Finally, depression is a highly prevalent condition, with recent figures that more than 10% of Australians are prescribed antidepressant medications (Stephenson et al., 2013; Brett et al., 2017). Medicinal cannabis prescribing for depressive disorders is low relative to other psychiatric indications, but is on the rise. This may reflect the lack of any RCTs specifically focused on the treatment of depression with cannabinoids (Scherma et al., 2020; Tibbo et al., 2021), as well as documented positive associations between cannabis use and depression, albeit with uncertainty of causal direction (Horwood et al., 2012; Bahorik et al., 2017; Hodgson et al., 2020). The available evidence for medicinal cannabis in treating depression is of poor quality and is restricted to several positive case reports involving dronabinol (Blaas, 2008) and other anecdotal observations of unregulated cannabis use in patients with complex psychiatric histories (Gruber et al., 1996). To date, SAS-B approvals for depressive disorders are primarily S8 products (87.3%; Table 2). There is no current evidence to support CBD-only prescribing for depression.

One alternative possible explanation for the low number of approvals for depressive disorders could be the overlap with comorbid chronic pain, which is highly prevalent in depressive

populations. However, it would not be captured in the SAS-B data (see MacPhail et al., 2022). Alternatively, the widespread use of medicinal cannabis products for anxiety disorders may also be inadvertently benefitting depression, which shows high co-morbidity with anxiety.

The growing use of medicinal cannabis can be seen as part of a broader movement within psychiatry toward use of unconventional therapies, or rather the return to some of the older options (including cannabis) ruled too radical in recent times, including a range of traditional "recreational" drugs, being accepted into clinical practice (Nutt, 2019). Examples include the use of ketamine and psilocybin for depression (Thomas et al., 2017; Rosenblat et al., 2019; Carhart-Harris et al., 2021; Goodwin et al., 2022) and MDMA for PTSD (Mitchell et al., 2021). This is perhaps a response to the obstinately dry pipeline of novel psychiatric medications from traditional pharmaceutical routes, and the ongoing use of traditional prescription psychotropics that are often older than the prescriber and the patient.

This is not to say that psychiatry should abandon caution and prescribe unregistered medicines as a first-line intervention. Indeed, official guidance on medicinal cannabis prescribing around psychiatric conditions is notably absent, meaning that clinicians have no readily available source of advice on rational prescribing for conditions such as anxiety, insomnia, PTSD and ASD. Prescribers struggle to find quality information to guide their use of the more than 360 medicinal cannabis products currently available under SAS-B within Australia. The TGA has produced guidance documents that outline the quality of the supportive clinical evidence for five different conditions: chronic non-cancer pain, epilepsy, multiple sclerosis, palliative care, and nausea and vomiting (Therapeutic Goods Administration, 2019). However, similar evidence-based guidance for psychiatric disorders is needed with some urgency, which is starting to be addressed by international peers at present yet has not been as much of a focus in the Australian context to date. Development of prescribing guidelines for MC has been completed by several groups in the UK, including NICE (National Institute for Health and Care Excellence, 2019) and the Medicinal Cannabis Clinicians Society (Medical Cannabis Clinicians' Society, 2021). Yet, these are still very generalist in nature and do not provide guidance specific to psychiatric indications using evidence-based conclusions.

Nor does this lack of guidance suggest that supervised use of medicinal cannabis for these conditions should be completely halted. The reality is that even when legal access pathways are available, many Australian patients with mental health conditions report self-medicating with cannabis (Lintzeris et al., 2022), which is perhaps a reflection of the uneasiness of some healthcare practitioners in administering and supervising use for these indications (Karanges et al., 2018). Unsupervised use of medicinal cannabis (prescribed or otherwise) may come with risk, and non-disclosure can ultimately affect quality of care received by patients (Cairns & Kelly, 2017; Stuart-Maver, 2020). Indeed, medicinal cannabis use under strict supervision of a healthcare practitioner may currently be the best option to balance these risks while providing appropriate care, given the general tolerability and safety under supervised use (Vickery et al., 2022). Medicinal cannabis for treating psychiatric indications is a rapidly evolving field with new studies being published regularly, hopefully providing greater clarity in the near future.

Conclusion

The purpose of this review was to present data on the current TGA approvals for medicinal cannabis products in Australia under the SAS-B scheme, their use for psychiatric indications, and to synthesize these data and relate back to the existing evidence base. We hope that our analysis will aid in transparency around current SAS-B prescribing practices in the psychiatric realm within Australia and stimulate further discussion, evaluation, and research into whether medicinal cannabis products represent effective standalone or adjunctive treatments for use within psychiatry. This issue is only likely to intensify in the coming months and years with the tremendous worldwide popularity and associated patient interest in using medicinal cannabis to treat a cornucopia of conditions. For this reason, the discussion must continue with the input of those in the academic and clinical community who are best placed to offer considered and balanced scientific views, with specific effort placed on facilitating high-quality RCTs, particularly where prescribing is disproportionate to existing clinical evidence of efficacy.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.tga.gov.au/sites/default/files/2022-12/foi-4020-01.XLSX.

Author contributions

EC, MB-P, and SM conducted the analysis. EC, MB, RC, and IM co-wrote the manuscript and conceived of the project. AM and PS were involved in manuscript writing, revisions and assisted with data classifications. All authors contributed to the article and approved the submitted version.

Funding statement

This work was supported by the Lambert Initiative for Cannabinoid Therapeutics at the University of Sydney by the philanthropic gift of Joy and Barry Lambert (Ref: G180048).

Conflict of interest

RC reports personal fees from Cannabis Consulting Australia Pty Ltd., personal fees from Biologics Research Institute Australia Pty Ltd., personal fees from University of Sydney, outside the submitted work;

IM reports grants from National Health and Medical Research Council of Australia, grants from National Institute of Health, grants from Wellcome Trust, grants and other from University of Sydney (Lambert Initiative), during the course of the study; personal fees from Janssen, outside the submitted work. In addition, IM has patents WO2018107216A1 WO2017004674A1 licensed to Kinoxis Therapeutics, a patent WO2011038451A1 issued, a patent WO2019071302 issued, a patent WO2019227167 issued, a patent AU2017904438 pending, and a patent AU2019051284 pending. PS is supported by an Investigator Grant from the NHMRC, and was on an expert advisory panel for Biogen Australia and Roche Australia in 2020 and 2021.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1142680/full#supplementary-material

References

Abel, E. L. (2005). Jacques joseph Moreau (1804-1884). *Am. J. Psychiatry* 162 (3), 458. doi:10.1176/appi.ajp.162.3.458

Appiah-Kusi, E., Petros, N., Wilson, R., Colizzi, M., Bossong, M. G., Valmaggia, L., et al. (2020). Effects of short-term cannabidiol treatment on response to social stress in subjects at clinical high risk of developing psychosis. *Psychopharmacol. Berl.* 237, 1121–1130. doi:10.1007/s00213-019-05442-6

Aran, A., Harel, M., Cassuto, H., Polyansky, L., Schnapp, A., Wattad, N., et al. (2021). Cannabinoid treatment for autism: A proof-of-concept randomized trial. *Mol. Autism* 12 (1), 6. doi:10.1186/s13229-021-00420-2

Arkell, T. R., Lintzeris, N., Kevin, R. C., Ramaekers, J. G., Vandrey, R., Irwin, C., et al. (2019). Cannabidiol (CBD) content in vaporized cannabis does not prevent tetrahydrocannabinol (THC)-induced impairment of driving and cognition. *Psychopharmacol. Berl.* 236 (9), 2713–2724. doi:10.1007/s00213-019-05246-8

Arseneault, L., Cannon, M., Poulton, R., Murray, R., Caspi, A., and Moffitt, T. E. (2002). Cannabis use in adolescence and risk for adult psychosis: Longitudinal prospective study. *BMJ* 325 (7374), 1212–1213. doi:10.1136/bmj.325.7374.1212

Australian Bureau of Statistics (2022). Population: Census. Retrieved 22 December 2022 from https://www.abs.gov.au/statistics/people/population/population-census/2021

Australian Institute of Health and Welfare (2020). National drug strategy household survey 2019. https://www.aihw.gov.au/reports/illicit-use-of-drugs/national-drug-strategy-household-survey-2019.

Australian Institute of Health and Welfare (2022). Prevalence and impact of mental illness. https://www.aihw.gov.au/mental-health/overview/mental-illness.

Bahji, A., Meyyappan, A. C., and Hawken, E. R. (2020). Efficacy and acceptability of cannabinoids for anxiety disorders in adults: A systematic review & meta-analysis. *J. Psychiatr. Res.* 129, 257–264. doi:10.1016/j.jpsychires.2020.07.030

Bahorik, A. L., Leibowitz, A., Sterling, S. A., Travis, A., Weisner, C., and Satre, D. D. (2017). Patterns of marijuana use among psychiatry patients with depression and its impact on recovery. *J. Affect Disord.* 213, 168–171. doi:10.1016/j.jad.2017.02.016

Bartoń, K. (2020). MuMIn: Multi-Model inference. https://CRAN.R-project.org/package=MuMIn.

Bawa, Z., Saini, B., McCartney, D., Bedoya-Perez, M., McLachlan, A. J., and McGregor, I. S. (2022). A cross-sectional survey exploring the knowledge, experiences and attitudes of Australian pharmacists toward medicinal cannabis. *Int. J. Clin. Pharm.* 45, 375–386. doi:10.1007/s11096-022-01519-z

Benson, M. J., Abelev, S. V., Corte, C. J., Connor, S. J., and McGregor, I. S. (2020). Attitudes and knowledge of Australian gastroenterologists around the use of medicinal cannabis for inflammatory bowel disease. *Crohn's Colitis* 360 (2), otaa045. doi:10.1093/crocol/otaa045

Bergamaschi, M. M., Queiroz, R. H., Chagas, M. H., de Oliveira, D. C., De Martinis, B. S., Kapczinski, F., et al. (2011). Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naive social phobia patients. *Neuropsychopharmacology* 36 (6), 1219–1226. doi:10.1038/npp.2011.6

Berger, M., Amminger, G. P., and McGregor, I. S. (2022a). Medicinal cannabis for the treatment of anxiety disorders. *Aust. J. Gen. Pract.* 51 (8), 586–592. doi:10.31128/AJGP-04-21-5936

Berger, M., Li, E., Rice, S., Davey, C. G., Ratheesh, A., Adams, S., et al. (2022b). Cannabidiol for treatment-resistant anxiety disorders in young people: An open-label trial. *J. Clin. Psychiatry* 83 (5), 21m14130. doi:10.4088/JCP.21m14130

Blaas, K. (2008). Treating depression with cannabinoids. *Cannabinoids* 3 (2), 8–10. https://www.cannabis-med.org/data/pdf/en_2008_02_2_0.pdf.

Black, N., Stockings, E., Campbell, G., Tran, L. T., Zagic, D., Hall, W. D., et al. (2019). Cannabinoids for the treatment of mental disorders and symptoms of mental disorders: A systematic review and meta-analysis. *Lancet Psychiatry* 6 (12), 995–1010. doi:10.1016/S2215-0366(19)30401-8

Boggs, D. L., Surti, T., Gupta, A., Gupta, S., Niciu, M., Pittman, B., et al. (2018). The effects of cannabidiol (CBD) on cognition and symptoms in outpatients with chronic schizophrenia a randomized placebo controlled trial. *Psychopharmacol. Berl.* 235 (7), 1923–1932. doi:10.1007/s00213-018-4885-9

Bolsoni, L. M., Crippa, J. A. S., Hallak, J. E. C., Guimaraes, F. S., and Zuardi, A. W. (2022). Effects of cannabidiol on symptoms induced by the recall of traumatic events in patients with posttraumatic stress disorder. *Psychopharmacol. Berl.* 239 (5), 1499–1507. doi:10.1007/s00213-021-06043-y

Bonaccorso, S., Ricciardi, A., Zangani, C., Chiappini, S., and Schifano, F. (2019). Cannabidiol (CBD) use in psychiatric disorders: A systematic review. *Neurotoxicology* 74, 282–298. doi:10.1016/j.neuro.2019.08.002

Botsford, S. L., Yang, S., and George, T. P. (2020). Cannabis and cannabinoids in mood and anxiety disorders: Impact on illness onset and course, and assessment of therapeutic potential. *Am. J. Addict.* 29 (1), 9–26. doi:10.1111/ajad.12963

Brett, J., Karanges, E. A., Daniels, B., Buckley, N. A., Schneider, C., Nassir, A., et al. (2017). Psychotropic medication use in Australia, 2007 to 2015: Changes in annual incidence, prevalence and treatment exposure. *Aust. N. Z. J. Psychiatry* 51 (10), 990–999. doi:10.1177/0004867417721018

Burnham, K. P., and Anderson, D. R. (2002). *A practical information-theoretic approach*. Berlin, Germany: Springer.

Cairns, E. A., and Kelly, M. E. M. (2017). Why support a separate medical access framework for cannabis? *CMAJ* 189 (28), E927-E928–E928. doi:10.1503/cmaj.170427

Cameron, C., Watson, D., and Robinson, J. (2014). Use of a synthetic cannabinoid in a correctional population for posttraumatic stress disorder-related insomnia and nightmares, chronic pain, harm reduction, and other indications: A retrospective evaluation. *J. Clin. Psychopharmacol.* 34 (5), 559–564. doi:10.1097/JCP.0000000000000180

Carhart-Harris, R., Giribaldi, B., Watts, R., Baker-Jones, M., Murphy-Beiner, A., Murphy, R., et al. (2021). Trial of psilocybin versus escitalopram for depression. *N. Engl. J. Med.* 384 (15), 1402–1411. doi:10.1056/NEJMoa2032994

Carlini, E. A., and Cunha, J. M. (1981). Hypnotic and antiepileptic effects of cannabidiol. *J. Clin. Pharmacol.* 21 (S1), 417S-427S–427S. doi:10.1002/j.1552-4604.1981.tb02622.x

Colizzi, M., Ruggeri, M., and Bhattacharyya, S. (2020). Unraveling the intoxicating and therapeutic effects of cannabis ingredients on psychosis and cognition. *Front. Psychol.* 11, 833. doi:10.3389/fpsyg.2020.00833

Cooper, R. E., Williams, E., Seegobin, S., Tye, C., Kuntsi, J., and Asherson, P. (2017). Cannabinoids in attention-deficit/hyperactivity disorder: A randomised-controlled trial. *Eur. Neuropsychopharmacol.* 27 (8), 795–808. doi:10.1016/j.euroneuro.2017.05.005

Cousens, K., and DiMascio, A. (1973). (- Delta 9 THC as an hypnotic. An experimental study of three dose levels. *Psychopharmacologia* 33 (4), 355–364. doi:10.1007/BF00437513

Crippa, J. A., Derenusson, G. N., Ferrari, T. B., Wichert-Ana, L., Duran, F. L., Martin-Santos, R., et al. (2011). Neural basis of anxiolytic effects of cannabidiol (CBD) in

generalized social anxiety disorder: A preliminary report. *J. Psychopharmacol.* 25 (1), 121–130. doi:10.1177/0269881110379283

D'Souza, D. C., DiForti, M., Ganesh, S., George, T. P., Hall, W., Hjorthoj, C., et al. (2022). Consensus paper of the WFSBP task force on cannabis, cannabinoids and psychosis. *World J. Biol. Psychiatry* 23 (10), 719–742. doi:10.1080/15622975.2022.2038797

Dahlgren, M. K., Lambros, A. M., Smith, R. T., Sagar, K. A., El-Abboud, C., and Gruber, S. A. (2022). Clinical and cognitive improvement following full-spectrum, high-cannabidiol treatment for anxiety: Open-label data from a two-stage, phase 2 clinical trial. *Commun. Med. (Lond)* 2 (1), 139. doi:10.1038/s43856-022-00202-8

DeLisi, L. E. (2008). The effect of cannabis on the brain: Can it cause brain anomalies that lead to increased risk for schizophrenia? *Curr. Opin. Psychiatry* 21 (2), 140–150. doi:10.1097/YCO.0b013e3282f51266

Devinsky, O., Cross, J. H., Laux, L., Marsh, E., Miller, I., Nabbout, R., et al. (2017). Trial of cannabidiol for drug-resistant seizures in the Dravet syndrome. *N. Engl. J. Med.* 376 (21), 2011–2020. doi:10.1056/NEJMoa1611618

Devinsky, O., Patel, A. D., Cross, J. H., Villanueva, V., Wirrell, E. C., Privitera, M., et al. (2018). Effect of cannabidiol on drop seizures in the lennox-gastaut syndrome. *N. Engl. J. Med.* 378 (20), 1888–1897. doi:10.1056/NEJMoa1714631

Di Forti, M., Quattrone, D., Freeman, T. P., Tripoli, G., Gayer-Anderson, C., Quigley, H., et al. (2019). The contribution of cannabis use to variation in the incidence of psychotic disorder across europe (EU-GEI): A multicentre case-control study. *Lancet Psychiatry* 6 (5), 427–436. doi:10.1016/S2215-0366(19)30048-3

Drug Science (2022). Access medical cannabis with project Twenty21. Retrieved 22 December 2022 from https://www.drugscience.org.uk/twenty21/

Efron, D. (2021). Potential therapeutic uses of cannabinoids to treat behavioural problems in children and adolescents with developmental disorders. *Aust. J. Gen. Pract.* 50 (6), 352–355. doi:10.31128/AJGP-01-21-5809

Elms, L., Shannon, S., Hughes, S., and Lewis, N. (2019). Cannabidiol in the treatment of post-traumatic stress disorder: A case series. *J. Altern. Complement. Med.* 25 (4), 392–397. doi:10.1089/acm.2018.0437

Englund, A., Oliver, D., Chesney, E., Chester, L., Wilson, J., Sovi, S., et al. (2022). Does cannabidiol make cannabis safer? A randomised, double-blind, cross-over trial of cannabis with four different CBD:THC ratios. *Neuropsychopharmacology* 48, 869–876. doi:10.1038/s41386-022-01478-z

Fischer, B., Robinson, T., Bullen, C., Curran, V., Jutras-Aswad, D., Medina-Mora, M. E., et al. (2022). Lower-risk cannabis use guidelines (lrcug) for reducing health harms from non-medical cannabis use: A comprehensive evidence and recommendations update. *Int. J. Drug Policy* 99, 103381. doi:10.1016/j.drugpo.2021.103381

Fletcher, S., Pawliuk, C., Ip, A., Huh, L., Rassekh, S. R., Oberlander, T. F., et al. (2021). Medicinal cannabis in children and adolescents with autism spectrum disorder: A scoping review. *Child. Care Health Dev.* 48, 33–44. doi:10.1111/cch.12909

Forsythe, M. L., and Boileau, A. J. (2021). Use of cannabinoids for the treatment of patients with post-traumatic stress disorder. *J. Basic Clin. Physiol. Pharmacol.* 33 (2), 121–132. doi:10.1515/jbcpp-2020-0279

Fraser, G. A. (2009). The use of a synthetic cannabinoid in the management of treatment-resistant nightmares in posttraumatic stress disorder (PTSD). *CNS Neurosci. Ther.* 15 (1), 84–88. doi:10.1111/j.1755-5949.2008.00071.x

Freeman, A. M., Petrilli, K., Lees, R., Hindocha, C., Mokrysz, C., Curran, H. V., et al. (2019). How does cannabidiol (CBD) influence the acute effects of delta-9-tetrahydrocannabinol (THC) in humans? A systematic review. *Neurosci. Biobehav Rev.* 107, 696–712. doi:10.1016/j.neubiorev.2019.09.036

Freeman, D., Dunn, G., Murray, R. M., Evans, N., Lister, R., Antley, A., et al. (2015). How cannabis causes paranoia: Using the intravenous administration of Δ9-tetrahydrocannabinol (THC) to identify key cognitive mechanisms leading to paranoia. *Schizophr. Bull.* 41 (2), 391–399. doi:10.1093/schbul/sbu098

Freeman, T. P., Hindocha, C., Baio, G., Shaban, N. D. C., Thomas, E. M., Astbury, D., et al. (2020). Cannabidiol for the treatment of cannabis use disorder: A phase 2a, double-blind, placebo-controlled, randomised, adaptive bayesian trial. *Lancet Psychiatry* 7 (10), 865–874. doi:10.1016/S2215-0366(20)30290-X

Goodwin, G. M., Aaronson, S. T., Alvarez, O., Arden, P. C., Baker, A., Bennett, J. C., et al. (2022). Single-dose psilocybin for a treatment-resistant episode of major depression. *N. Engl. J. Med.* 387 (18), 1637–1648. doi:10.1056/NEJMoa2206443

Gorfinkel, L. R., Stohl, M., and Hasin, D. (2020). Association of depression with past-month cannabis use among US adults aged 20 to 59 Years, 2005 to 2016. *JAMA Netw. Open* 3 (8), e2013802. doi:10.1001/jamanetworkopen.2020.13802

Gruber, A. J., Pope, H. G., and Brown, M. E. (1996). Do patients use marijuana as an antidepressant? *Depression* 4 (2), 77–80. doi:10.1002/(SICI)1522-7162

Gulbransen, G., Xu, W., and Arroll, B. (2020). Cannabidiol prescription in clinical practice: An audit on the first 400 patients in New Zealand. *BJGP Open* 4. doi:10.3399/bjgpopen20X101010

Hay, G. L., Baracz, S. J., Everett, N. A., Roberts, J., Costa, P. A., Arnold, J. C., et al. (2018). Cannabidiol treatment reduces the motivation to self-administer methamphetamine and methamphetamine-primed relapse in rats. *J. Psychopharmacol.* 32 (12), 1369–1378. doi:10.1177/0269881118799954

Hill, M. (2015). Perspective: Be clear about the real risks. *Nature* 525 (7570), S14. doi:10.1038/525S14a

Hindocha, C., Cousijn, J., Rall, M., and Bloomfield, M. A. P. (2020). The effectiveness of cannabinoids in the treatment of posttraumatic stress disorder (PTSD): A systematic review. *J. Dual Diagn* 16 (1), 120–139. doi:10.1080/15504263.2019.1652380

Hoch, E., Niemann, D., von Keller, R., Schneider, M., Friemel, C. M., Preuss, U. W., et al. (2019). How effective and safe is medical cannabis as a treatment of mental disorders? A systematic review. *Eur. Arch. Psychiatry Clin. Neurosci.* 269 (1), 87–105. doi:10.1007/s00406-019-00984-4

Hodgson, K., Coleman, J. R. I., Hagenaars, S. P., Purves, K. L., Glanville, K., Choi, S. W., et al. (2020). Major depressive disorder working group of the psychiatric genomics cannabis use, depression and self-harm: Phenotypic and genetic relationships. *Addiction* 115 (3), 482–492. doi:10.1111/add.14845

Horwood, L. J., Fergusson, D. M., Coffey, C., Patton, G. C., Tait, R., Smart, D., et al. (2012). Cannabis and depression: An integrative data analysis of four australasian cohorts. *Drug Alcohol Depend.* 126 (3), 369–378. doi:10.1016/j.drugalcdep.2012.06.002

Hothorn, T., Hornik, K., van de Wiel, M. A., and Zeileis, A. (2006). A lego system for conditional inference. *Am. Statistician* 60 (3), 257–263. doi:10.1198/000313006x118430

Hutten, N., Arkell, T. R., Vinckenbosch, F., Schepers, J., Kevin, R. C., Theunissen, E. L., et al. (2022). Cannabis containing equivalent concentrations of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) induces less state anxiety than THC-dominant cannabis. *Psychopharmacol. Berl.* 239 (11), 3731–3741. doi:10.1007/s00213-022-06248-9

Jetly, R., Heber, A., Fraser, G., and Boisvert, D. (2015). The efficacy of nabilone, a synthetic cannabinoid, in the treatment of PTSD-associated nightmares: A preliminary randomized, double-blind, placebo-controlled cross-over design study. *Psychoneuroendocrinology* 51, 585–588. doi:10.1016/j.psyneuen.2014.11.002

Karanges, E. A., Suraev, A., Elias, N., Manocha, R., and McGregor, I. S. (2018). Knowledge and attitudes of Australian general practitioners towards medicinal cannabis: A cross-sectional survey. *BMJ Open* 8 (7), e022101. doi:10.1136/bmjopen-2018-022101

Kassambara, A. (2020). ggpubr: 'ggplot2' based publication ready plots. R package version 0.4.0. https://rpkgs.datanovia.com/ggpubr/.

Khan, R., Naveed, S., Mian, N., Fida, A., Raafey, M. A., and Aedma, K. K. (2020). The therapeutic role of cannabidiol in mental health: A systematic review. *J. Cannabis Res.* 2 (1), 2. doi:10.1186/s42238-019-0012-y

Kloiber, S., Matheson, J., Kim, H. K., and Le Foll, B. (2021). "Cannabinoid drugs in mental health disorders," in *NeuroPsychopharmacotherapy*, 1–35. doi:10.1007/978-3-319-56015-1_465-1

Laux, L. C., Bebin, E. M., Checketts, D., Chez, M., Flamini, R., Marsh, E. D., et al. (2019). Long-term safety and efficacy of cannabidiol in children and adults with treatment resistant Lennox-Gastaut syndrome or Dravet syndrome: Expanded access program results. *Epilepsy Res.* 154, 13–20. doi:10.1016/j.eplepsyres.2019.03.015

Lavender, I., McGregor, I. S., Suraev, A., Grunstein, R. R., and Hoyos, C. M. (2022). Cannabinoids, insomnia, and other sleep disorders. *Chest* 162 (2), 452–465. doi:10.1016/j.chest.2022.04.151

Leweke, F. M., Piomelli, D., Pahlisch, F., Muhl, D., Gerth, C. W., Hoyer, C., et al. (2012). Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl. Psychiatry* 2, e94. doi:10.1038/tp.2012.15

Linares, I. M., Zuardi, A. W., Pereira, L. C., Queiroz, R. H., Mechoulam, R., Guimaraes, F. S., et al. (2019). Cannabidiol presents an inverted U-shaped dose-response curve in a simulated public speaking test. *Braz. J. Psychiatry* 41 (1), 9–14. doi:10.1590/1516-4446-2017-0015

Lintzeris, N., Mills, L., Abelev, S. V., Suraev, A., Arnold, J. C., and McGregor, I. S. (2022). Medical cannabis use in Australia: Consumer experiences from the online cannabis as medicine survey 2020 (CAMS-20). *Harm Reduct. J.* 19 (1), 88. doi:10.1186/s12954-022-00666-w

Loflin, M., Earleywine, M., and Bonn-Miller, M. (2017). Medicinal versus recreational cannabis use: Patterns of cannabis use, alcohol use, and cued-arousal among veterans who screen positive for PTSD. *Addict. Behav.* 68, 18–23. doi:10.1016/j.addbeh.2017.01.008

MacPhail, S. L., Bedoya-Perez, M. A., Cohen, R., Kotsirilos, V., McGregor, I. S., and Cairns, E. A. (2022). Medicinal cannabis prescribing in Australia: An analysis of trends over the first five years. *Front. Pharmacol.* 13, 885655. doi:10.3389/fphar.2022.885655

Martin-Santos, R., Crippa, J. A., Batalla, A., Bhattacharyya, S., Atakan, Z., Borgwardt, S., et al. (2012). Acute effects of a single, oral dose of d9-tetrahydrocannabinol (THC) and cannabidiol (CBD) administration in healthy volunteers. *Curr. Pharm. Des.* 18 (32), 4966–4979. doi:10.2174/138161212802884780

Masataka, N. (2019). Anxiolytic effects of repeated cannabidiol treatment in teenagers with social anxiety disorders. *Front. Psychol.* 10, 2466. doi:10.3389/fpsyg.2019.02466

Mashiah, M. "Medical cannabis as a treatment for chronic combat PTSD," in Proceedings of the Promising results in an open pilot study Patients Out of Time Conference, Tucson, Arizona, USA, April 2012. https://maps.org/research-archive/presentations/Mashiah-MotiApril27.pdf.

McGuire, P., Robson, P., Cubala, W. J., Vasile, D., Morrison, P. D., Barron, R., et al. (2018). Cannabidiol (CBD) as an adjunctive therapy in schizophrenia: A multicenter randomized controlled trial. *Am. J. Psychiatry* 175 (3), 225–231. doi:10.1176/appi.ajp.2017.17030325

Medical Cannabis Clinicians' Society (2021). Recommendations and guidance on medical cannabis under prescription. https://www.ukmccs.org/wp-content/uploads/2021/03/Recommendations-and-Guidance-on-Medical-Cannabis-under-Prescription-3rd-Edition-2021-reduc.pdf.

Mitchell, J. M., Bogenschutz, M., Lilienstein, A., Harrison, C., Kleiman, S., Parker-Guilbert, K., et al. (2021). MDMA-Assisted therapy for severe PTSD: A randomized, double-blind, placebo-controlled phase 3 study. *Nat. Med.* 27 (6), 1025–1033. doi:10.1038/s41591-021-01336-3

Moore, D. S., and Kirkland, S. (2013). *The basic practice of statistics*. New York, NY, USA: W. H. Freeman.

National Academies of Sciences, E., and Medicine (2017). *The health effects of cannabis and cannabinoids: The current state of evidence and recommendations for research*. Washington, D.C., United States: National Academies Press.

National Institute for Health and Care Excellence (2019). Cannabis-based medicinal products. Retrieved 22 December 2022 from https://www.nice.org.uk/guidance/ng144

Nicholson, A. N., Turner, C., Stone, B. M., and Robson, P. J. (2004). Effect of Delta-9-tetrahydrocannabinol and cannabidiol on nocturnal sleep and early-morning behavior in young adults. *J. Clin. Psychopharmacol.* 24 (3), 305–313. doi:10.1097/01.jcp.0000125688.05091.8f

Nutt, D. (2019). Psychedelic drugs-a new era in psychiatry? *Dialogues Clin. Neurosci.* 21 (2), 139–147. doi:10.31887/DCNS.2019.21.2/dnutt

Onaemo, V. N., Fawehinmi, T. O., and D'Arcy, C. (2021). Comorbid cannabis use disorder with major depression and generalized anxiety disorder: A systematic review with meta-analysis of nationally representative epidemiological surveys. *J. Affect Disord.* 281, 467–475. doi:10.1016/j.jad.2020.12.043

Orsolini, L., Chiappini, S., Volpe, U., Berardis, D., Latini, R., Papanti, G. D., et al. (2019). Use of medicinal cannabis and synthetic cannabinoids in post-traumatic stress disorder (PTSD): A systematic review. *Med. Kaunas.* 55 (9), 525. doi:10.3390/medicina55090525

Pasman, J. A., Verweij, K. J. H., Gerring, Z., Stringer, S., Sanchez-Roige, S., Treur, J. L., et al. (2018). GWAS of lifetime cannabis use reveals new risk loci, genetic overlap with psychiatric traits, and a causal influence of schizophrenia. *Nat. Neurosci.* 21 (9), 1161–1170. doi:10.1038/s41593-018-0206-1

Passie, T., Emrich, H. M., Karst, M., Brandt, S. D., and Halpern, J. H. (2012). Mitigation of post-traumatic stress symptoms by cannabis resin: A review of the clinical and neurobiological evidence. *Drug Test. Analysis* 4 (7-8), 649–659. doi:10.1002/dta.1377

Pauli, C. S., Conroy, M., Vanden Heuvel, B. D., and Park, S. H. (2020). Cannabidiol drugs clinical trial outcomes and adverse effects. *Front. Pharmacol.* 11, 63. doi:10.3389/fphar.2020.00063

Quinn, H. R., Matsumoto, I., Callaghan, P. D., Long, L. E., Arnold, J. C., Gunasekaran, N., et al. (2008). Adolescent rats find repeated Delta(9)-THC less aversive than adult rats but display greater residual cognitive deficits and changes in hippocampal protein expression following exposure. *Neuropsychopharmacology* 33 (5), 1113–1126. doi:10.1038/sj.npp.1301475

R Core Team (2022). "R: A language and environment for statistical computing," in *R foundation for statistical computing* (Vienna, Austria: R Core Team). https://www.R-project.org/.

Ramar, K., Rosen, I. M., Kirsch, D. B., Chervin, R. D., Carden, K. A., Aurora, R. N., et al. (2018). Medical cannabis and the treatment of obstructive sleep apnea: An American Academy of sleep medicine position statement. *J. Clin. Sleep. Med.* 14 (4), 679–681. doi:10.5664/jcsm.7070

Rehman, Y., Saini, A., Huang, S., Sood, E., Gill, R., and Yanikomeroglu, S. (2021). Cannabis in the management of PTSD: A systematic review. *AIMS Neurosci.* 8 (3), 414–434. doi:10.3934/Neuroscience.2021022

Roitman, P., Mechoulam, R., Cooper-Kazaz, R., and Shalev, A. (2014). Preliminary, open-label, pilot study of add-on oral Δ9-tetrahydrocannabinol in chronic post-traumatic stress disorder. *Clin. Drug Investig.* 34 (8), 587–591. doi:10.1007/s40261-014-0212-3

Rosenblat, J. D., Carvalho, A. F., Li, M., Lee, Y., Subramanieapillai, M., and McIntyre, R. S. (2019). Oral ketamine for depression: A systematic review. *J. Clin. Psychiatry* 80 (3), 18r12475. doi:10.4088/JCP.18r12475

Sakal, C., Lynskey, M., Schlag, A. K., and Nutt, D. J. (2022). Developing a real-world evidence base for prescribed cannabis in the United Kingdom: Preliminary findings from project Twenty21. *Psychopharmacol. Berl.* 239 (5), 1147–1155. doi:10.1007/s00213-021-05855-2

Sarris, J., Sinclair, J., Karamacoska, D., Davidson, M., and Firth, J. (2020). Medicinal cannabis for psychiatric disorders: A clinically-focused systematic review. *BMC Psychiatry* 20 (1), 24. doi:10.1186/s12888-019-2409-8

Scherma, M., Muntoni, A. L., Riedel, G., Fratta, W., and Fadda, P. (2020). Cannabinoids and their therapeutic applications in mental disorders. *Dialogues Clin. Neurosci.* 22 (3), 271–279. doi:10.31887/DCNS.2020.22.3/pfadda

Shannon, S., Lewis, N., Lee, H., and Hughes, S. (2019). Cannabidiol in anxiety and sleep: A large case series. *Perm. J.* 23, 18–041. doi:10.7812/TPP/18-041

Shannon, S., and Opila-Lehman, J. (2016). Effectiveness of cannabidiol oil for pediatric anxiety and insomnia as part of posttraumatic stress disorder: A case report. *Perm. J.* 20 (4), 16–005. doi:10.7812/TPP/16-005

Spinella, T. C., Stewart, S. H., Naugler, J., Yakovenko, I., and Barrett, S. P. (2021). Evaluating cannabidiol (CBD) expectancy effects on acute stress and anxiety in healthy adults: A randomized crossover study. *Psychopharmacol. Berl.* 238 (7), 1965–1977. doi:10.1007/s00213-021-05823-w

Stanciu, C. N., Brunette, M. F., Teja, N., and Budney, A. J. (2021). Evidence for use of cannabinoids in mood disorders, anxiety disorders, and PTSD: A systematic review. *Psychiatr. Serv.* 72 (4), 429–436. doi:10.1176/appi.ps.202000189

Steardo, L., Jr., Carbone, E. A., Menculini, G., Moretti, P., Steardo, L., and Tortorella, A. (2021). Endocannabinoid system as therapeutic target of PTSD: A systematic review. *Life (Basel)* 11 (3), 214. doi:10.3390/life11030214

Stephenson, C. P., Karanges, E., and McGregor, I. S. (2013). Trends in the utilisation of psychotropic medications in Australia from 2000 to 2011. *Aust. N. Z. J. Psychiatry* 47 (1), 74–87. doi:10.1177/0004867412466595

Stuart-Maver, S. L. (2020). Working with clients who self-medicate using cannabis: Ethical and clinical considerations for psychologists. *Prof. Psychol. Res. Pract.* 51 (1), 77–84. doi:10.1037/pro0000269

Suraev, A., McGregor, I., Marshall, N., D'Rozario, A., Kao, C., Gordon, C., et al. (2022). O006 acute effects of combined cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) in insomnia disorder: A randomised, placebo-controlled trial using high-density eeg. *Sleep. Adv.* 3, A3–A4. doi:10.1093/sleepadvances/zpac029.005

Suraev, A. S., Marshall, N. S., Vandrey, R., McCartney, D., Benson, M. J., McGregor, I. S., et al. (2020). Cannabinoid therapies in the management of sleep disorders: A systematic review of preclinical and clinical studies. *Sleep. Med. Rev.* 53, 101339. doi:10.1016/j.smrv.2020.101339

Telch, M. J., Fischer, C. M., Zaizar, E. D., Rubin, M., and Papini, S. (2022). Use of Cannabidiol (CBD) oil in the treatment of PTSD: Study design and rationale for a placebo-controlled randomized clinical trial. *Contemp. Clin. Trials* 122, 106933. doi:10.1016/j.cct.2022.106933

Therapeutic Goods Administration (2019). Medicinal cannabis - guidance documents. https://www.tga.gov.au/medicinal-cannabis-guidance-documents.

Therapeutic Goods Administration (2022a). Medicinal cannabis authorised prescriber scheme data. Retrieved 6 May 2023 from https://www.tga.gov.au/products/unapproved-therapeutic-goods/medicinal-cannabis-hub/medicinal-cannabis-access-pathways-and-patient-access-data/medicinal-cannabis-authorised-prescriber-scheme-data

Therapeutic Goods Administration (2022b). Medicinal cannabis products by active ingredients. Retrieved 22 December 2022 from https://www.tga.gov.au/medicinal-cannabis-products-active-ingredient

Thoen, E. (2020). padr: Quickly get datetime data ready for analysis. https://github.com/EdwinTh/padr.

Thomas, K., Malcolm, B., and Lastra, D. (2017). Psilocybin-assisted therapy: A review of a novel treatment for psychiatric disorders. *J. Psychoact. Drugs* 49 (5), 446–455. doi:10.1080/02791072.2017.1320734

Tibbo, P. G., McKee, K. A., Meyer, J. H., Crocker, C. E., Aitchison, K. J., Lam, R. W., et al. (2021). Are there therapeutic benefits of cannabinoid products in adult mental illness? *Can. J. Psychiatry* 66 (2), 185–194. doi:10.1177/0706743720945525

Trezza, V., Cuomo, V., and Vanderschuren, L. J. (2008). Cannabis and the developing brain: Insights from behavior. *Eur. J. Pharmacol.* 585 (2-3), 441–452. doi:10.1016/j.ejphar.2008.01.058

Turna, J., Syan, S. K., Frey, B. N., Rush, B., Costello, M. J., Weiss, M., et al. (2019). Cannabidiol as a novel candidate alcohol use disorder pharmacotherapy: A systematic review. *Alcohol Clin. Exp. Res.* 43 (4), 550–563. doi:10.1111/acer.13964

Vaissie, P., Monge, A., and Husson, F. (2021). Factoshiny: Perform factorial analysis from 'FactoMineR' with a shiny application. https://CRAN.R-project.org/package=Factoshiny.

Venables, W. N., and Ripley, B. D. (2002). *Modern applied statistics with S*. 4 ed. Berlin, Germany: Springer.

Vickery, A. W., Roth, S., Ernenwein, T., Kennedy, J., and Washer, P. (2022). A large Australian longitudinal cohort registry demonstrates sustained safety and efficacy of oral medicinal cannabis for at least two years. *PLoS One* 17 (11), e0272241. doi:10.1371/journal.pone.0272241

Walsh, J. H., Maddison, K. J., Rankin, T., Murray, K., McArdle, N., Ree, M. J., et al. (2021). Treating insomnia symptoms with medicinal cannabis: A randomized, crossover trial of the efficacy of a cannabinoid medicine compared with placebo. *Sleep* 44 (11), zsab149. doi:10.1093/sleep/zsab149

Ware, M. A., Fitzcharles, M. A., Joseph, L., and Shir, Y. (2010). The effects of nabilone on sleep in fibromyalgia: Results of a randomized controlled trial. *Anesth. Analg.* 110 (2), 604–610. doi:10.1213/ANE.0b013e3181c76f70

Weiland, B. J., Thayer, R. E., Depue, B. E., Sabbineni, A., Bryan, A. D., and Hutchison, K. E. (2015). Daily marijuana use is not associated with brain morphometric measures in adolescents or adults. *J. Neurosci.* 35 (4), 1505–1512. doi:10.1523/JNEUROSCI.2946-14.2015

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., et al. (2019). Welcome to the tidyverse. *J. Open Source Softw.* 4 (43), 1686. doi:10.21105/joss.01686

Wickham, H., François, R., Henry, L., and Müller, K. (2021). dplyr: a grammar of data manipulation. https://dplyr.tidyverse.org/reference/dplyr-package.html.

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Berlin, Germany: Springer.

Wilke, C. (2020). cowplot: streamlined plot theme and plot annotations for 'ggplot2'. https://wilkelab.org/cowplot/.

Wilkinson, S. T., Stefanovics, E., and Rosenheck, R. A. (2015). Marijuana use is associated with worse outcomes in symptom severity and violent behavior in patients with posttraumatic stress disorder. *J. Clin. Psychiatry* 76 (9), 1174–1180. doi:10.4088/JCP.14m09475

Zalai, D. C., Hussain, N., and Shapiro, C. M. (2015). Does cannabinoid really improve sleep? Testing the sleep effects of nabilone in chronic pain patients: A placebo-controlled, randomized, pilot study. *Psychother. Psychosom.* 84, 81. doi:10.1159/000438780

Zamarripa, C. A., Spindle, T. R., Surujunarain, R., Weerts, E. M., Bansal, S., Unadkat, J. D., et al. (2023). Assessment of orally administered d9-tetrahydrocannabinol when coadministered with cannabidiol on d9-tetrahydrocannabinol pharmacokinetics and pharmacodynamics in healthy adults: A randomized clinical trial. *JAMA Netw. Open* 6 (2), e2254752. doi:10.1001/jamanetworkopen.2022.54752

Zuardi, A., Crippa, J., Dursun, S., Morais, S., Vilela, J., Sanches, R., et al. (2010). Cannabidiol was ineffective for manic episode of bipolar affective disorder. *J. Psychopharmacol.* 24 (1), 135–137. doi:10.1177/0269881108096521

frontiers | Frontiers in Pharmacology

TYPE Opinion
PUBLISHED 22 September 2023
DOI 10.3389/fphar.2023.1210202

OPEN ACCESS

EDITED BY
Francesca Baratta,
University of Turin, Italy

REVIEWED BY
Gonzalo Recabarren-Gajardo,
Pontificia Universidad Católica de Chile, Chile

*CORRESPONDENCE
Daniel Rojas-Valverde,
drojasv@hotmail.com

RECEIVED 21 April 2023
ACCEPTED 13 September 2023
PUBLISHED 22 September 2023

CITATION
Rojas-Valverde D and Fallas-Campos A (2023), Cannabidiol in sports: insights on how CBD could improve performance and recovery.
Front. Pharmacol. 14:1210202.
doi: 10.3389/fphar.2023.1210202

Cannabidiol in sports: insights on how CBD could improve performance and recovery

Daniel Rojas-Valverde[1,2]* and Andrea Fallas-Campos[3]

[1]Sport Injury Clinic (Rehab Readapt), Escuela Ciencias del Movimiento Humano y Calidad de Vida (CIEMHCAVI), Universidad Nacional, Heredia, Costa Rica, [2]Núcleo de Estudios para el Alto Rendimiento y la Salud (CIDISAD-NARS), Escuela Ciencias del Movimiento Humano y Calidad de Vida (CIEMHCAVI), Universidad Nacional, Heredia, Costa Rica, [3]Núcleo de Estudios para el Alto Rendimiento y la Salud (ACUAUNA-NARS), Escuela Ciencias del Movimiento Humano y Calidad de Vida (CIEMHCAVI), Universidad Nacional, Heredia, Costa Rica

KEYWORDS

cannabis, THC, rest, stress, training, anti-inflammatory, ergogenic aid

What is cannabidiol (CBD)?

Cannabidiol is popularly known as CBD, a substance that is part of the cannabinoids, chemical components extracted from the cannabis or hemp plant. Of all the chemical substances extracted from cannabis, some are legal, and others are not. CBD's consumption, sale, and distribution are permitted and legal in some countries worldwide, such as the United States, Spain, Germany, China, Uruguay, Costa Rica, and Morocco.

The World Anti-Doping Agency, the institution controlling prohibited substances in sports worldwide, has accepted CBD among professional athletes (Nichols and Kaplan, 2019). Normally, CBD can be consumed in multiple products, in drops of oil, processed foods, drinks and other products (Lim et al., 2020) that athletes can find in a supermarket or specialised sports store. For this reason and its apparent benefits, the consumption of CBD has increased significantly among athletes (Docter et al., 2020). This has fueled a race to study its properties, benefits and risks for the health and performance of athletes.

Coaches, athletes, doctors, therapists, and scientists are constantly concerned with finding ways to improve the performance of athletes by making athletes faster, more resistant, more agile, rest and recover better from efforts and feel better. Athletes try a series of substances, technologies, and training methodologies to win (Bampouras et al., 2012). In the case of CBD, the studies that have been carried out so far are insufficient to adjudicate ergogenic, ergolytic, and there is a lack of experimentation in humans, especially in its effects on athletes and physically active people (Kennedy, 2017; Maurer et al., 2020; McCartney et al., 2020; Burr et al., 2021; Rojas-Valverde, 2021). Despite this lack of knowledge on the effects on athlete's performance and health, based on its impact on other populations and health problems, some potential benefits should be more in-depth analysed.

Based on what is currently known, CBD has potential benefits and properties that could help the athlete feel better when facing competition (Kennedy, 2017; McCartney et al., 2020; Rojas-Valverde, 2021). Among these benefits, the consumption of CBD could make athletes rest better (e.g., improve sleep latency, sleep continuity, subjective sleep quality and reduce nightmares and insomnia) (Russo et al., 2007; Choi et al., 2020; Mondino et al., 2021; Ranum et al., 2023), reduce their stress and feel better in the face of competition and training (anxiolytic and antidepressant) (Narayan et al., 2022), can deflate their muscles after the damage caused by physical exertion (anti-inflammatory) (Kennedy, 2017; Gamelin et al., 2020; Villanueva et al., 2022; Stone et al., 2023), and reduce pain caused by high physical

demands (pain and soreness reliever) (see Figure 1) (Kennedy, 2017; Gamelin et al., 2020; Henson et al., 2022).

What causes CBD in the body of athletes?

CBD is a natural substance that causes changes and alterations at the physiological and cognitive (mental and emotional) levels (Stout and Cimino, 2014). These changes appear because CBD influences the function of an endocannabinoid system, which is responsible for maintaining homeostasis (Nichols and Kaplan, 2019). This system participates in processes related to neurogenesis, brain plasticity, control mode, dopamine release, and fatty acid hydrolase release. These functions, therefore, regulate how we feel emotionally, how the brain learns and multiplies its nerve connection networks, controls inflammation (anti-inflammatory) and how we perceive pain (analgesic) (Rojas-Valverde, 2021). CBD intake increases oxygen consumption and pleasure ratings during endurance running (Sahinovic et al., 2022). Also, preclinical studies have shown how CBD could protect myocardial injury during intense exercise, demonstrating anti-inflammatory, anti-apoptosis and antioxidative stress effects (Zhang et al., 2022).

The cannabis system enables numerous effects during physical exertion, including sensations of joy, calm, and euphoria (Carek et al., 2011). Endocannabinoids, such as anandamide and 2-arachidonoylglycerol (2AG), behave as cannabinoids by activating cannabinoid receptors called type-1 (CB1) and type-2 (CB2) receptors. These molecules, comparable to N-acyl ethanolamine's (De Petrocellis and Di Marzo, 2009), generate benefits similar to exercises, such as hunger control, inflammation reduction, anxiety relief, and prevention of excessive cell proliferation. CBD inhibits the degradation and absorption of endocannabinoids such as anandamide, increasing endocannabinoids' binding to their receptors. CB1 receptors are located in the central nervous system, whereas CB2 receptors are found in the peripheral nervous system.

Ccannabinoids and endocannabinoids are required for the release of brain-derived neurotrophic factor, which aids in processes such as neurogenesis and neural plasticity. They also play a role in releasing glucocorticoids, which help regulate mood by alleviating symptoms of melancholy and anxiety. Cannabis also stimulates dopamine release, resulting in a sensation of pleasure. Furthermore, they are linked to fatty acid amide hydrolase release, which results in analgesic effects. Notably, these reactions are consistent with the beneficial effects of exercise (Tantimonaco et al., 2014). Stimuli that activate TRPV1 ion channels (Vanilloid receptors) cause these actions, which result in antinociceptive effects (Gochman et al., 2023). Stimuli targeting CB1 and CB2 receptors elicit relaxation through neurodepression and cytokine release inhibition, respectively (Jean-Gilles et al., 2015). Furthermore, the stimulation of 5HT1A receptors promotes serotonin absorption in postsynaptic neurons, which helps to regulate mood states (Resstel et al., 2009). Figure 1 is an in-depth representation of the potential physiological pathways and the interactions between enzymes and receptors with CBD in the human body.

New evidence has suggested that in humans, CBD intake could improve satellite cell differentiation in muscles, improving muscle recovery (e.g., muscle damage attenuation) and performance (e.g., strength) (Schouten et al., 2022). Also, recent findings demonstrate modest yet meaningful effects on muscle damage and recovery (reduction in creatine kinase and myoglobin) within a 72-h after 60 mg of CBD supplementation (Isenmann et al., 2021). The evidence is contradictory in this sense, and the debate is more open than ever (Cochrane-Snyman et al., 2021; Crossland et al., 2022; Stone et al., 2023), which is why more quantity, quality and variety of specific studies on sport and exercise are necessary. This recent data gives promissory insights on using CBD as a performance enhancer and recovery aid, even though serious doubts about its use (e.g., dose administration) and safety must be carefully addressed.

CBD to improve sleep quality

Athletes frequently overreact because of high training loads and inadequate recovery between efforts. These conditions can cause sleep disturbances or moments in which the athlete cannot rest comfortably, impacting sleep quality or recovery. CBD appears to regulate the cycle in which the body stays awake or asleep, which is essential for an athlete's recovery (Burstein, 2015; Hill et al., 2017). One of the advantages of CBD consumption is its potential to enhance sleep in athletes. This includes improvements in sleep initiation, uninterrupted sleep, subjective sleep quality, as well as a reduction in nightmares and insomnia symptoms (Russo et al., 2007; Choi et al., 2020; Mondino et al., 2021; Ranum et al., 2023). In addition, some substances promote sleep controlled by the endocannabinoid system, which we can activate by consuming CBD (McCartney et al., 2020; Rojas-Valverde, 2021).

Sleep management requires a precise balance of neurotransmitters, and CBD's actions on the endocannabinoid system contribute to this balance. CBD interacts with adenosine receptors, which is significant since adenosine is a neurotransmitter that promotes sleep and relaxation. CBD promotes tranquillity and preparedness for sleep by boosting adenosine signalling. Furthermore, CBD's effect on GABAergic neurotransmission adds to its sleep-enhancing properties (Kesner and Lovinger, 2020; Kaul et al., 2021). GABA is an inhibitory neurotransmitter that promotes relaxation and drowsiness by lowering neuronal excitability. CBD's effect on GABA receptors can promote deeper, more comfortable sleep. Furthermore, CBD's ability to relieve anxiety and stress, which are significant causes of sleep disruption, indirectly supports greater sleep quality (Blessing et al., 2015; Moltke and Hindocha, 2021; Ortiz Rios et al., 2022). CBD provides a biological foundation for its action via modifying endocannabinoid system signalling, increasing adenosine effects, and regulating GABAergic neurotransmission (Zou & Kumar, 2018; Yarar, 2020; Martinez Naya et al., 2023).

CBD to reduce stress and regulate mood

Usually, due to athlete's significant effort during their sports practice, they suffer from fatigue, which can lead them to situations

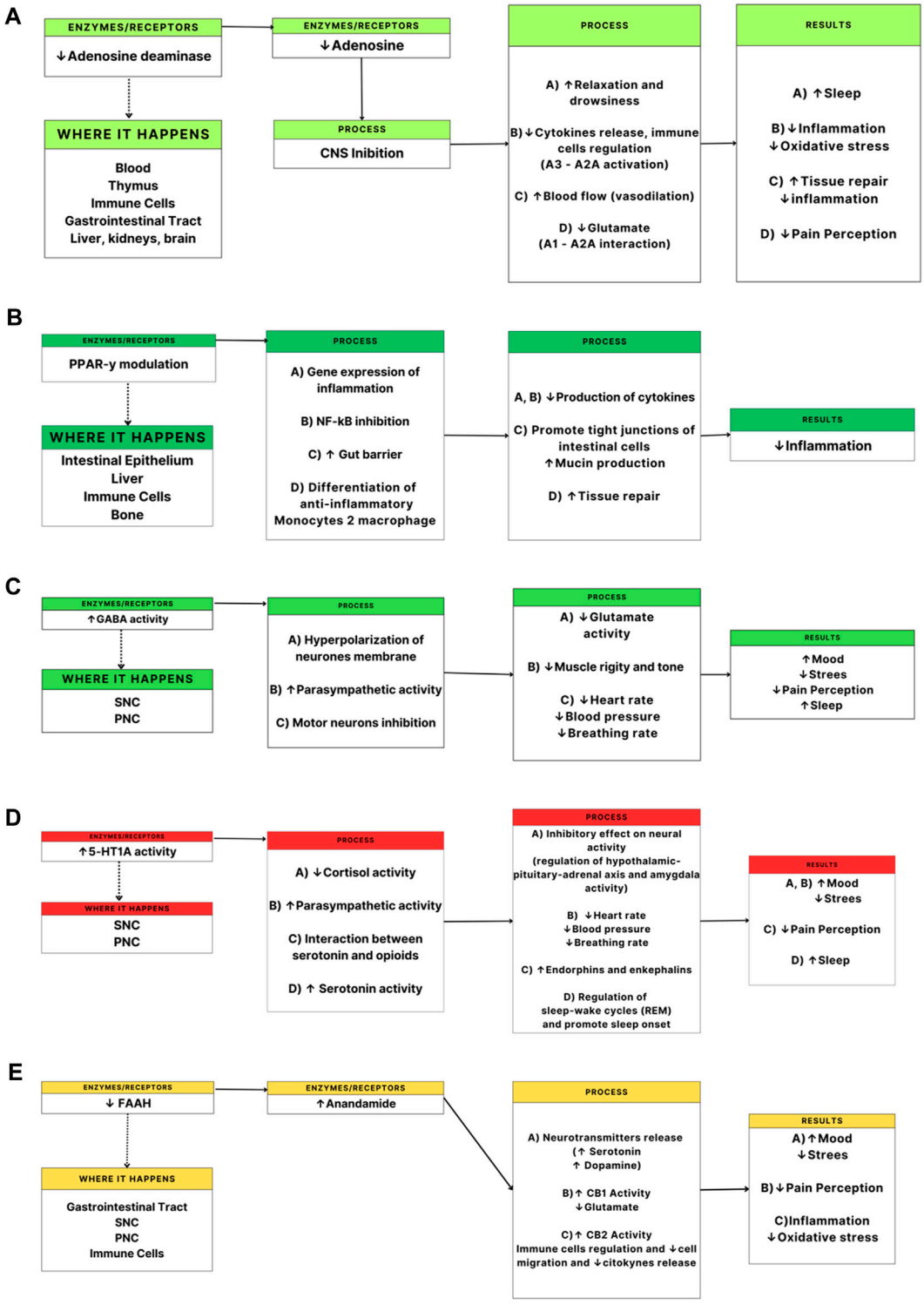

FIGURE 1
Potential benefits of cannabidiol (CBD) intake on sports performance and recovery: physiological pathways and enzymes and receptors interaction. **(A)** adenosine deaminase, **(B)**. PPAR-*y*: gamma peroxisome proliferator-activated receptors, **(C)**. GABA: gamma-aminobutyric acid, **(D)**. 5-HT1A: 5-hydroxytryptamine receptor 1A, **(E)**. FAAH: fatty acid amide hydrolase. CNS: central nervous system, PNS: peripheral nervous system.

where they do not feel very well emotionally. The ability of CBD to regulate the athlete's mood is being studied (Kasper et al., 2020).

CBD can boost anandamide signalling, an endocannabinoid related to emotions of wellbeing, by preventing its absorption and breakdown, resulting in higher levels in the brain (Leweke et al., 2012; Henson et al., 2022). CBD has also been demonstrated to interact with serotonin receptors, including the 5-HT1A receptor, which regulates mood. Research findings indicate that CBD has been

found to decrease anxiety levels by activating the 5-HT1A receptors and restoring impaired neurotransmission of the 5-HT1A (serotonin) system (De Gregorio et al., 2019). CBD can help serotonin transmission by attaching to these receptors. Serotonin is a neurotransmitter that is directly tied to mood and emotions. Furthermore, CBD has been shown to influence the hypothalamic-pituitary-adrenal axis, a critical mechanism in the body's stress response. CBD reduces stress response by inhibiting the production of stress hormones such as cortisol. Overall, CBD's capacity to modify endocannabinoid system function, increase anandamide signalling, interact with serotonin receptors, and influence stress hormone release all contribute to its potential for pain relief (Viudez-Martínez et al., 2018; Yarar, 2020; Lookfong et al., 2023).

CBD effects on anxiety seem to depend on dosage; 300 mg is more effective than 150 or 600 mg for reducing anxiety-related symptoms (Linares et al., 2019). There is no evidence of reduced anxiety or mood regulation in sports. Still, it seems that CBD could have certain properties that can be anxiolytic and anti-depressive (Murillo-Rodríguez et al., 2020) that some athletes suffer due to the pressure they always have to be better and win, as well as the frustration they may suffer from not achieving certain goals (McCartney et al., 2020; Rojas-Valverde, 2021).

CBD to reduce inflammation and oxidative stress

Inflammation and oxidative stress are two processes that intervene in people's general health (McPartland et al., 2015). These two processes are normally triggered after exercise in athletes, and as we can control them, the athlete will feel more recovered and be more prepared to exert effort again. Inflammation is caused because, during exercise, the muscles suffer tension that causes damage, and by becoming inflamed, the body initiates the processes to repair that damage (McCartney et al., 2020; Rojas-Valverde, 2021).

Inflammation is necessary to recover from significant efforts. Still, excess inflammation could cause problems in our digestive and musculoskeletal systems and other systems due to the damage to tissues and organs that this causes (McCartney et al., 2020); that is why controlling it is optimal. CBD in athletes could regulate inflammatory processes by reducing substances that usually cause unwanted increases in inflammation, such as cytokines and cortisol (Zuardi et al., 1993). In addition to muscle and digestive inflammation, CBD reduces oxidative stress and neuroinflammation (Atalay et al., 2019; Sahinovic et al., 2022). In this regard, 300 mg of CBD has been shown to induce glucocorticoid regulation, such as cortisol in humans, a key regulator of the inflammatory response to injury (Zuardi et al., 1993).

Based on recent evidence, 10 mg/kg of CBD could attenuate inflammation (e.g., IL-6, IL-1 and tumour necrosis factor α) after fatiguing eccentric exercise by activating cannabinoid receptor two (Stone et al., 2023). This is based on CBD's interactions with inflammation-controlling receptors (CB1 cannabinoid, CB2 cannabinoid, adenosine A2A), its cytokine level-reducing actions, and its moderation of immune cell activity, thus mitigating collateral tissue inflammation (Booz, 2011; Burstein, 2015; A. J; Hill et al., 2012). Moreover, CBD's potential to enhance the release of arachidonic acid could improve healing by regulating growth signals mediated by pro-resolving substances (e.g., lipoxin A4 and 15d-PGJ2) (Burstein, 2015).

CBD to reduce the pain

CBD appears to have analgesic properties and bone that can decrease pain (Marques Azzini et al., 2023). Due to exercise, athletes usually feel pain from the effort and the damage caused to their bodies when they reach the limit. Running, pedalling, jumping, changing directions, hitting, and kicking generate muscle breakdown that causes inflammation, which can become painful.

For example, Sativex, THC, and CBD have been licensed to treat central and peripheral neuropathic pain. This pain condition is linked to activated microglia and a subsequent cascade of proinflammatory cytokines, including IL-6, IL-1, and TNF (Booz, 2011). In addition to its neuroprotective properties, this effect was discovered in a recent systematic analysis of the result of CBD consumption in connection to its prospective usage as a performance-enhancing agent (McCartney et al., 2020). It is currently unknown how CBD interacts with the pain cascade and pathways (Anthony et al., 2020). Still, it is suggested that serotonin and opioid interactions could have a great role in endorphins and enkephalins release and reduction of glutamate release via the interaction of adenosine 1 and A2A, leading to pain reduction (Navarrete et al., 2021; Peng et al., 2022). CBD has demonstrated its ability to cure and control pain in illnesses and pain disorders, and based on this information, CBD appears to have a possible effect on reducing swelling and avoiding soreness after hard activity (Sahinovic et al., 2022), but further research is needed to make a definitive declaration.

CBD, in a specific manner, interferes with neuronal communication, preventing the transmission of information related to pain (e.g., inhibition of neurotransmitter activity). As a result, the pain sensation is not perceived as it typically would be (McCartney et al., 2020; Rojas-Valverde, 2021). There is evidence of using CBD for chronic and acute pain management (Alaia et al., 2022; Marques Azzini et al., 2023). CBD can promote analgesia by activating transient receptor potential cation channel subfamily V (TRPV1) and serotonin receptors (Naik and Trojian, 2021). The latest scientific data found a pain-reliever effect of topical application (2*10 mg/day) of CBD in elite athletes with only minor side effects (e.g., dry skin) (Hall et al., 2023).

What care should we have, and what remains to be demonstrated scientifically?

We must be careful to consume CBD products that official health institutions approve. Because CBD is illegal in certain countries, it is normal to find products with other substances that can cause unwanted side effects or could represent a legal issue for athletes. Concerns around athlete doping are raised because certain CBD products include THC and other cannabinoids (Hazekamp, 2018; Evans, 2020; Johnson et al., 2022). When utilising CBD products, athletes should take caution and make

sure they are using reliable, independently tested goods that verify there is no THC or other illegal cannabinoids present.

In addition, it is important to consider that CBD is unlike any other food, so the amount we consume must be regulated. Scientists are still unsure how much dose is needed to cause certain reactions in the body (McCartney et al., 2020; Rojas-Valverde, 2021). Also, recent evidence in humans still shows highly variable dosing and methodological concerns that should be addressed when consuming CBD products (Schouten et al., 2022). In exercise and sport-related evidence, the dose could be a key in finding performance or recovery benefits. For example, 2 and 5 mg/kg seem ineffective for these purposes, but 10 mg/kg is (Crossland et al., 2022; Stone et al., 2023), even higher doses of CBD (25 mg/kg) seem secure for consumption in humans and its effects could be studied in future studies (Grotenhermen et al., 2017). Also, the drug-drug interaction of CBD with other drugs should be explored when used for athletic purposes (Lopera et al., 2022). When discussing and advocating the use of CBD, professionals working with the sports community must consider any potential legal, medical, and ethical concerns.

Future research recommendations

With the growing interest in the use of CBD in athlete recovery, more research is warranted to understand its physiological mechanism of action, potential benefits, and intended safety and efficacy profile when consuming CBD before, during, and after training or competition. Future sports science and medicine research should focus on understanding the role of CBD in physiological mechanisms such as the inflammatory cascade, neuroprotection, analgesic and anxiolytic pathways, muscular enhancement, and neuromechanical function.

New randomised controlled trials with a placebo should consider different fatigue and damage etiologies, individualities, disciplines, needs and special characteristics. Other potential research areas include optimal dosing based on physical and physiological load, efficacy concerning administration timing, chronic and acute effects, cumulative responses with different recovery strategies, differences in tolerance and effectiveness by sex, professional level, fitness level, and other individual conditions and situational factors. Furthermore, more information is needed to understand CBD's inflammatory signalling as an essential factor in the recovery process. The effectiveness of CBD compared to conventional medications should be evaluated.

Conclusion

CBD appears to have anti-inflammatory, neuroprotective, analgesic, anxiolytic, and potentially recovery-mediating properties in athletes, but more scientific evidence is needed to confirm these effects. Confirmatory analyses using randomised controlled trials with placebo are necessary to test the acute and chronic effects of different dosage prescriptions. These studies must consider fundamental sport-specific particularities, such as the diverse biological and situational conditions that contribute to fatigue, the characteristics of each discipline during training and competition, the individual peculiarities of athletes, their tolerance and response to CBD intake, and the combined effect of CBD administration with other physical and nutritional aids.

Given the relatively common use of cannabis and CBD among athletes, there is a clear need to improve the scientific understanding of the effects of CBD use on athlete recovery and performance. Further scientific progress is necessary, primarily through the execution of experimental trials, to better understand critical positive and negative outcomes for the ultimate benefit of athlete recovery and performance. Furthermore, resulting evidence could provide new clinical guidance for prescribing CBD during the athlete recovery process and other potential applications. The potential therapeutic benefits of CBD administration have been minimised for years, but the actual scenario could increase knowledge about this natural compound and its effects. Additionally, from an administrative point of view, adopting a clearer and more global policy for the use of cannabis in sports should be considered.

Author contributions

DR-V conceived the idea and wrote the first draft of the manuscript. DR-V and AF-C contributed equally to the critical review and final approval of the manuscript.

Funding

Vicerectory of Research of the National University of Costa Rica.

Conflict of interest

The 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.

Publisher's note

References

Alaia, M. J., Hurley, E. T., Vasavada, K., Markus, D. H., Britton, B., Gonzalez-Lomas, G., et al. (2022). Buccally absorbed cannabidiol shows significantly superior pain control and improved satisfaction immediately after arthroscopic rotator cuff repair: A placebo-controlled, double-blinded, randomized trial. *Am. J. Sports Med.* 50 (11), 3056–3063. doi:10.1177/03635465221109573

Anthony, A. T., Rahmat, S., Sangle, P., Sandhu, O., and Khan, S. (2020). Cannabinoid receptors and their relationship with chronic pain: A narrative review. *Cureus* 12 (9), e10436. doi:10.7759/cureus.10436

Atalay, S., Jarocka-Karpowicz, I., and Skrzydlewska, E. (2019). Antioxidative and anti-inflammatory properties of cannabidiol. *Antioxidants (Basel, Switz.* 9 (1), 21. doi:10.3390/antiox9010021

Bampouras, M. T., Cronin, C., and Miller, K. P. (2012). Performance analytic processes in elite sport practice: An exploratory investigation of the perspectives of a sport scientist, coach and athlete. *Int. J. Perform. Analysis Sport* 12 (2), 468–483. doi:10.1080/24748668.2012.11868611

Blessing, E. M., Steenkamp, M. M., Manzanares, J., and Marmar, C. R. (2015). Cannabidiol as a potential treatment for anxiety disorders. *Neurotherapeutics* 12 (4), 825–836. doi:10.1007/s13311-015-0387-1

Booz, G. W. (2011). Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress. *Free Radic. Biol. Med.* 51 (5), 1054–1061. doi:10.1016/j.freeradbiomed.2011.01.007

Burr, J. F., Cheung, C. P., Kasper, A. M., Gillham, S. H., and Close, G. L. (2021). Cannabis and athletic performance. *Sports Med. Auckl. N.Z.)* 51 (1), 75–87. doi:10.1007/s40279-021-01505-x

Burstein, S. (2015). Cannabidiol (CBD) and its analogs: A review of their effects on inflammation. *Bioorg. Med. Chem.* 23 (7), 1377–1385. doi:10.1016/j.bmc.2015.01.059

Carek, P. J., Laibstain, S. E., and Carek, S. M. (2011). Exercise for the treatment of depression and anxiety. *Int. J. Psychiatry Med.* 41 (1), 15–28. doi:10.2190/PM.41.1.c

Choi, S., Huang, B. C., and Gamaldo, C. E. (2020). Therapeutic uses of cannabis on sleep disorders and related conditions. *J. Clin. Neurophysiology* 37 (1), 39–49. doi:10.1097/WNP.0000000000000617

Cochrane-Snyman, K. C., Cruz, C., Morales, J., and Coles, M. (2021). The effects of cannabidiol oil on noninvasive measures of muscle damage in men. *Med. Sci. Sports Exerc.* 53 (7), 1460–1472. doi:10.1249/MSS.0000000000002606

Crossland, B. W., Rigby, B. R., Duplanty, A. A., King, G. A., Juma, S., Levine, N. A., et al. (2022). Acute supplementation with cannabidiol does not attenuate inflammation or improve measures of performance following strenuous exercise. *Healthc. (Basel, Switz.* 10 (6), 1133. doi:10.3390/healthcare10061133

De Gregorio, D., McLaughlin, R. J., Posa, L., Ochoa-Sanchez, R., Enns, J., Lopez-Canul, M., et al. (2019). Cannabidiol modulates serotonergic transmission and reverses both allodynia and anxiety-like behavior in a model of neuropathic pain. *Pain* 160 (1), 136–150. doi:10.1097/j.pain.0000000000001386

De Petrocellis, L., and Di Marzo, V. (2009). An introduction to the endocannabinoid system: From the early to the latest concepts. *Best Pract. Res. Clin. Endocrinol. Metabolism* 23 (1), 1–15. doi:10.1016/j.beem.2008.10.013

Docter, S., Khan, M., Gohal, C., Ravi, B., Bhandari, M., Gandhi, R., et al. (2020). Cannabis use and sport: A systematic review. *Sports Health* 12 (2), 189–199. doi:10.1177/1941738120901670

Evans, D. G. (2020). Medical fraud, mislabeling, contamination: All common in CBD products. *Mo. Med.* 117 (5), 394–399.

Gamelin, F.-X., Cuvelier, G., Mendes, A., Aucouturier, J., Berthoin, S., Di Marzo, V., et al. (2020). Cannabidiol in sport: Ergogenic or else? *Pharmacol. Res.* 156, 104764. doi:10.1016/j.phrs.2020.104764

Gochman, A., Tan, X., Bae, C., Chen, H., Swartz, K. J., and Jara-Oseguera, A. (2023). Cannabidiol sensitizes TRPV2 channels to activation by 2-APB. bioRxiv, 2023.01.27.525817. doi:10.1101/2023.01.27.525817

Grotenhermen, F., Russo, E., and Zuardi, A. W. (2017). Even High Doses of Oral Cannabidol Do Not Cause THC-Like Effects in Humans: Comment on Merrick et al. Cannabis and Cannabinoid Research 2016;1(1):102-112; 10.1089/can.2015.0004. *Cannabis Cannabinoid Res.* 2 (1), 1–4. doi:10.1089/can.2016.0036

Hall, N., James, B., Bhuiyan, M. A. N., Crane, E., Falgout, C., and Murnane, K. S. (2023). Topical cannabidiol is well tolerated in individuals with a history of elite physical performance and chronic lower extremity pain. *J. Cannabis Res.* 5 (1), 11. doi:10.1186/s42238-023-00179-8

Hazekamp, A. (2018). The trouble with CBD oil. *Med. Cannabis Cannabinoids* 1 (1), 65–72. doi:10.1159/000489287

Henson, J. D., Vitetta, L., and Hall, S. (2022). Tetrahydrocannabinol and cannabidiol medicines for chronic pain and mental health conditions. *Inflammopharmacology* 30 (4), 1167–1178. doi:10.1007/s10787-022-01020-z

Hill, A. J., Williams, C. M., Whalley, B. J., and Stephens, G. J. (2012). Phytocannabinoids as novel therapeutic agents in CNS disorders. *Pharmacol. Ther.* 133 (1), 79–97. doi:10.1016/j.pharmthera.2011.09.002

Hill, K. P., Palastro, M. D., Johnson, B., and Ditre, J. W. (2017). Cannabis and pain: A clinical review. *Cannabis Cannabinoid Res.* 2 (1), 96–104. doi:10.1089/can.2017.0017

Isenmann, E., Veit, S., Starke, L., Flenker, U., and Diel, P. (2021). Effects of cannabidiol supplementation on skeletal muscle regeneration after intensive resistance training. *Nutrients* 13 (9), 3028. doi:10.3390/nu13093028

Jean-Gilles, L., Braitch, M., Latif, M. L., Aram, J., Fahey, A. J., Edwards, L. J., et al. (2015). Effects of pro-inflammatory cytokines on cannabinoid CB1 and CB2 receptors in immune cells. *Acta Physiol. Oxf. Engl.* 214 (1), 63–74. doi:10.1111/apha.12474

Johnson, E., Kilgore, M., and Babalonis, S. (2022). Cannabidiol (CBD) product contamination: Quantitative analysis of Δ9-tetrahydrocannabinol (Δ9-THC) concentrations found in commercially available CBD products. *Drug Alcohol Dependence* 237, 109522. doi:10.1016/j.drugalcdep.2022.109522

Kasper, A. M., Sparks, S. A., Hooks, M., Skeer, M., Webb, B., Nia, H., et al. (2020). High prevalence of cannabidiol use within male professional rugby union and league players: A quest for pain relief and enhanced recovery. *Int. J. Sport Nutr. Exerc. Metabolism* 30, 315–322. doi:10.1123/ijsnem.2020-0151

Kaul, M., Zee, P. C., and Sahni, A. S. (2021). Effects of cannabinoids on sleep and their therapeutic potential for sleep disorders. *Neurotherapeutics* 18 (1), 217–227. doi:10.1007/s13311-021-01013-w

Kennedy, M. (2017). Cannabis: Exercise performance and sport. A systematic review. *J. Sci. Med. Sport* 20, 825–829. doi:10.1016/j.jsams.2017.03.012

Kesner, A. J., and Lovinger, D. M. (2020). Cannabinoids, endocannabinoids and sleep. *Front. Mol. Neurosci.* 13, 125. doi:10.3389/fnmol.2020.00125

Leweke, F., Piomelli, D., Pahlisch, F., Muhl, D., Gerth, C. W., Hoyer, C., et al. (2012). Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl. Psychiatry* 2, e94. doi:10.1038/tp.2012.15

Lim, S. Y., Sharan, S., and Woo, S. (2020). Model-based analysis of cannabidiol dose-exposure relationship and bioavailability. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* 40 (4), 291–300. doi:10.1002/phar.2377

Linares, I. M., Zuardi, A. W., Pereira, L. C., Queiroz, R. H., Mechoulam, R., Guimarães, F. S., et al. (2019). Cannabidiol presents an inverted U-shaped dose-response curve in a simulated public speaking test. *Rev. Bras. De. Psiquiatr. (Sao Paulo, Braz. 1999)* 41 (1), 9–14. doi:10.1590/1516-4446-2017-0015

Lookfong, N. A., Raup-Konsavage, W. M., and Silberman, Y. (2023). Potential utility of cannabidiol in stress-related disorders. *Cannabis Cannabinoid Res.* 8 (2), 230–240. doi:10.1089/can.2022.0130

Lopera, V., Rodríguez, A., and Amariles, P. (2022). Clinical relevance of drug interactions with cannabis: A systematic review. *J. Clin. Med.* 11 (5), 1154. doi:10.3390/jcm11051154

Marques Azzini, G. O., Marques Azzini, V. O., Santos, G. S., Visoni, S., Fusco, M. A., Beker, N. S., et al. (2023). Cannabidiol for musculoskeletal regenerative medicine. *Exp. Biol. Med. (Maywood, N.J.)* 248 (5), 445–455. doi:10.1177/15353702231162086

Martinez Naya, N., Kelly, J., Corna, G., Golino, M., Abbate, A., and Toldo, S. (2023). Molecular and cellular mechanisms of action of cannabidiol. *Molecules* 28 (16), 5980. doi:10.3390/molecules28165980

Maurer, G. E., Mathews, N. M., Schleich, K. T., Slayman, T. G., and Marcussen, B. L. (2020). Understanding cannabis-based therapeutics in sports medicine. *Sports Health* 12 (6), 540–546. doi:10.1177/1941738120956604

McCartney, D., Benson, M. J., Desbrow, B., Irwin, C., Suraev, A., and McGregor, I. S. (2020). Cannabidiol and sports performance: A narrative review of relevant evidence and recommendations for future research. *Sports Med. - Open* 6 (1), 27. doi:10.1186/s40798-020-00251-0

McPartland, J. M., Duncan, M., Marzo, V. D., and Pertwee, R. G. (2015). Are cannabidiol and δ9-tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. *Br. J. Pharmacol.* 172 (3), 737–753. doi:10.1111/bph.12944

Moltke, J., and Hindocha, C. (2021). Reasons for cannabidiol use: A cross-sectional study of CBD users, focusing on self-perceived stress, anxiety, and sleep problems. *J. Cannabis Res.* 3 (1), 5. doi:10.1186/s42238-021-00061-5

Mondino, A., Cavelli, M., González, J., Murillo-Rodriguez, E., Torterolo, P., and Falconi, A. (2021). Effects of cannabis consumption on sleep. *Adv. Exp. Med. Biol.* 1297, 147–162. doi:10.1007/978-3-030-61663-2_11

Murillo-Rodríguez, E., Budde, H., Veras, A. B., Rocha, N. B., Telles-Correia, D., Monteiro, D., et al. (2020). The endocannabinoid system may modulate sleep disorders in aging. *Curr. Neuropharmacol.* 18 (2), 97–108. doi:10.2174/1570159X17666190801155922

Naik, H., and Trojian, T. H. (2021). Therapeutic potential for cannabinoids in sports medicine: Current literature review. *Curr. Sports Med. Rep.* 20 (7), 345–350. doi:10.1249/JSR.0000000000000858

Narayan, A. J., Downey, L. A., Manning, B., and Hayley, A. C. (2022). Cannabinoid treatments for anxiety: A systematic review and consideration of the impact of sleep disturbance. *Neurosci. Biobehav. Rev.* 143, 104941. doi:10.1016/j.neubiorev.2022.104941

Navarrete, F., García-Gutiérrez, M. S., Gasparyan, A., Austrich-Olivares, A., and Manzanares, J. (2021). Role of cannabidiol in the therapeutic intervention for substance use disorders. *Front. Pharmacol.* 12, 626010. doi:10.3389/fphar.2021.626010

Nichols, J. M., and Kaplan, B. L. F. (2019). Immune responses regulated by cannabidiol. *Cannabis Cannabinoid Res.* 5 (1), 12–31. doi:10.1089/can.2018.0073

Ortiz Rios, F. C., Dávila Ruiz, I. G., and Sacal Dumani, E. (2022). Cannabidiol as a personalized treatment for anxiety: Clinical cases in Mexico. *Drugs Context* 11, 1–14. doi:10.7573/dic.2022-3-2

Peng, J., Fan, M., An, C., Ni, F., Huang, W., and Luo, J. (2022). A narrative review of molecular mechanism and therapeutic effect of cannabidiol (CBD). *Basic & Clin. Pharmacol. Toxicol.* 130 (4), 439–456. doi:10.1111/bcpt.13710

Ranum, R. M., Whipple, M. O., Croghan, I., Bauer, B., Toussaint, L. L., and Vincent, A. (2023). Use of cannabidiol in the management of insomnia: A systematic review. *Cannabis Cannabinoid Res.* 8 (2), 213–229. doi:10.1089/can.2022.0122

Resstel, L. B., Tavares, R. F., Lisboa, S. F., Joca, S. R., Corrêa, F. M., and Guimarães, F. S. (2009). 5-HT1A receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats. *Br. J. Pharmacol.* 156 (1), 181–188. doi:10.1111/j.1476-5381.2008.00046.x

Rojas-Valverde, D. (2021). Potential role of cannabidiol on sports recovery: A narrative review. *Front. Physiology* 12, 722550. doi:10.3389/fphys.2021.722550

Russo, E. B., Guy, G. W., and Robson, P. J. (2007). Cannabis, pain, and sleep: Lessons from therapeutic clinical trials of Sativex®, a cannabis-based medicine. *Chem. Biodivers.* 4 (8), 1729–1743. doi:10.1002/cbdv.200790150

Sahinovic, A., Irwin, C., Doohan, P. T., Kevin, R. C., Cox, A. J., Lau, N. S., et al. (2022). Effects of cannabidiol on exercise physiology and bioenergetics: A randomised controlled pilot trial. *Sports Med. - Open* 8 (1), 27. doi:10.1186/s40798-022-00417-y

Schouten, M., Dalle, S., and Koppo, K. (2022). Molecular mechanisms through which cannabidiol may affect skeletal muscle metabolism, inflammation, tissue regeneration, and anabolism: A narrative review. *Cannabis Cannabinoid Res.* 7 (6), 745–757. doi:10.1089/can.2022.0220

Stone, W. J., Tolusso, D. V., Pancheco, G., Brgoch, S., and Nguyen, V. T. (2023). A pilot study on cannabidiol (CBD) and eccentric exercise: Impact on inflammation, performance, and pain. *Int. J. Exerc. Sci.* 16 (2), 109–117.

Stout, S. M., and Cimino, N. M. (2014). Exogenous cannabinoids as substrates, inhibitors, and inducers of human drug metabolizing enzymes: A systematic review. *Drug Metab. Rev.* 46 (1), 86–95. doi:10.3109/03602532.2013.849268

Tantimonaco, M., Ceci, R., Sabatini, S., Catani, M. V., Rossi, A., Gasperi, V., et al. (2014). Physical activity and the endocannabinoid system: An overview. *Cell. Mol. Life Sci.* 71 (14), 2681–2698. doi:10.1007/s00018-014-1575-6

Villanueva, M. R. B., Joshaghani, N., Villa, N., Badla, O., Goit, R., Saddik, S. E., et al. (2022). Efficacy, safety, and regulation of cannabidiol on chronic pain: A systematic review. *Cureus* 14 (7), e26913. doi:10.7759/cureus.26913

Viudez-Martínez, A., García-Gutiérrez, M. S., and Manzanares, J. (2018). Cannabidiol regulates the expression of hypothalamus-pituitary-adrenal axis-related genes in response to acute restraint stress. *J. Psychopharmacol.* 32 (12), 1379–1384. doi:10.1177/0269881118805495

Yarar, E. (2020). Role and function of endocannabinoid system in major depressive disease. *Med. Cannabis Cannabinoids* 4 (1), 1–12. doi:10.1159/000511979

Zhang, J., Luo, Z., Zhang, Z., Zhao, M., Tong, C., Cong, P., et al. (2022). Protective effect and mechanism of cannabidiol on myocardial injury in exhaustive exercise training mice. *Chemico-Biological Interact.* 365, 110079. doi:10.1016/j.cbi.2022.110079

Zou, S., and Kumar, U. (2018). Cannabinoid receptors and the endocannabinoid system: Signaling and function in the central nervous system. *Int. J. Mol. Sci.* 19 (3), 833. doi:10.3390/ijms19030833

Zuardi, A. W., Guimarães, F. S., and Moreira, A. C. (1993). Effect of cannabidiol on plasma prolactin, growth hormone and cortisol in human volunteers. *Braz J. Med. Biol. Res.* 26 (2), 213–217.

Frontiers in Pharmacology

Explores the interactions between chemicals and living beings

The most cited journal in its field, which advances access to pharmacological discoveries to prevent and treat human disease.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact