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Global excellence in ethnopharmacology: Africa

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Global excellence in ethnopharmacology: Africa

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Editorial: Global excellence in ethnopharmacology: Africa

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Editorial on the Research Topic
Global excellence in ethnopharmacology: Africa

African Traditional Medicine (ATM) has a rich history of use in the continent dating back to the dawn of humanity. This knowledge has been preserved and passed down through generations via oral traditions and today ATM continues to thrive, with traditional healers outnumbering conventional doctors and pharmacists in sub-Saharan Africa. Thus, traditional medicine remains a vital, though often overlooked, component of the continent's healthcare system.

At the heart of ATM are the plants that form the foundation of traditional healing practices. Many of these plants have deep roots in African ethnobotany. For example, the aloe family, particularly *Aloe vera* (L.) Burm. f. (syn *Aloe barbadensis* Mill.) (originally misattributed to the West Indies) and *Aloe ferox* Mill. (Cape aloes), have played a significant role in both traditional and global medicine. *A. ferox* was once the only species with an official monograph in the British Pharmacopoeia, while gum arabica obtained from *Senegalia senegal* L. Britton (syn *Acaia senegal* (L.) Willd.) from East Africa remains a key ingredient in pharmaceuticals and consumer goods. Africa's plant diversity comprises approximately 45,000 vascular plant species, including 6,300 endemic to South Africa's Cape region and 8,600 to Madagascar.

Despite this rich botanical and cultural heritage, Africa's contribution to global pharmaceutical medicine remains limited. Some important drugs like tubocurarine, camptothecin, and yohimbine were derived from African medicinal plants. However, much of the contemporary research in ethnopharmacology by African scientists remain under published, often confined to academic theses publications available only in hard copy at universities. Efforts to publish these findings in international journals or commercialize them into herbal or pharmaceutical products are fragmented, mainly hampered by a lack of research infrastructure and funding.

This Research Topic on Global Excellence in Ethnopharmacology in Africa has highlighted groundbreaking studies on the pharmacological and biological effects of plants, fungi, animals, microorganisms, and minerals used in ATM for both human and livestock health. The Research Topic features nine high-quality articles from across the continent, including five original research papers and four review articles (Mambou et al.; Beressa et al.; Nyazema et al.; Okumu et al.; Setlhare et al.; Brendler et al.; Pretorius and Smith, 2023; Irungu et al.; Smith et al. Topics covered range from the anti-epileptic effects of *Mimosa pudica* L. extracts to ethnobotanical studies in Ethiopia and Kenya, immunological research on South Africa's anti-HIV product Nkabinde, and the well-known herbal remedy

Umckaloabo from Lesotho. The review papers focus on disease conditions critical to Africa including malaria, COVID-19, as well as on translational ethnomedicine, and the use and misuse of psychoactive plants.

These contributions offer a glimpse into the vibrant and multidisciplinary field of ethnopharmacology in Africa. They lay the groundwork for future research, particularly in areas that would further the development of these botanicals for purposes such as commercial exploitation, intellectual property rights, access, and benefit-sharing, and improving access to medicines for African patients. This is especially crucial in an era of diminishing resources for global health, where traditional medicine can play a pivotal role in addressing healthcare challenges and needs.

Ethnopharmacological research in Africa should focus not only on documenting the known herbal materials and practises but also on developing herbal based products to improve the health of its people. Presently the depth of such research is questionable as there is often use of inappropriate *in vitro* models and minimal progression in the use of newer techniques of compound isolation and new approach methodologies (NAMs) for the assessment of efficacy and safety of these natural products. Research on herbal products is acutely hampered by the lack of good laboratory infrastructure and strategic collaborations. In countries like South Africa, stringent regulations governing the research on indigenous genetic resources have pushed researchers and potential collaborators to refocus their research efforts on non-indigenous species or botanicals from other countries that maybe more welcoming of such research.

Thus, in the context of all this what is required is capacity building for the next-generation of researchers. This could be achieved by working with some of the existing organizations interested in natural product research such as the Association of African Medicinal Plant Standards (AAMPS), GA – African Research Network, NAPRECA, in association with journals such as Frontiers. Capacity building should focus on the key pillars of pharmacognosy, i.e., taxonomy and documentation of African flora and ethnobotanical use, phytochemistry, pharmacological and toxicological research (including the use of appropriate and valid assays) and commercial development and regulatory science of herbal products.

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Potential pharmacokinetic interactions with concurrent use of herbal medicines and a ritonavir-boosted COVID-19 protease inhibitor in low and middle-income countries

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The COVID-19 pandemic sparked the development of novel anti-viral drugs that have shown to be effective in reducing both fatality and hospitalization rates in patients with elevated risk for COVID-19 related morbidity or mortality. Currently, nirmatrelvir/ritonavir (Paxlovid™) fixed-dose combination is recommended by the World Health Organization for treatment of COVID-19. The ritonavir component is an inhibitor of cytochrome P450 (CYP) 3A, which is used in this combination to achieve needed therapeutic concentrations of nirmatrelvir. Because of the critical pharmacokinetic effect of this mechanism of action for Paxlovid™, co-administration with needed medications that inhibit or induce CYP3A is contraindicated, reflecting concern for interactions with the potential to alter the efficacy or safety of co-administered drugs that are also metabolized by CYP3A. Some herbal medicines are known to interact with drug metabolizing enzymes and transporters, including but not limited to inhibition or induction of CYP3A and P-glycoprotein. As access to these COVID-19 medications has increased in low- and middle-income countries (LMICs), understanding the potential for herb-drug interactions within these regions is important. Many studies have evaluated the utility of herbal medicines for COVID-19 treatments, yet information on potential herb-drug interactions involving Paxlovid™, specifically with herbal medicines commonly used in LMICs, is lacking. This review presents data on regionally-relevant herbal medicine use (particularly those promoted as treatments for COVID-19) and mechanism of action data on

herbal medicines to highlight the potential for herbal medicine interaction Herb-drug interaction mediated by ritonavir-boosted antiviral protease inhibitors This work highlights potential areas for future experimental studies and data collection, identifies herbal medicines for inclusion in future listings of regionally diverse potential HDIs and underscores areas for LMIC-focused provider-patient communication. This overview is presented to support governments and health protection entities as they prepare for an increase of availability and use of Paxlovid™.

KEYWORDS

COVID-19, herb-drug interaction (HDI), drug-drug interaction (DDI), PAXLOVID, herbal medicine

Introduction

The development of novel anti-viral drugs represents a critical step forward in the global control of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), also known as COVID-19. Access to these medications for use by patients at elevated risk for COVID-19-related morbidity or mortality has dramatically lowered hospitalization and fatality rates, and reduced the overall burden of illness as well (Petty and Malani, 2022). Of these novel drug products, nirmatrelvir/ritonavir (Paxlovid™) fixed-dose combination is recommended by the World Health Organization for treatment of COVID-19 (WHO, 2022). Although access to this and other COVID-19 treatments is limited (Usher, 2022), progress has been made to increase access in 95 low- and middle-income countries (LMICs) via a partnership between the manufacturer and the Medicines Patent Pool (Pfizer, 2021).

Ritonavir, a protease inhibitor with antiviral properties, has been used for decades as part of HIV Highly Active Antiretroviral Therapy (HAART). Ritonavir acts as a pharmacokinetic boosting agent for other antiviral agents contained in both HAART and Paxlovid™ by inhibiting the prominent drug-metabolizing enzyme cytochrome P450 (CYP) 3A, facilitating the achievement of therapeutic concentrations by other co-administered antiviral drugs metabolized by CYP3A (Croxtall and Perry, 2010). Because of this mechanism of action for Paxlovid™, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) labelling states that co-administration with certain medications that inhibit or induce CYP3A is contraindicated. This designation reflects concern for potential drug-drug interactions (DDIs) that may alter the efficacy of ritonavir or nirmatrelvir or alter the efficacy or safety of co-administered drugs metabolized by CYP3A (Marzolini et al., 2022). In addition to pharmaceutical medications, some herbal medicines (Gurley, 2012; Sprouse and van Breemen, 2016) are known to interact with drug metabolizing enzymes and transporters, including but not limited to inhibition or induction of CYP3A and the apically located efflux transporter P-glycoprotein (P-gp). As of the writing of this manuscript, only one herbal medicine, St. John's Wort (*Hypericum perforatum*), is included in the list of contraindicated substances highlighted in the FDA or EMA fact sheets for healthcare providers (USFDA, 2022a; Paxlovid, 2022). As Paxlovid™ becomes more globally available—particularly throughout the African and Asian continents—it will be critical to contextualize it within a background of concomitant herbal medicine use in those regions. Such use is both substantially greater and distinct from that in North America and Europe. Use of herbal medicines and supplements during the COVID-19 pandemic increased significantly, with several countries actively promoting herbal treatments combined with pharmaceuticals (Ang et al., 2020) and prompting the Africa Centers for Disease Control and Prevention to issue a warning statement about herbal medicines as treatments for COVID-19 (Africa Centers for Disease Control and Prevention, 2020). Although many studies have evaluated the utility of herbal medicines for COVID-19 treatments, particularly in LMICs (Dwarka et al., 2020; Adeleye et al., 2021; Attah et al., 2021; Beressa et al., 2021; Gajewski et al., 2021; Mphekgwana et al., 2021; Akindele et al., 2022), well characterized and readily accessible information on potential herb-drug interactions (HDIs) involving Paxlovid™ is lacking (Bertuccioli et al., 2022). Conceptually Paxlovid™ interaction with herbs and other drugs may involve the pathways in Figure 1 and could impact systemic and/or tissue concentrations of nirmatrelvir (NMV), ritonavir (RTV), NMV metabolite(s) (NMV-M), and/or RTV metabolite(s) (RTV-M). PXR, pregnane X receptor; CYP3A4, cytochrome P450 3A4.

To the authors' knowledge, this review is the first to present data on regionally relevant herbal medicine use (particularly those promoted as treatments for COVID-19) in LMICs and mechanistic data on herbal medicines to discuss the potential for ritonavir-boosted antiviral protease inhibitor-mediated HDIs. This work highlights potential areas for future experimental studies and data collection, identifies herbal medicines for inclusion in future listings of regionally diverse potential HDIs, and suggests areas for LMIC-focused provider-patient communication. This summary aims to support governments and health protection entities as they prepare for the global availability and use of Paxlovid™.

Because the current Emergency Use Authorizations in the United States for Paxlovid™ are focused on CYP3A-mediated interactions (USFDA M., 2022; Paxlovid, 2022), we direct our efforts on this mechanism, as most available information for herbal medicines is related to CYP3A (or CYPs more broadly). Other mechanisms underlying potential HDIs (e.g., inhibition or induction of P-gp and other transporters) are recognized, and additional research is needed to further explore these pathways. This review highlights the potential for herbal medicines to alter the efficacy or safety of nirmatrelvir/ritonavir. The reverse situation, wherein nirmatrelvir/ritonavir could alter the efficacy or safety of the herbal medicine, is also possible but is beyond the scope of this review.

This work is not a systematic review of potential novel HDIs with COVID-19 treatments but rather presents data derived from a limited pool of clinical and nonclinical studies for herbal medicines identified as commonly used in parts of Africa or Asia, or those used or proposed for use in the treatment of COVID-19 in these regions. This review draws on data from the peer-reviewed literature, as well

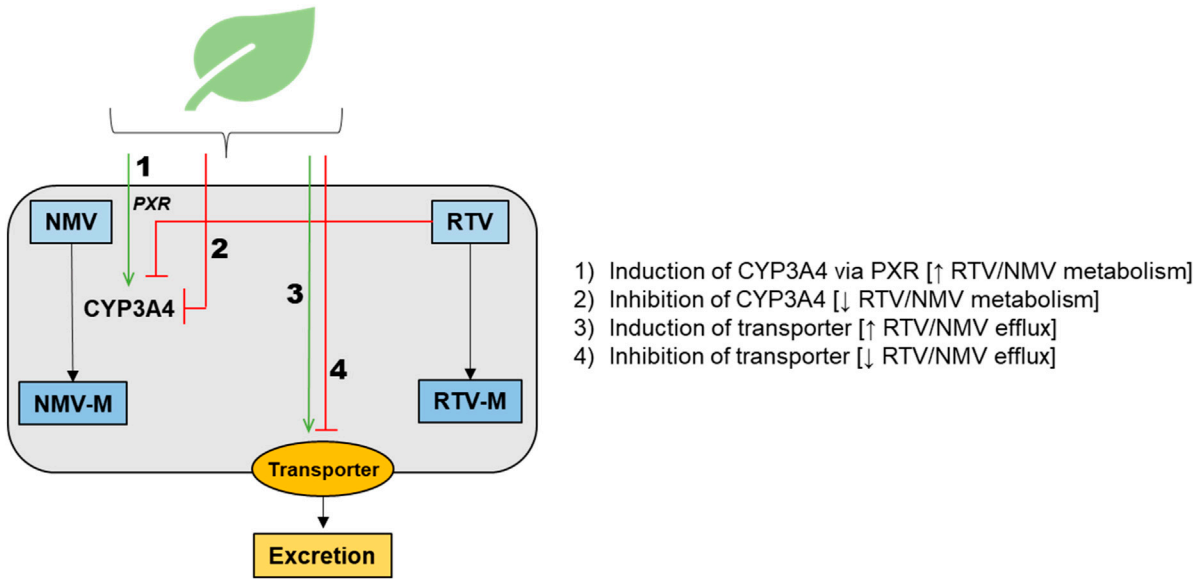

FIGURE 1
Schematic representation of the main potential mechanisms for herb-drug interactions involving Paxlovid™, highlighting pathways that could impact systemic and/or tissue concentrations of nirmatrelvir (NMV), ritonavir (RTV), NMV metabolite(s) (NMV-M), and/or RTV metabolite(s) (RTV-M). PXR, pregnane X receptor; CYP3A4, cytochrome P450 3A4.

as expert opinion from pharmacognosists, pharmacologists, toxicologists, researchers—including in-country experts in these areas—to inform initial case studies on potential HDIs involving herbal medicines and Paxlovid™ in low and middle-income regions in Africa and Asia. The description of plants as treatments in this review are not meant to support the reported efficacy of the plants, but to describe their use and discuss potential interactions (Collins et al., 2020; Mitchell et al., 2022). Herbal medicines and preparations derived from them (e.g., extracts or tinctures) are complex mixtures, containing hundreds to thousands of individual chemicals (or phytochemicals). This natural complexity (and variability) makes it difficult to assess the efficacy, safety, or herbal-drug interactions of any one preparation without sufficient chemical characterization (Collins et al., 2020; Mitchell et al., 2022). However, we hope this review can stimulate further research and potentially alert those taking herbal medicines and anti-virals to treat COVID-19 about potential interactions with Paxlovid.

Methods

The author team identified a list of herbal medicines and plants for potential inclusion in this article based upon the following criteria:

- Expert opinion of author team based on local practices and availability in their county or region.
- Initial data of common use in LMICs in Africa and Asia.
- Initial data of promotion or use specifically as a treatment for COVID-19.
- Potential for pharmacokinetic interactions with Paxlovid™ based on available mechanistic or clinical evidence (e.g., modelling of known phytochemicals and *in vitro*, *in vivo*, or clinical data).

The herbal medicines/plants on this list were then further characterized via a non-systematic literature search to identify HDIs associated with the plant, mechanistic studies on the plant, and adverse event reports associated with the plant's use. The non-systematic literature review informs on HDIs rather than give an all-compassing review of each botanical. The search was conducted in English using the following sources: PubMed, Web of Science, Google Scholar, Science Direct, China National Knowledge Infrastructure, and the online ethnobotanical database. This activity was reviewed by CDC and was conducted consistent with applicable federal law and CDC policy.[1]

Results

A summary of the plants examined in this review is presented in Table 1.

Andrographis (*Andrographis paniculata*)

Andrographis paniculata, also known as green chiretta, belongs to the Acanthaceae family, has Ayurvedic origins, and is native to India and Sri Lanka. During the COVID-19 pandemic, China, Thailand, and other countries incorporated this herbal medicine into treatment guidelines and added it to essential medicine lists (Intharuksa et al., 2022). *A. paniculata* leaf extract has been used to

1 See, e.g., 45 C.F.R. part 46, 21 C.F.R. part 56; 42 U.S.C. §241(d); 5 U.S.C. §552a; 44 U.S.C. §3501 et seq.

TABLE 1 Summary of Herbal Medicines highlighted in this review.

Scientific name	Common Name(s)	Native region(s)	Regions/countries commonly used	Plant parts(s)/ Preparation	Common traditional (non-COVID-19) therapeutic use(s)/Biological activities
Andrographis paniculate	Green chiretta	India Sri Lanka	Southeast Asia (Thailand), China	Leaf extract	• Stomachache
					• Inflammation
					• Pyrexia
					• Fevers
					• Loss of appetite
					• Irregular stools
					• Diarrhoea
Artemisia annua	Sweet wormwood	Asia	Asia; Africa	Dried leaves; tea	• Malaria
					• Inflammation (e.g., osteoarthritis)
					• Viral infections
					• Bacterial infections
					• Fevers
					• Cancer
Bowiea volubilis	Climbing onion	Southern Africa	Southern Africa	Decoction of crushed fresh bulbs & water	• Dermatological
					• Sore eyes
					• Urinary complications
					• Infertility
					• Abortion
Curcuma longa	Turmeric	South Asia	Widespread	Dried rhizomes	• Ulcers
					• Allergy
					• Pain
					• Inflammation
					• Liver disorders
Glycyrrhizae radix et rhioma	Licorice	West Asia, North Africa, S. Europe	China	Dried root; dried rhizome	• Allergies
					• Inflammation
					• Viral infections
					• Cancer
Harpagophytum procumbens	Devil's claw	Southern Africa	Southern Africa	Dried root/tuber extract	• Rheumatoid arthritis
					• Osteo arthritis
					• Pain
					• Kidney inflammation
					• Dyspepsia
					• Fever
					• Wound healing
Hypoxis haemercollidea	African potato	Southern Africa	Southern Africa	Decoction of corms	• HIV/AIDS
					• Tuberculosis
					• Cancer

(Continued on following page)

TABLE 1 (*Continued*) Summary of Herbal Medicines highlighted in this review.

Scientific name	Common Name(s)	Native region(s)	Regions/countries commonly used	Plant parts(s)/ Preparation	Common traditional (non-COVID-19) therapeutic use(s)/Biological activities
					• Headache
					• Dizziness
					• Ulcers
					• Seizures
					• Depression
					• Anxiety
Lessertia frutecens	Cancer bush	Southern Africa	Southern Africa	Decoctions from leaves or bark	• Cancer
					• Viral infections; specifically, HIV/AIDS
Moringa oleifera	Moringa	India	Widespread	Leaf powder: other parts also used	• Diabetes
					• Inflammation
Uncaria tomentosa	Cat's claw	South/Central America	Widespread	Bark	• Viral infection
					• Inflammation
Vernonia amygdalina	Bitter leaf	Southern Africa	Southern Africa	Leaf infusion	• Diarrhoea
					• Constipation
					• Stomachache
					• Malaria
					• Diabetes
					• Cancer
					• Pyrexia

treat ailments such as stomachache, inflammation, pyrexia, loss of appetite, irregular stools, and diarrhoea. The aerial parts have been documented in the treatment of the common cold, hypertension, malaria, and snakebites (Okhuarobo et al., 2014). *A. paniculata* has the potential for HDIs, particularly when taken in combination with drugs metabolized by CYP3A, CYP2C9, and CYP2C19, through both induction and inhibition (Sundhani et al., 2022). Balap et al. (2017) reported that co-administration of *A. paniculata* extract with naproxen, a CYP2C9 substrate in humans, decreased systemic exposure to naproxen in rat models. Systemic concentrations of drugs metabolized by CYP1A2 increased after co-administration with *A. paniculata* in rat models (Chien et al., 2010). With the increased use of this herbal medicine during the COVID-19 pandemic, HDIs with Paxlovid™ through CYP inhibition and induction, are possible.

Sweet wormwood (*Artemisia annua*)

Artemisia annua L., also known as sweet wormwood, is a member of the Asteraceae family and has a long history as treatment for various diseases. Extracts and isolated phytochemicals of *A. annua* have been reported to have anti-viral, pro-apoptotic, anti-inflammatory, anti-pyretic, antioxidant, and immunomodulatory activities (Soni et al., 2022). The sesquiterpenes produced by *A. annua*, artemisinin and artesunate, have shown high efficacy against multi-drug resistant malaria-causing parasites, resulting in artemisinin-based combination therapies for the treatment of *P. falciparum* malaria (Bosman et al., 2007). Artemisinin is known to be both an inducer and a substrate of CYP3A4 and CYP2B6 (Xing et al., 2012), leading to concerns about herb-drug interactions with artemisinin-based combination therapy. Based on its relatively broad range of biological activities, *A. annua* has also been used for the treatment of rheumatoid arthritis, systemic lupus erythematosus, allergic contact dermatitis, and has been evaluated for treatment of COVID-19 (Fuzimoto, 2021; Soni et al., 2022). *A. annua* extracts demonstrated inhibition of CYP3A4 activity (measured via 6ß-(OH)-testosterone) in Caco-2 cell monolayers, whereas artemisinin alone had no effect (Melillo de Magalhães et al., 2012). The same study also showed inhibition as well as a slight induction of CYP1A1 activity (measured via 7-ethoxyresorufin-O-deethylase) in Caco-2 cell monolayers (Melillo de Magalhães et al., 2012). *A. annua* tea infusions showed inhibition of CYP2B6 and CYP3A4 activity in HepaRG cells using P450-Glo assays (Kane et al., 2022). Based on the data available, HDIs with Paxlovid™ through CYP inhibition and induction with *A. annua* are possible.

Climbing onion (*Bowiea volubilis*)

Bowiea volubilis Harv. ex Hook.f. is a perennial, succulent plant commonly known as climbing onion, that belongs to the family Asparagaceae and is widely grown in dry regions of eastern and southern Africa (Ramarumo et al., 2019). Among the tribes of Southern Africa, the bulbous medicinal plant is traditionally used to treat various medical conditions, including dermatological disorders, sore eyes, urinary complications, infertility, and facilitation and induction of abortion (Steenkamp, 2003). Most preparations for therapeutic use involve mixing crushed fresh bulbs with water to form a decoction. A study by Fasinu et al. (2014) (Fasinu et al., 2014) showed that aqueous extracts of *Bowiea volubilis* inhibited the activity of CYP1A2 (phenacetin O-deethylation) and CYP3A4 (testosterone 6β-hydroxylation) in human liver microsomes, suggesting the potential for HDIs if these concentrations are attained *in vivo*. Studies have reported that all parts of *B. volubilis* contain alkaloids and several cardiac glycosides of the scillaren type (Mulholland et al., 2013). The mechanism of action of cardiac glycosides involves binding and inhibition of Na^+/K^+-ATPase with high selectivity and affinity. The CYP inhibitory activity of *B. volubilis* may be attributed to the alkaloids, which are substrates of human CYPs. A study by Salminen et al. (2011) showed that structurally related alkaloids from plants inhibited major human drug metabolizing CYPs (CYP3A4, CYP2D6, CYP2C19). Further research is needed to determine whether *B. volubilis* precipitates interactions with Paxlovid™ in patients and potential clinical consequences.

Turmeric (*Curcuma longa*)

Turmeric is obtained from the rhizomes of *C. longa* L., a plant of the Zingiberaceae family that is native to tropical South Asia. The rhizomes are yellow-brown and are usually dried and ground to a yellow powder for use as a spice, herbal medicine, or dietary supplement. Ayurvedic medicine, Chinese medicine, and various pharmacopoeias describe uses of turmeric that include treatment of peptic ulcers, allergy, pain and inflammation, and liver disorders (Razavi et al., 2021). Turmeric is also under investigation for potential anti-SARS-CoV-2 activity and possible value in mitigating the COVID-19 cytokine storm (Rattis et al., 2021). The rhizomes of *Curcuma longa* contain monoterpenes and sesquiterpenes, including turmerone, arturmerone, and zingiberene, polysaccharides and curcuminoids. Turmeric dietary supplements usually contain extracts enriched in curcuminoids (up to 95% by weight) (Cheng et al., 2019). Some preclinical and clinical studies suggest that turmeric may produce pharmacokinetic HDIs. In a prospective study of 60 breast cancer patients, co-administration of turmeric with paclitaxel reduced serum AUC and C_{max} of paclitaxel by 7.7% and 12.1%, respectively (Kalluru et al., 2022). Although probably caused by induction of CYP3A4 activity, the effect was not considered clinically relevant. A study involving a human colorectal adenocarcinoma cell line showed curcumin inhibits P-gp, indicating the potential to alter the pharmacokinetics of drugs that are substrates for this efflux transporter (Flory et al., 2021). A clinical study involving eight healthy adult participants showed that curcumin increased the area under the curve (AUC) of sulfasalazine, a substrate for BCRP (Kusuhara et al., 2012). Several uridine 5′-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGTs), including hepatic UGT1A1 and intestinal UGT1A8 and UGT1A10, metabolize curcumin to curcumin-*O*-glucuronide, which is a substrate for the basolaterally located efflux transporter MRP3 (ABCC3) (Jia et al., 2020). Therefore, pharmacokinetic turmeric-drug interactions may occur for drugs that are also substrates for MRP3 or these UGTs.

Licorice (*Glycyrrhizae radix et rhizoma*)

Glycyrrhizae radix et rhizoma (Chinese name, Gan cao), also known as licorice, is the dried root and rhizome of *Glycyrrhiza uralensis* Fisch., *Glycyrrhizae inflata* Bat, or *Glycyrrhizae glabra* L, and is a commonly used herbal medicine native to West Asia, North Africa, and Southern Europe (Wang et al., 2013; Liu et al., 2021). Licorice is used as an herbal medicine, dietary supplement, and food flavouring (Omar et al., 2012), and has been reported to exhibit various bioactivities, including anti-allergy, anti-inflammation, anti-virus, and anti-oxidant effects (Zhao et al., 2017). Studies have shown that licorice extracts or its bioactive phytochemicals can inhibit SARS-CoV-2 infection by affecting entry and replication of the virus, having potential for the treatment of COVID-19 (Sprouse and van Breemen, 2016; Yi et al., 2022). Various natural products and Chinese herbal formulae against COVID-19 are recommended by the Guidelines of Diagnosis and Treatment for COVID-19 issued by the National Health Commission of China. Licorice is one of the most frequently involved herbs in these herbal formulae or preparations against COVID-19, increasing risk for HDIs for anti-COVID-19 drugs (Lyu et al., 2021), and has been reported to modulate several CYP isoforms *in vitro*, including CYP3A4, CYP2C9, and CYP2E1 (Wang et al., 2013). Licorice extract was shown to activate human pregnane X receptor (PXR) (Haron et al., 2022) and induce CYP3A4 activity in human hepatoma cells (Wang et al., 2012). Regarding drug transporters, glycyrrhizin, one of the main bioactive ingredients of licorice, was shown to inhibit MRP2's uptake of S-(2,4-dinitrophenyl)-glutathione and induce P-gp. (Feng et al., 2015). Conversely, ritonavir is an inducer of uridine diphosphate (UDP)-glucuronyltransferase (UGT) and an inhibitor of P-gp and breast cancer resistance protein (BCRP), which may decrease efflux and in turn increase the metabolism of licorice phytochemicals (Marzolini et al., 2022). Given that licorice is one of the most used herbal medicines, in addition to a promoted treatment against COVID-19, there is potential concern regarding interactions with Paxlovid™.

Devil's claw (*Harpagophytum procumbens*)

Harpagophytum procumbens is a weedy, perennial, and herbaceous plant that is geographically distributed in the Kalahari Desert of Southern Africa. (Mncwangi et al., 2012). Harpogoside was isolated as the major phytochemical from *H. procumbens* tubers, amongst other iridoid glycosides. Both the pure isolated phytochemical (harpagoside) and extracts from the plant have shown potent anti-rheumatic, anti-inflammatory and

analgesic effects (Georgiev et al., 2013). Products containing devil's claw extract are widely used internationally by patients suffering from chronic low back pain and/or other chronic inflammatory diseases (Grant et al., 2007). Unger and Frank (2004) (Unger and Frank, 2004) conducted a study using baculovirus-infected insect cells to measure inhibition of the activity of six major CYPs by an *H. procumbens* extract, amongst other herbal extracts using LC/LC/MS. Inhibition of CYP1A2 and CYP2D6 by *H. procumbens* extract was comparably low, whereas CYP2C8, CYP2C9, CYP2C19 and CYP3A4 showed higher levels of inhibition. In another study, the effects of three commercially available devil's claw products on P-gp activity were tested in a human proximal tubule (HK-2) cell line. (Romiti et al., 2009). Two of the products inhibited P-gp activity in calcein-AM tests and decreased protein expression via Western blot, albeit to a lower extent than the positive control, verapamil. These commercial devil's claw products also inhibited esterase activity (via free calcein measurements), which may have resulted in an underestimation of their true P-gp inhibitory effects. However, harpagoside alone did not inhibit P-gp activity nor esterase activity (Romiti et al., 2009). Due to these *in vitro* interactions observed for devil's claw through CYP and P-gp inhibition, Bordes et al. (2020) (Bordes et al., 2020) noted that *H. procumbens* may increase plasma concentrations of anti-retroviral drugs in patients, including those containing ritonavir, when administered concomitantly.

African potato (*Hypoxis haemercollidea*)

Hypoxis haemercollidea Fisch., C.A. Mey. (Hypoxidaceae), also known as African potato, is a tuberous perennial with star shaped yellow flowers that is indigenous to Southern Africa (South Africa, Lesotho, Eswatini, Zimbabwe, Botswana, and Mozambique) (Matyanga et al., 2020). Many species of *Hypoxis* are used as herbal medicines and are often morphologically similar (Ncube et al., 2013). Of these, *H. haermercollidea* is the best known and most studied. Decoction of *H. haermercollidea* corms is the most prominent herbal medicine preparation specifically used to treat HIV/AIDS as an immunomodulator (Mills et al., 2005a) and is also used to treat tuberculosis, cancer, headache, dizziness, ulcers, seizure disorders, depression, and anxiety (Ncube et al., 2013). Hypoxoside is the main phytochemical in African potato, which is readily converted to rooperol by β-glucosidase. Rooperol is the biologically active phytochemical that is attributed to the medicinal properties of the plant (Drewes and Khan, 2004; Mills et al., 2005a). Other notable biologically active phytochemicals are phytosterols and their glycosides, specifically β-sitosterol (Mills et al., 2005a; Nair et al., 2007; Matyanga et al., 2020). HDIs involving *H. haemercollidea* have been investigated, most focusing on antiretroviral drugs. African potato extracts mainly inhibited CYP3A4 (Mills et al., 2005b; Nair et al., 2007; Gwaza et al., 2009), as well as CYP1A2, 2A6, 2B6, 2C9, 3A5, and 2D6 in vitro assays (Nair et al., 2007; Gwaza et al., 2009; Fasinu et al., 2013). African potato extract showed dose-dependent activation of PXR (Mills et al., 2005b). For extracts high in hypoxoside, P-gp-mediated efflux increased compared to the ritonavir control (Nair et al., 2006). The P-gp-mediated efflux of nevirapine across human intestinal epithelial cells increased significantly in the presence of an African potato extract compared to vehicle (Brown et al., 2008), whereas the P-gp-mediated efflux of indinavir decreased substantially in the presence of *H. haemercollidea* (Havenga et al., 2018) compared to controls. Other studies showed no induction of P-gp at the concentrations tested (Gwaza et al., 2009; Fasinu et al., 2013). Co-administration of African potato with ART in human clinical trials did not alter the pharmacokinetics of efavirenz (Mogatle et al., 2008) nor lopinavir/ritonavir (Gwaza et al., 2013), which are substrates for and inhibitors of CYP2B6 and CYP3A4, respectively. Although the Gwaza et al. (2013) study showed no change in drug pharmacokinetics with concomitant use of *Hypoxis obtusa*, additional studies are needed to further elucidate potential concern with use of *H. haemercollidea* extracts co-administered with Paxlovid™, particularly regarding potential P-gp-mediated interactions.

Cancer bush (*Lessertia frutecens*)

Lessertia frutecens (L.) Goldblatt & J.C.Manning (previously *Sutherlandia frutecens* (L.)., R.Br) also known as cancer bush or Sutherlandia, is a flowering shrub that is a member of the Fabaceae family (Mills et al., 2005a; van Wyk and Albrecht, 2008). The plant is indigenous to South Africa, Lesotho, southern Namibia, and south-eastern Botswana and has been used by a wide range of cultures as a medicinal plant (van Wyk and Albrecht, 2008). Cancer bush has been used mainly to treat internal cancers—hence the name—as well as diabetes, inflammation, and infections, and to promote wound healing (van Wyk and Albrecht, 2008). More recently, *Lessertia frutecens* has gained popularity as antiviral treatment, specifically against HIV/AIDS as an immunomodulator (Mills et al., 2005a; Babb et al., 2007; Sibanda et al., 2016). Traditionally, decoctions or infusions are made from the leaves or bark (van Wyk and Albrecht, 2008). The main phytochemicals of *L. frutescens* are L-canavanine, D-pinitol, and gamma-aminobutyric acid (Mills et al., 2005a; van Wyk and Albrecht, 2008). Although research is limited, *Lessertia* has shown the potential to precipitate HDIs, specifically with antiretroviral drugs. Both water and ethanolic extracts showed inhibition of CYP3A4 (via conversion of dibenzylfluorescein to fluorescein) and activation of hPXR (via CYP3A4 luciferase reporter gene construct) and inhibited P-gp activity (via orthovanadate sensitive release of phosphate and adenosine triphosphatase activity) in HepG2 cells (Mills et al., 2005b). Similarly, *Lessertia* extracts inhibited P-gp in MDCK-MDR1 cells, reducing amprenavir efflux (Katerere, 2018), and modestly inhibited P-gp in Caco-2 cells using nevirapine as the substrate (Brown et al., 2008). In the same study by Brown et al. (2008), inhibition of P-gp by L-canavanine, a main phytochemical of *L. frutescens*, was observed. Extracts of *L. frutecens* inhibited a range of CYPs but mainly CYP3A4 and CYP3A5 (Fasinu et al., 2013). In LS-180 cells, CYP3A4 decreased after a short-term exposure to a *Lessertia* aqueous extract (less than 5 days); however, after 5 days of exposure, CYP3A4 activity increased 2-3 fold (Minocha et al., 2011). The lack of increase in CYP3A4 mRNA expression implied a post-transcriptional mechanism. In the same study, the pharmacokinetics of nevirapine were altered in rats. Specifically, the area under the curve (AUC) and maximum plasma concentration (C_{max}) decreased by 50% after 5 days of treatment with *Lessertia*

(Minocha et al., 2011). Similarly, the AUC and C_{max} of atazanavir decreased in human participants (n = 12) after a single dose of Sutherlandia tablets (Müller et al., 2013). The activation of CYP3A4 and inhibition of P-gp by *L. frutecens* has the potential to elicit sub-therapeutic effects of the drugs administered, which may lead to either treatment failure or drug resistance. Given these potential interactions with CYPs and related pathways, there is evidence for potential HDIs with *Lessertia.*

Moringa *(Moringa oleifera)*

Moringa oleifera, commonly known as moringa, is a tree that is native to India and widely cultivated throughout the tropics and subtropics because of its adaptability to different climatic conditions. (Kashyap et al., 2022; Liu et al., 2022). Previously reported analgesic, anti-inflammatory, antipyretic and immune boosting properties render moringa a potentially viable treatment for the management of COVID-19 (Chikowe et al., 2021; Nuertey et al., 2022; Siddiqui et al., 2022). However, some preclinical and clinical safety data indicate the potential for HDIs. Inhibition of CYP1A2 and CYP3A activity in pooled human liver microsomes has been reported. Using testosterone as the probe substrate, methanolic leaf extracts of *M. oleifera* inhibited CYP3A activity modestly, suggesting low interaction risk. Likewise, aqueous extracts showed weak CYP3A inhibition (Monera et al., 2008). Using fluorometric probes, methanolic extracts of *M. oleifera* leaves inhibited CYP3A and CYP1A2 activity (Awortwe et al., 2014), and aqueous extracts of *M. oleifera* leaves inhibited CYP1A2 and CYP2C9 activity. Inhibition of CYP3A and CYP1A2 activity was considered most likely because the IC_{50}s were higher than the minimum estimated dose warranting further investigation (Awortwe et al., 2014; Showande et al., 2019). Additionally, inhibitory effects of an ethanolic extract of moringa leaves on CYP1A2 and CYP3A4 activity were demonstrated *in vitro* (Taesotikul et al., 2010; Ahmmed et al., 2015). Two human clinical trials assessing the interaction between moringa and pharmaceutical drugs compared AUC and C_{max} of nevirapine with and without moringa coadministration, showing that moringa had no effect on the steady-state pharmacokinetics of the drug based on the FDA bioequivalence approach (USFDA, 2016; Monera-Penduka et al., 2017). In contrast, coadministration of moringa with amodiaquine resulted in a significant decrease (p = 0.037) in the time for amodiaquine to reach C_{max}. Several moringa phytochemicals have demonstrated antispasmodic activity, including glucomorigin, which may slow absorption, thus increasing time to reach C_{max}. In addition, other phytochemicals could form insoluble complexes affecting both C_{max} and the time to reach C_{max} (Olawoye et al., 2018). Although additional data are needed, some preclinical and clinical safety data indicate the potential for interactions between moringa and Paxlovid™.

Cat's claw (*Uncaria tomentosa*)

Cat's claw (*Uncaria tomentosa*) has shown beneficial effects against inflammation and viral infection, including COVID-19 (Steinberg, 1995; Erowele and Kalejaiye, 2009; Yuan et al., 2014; Yepes-Perez et al., 2021). Multiple *in vitro* studies have demonstrated cat's claw as a CYP3A inhibitor (Budzinski et al., 2000; Sato et al., 2015; Weiss, 2018), but the specific phytochemicals have not been identified. A clinical study showed cat's claw to inhibit CYP3A, as evidenced by increased plasma concentrations of antiretroviral drugs that are CYP3A substrates, including ritonavir (López Galera et al., 2008). In addition, a cell-based study showed that an extract of cat's claw upregulated the protein expression of CYP2J2, CYP3A4, UGT1A3, UGT1A9, P-gp, and the apically located uptake transporter organic anion transporting polypeptide (OATP) 1B1 (SLCO1B1) (Weiss, 2018). Both nirmatrelvir and ritonavir are CYP3A substrates (Kumar et al., 1996; Li et al., 2011; Eng et al., 2022), suggesting risk for HDIs between cat's claw and Paxlovid™ via CYP3A inhibition or induction.

Bitter leaf (*Vernonia amygdalina*)

Vernonia amygdalina is a small, perennial, rapidly regenerating shrub that grows wild in many sub-Saharan African countries, particularly Nigeria, Cameroon, South Africa, and Zimbabwe. Commonly known as bitter leaf, *Vernonia amygdalina* is widely consumed as a green leafy vegetable. An infusion of *V. amygdalina* leaves, sometimes with the young stems, is used to treat gastrointestinal tract conditions such as diarrhoea, constipation, and stomachache caused by helminthic, protozoal, and bacterial infection. *Vernonia amygdalina* is also used to manage malaria, diabetes, and cancer (Toyang and Verpoorte, 2013; Oyeyemi et al., 2018; Gyebi et al., 2021). Its use in managing fever and colds made it a candidate for screening as a potential source for a COVID-19 drug target (Gyebi et al., 2021). *Vernonia amygdalina* contains saponins, alkaloids, sesquiterpenes, and steroid glycosides, which are responsible for the plant's biological activity (Farombi and Owoeye, 2011). *Vernonia amygdalina* has been shown to inhibit P-gp activity *in vitro* and to increase systemic exposure to the P-gp probe drug digoxin *in vivo*. Using Caco-2 cells, aqueous extracts of *V. amygdalina* leaves significantly inhibited P-gp-mediated efflux digoxin ($p < 0.01$) relative to control (Farombi and Owoeye, 2011; Oga et al., 2012). Using an isolated rat ileum model, *V. amygdalina* increased digoxin permeability in the mucosal-to-serosal direction by 43% compared to control (Oga et al., 2013). When *V. amygdalina* was co-administered with digoxin to rats, total plasma digoxin AUC was 2.1 times higher than that after digoxin was administered with vehicle. Caution may be warranted when *V. amygdalina* is co-consumed with other P-gp substrate drugs' however, further research is needed to determine whether these pre-clinical results translate to the clinical setting.

Discussion/conclusion

Herbal medicines are used around the world in the treatment and prevention of disease. The rollout of Paxlovid™ during the COVID-19 pandemic has prevented hospitalizations and saved lives and will continue to as it becomes more globally available (Shah et al., 2022; Wong et al., 2022). Comprehensive resources have been

prepared to help address challenges that the use of Paxlovid™ presents. For example, the United States Food and Drug Administration (FDA) created an interaction checklist and the University of Liverpool designed a COVID-19 drug interaction tool (USFDA, 2022c; Liverpool, 2022). However, these resources do not account for the impact of HDIs on patient care beyond St. John's wort.

The herbal medicines highlighted above, although not comprehensive, demonstrate the potential for interactions with ritonavir-containing medications, including Paxlovid™, particularly through CYP induction or inhibition. Because herbal medicine use varies across regions, public health bodies and ministries of health may consider creating an interaction checklist (similar to FDA's or Liverpool's checklist (USFDA, 2022c; Liverpool, 2022)) and include commonly used herbal medicines that have potential HDIs with Paxlovid™ alongside drug-drug interactions. These checklists can be accompanied with education for both the public and healthcare providers to raise awareness of potential HDIs by encouraging patients to report herbal medicine use to their providers and by equipping providers with tools and knowledge to manage such HDIs.

Extensive *in vitro* and *in silico* data are available for HDIs involving herbal medicines such as *V. amygdalina*, and *L. frutecens*. However, how these data and results translate to the clinical settings remain unknown. As Paxlovid™ is scaled up globally and the concurrent use of antivirals and herbal medicines increases, additional research can help elucidate the clinical impact of HDIs. Prospective and retrospective studies can add to the understanding of HDIs. Predictive modelling based on available non-clinical data by applying established tools (e.g., physiology-based pharmacokinetic modelling) may also inform HDIs with Paxlovid. Without this critical information, adverse events can result, and systemic concentrations can vary (both for Paxlovid™ and herbal medicine phytochemicals), which may lead to drug resistance, low patient adherence, and failed therapy.

Study limitations include a non-systematic review methodology, however, the information provided in this manuscript raises awareness of potential HDIs and is not meant to be a comprehensive review.

With the likelihood of future SARS-CoV-2 variants emerging, Paxlovid™ represents an effective therapeutic tool to prevent death and strain on healthcare systems. Raising awareness and furthering the understanding of HDIs is crucial as the global Paxlovid™ rollout continues.

Author contributions

DS, CM, SP, and ME contributed to conception and design of the study. DS and ME wrote the first draft of the manuscript. DS, HB, JH, XM, CM, KN, TM-P, HO-R, MP, SP, WP, RV, and ME wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Author Disclaimer

The findings and conclusions of this article are those of the authors and do not necessarily represent the official position of the US Centers for Disease Control and Prevention (CDC).

Conflict of interest

HO-R was employed by United States Pharmacopeia.

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

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Potential of medicinal plants as antimalarial agents: a review of work done at Kenya Medical Research Institute

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Background: Medicinal plants have traditionally been used as remedies against malaria. The present review attempted to compile data on scientific research evidence on antimalarial medicinal plants screened at Kenya Medical Research Institute (KEMRI), Center for Traditional Medicine and Drug (CTMDR) Research from January 2003 to December 2021.

Methods: A systematic review was conducted using a predefined protocol based on PRISMA. Search was performed in Google Scholar and PubMed. One hundred and eight journal articles were identified 37 of which published on antimalarial/antiplasmodial work. Thirty journal articles with at least one author from KEMRI-CTMDR and accessible in full were selected for analysis. Relevant data was captured in MS Excel format and descriptive statistics, percentages and tables used to summarize the findings.

Results: Assessment of individual plant species was considered as an independent study resulting in 1170 antiplasmodial/antimalarial tests done from 197 plant species. One hundred and fifty plant species were screened *in vitro*, one *in vivo* and 46 were both *in vivo* and *in vitro*. Three hundred and forty-four of tests reported good activity (IC_{50} < 10 µg/mL or parasite suppression rate of ≥50%), 414 moderate activity (IC_{50} values of 10–49 µg/mL or parasite suppression rate of 30%–49%) and 412 were reports of inactivity (IC_{50} > 50 µg/mL or parasite suppression rate of <30%). *Fuerstia africana* and *Ludwigia erecta* were reported to have the highest activities, with IC_{50} < 1 µg/mL against *Plasmodium falciparum* D6 strain and chemosuppression in mice at an oral dose of 100 mg/kg, was reported as 61.9% and 65.3% respectively. Fifty five antimalarial/antiplasmodial active compounds isolated from eight plant species were reported with resinone (**39**) having the best activity (IC_{50} < 1 µg/mL).

Conclusion: Though 344 of tests reported promising antimalarial activity, it was noted that there was limited evaluation of these plants in animal models, with only 9.0% (105/1170) studies and no clinical trials. This highlights an important research gap emphasizing the need for drug development studies that aim to progress

Abbreviations: KEMRI, Kenya Medical Research Institute; CTMDR, Center for Traditional Medicine and Drug Research; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis; WHO, World Health Organization; DNMP, Division of National Malaria Program; ICF, International Classification of Functioning disability, and health; IPTp, Intermittent Preventive Treatment during pregnancy; ACT, Artemisinin Combination Therapy; DCM, Dichloromethane; Pet ether, Petroleum ether; EtOAc, ethyl acetate.

study findings from preclinical to clinical studies. There is still need for extensive research on promising plant species aimed at developing new plant based antimalarial drugs.

KEYWORDS

malaria, medicinal plants, antimalarial, antiplasmodial, *Plasmodium falciparum*, drug discovery, cytotoxicity

1 Introduction

Morbidity and mortality caused by malaria is still a public health concern despite the fact that it is a curable and preventable disease. World Malaria Report of 2022 reveals that between 2019 and 2020, estimated malaria cases increased from 218 to 232 million, and deaths from 544 000 to 599 000 in the World Health Organization (WHO) African Region WHO (2022). In Kenya, malaria remains a major public health problem accounting for an estimated 13%–15% of outpatient consultations. The *Plasmodium falciparum* parasite, which causes the most severe form of the disease, accounts for more than 99% of infections. Malaria prevalence in Kenya varies considerably by season and across different geographic zones. This is because transmission and infection risks are mainly determined by altitude, rainfall patterns and temperature (Division of National Malaria Programme DNMP [Kenya] and International Classification of Functioning, Disability, and Health ICF, 2021). Key malaria control and prevention strategies that have been employed in Kenya include use of insecticide treated nets, intermittent preventive treatment during pregnancy (IPTp) using sulfadoxine pyrimethamine, indoor residual spraying and adoption of artemisinin combination therapy (ACT). However, the adaptation of the mosquitoes to insecticides and emergence and spread of drug resistant parasites, especially *P. falciparum*, is a drawback to these interventions. The WHO (2022) confirmed emergency of partial resistance to artemisinin drugs in some African countries, namely,: Rwanda, Eritrea, and Uganda. The possibility of the spread of artemisinin resistant parasites to other malaria endemic regions in Africa is inevitable. Therefore, the challenge to eliminate malaria remains significant hence the need for new agents that are cheap, safe, readily available, active against sensitive and drug resistant *Plasmodium* parasites or act in combination with existing drugs.

Medicinal plants have played a major role in discovery and development of antimalarial drugs. It is expected that medicinal plants would still serve as a source of new drug leads given their chemodiversity (Batista et al., 2009). Several studies have documented medicinal plants used in management of malaria by various local communities in Kenya (Muthaura et al., 2007a; Njoroge and Bussmann, 2007; Gathirwa et al., 2011; Mukungu et al., 2016). Continued research on Kenyan medicinal plants has offered plants extracts and purified secondary metabolites with potent antimalarialantiplasmodial activities (Muiva et al., 2009; Irungu et al., 2014; 2015; Muthaura et al., 2015b).

In this review we summarize research evidence on toxicity, cytotoxicity, antimalarial and antiplasmodial properties of medicinal plant extracts and secondary metabolites evaluated at Kenya Medical Research Institute, Center for Traditional Medicine and Drug Research (KEMRI-CTMDR) between January 2003 and December 2021. This review covers a period within which there was increased research activities on screening medicinal plants for antimalarial properties providing a recent outlook of our drug discovery efforts. We acknowledge that other Kenyan institution have documented medicinal plants with antimalarial activity. However, this review chose to exclusively focus on work done at KEMRI due to its renowned expertise in human health research, including rationalization of traditional medicine in Kenya.

2 Methods

2.1 Systematic review

A systematic review was conducted using a predefined protocol based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Page et al., 2021) guidelines including; literature search to identify potential articles, assessing the relevance of the articles quality and data extraction. The search was performed in Google Scholar and PubMed covering the period January 2003 to

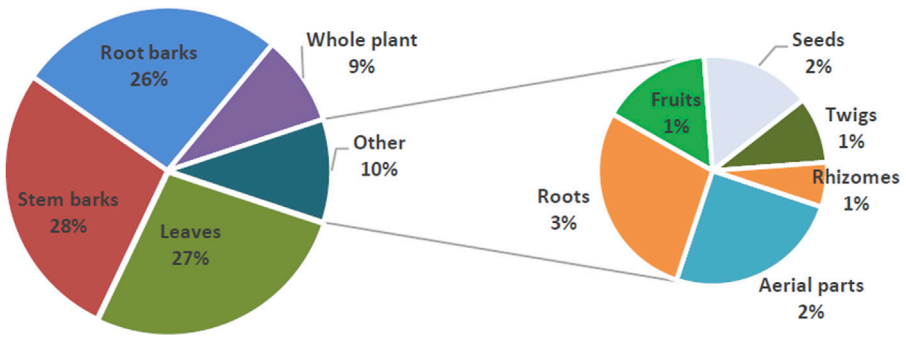

FIGURE 1
Frequency distribution of plant parts used to prepare extracts.

TABLE 1 Site of plant collection, nature of collaboration and journal published.

Collection site	Nature of collaboration	Journal published	References
Meru, Kenya	International	*Fitoterapia*	Rukunga et al. (2008)
Meru, Kenya	Local	*Journal of Ethnopharmacology*	Gathirwa et al. (2008)
Meru, Kenya	local	*Journal of Ethnopharmacology*	Muthaura et al. (2015b)
Meru, Kenya	local	*Journal of Natural Medicines*	Gathirwa et al. (2007)
Kilifi, Kenya	Local	*Journal of Ethnopharmacology*	Rukunga et al. (2009)
Kilifi, Kenya	International	*Journal of Ethnopharmacology*	Gathirwa et al. (2011)
Kajiado, Embu, Baringo; Kenya	Local	*African Journal of Pharmacology and Therapeutics*	Rotich et al. (2015)
Kajiado, Kenya	International	*Natural Product Research*	Muiva-Mutisya et al. (2017)
Kajiado, Kenya	Local	*South African Journal of Botany*	Kigondu et al. (2011)
Mombasa, Kenya	Local	*Journal of Pathogens*	Udu et al. (2021)
Meru and Mombasa; Kenya	Local	*South African Journal of Botany*	Irungu et al. (2007)
Kisumu, Kenya	Local	*Journal of Ethnopharmacology*	Orwa et al. (2013)
Kwale, Kenya	Local	*African Journal of Health Sciences*	Nyangacha et al. (2012)
Meru, Kenya	Local	*Phytotherapy Research*	Muthaura et al. (2007b)
Kwale, Kenya	Local	*Journal of Ethnopharmacology*	Muthaura et al. (2015b)
Central Kenya	Local	*Journal of Ethnopharmacology*	Kigondu et al. (2009)
West Pokot, Kenya	Local	*European Journal of Medicinal Plants*	Wachira et al. (2018)
Machakos, Kenya	International	*African Journal of Traditional, Complementary and Alternative Medicines*	Mutai et al. (2008)
Makueni, Kenya	International	*Phytochemistry Letters*	Muiva et al. (2009)
Nandi, Kenya	Local	*African Journal of Pharmacology and Therapeutics*	Jeruto et al. (2015)
Nairobi, Kenya	International	*Journal of Ethnopharmacology*	Irungu et al. (2015)
Kiambu, Kenya	International	*Molecules*	(Irungu et al., 2014)
Arusha, Tanzania	International	*Journal of Medicinal Plants Research*	(Ng'etich et al., 2020))
Arusha, Tanzania	Local	*African Journal of Pharmacology and Therapeutics*	Kangethe et al. (2016)
Baka Pygmies of the Dja Biosphere Reserve in Cameroon	International	*Journal of Natural Products*	Fotie et al. (2006)
Uganda	Local	*Journal of Ethnopharmacology*	Obbo et al. (2019)
Meru and Kilifi, Kenya	Local	*Journal of Ethnopharmacology*	Kirira et al. (2006)
OAU campus, Ile-Ife, Nigeria	International	*Journal of Herbs, Spices and Medicinal Plants*	Adebajo et al. (2013)
Ngong' forest, Kajiado County in Kenya	Local	*African Journal of Pharmacology and Therapeutics*	Irungu et al. (2012)
Nairobi, Kenya	International	*Acta Tropica*	Yenesew et al. (2012)

December 2021 and was limited to original English journal articles whose full text were accessible. Literature search was performed using key terms such as: Kenyan medicinal plants with antiplasmodial/antimalarial activities, antimalarial studies at Kenya Medical Research Institute (KEMRI), Center for Traditional Medicine and Drug Research (CTMDR), Kenyan antimalarial herbal remedies. We also searched with individual names of past and present Research Scientists working at KEMRI-CTMDR.

2.2 Inclusion and exclusion criteria

After a web search on pharmacological activities of medicinal plants screened at KEMRI-CTMDR, 108 journal articles were identified, 37 of which reported on antimalarial/antiplasmodial activities. Seven articles, did not meet our inclusion criteria since they either did not have an author from CTMDR, reported on synthetic compounds, were non-open access or were partially accessed (abstract only) as shown in Figure 1.

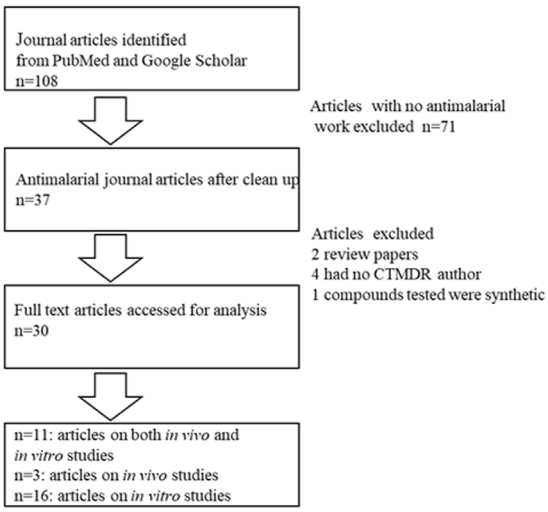

FIGURE 2
Flow chart describing journal articles selection strategy.

Thirty journal articles that met our selection criteria were selected for analysis.

2.3 Data screening and extraction

The articles were further analyzed based on the originality of reported data determined by the description of the study design. Data was captured in excel format and the following information from each eligible journal article was extracted; article title, plant botanical name, family, plant collection site, part(s) of the plant used, type of study (*in vitro* or *in vivo*), *Plasmodium* strain tested, IC_{50} values,% chemosuppression, isolated compound (s), cytotoxicity (CC_{50} or IC_{50}), toxicity (LD_{50}) and extraction solvent used. Descriptive statistics was used to summarize the findings.

3 Results

3.1 Medicinal plants screened for antimalarial/antiplasmodial activity

KEMRI-CTMDR Research Scientists have published data on 197 plant species and their potential antimalarial/antiplasmodial activities within a period of 18 years (January 2003 to December 2021). An extensive search and abstract screening within this period revealed 30 articles with at least one researcher from KEMRI-CTMDR as the main/co-author. Out of the 30 journal articles considered in this review, 66.7% (20/30) of the studies were done by local collaborators compared to international collaborators at 33.3% (10/30). The most preferred journal was *Journal of Ethnopharmacology* with 11 publications out of the 30 articles analyzed (Table 1). Twenty six out of the 30 articles, focused on plants that were collected from within Kenya while four articles investigated plant materials that were collected from outside Kenya (Table 1).

In this paper, each assessment of a plant species was treated as a separate study, which means that depending on the number of plant species examined, an article could encompass multiple studies. In total there were 1170 antiplasmodial/antimalarial tests done from 197 plant species. One hundred and fifty (76.1%) plant species were screened *in vitro*, one (0.5%) *in vivo* and 46 (23.4%) were both *in vivo* and *in vitro*. Majority of studies reported crude extracts except three, where fraction blends obtained separately from *Gongronema latifolium* (Benth.) K. Schum, *Artemisia annua* L. and *Lippia kituiensis* Vatke were evaluated (Adebajo et al., 2013; Kangethe et al., 2016; Ng'etich et al., 2020).

3.2 Diversity of plants evaluated

Of the 197 plants species, the most studied plant families were Asteraceae 16 (8.1%), Verbenaceae, 9 (4.6%), Rubiaceae, 8 (4%), Fabaceae, 7 (3.6%) and Leguminosae, 7 (3.6%). The most investigated plant species were; *Rotheca myricoides* (Hochst.) Steane and Mabb *Azadirachta indica* A. Juss., *Rhus natalensis* Bernh. ex Krauss, *Turraea robusta* (Hochst.) Benth., *Ximenia americana* L., *Vernonia auriculifera* Hiern, *Toddalia asiatica* (L.) Lam., *Maytenus undata* (Thunb.) Blakelock, *Lannea schweinfurthii* (Engl.) Engl., *Zanthoxylum chalybeum* Engl., *Harrisonia abyssinica* Oliv. *Fuerstia africana* Oliv. and *Asparagus racemosus* Willd. Leaves, 85 (27%), stem barks, 87 (28%), root barks, 83 (26%) and whole plant 28 (9%) were the most common parts of the plants used to prepare extracts (Figure 2). Crude extracts dominated in the tests compared to tests done using isolated compounds at 1072 (91.6%) and 98 (8.4%), respectively. Moreover, a majority of the extracts were organic 401 (67.3%) compared to aqueous extracts 195 (32.7%). In ascending order: 1:1 mixture dichloromethane: methanol, 9 (1.5%), hexane, 13 (2.2%), chloroform, 13 (2.2%), petroleum ether, 17 (2.9%), ethyl acetate, 18 (3%), dichloromethane, 19 (3.2%), water, 195 (32.7%) and methanol 309 (51.8%) were the most frequent extraction solvents used.

3.3 *In vitro* and *in vivo* activities of plant extracts

The activities were divided into three categories; good (IC_{50} values <10 µg/mL or suppression rate of ≥50%), moderate (IC_{50} values of 10 µg/mL–49 µg/mL or suppression rate of 30%–49%) and inactive (IC_{50} values ˃ 50 µg/mL or suppression rate of <30%) (Waiganjo et al., 2020). In general, 344 (29.4%) of the antiplasmodial tests reported good activity, 414 (35.4%) moderate activity and 412 (35.2%) were reports of inactivity. For the *in vitro* tests (Supplementary Table S1), inactive reports were the majority 386 (33%) followed by moderate activity 379 (32.4%) and good activity 300 (25.6%). Of the 300 *in vitro* studies with good activity, 177 (59%) were active with IC_{50} between 5 and 10 µg/mL while 123 (41%) were highly active with IC_{50} < 5 µg/mL (Table 2). On the other hand, a majority of *in vivo* tests reported good activity 44 (41.9%) (Table 3) followed by moderate activity 35 (33.3%) and 26 (24.8%) reported inactivity (Supplementary Table S2). Plant species that were commonly reported to display promising antiplasmodial activities in different studies included; *T. robusta* which exhibited good antiplasmodial activity in 8 out of 13 tests (61.5%), *T. asiatica,* 14 out of 26 tests (53.8%), *Erythrina burttii* Baker f., 12 out of 22 tests (54.5%) and *M. undata,* 10 out of 14 tests (71.4%).

TABLE 2 Plant extracts with highest antiplasmodial activity (IC_{50} < 5 μg/ml).

Plant screened	Plant family	Part used	Solvent used	Parasite strain	IC_{50} ug/ml	Cytotoxicity/LD_{50}	References
Holarrhena floribunda	Apocynaceae	Stem bark	Aqueous extract	W2	1.02	n.d	Fotie et al. (2006)
			Ethanoic extract	D6	4.33	n.d	
			Chloroform	W2	2.29	n.d	
Harrisonia abyssinica	Simaroubaceae	Stem barks	DCM	K1	4.4	n.d	Irungu et al. (2007)
Vernonia lasiopus	Asteraceae	Root barks	DCM	K1	4.7	>90 μg/mL	
				NF54	4.9		
Warbugia ugandensis	Canellaceae	Stem barks	DCM	K1	1.4	0.34 μg/mL	
				NF54	2.2		
Maytenus undata	Celastraceae	Leaves	Water	D6	0.95	n.d	Muthaura et al. (2015a)
				W2	1.9	n.d	
		Root barks	Methanol	W2	4.9	n.d	
			Methanol	D6	4.4	n.d	
Maytenus senegalensis	Celastraceae	Root barks	Methanol	D6	4.7	n.d	
Tabernaemontana pachysiphon	Apocynaceae	Fruits	Water	D6	4.8	n.d	
				W2	3.4	n.d	
			Methanol	D6	3.9	n.d	
Vernonia amygdalina	Asteraceae	Leaves	Water	W2	3.8	n.d	
			Methanol	D6	4.9	n.d	
Warburgia stuhlmannii	Canellaceae	Stem barks	Methanol	D6	1.8	n.d	
				W2	2.3	n.d	
Zehneria scabra	Cucurbitaceae	Whole plant	Methanol	W2	1.8	n.d	
Ziziphus mucronata	Rhamnaceae		methanol	D6	4.4	n.d	
Zanthoxylum chalybeum	Rutaceae	Root barks	Water	W2	3.1	n.d	
			Methanol	D6	3.7	n.d	
				W2	2.9	n.d	
Zanthoxylum chalybeum	Rutaceae	Root barks	Water (K)	ENT30	2.32	n.d	Rukunga et al. (2009)
			Methanol (K)	ENT30	3.14	n.d	
			Water (T)	NF54	3.65	n.d	
				ENT30	2.88	n.d	
Cyperus articulatus	Cyperaceae	Rhizomes	Methanol	NF54	4.84	n.d	
Erythrina burtii	Fabaceae	Root barks	Acetone	D6	0.97	n.d	Yenesew et al. (2012)
				W2	1.73	n.d	
		stem barks		D6	2.6	n.d	
				W2	2.9	n.d	
Cyperus articulatus	Cyperaceae	Rhizomes	Methanol	NF54	4.8	n.d	Rukunga et al. (2008)

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TABLE 2 (*Continued*) Plant extracts with highest antiplasmodial activity (IC_{50} < 5 μg/ml).

Plant screened	Plant family	Part used	Solvent used	Parasite strain	IC_{50} ug/ml	Cytotoxicity/LD_{50}	References
			Chloroform	NF54	2.1	n.d	
				ENT 30	3.3	n.d	
Fagaropsis angolensis	Rutaceae	Stem bark	Methanol	NF 54	4.68	brine shrimp nauplii 57.09 μg/mL	Kirira et al. (2006)
Zanthoxylum usambarense	Rutaceae	Stem bark	Methanol	NF 54	3.2	97.66 μg/mL	
Suregada zanzibariensis	Euphorbiaceae	Leaves	Methanol	D6	4.66	HELF cells >1000	Kigondu et al. (2009)
				W2	1.82		
Schkuhria pinnata	Asteraceae	Aerial	Pet ether	K1	2.46	>12.20 μg/mL	Obbo et al. (2019)
V. lasiopus	Asteraceae	Leaves	Chloroform	K39	1.2	n.d	Muregi et al. (2003)
			EtOAc	K39	1	n.d	
			Methanol	K39	3.2	n.d	
Boscia salicifolia	Rubiaceae	stem barks	water	D6	3.6	n.d	Muthaura et al. (2015a)
			methanol	D6	1.1	n.d	
		leaves	methanol	D6	4.4	n.d	
Commiphora schimperi	Burseraceae	stem barks	methanol	D6	3.9	n.d	
Artemesia afra	Asteraceae	leaves	water	W2	4.6	n.d	Muthaura et al. (2015b)
			Methanol	W2	3.9	n.d	
		stem barks	water	W2	4.1	n.d	
			Methanol	W2	1.2	n.d	
Artemisia annua	Asteraceae	Leaves	methanol	D6	4.7	n.d	
Clerodendrum rotundifolium	Verbenaceae	leaves	DCM	D6	3.9	n.d	
Croton macrostachyus	Euphorbiaceae	stem bark	methanol	D6	3.8	n.d	
Cyperus articulatus	Cyperaceae	tuber	methanol	D6	4.8	n.d	
Fagaropsis angolensis	Rutaceae	stem barks	methanol	D6	4.2	n.d	
Hypoestes forskaolii	Acanthaceae	root barks	methanol	D6	4.3	n.d	
Maytenus heterophylla	Celastraceae	Root barks	methanol	D6	1.8	n.d	
				W2	3.9	n.d	
Maytenus obtusifolia	Celastraceae	root bark	methanol	D6	<1.9	n.d	
Parinari curatellifolia	Chrysobalanaceae	root bark	methanol	W2	3.9	n.d	
Rubia cordifolia	Rubiaceae	whole plant	methanol	D6	<5	n.d	
				W2	<5	n.d	
Stephania abbyssinica	Menispermaceae	root barks	methanol	D6	4.7	n.d	
		leaves		D6	4.7	n.d	
Turrea robusta	Meliaceae	stem barks	methanol	D6	2.1	n.d	
Warburgia ugandensis	Canellaceae	root bark		W2	4.1	n.d	
Zanthoxylum usambarense	Rutaceae	root barks	methanol	D6	3.2	n.d	
Schkuhria pinnata	Compositae	Whole plant	methanol	D6	1.3	n.d	

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TABLE 2 (*Continued*) Plant extracts with highest antiplasmodial activity (IC_{50} < 5 μg/ml).

Plant screened	Plant family	Part used	Solvent used	Parasite strain	IC_{50} ug/ml	Cytotoxicity/LD_{50}	References
Clerodendrum eriophyllum	Verbenaceae	Leaves	methanol	D6	1.8	n.d	
				W2	3.9	n.d	
Fuerstia africana	Lamiaceae	Whole plant	Methanol	D6	0.98	954.7 μg/mL	Muthaura et al. (2007b)
				W2	2.4		
Schkuhria pinnata	Asteraceae	whole plant	Methanol	D6	1.3	161.5 μg/mL	
Boscia angustifolia	Capparaceae	Leaves	water	D6	1.42	6720 μg/mL	
				W2	4.77		
Boscia angustifolia	Capparaceae	stem barks	water	D6	1.4	n.d	Muthaura et al. (2015a)
				W2	4.7	n.d	
Ludwigia erecta	Onagraceae	Leaves	Methanol	D6	4.1	VERO cells 544.3 μg/mL	Muthaura et al. (2007b)
			water	D6	0.93	3283.6 μg/mL	
				W2	1.61		
Teclea nobilis	Rutaceae	Stem barks	Methanol	D6	3.9	n.d	Muthaura et al. (2015b)
		root barks	Methanol	D6	4.5	n.d	
Ludwigia erecta	Onagraceae	whole plant	water	D6	0.9	n.d	
				W2	1.6	n.d	
			methanol	D6	4.1	n.d	
Toddalia asiatica	Rutaceae	Fruits	Ethyl acetate	W2	1.87	Vero 199 Cells >100 μg/mL	Orwa et al. (2013)
				D6	4.01	>100 μg/mL	
		Root bark	Methanol	W2	2.49	>100 μg/mL	
			Water	W2	2.43	>100 μg/mL	
				D6	1.98	>100 μg/mL	
		Leaves	Ethyl acetate	D6	2.72	>100 μg/mL	
Fuerstia africana	Lamiaceae	Whole plant	methanol	D6	0.9	n.d	Muthaura et al. (2015a)
				W2	2.4	n.d	
Pentas lanceolata	Rubiaceae	Aerial parts	Water	D6	3.744	≥100 μg/mL	Rotich et al. (2015)
Fuerstia africana	Lamiaceae	Aerial parts	Water	D6	1.84	≥100 μg/mL	
Ximenia americana	Olacaceae	Stem barks	Water	D6	2.108	≥100 μg/mL	
Premna chrysoclada	Verbenaceae	Stems	Methanol	D6	0.75	Vero E6 Cells >100/mL	Gathirwa et al. (2011)
Flueggea virosa	Euphorbiaceae	Leaves	methanol	D6	2.2	n.d	Muthaura et al. (2015a)
				W2	3.6	n.d	
Turraea robusta	Meliaceae	Root barks	Methanol	D6	2.09	24.38 μg/mL	Gathirwa et al. (2008)
Turraea robusta	Meliaceae	Root barks	Methanol	K1	3.5	n.d	Irungu et al. (2007)
				NF54	2.4		
Turraea robusta	Meliaceae	Stem barks	DCM: methanol	W2	2.87	VERO cells 21.9 μg/mL	Irungu et al. (2015)
				D6	2.3	4TI 5.3 μg/ml	

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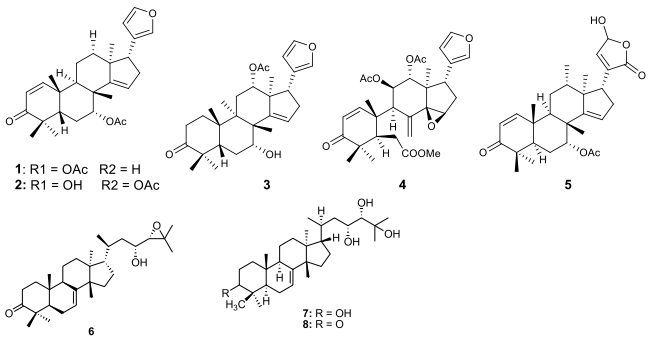
TABLE 2 (*Continued*) Plant extracts with highest antiplasmodial activity (IC_{50} < 5 μg/ml).

Plant screened	Plant family	Part used	Solvent used	Parasite strain	IC_{50} ug/ml	Cytotoxicity/LD_{50}	References
Artemisia afra	Asteraceae	Leaves	Methanol	W2	3.98	Vero cells 594.8 5 μg/mL	Gathirwa et al. (2007)
			Water	W2	4.65	2825.21 μg/mL	
Boscia salicifolia	Capparidaceae	Stem barks	Methanol	D6	1.04	304.92 μg/mL	
			Water	D6	3.65	1683.95 μg/mL	
Catharanthus roseus	Apocynaceae	Leaves	Methanol	D6	4.65	167.52 μg/mL	
			methanol	D6	4.6	n.d	Muthaura et al. (2015a)
Clutia robusta	Euphorbiaceae	leaves	methanol	D6	3.4	n.d	Muthaura et al. (2015a)
Clutia robusta	Euphorbiaceae	Leaves	Methanol	D6	3.41	460.29 μg/mL	Gathirwa et al. (2007)
Rotheca myricoides	Verbenaceae	root barks	methanol	D6	4.7	n.d	Muthaura et al. (2015a)
				W2	4.3	n.d	
Acacia mellifera	Leguminosae	Root barks	DCM	W2	4.2	n.d	Muthaura et al. (2015a)
		Leaves	Methanol	D6	3.9	n.d	
Sericocomopsis hilde brandtii	Amaranthacea	Aerial parts	Methanol	D6	3.15	≥100 μg/mL	Rotich et al. (2015)
				D6	4	≥100 μg/mL	
Sericocomopsis hilde brandtii	Amaranthacea	Root barks	Water	D6	2.12	≥100 μg/mL	Kigondu et al. (2011)
Fuerstia africana	Lamiaceae	Aerial parts	Pet ether	D6	1.56	n.d	
				W2	2.5		
		Roots	Pet ether	D6	4.6		
Fuerstia africana	Lamiaceae	Whole plant	methanol	D6	0.9	n.d	Muthaura et al. (2015a)
				W2	2.4	n.d	

DCM, dichloromethane; pet ether = Petroleum ether; K = *zanthoxylum chalybeum* collected from kilifi county kenya; T = *zanthoxylum chalybeum* collected from tharaka nithi county kenya; EtOAc, ethyl acetate; n. d = not done.

3.4 *In vitro* and *in vivo* activities of isolated compounds

Fifty five antimalarial/antiplasmodial active compounds isolated from eight plant species were reported. Of the 55 compounds, 7 (12.7%) and 48 (87.3%) were evaluated *in vivo* and *in vitro,* respectively. Twenty two of 55 (40%) compounds exhibited moderate activity while 16 (29%) were inactive. The most active compounds (IC_{50} values ≤10 μg/mL) were 17 (i.e., **5, 25, 26, 27, 28, 29, 31, 32, 34, 37, 40, 41, 42, 44, 46, 48**) (Table 4) with resinone (**39**) having the best activity (IC_{50} < 1 μg/mL).



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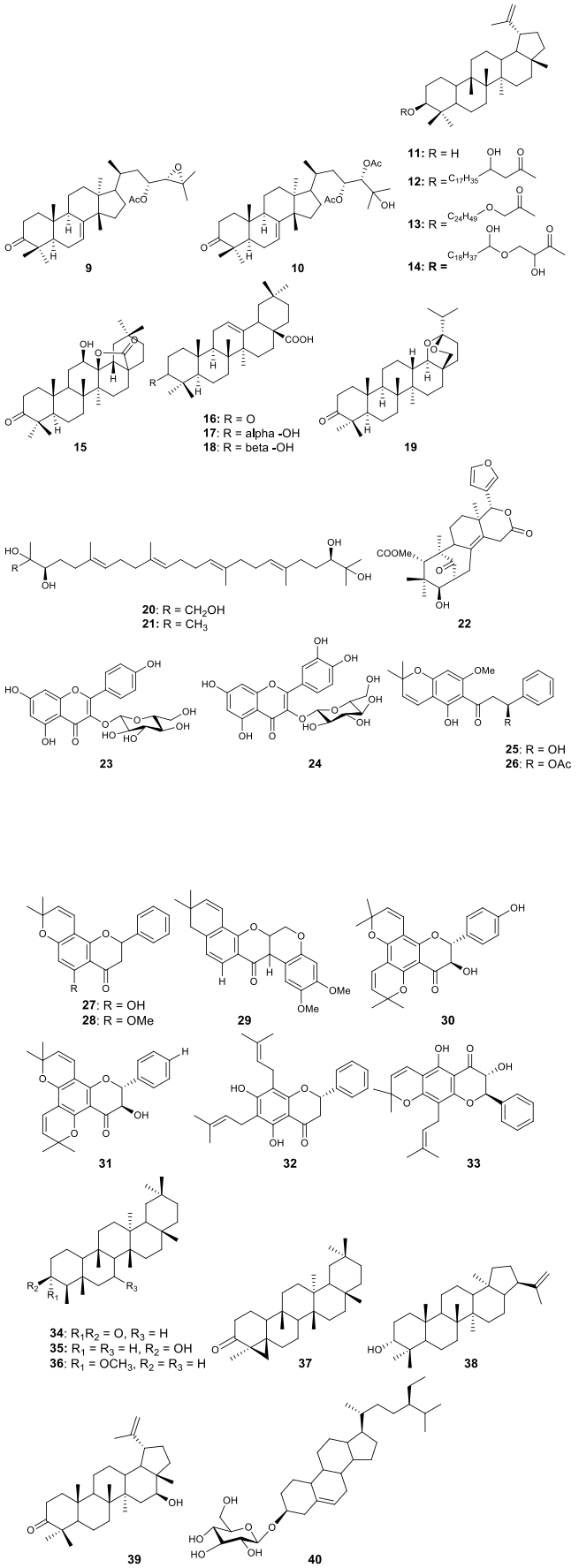

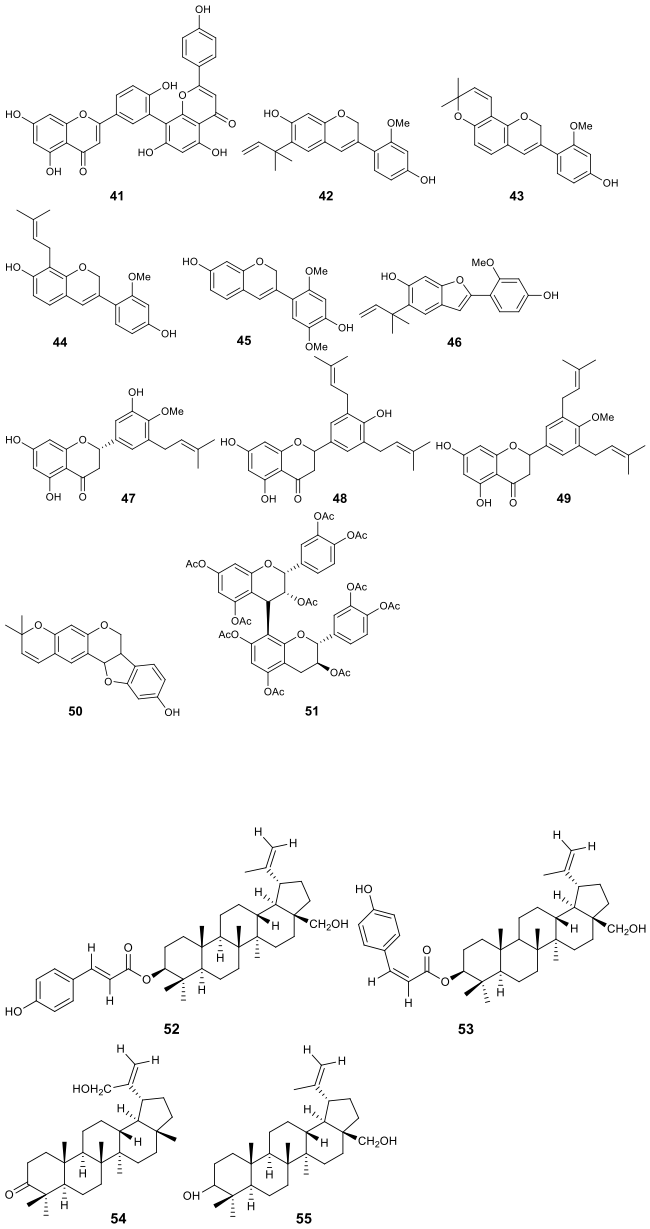

3.5 Cytotoxicity of plant extracts and compounds evaluated for antimalarial and antiplasmodial activity

In this review, a promising antimalarial extract was classified as lacking cytotoxicity to the mammalian cells by displaying an IC_{50} value greater than 90 μg/mL (Irungu et al., 2007). In general, there were 210 cytotoxicity tests from 40 plants. Out of the 40 plant species 14 (35%) had some degree of cytotoxicity across different studies. Plant families with the most cytotoxic (CC_{50} < 10 μg/mL) plant species were Meliaceae, Cucurbitaceae, Canellaceae, Asclepiadaceae, Asparagaceae and Lamiaceae. Fourteen (35%) of the plants tested were cytotoxic (CC_{50} < 10 μg/mL) and 8 (20%) demonstrated some toxicity levels (LD_{50} 100 mg/kg) in mice. The plants with good and

TABLE 3 Plant extracts with highest antimalarial activity (chemosuppression ≥50%).

Plant screened	Family	Part used	Solvent used	Parasite suppression (%) (dose)	LD_{50}	References
Premna chrysoclada	Verbenaceae	Stems	Methanol	65.08 (250 mg/kg)	n.d	Gathirwa et al. (2011)
		Leaves	Methanol	65.08 (250 mg/kg)	n.d	
Flueggea virosa	Euphorbiaceae	Roots	Methanol	68.55 (250 mg/kg)	n.d	
Azadirachta indica	Meliaceae	Leaves	Methanol	89.16 (250 mg/kg)	n.d	
Rhus natalensis	Anacardiaceae	Leaves	Methanol	82.7 (250 mg/kg)	n.d	
Grewia plagiophylla	Tiliaceae	Leaves	Methanol	77.9 (250 mg/kg)	n.d	
Hoslundia opposita	Labietaceae	Roots	Methanol	79.67 (250 mg/kg)	n.d	
		Aerial parts	Methanol	55.05 (250 mg/kg)	n.d	
Combretum padoides	Combretaceae	Roots	Methanol	50.56 (250 mg/kg	n.d	
		Stem barks	Water	83.08 (250 mg/kg)	n.d	
			Methanol	91.37 (250 mg/kg)	n.d	
Allophylus pervillei	Sapindaceae	Stem barks	Methanol	62.1 (250 mg/kg)	n.d	
Lannea schweinfurthii	Anacardiaceae	stem barks	water	83.08 (100 mg/kg)	n.d	Gathirwa et al. (2008)
			methanol	91.37 (100 mg/kg)	n.d	
Sclerocarya birrea	Anacardiaceae	stem barks	water	66.51 (100 mg/kg)	n.d	
			methanol	63.49 (100 mg/kg)	n.d	
Turraea robusta	Meliaceae	Root barks	Water	63.8 (100 mg/kg)	n.d	
			Methanol	78.2 (100 mg/kg)	n.d	
Artemisia afra	Asteraceae	Leaves	Methanol	77.45 (100 mg/kg)	n.d	Gathirwa et al. (2007)
		Leaves	Water	70.25 (100 mg/kg)	n.d	
Boscia salicifolia	Capparidaceae	Stem barks	Methanol	86.5 (100 mg/kg)	n.d	
Rhus natalensis	Anacardiaceae	Stem barks	Methanol	56.24 (100 mg/kg)	n.d	
Rhus natalensis	Anacardiaceae	Stem barks	Water	83.15 (100 mg/kg)	n.d	
Rotheca myricoides	Verbenaceae	Leaves	Methanol	82.17 (800 mg/kg)	n.d	Jeruto et al. (2015)
Rotheca myricoides	Verbenaceae	Root barks	Methanol	61.18 (800 mg/kg)	n.d	
Asparagus racemosus	Asparagaceae	Leaves	Methanol	54.35 (800 mg/kg)	n.d	
Pentas lanceolata	Rubiaceae	Aerial parts	Methanol	64.9 (500 mg/kg)	>5000 mg/Kg	Rotich et al. (2015)
Ximenia americana	Olacaceae	Stem barks	Water	54.9 (500 mg/kg)	n.d	
			Methanol	50.8 (500 mg/kg)	n.d	
Turraea mombassana	Meliaceae	Leaves	Methanol	52.86 (800 mg/kg)	>5000 mg/kg	Nyangacha et al. (2012)
Ludwigia erecta	Onagraceae	Leaves	Methanol	65.28 (100 mg/kg)	>100 mg/kg	Muthaura et al. (2007b)
Boscia angustifolia	Capparaceae	Leaves	Methanol	60.12 (100 mg/kg)	>100 mg/kg	
Pittosporum viridiflorum	Pittosporaceae	Leaves	Methanol	54.77 (100 mg/kg)	>100 mg/kg	
			Water	89.76 (100 mg/kg)	1000 mg/kg	
Clutia abyssinica	Euphorbiaceae	Leaves	Water	71.69 (100 mg/kg)	>5000 mg/kg	
Fuerstia africana	Lamiaceae	Whole plant	Methanol	61.85 (100 mg/kg)	>100 mg/kg	
Schkuhria pinnata	Asteraceae	whole plant	Water	64.22 (100 mg/kg)	>5000 mg/kg	
Clerodendrum eriophyllum	Verbenaceae	Root bark	Methanol	90.13 (100 mg/kg)	>100 mg/kg	
			Water	61.54 (100 mg/kg)	>5000 mg/kg	

(Continued on following page)

TABLE 3 (*Continued*) Plant extracts with highest antimalarial activity (chemosuppression ≥50%).

Plant screened	Family	Part used	Solvent used	Parasite suppression (%) (dose)	LD_{50}	References
Clausena anisata	Rutaceae	Stem barks	Hexane	56.7 (500 mg/kg)	4166.7 mg/kg	Irungu et al. (2012)
			Chloroform	73.4 (500 mg/kg)	4166.7 mg/kg	

DCM, dichloromethane; pet ether = Petroleum ether; EtOAc, ethyl acetate; n. d = not done. Parasite strain for all *in vivo* studies: *Plasmodium berghei ANKA*

moderate antiplasmodial activity demonstrated some degree of cytotoxicity of 10% and 7.5%, respectively. Organic extracts especially methanol, petroleum ether, dichloromethane: 1; 1 mixture of methanol and dichloromethane were reported to have the highest degree of cytotoxicity (CC_{50} < 10 μg/mL). The most cytotoxic was dichloromethane extract from *Warburgia ugandensis* Sprague with CC_{50} 0.34 μg/mL against L6, rat skeletal myoblast cells (Irungu et al., 2007). The most cytotoxic compounds were azadironolide (**5**) with CC_{50} of 8.5 μg/mL (HEp2 cells), oleanonic acid (**16**) with CC_{50} of 1.4 μM (HEp2 cells), 12α-acetoxy-7-deacetylazadirone (**2**) with CC_{50} of 4.3 μM (HEp2 cells), niloticin (**6**) with CC_{50} of 6.9 μM (HEp2 cells), hispidol B (**7**) with CC_{50} of 7.4 μM (HEp2 cells), amentoflavone (**41**) with CC_{50} of 0.34 μg/mL (L6 cells) and piscidinol A (**8**) with CC_{50} of 8.4 μM (HEp2 cells). The mentioned compounds had promising antiplasmodial activity, with azadironolide (**5**) being the most active. The most frequently used cells for cytotoxicity determination were Vero cells and HEp-2 cells.

4 Discussion

The global concern over the increasing resistance to primary antimalarial medications necessitates a boost in research efforts to discover and develop new drugs for malaria. The escalating resistance rates emphasize the urgency of accelerating the exploration and development of novel antimalarial drugs. It is evident from this review that a lot needs to be done towards the discovery of new antimalarial drugs. While numerous plant species have shown promising antiplasmodial effects, there has been limited evaluation of these plants in animal models, with only 9% (105/1170) *in vivo* studies and no clinical trial conducted. This highlights the importance of conducting comprehensive preclinical and clinical research. Pre-clinical and clinical research are a significant next step to determine the prospects of these promising medicinal plants (Al Rashid et al., 2020).

The majority of studies (91.6%) included in the analysis utilized crude plant extracts rather than pure compounds for their investigations. Such preference for crude extracts can be attributed to insufficient infrastructure required to process plant materials and extract pure compounds as well as an attempt to mimic the traditional preparation of plant remedies using alcoholic beverages. Preference for leaves, stem barks and root barks (Figure 2), can be attributed to their abundance and the local communities' indigenous knowledge and skills on their uses (Umair et al., 2019). Additionally, the preference for harvesting these plant parts is influenced by their lower impact on the overall health and sustainability of medicinal plant populations (Araya et al., 2015).

In this review, the IC_{50} values below 10 μg/mL were regarded as the threshold for significant antimalarial activity. This cutoff is considered as the minimum requirement for preliminary positive result in screening of potential antimalarial plant extracts (Mohammed et al., 2014). A total of 151 plant species belonging to 48 families exhibited moderate to good antiplasmodial activity. Among the most extensively studied plant families were Asteraceae, Verbenaceae, Fabaceae, Euphorbiaceae, Rubiaceae, and Leguminosae while families with the highest number of active plants were Apocynaceae, Celestraceae, Euphorbiaceae and Rutaceae. These findings suggest that greater attention should be given to plants whose extracts were promising for the discovery of antimalarial drug leads. Regarding individual plant species, notable ones that have received significant research attention include *R. myricoides, A. indica, R. natalensis, T. robusta, X. americana, T. asiatica, M. undata, L. schweinfurthii, Z. chalybeum, H. abyssinica, F. africana*, *A. racemosus* and *T. robusta.* Their extracts have consistently demonstrated significant antiplasmodial activities in multiple studies (Gathirwa et al., 2007; 2008; 2011; Orwa et al., 2013; Muthaura et al., 2015a; Jeruto et al., 2015; Rotich et al., 2015). Therefore, further preclinical evaluation of these plant species is recommended. This review has identified most potent plant extracts with significant activity against *P. falciparum*, exhibiting an IC_{50} value of ≤1 μg/mL and/or a parasite suppression rate above 90%. The plant species whose extracts were classified as most potent include *Combretum padoides* Engl. and Diels, *L. schweinfurthii*, *Clerodendrum eriophyllum* (Hochst.) Vatke, *Holarrhena floribunda* (G. Don) T. Durand and Schinz, *M. undata*, *E. burtii*, *Vernonia lasiopus* O. Hoffm., *F. africana*, *Ludwigia erecta* (L.) H. Hara, *Boscia salicifolia* Oliv., and *Premna chrysoclada* (Bojer) Gürke. Furthermore, studies conducted by other researchers (Machumi, 2010; Machumi et al., 2010; Muganga et al., 2010; Zofou et al., 2013; Hoekou et al., 2017; Elhaj et al., 2021) have also reported good antiplasmodial activities of *C. eriophyllum*, *H. floribunda*, *V. lasiopus*, *F. africana*, and *L. erecta*.

In this review, we documented compounds that are reported to possess other pharmacological activities such as obovatin (**27**) which has shown great potential as an antibacterial agent (Akter et al., 2016). Other studies have demonstrated antiplasmodial and anticancer activities of compounds captured in this review, deguelin (**29**), (Varughese et al., 2019; Buyinza, 2020), friedelin (**34**) (Prabhu et al., 2011; Emsen et al., 2018; Joshi et al., 2022; Wuttikit and Thanakijcharoenpath, 2023), and epifriedelanol (**35**) (Kundu et al., 2000; Gashu, 2022; Wuttikit and Thanakijcharoenpath, 2023). The antiplasmodial activity of friedelin (**34**) was found to be lower in a study by Sadeghpour et al. (2006) compared to other research cited in the current review (Sadeghpour et al., 2006). In summary, these compounds

TABLE 4 Compounds with highest antiplasmodial activity (IC_{50} ≤ 10 μg/mL).

Plant screened	Compound isolated	Class	Parasite strain	IC_{50}	Cytotoxicity	References
Turraea nilotica	Azadironolide **(5)**	Terpenoid	D6	2.4 μM	4TI 14.7 μg/mL	Irungu et al. (2015)
			W2	1.1 μM	HEp2 8.5 μg/mL	
					Vero	
					27.6 μg/mL	
Tephrosia elata	Elatadihydrochalcone **(25)**	Flavonoids	D6	8.4 μg/mL		Muiva et al. (2009)
			W2	8.6 μg/mL		
			D6	2.8 μg/mL		
			W2	5.5 μg/mL		
	Acetoxyelatadihydrochalcone **(26)**		D6	9.6 μg/mL		
	Obovatin **(27)**		D6	4.9 μg/mL		
			W2	6.4 μg/mL		
	Obovatin methyl ether **(28)**		D6	3.8 μg/mL		
			W2	4.4 μg/mL		
	Deguelin **(29)**		D6	6.3 μg/mL		
			W2	8.9 μg/mL		
Tephrosia subtriflora	MS-II **(31)**	Flavanol	D6	4.6 μM	Vero >247.5 μM	Muiva-Mutisya et al. (2018)
			3D7	1.7 μM	HEp 2 > 247.5 μM	
			KSM	1.5 μM		
			F32-TEM	1.4 μM		
	Spinosaflavanone B **(32)**	Flavanone	D6	5.9 μM	n.d	
			3D7	5.5 μM	n.d	
			KSM	6.6 μM		
Drypetes gerrardii	Friedelin **(34)**	Terpenoids	K1	4.8 μg/mL	L6	Ng′ang′a et al. (2012)
					>90 μg/mL	
	5 β,24-cyclofriedelan-3-one **(37)**		K1	2.2 μg/mL	21.2 μg/mL	
	Resinone **(39)**		K1	0.09 μg/mL	84.8 μg/mL	
	β - Sitosterol glucopyranoside **(40)**		K1	5.4 μg/mL	14.3 μg/mL	
	Amentoflavone **(41)**		K1	2.6 μg/mL	0.34 μg/mL	
Erythrina burtii	Burttinol-A **(42)**	Isoflav-3-enes	D6	7.6 μM	n.d	Yenesew et al. (2012)
			W2	8.5 μM		
	Burttinol-C **(44)**		D6	9.3 μM		
			W2	9.1 μM		
	Burttinol-D **(46)**	2-Arylbenzofuran	D6	4.0 μM		
			W2	6.1 μM		
	Abyssinone V **(48)**	Flavanones	D6	5.7 μM		
			W2	6.6 μM		

have demonstrated promising antiplasmodial activity and are thus valuable candidates for further antimalarial drug development.

Our review has demonstrated that majority of investigated plants have promising antiplasmodial activity. However, when the same plants were tested in a mouse model, their activity

against malaria parasites decreased in most cases, with many plants showing no activity at all. For instance, Rotich et al. (2015) and Gathirwa et al. (2011) reported that *Uvaria acuminate* Oliv. and *F. africana*, displayed good antiplasmodial activity (IC_{50} < 10 μg/mL) but were inactive *in vivo* (chemosupressiom at 27.0% and 27.9%, respectively). The observed variations could be explained by the fact that *in vitro* studies involved direct contact between the extracts and the parasite, while for *in vivo* studies activity of the extracts/compounds might have been altered by metabolism. Nevertheless, a few studies have shown that plant activity can actually increase from *in vitro* to *in vivo*. For example, Muthaura et al. (2007b) demonstrated that *Pittosporum viridiflorum* Sims exhibited moderate activity *in vitro* [IC_{50} 18.9 μg/mL and 17.7 μg/ml against D6 and W2 strains, respectively] but showed good activity *in vivo* with chemosuppression of 54.8% (Muthaura et al., 2007b). These findings suggest that plants could still possess significant antimalarial properties in animal models even if they do not show activity *in vitro*. Apparently, researchers proceed to *in vivo* studies only when they observe substantial antiplasmodial activity. This may explain the limited number of *in vivo* studies documented in this review. Despite the unsatisfactory outcomes observed *in vitro*, it still remains crucial to examine the antimalarial properties of plants through *in vivo* studies.

The present study identified significant inter study variations in the antiplasmodial activity of various plant species. Notably, considerable variation was observed for species such as *P. chrysoclada, F. virosa, Grewia plagiophylla* Burret*, T. robusta, R. myricoides, A. racemosus, Vangueria acutiloba* K. Schum.*, C. eriophyllum, H. abyssinica, V. lasiopus, W. ugandensis, Ajuga remota* Benth.*, Tabernaemontana pachysiphon* Stapf*, Uvaria lucida* Benth.*, Uvaria scheffleri* Engl. and Diels*, Vitex strickeri* Moldenke*, Warburgia stuhlmannii* Engl. and *Cyperus articulatus* L. (Muthaura et al., 2007a; Muthaura et al., 2015a; Muthaura et al., 2015b; Irungu et al., 2007; Gathirwa et al., 2008; Gathirwa et al., 2011; Rukunga et al., 2008; Jeruto et al., 2015). Several factors may account for these differences, including variations in the extraction solvent used, which affects the yield and composition of extracted metabolites. Dichloromethane, for instance, primarily extracts apolar metabolites, while methanol extracts a range of polar to moderately apolar metabolites and water extracts polar metabolites. The choice of plant parts used in the studies also contributed to the observed variations, as certain parts may contain higher concentrations of specific active metabolites. Additionally, differences in extraction yields can arise due to the varying accumulation of active metabolites in different plant parts. Also, the location, environmental factors and season (dry and rainy seasons) have significant effect on the accumulation of various phytochemicals present in medicinal plants. During the dry season, there is a decrease in water and nutrient supply to plants. Nutritional stress can result in the accumulation of osmo-protectants to stabilize proteins structure and maintain membrane integrity and scavenge reactive oxygen species (ROS), with biomass and secondary metabolites production (Niinemets, 2016). Phenolic compounds including coumarins, flavonoids, cinnamic acids and lignans, as well as plant hormones such as auxins, salicylic acid, cytokinin, ethylene, gibberellic acid and jasmonic acid are involved in modulation of developmental processes in plants and determine plant responses to environmental stresses (Fang et al., 2011; Fayez and Bazaid, 2014; Giménez et al., 2014). On the other hand, plants growing in lower temperatures develop significant adjustments in several physiological and biochemical processes that enable them to survive under low temperature stress, and this causes inhibition in the synthesis and storage of secondary metabolites (Verma and Shukla, 2015).

Another factor that may contribute to the observed inter study variation is the strain of *Plasmodium* used in the experiments. Studies employing chloroquine-sensitive strains of the parasite, such as *P. falciparum* 3D7, D6, and NF54, tend to report higher antiplasmodial activity compared to studies utilizing chloroquine-resistant strains like W2, K39, ENT30, or K1. This variation in strain susceptibility to the tested extract/compound can influence the reported outcomes and contribute to the differences observed across studies. It is worth noting that the variation in the antiplasmodial activity of *Turraea nilotica* Hochst. ex Benth (Irungu et al., 2015). observed with pure compounds highlights an important issue. Even extracts that initially show low potency and might be disregarded during the initial screening process for further development may still contain active components with therapeutic potential, as mentioned by Kuria et al. (2001). In the given example, the preliminary analysis of the crude extract demonstrates an IC_{50} value of 59 μg/mL for the D6 strain and 47.4 μg/mL for the W2 strain, as indicated in Supplementary Table S1. However, within the same extract, there is a highly activeepimeric mixture, azadironolide (**51**) that exhibited an IC_{50} value of less than 5 μg/mL.

Data collated in this review showed 14 out of 40 (35%) plant species, exhibited high level of cytotoxicity (CC_{50} < 10 μg/mL). The plant families Meliaceae, Cucurbitaceae, Asclepiadaceae, Asparagaceae, Canellaceae and Lamiaceae were found to have the highest number of cytotoxic plant species. The most cytotoxic plants identified were *W. ugandensis, X. americana* and *Khaya anthotheca* (Welw.). Interestingly, *W. ugandensis* and *X. americana* have shown promising antiplasmodial/antimalarial activity in certain studies (Irungu et al., 2007; Muthaura et al., 2015b). This suggests that the observed strong antiplasmodial effects could probably be as a result of cytotoxicity rather than direct activity against the parasites themselves (Irungu et al., 2007). Other plants with significant cytotoxicity but also exhibiting moderate to good antiplasmodial/antimalarial properties include *Vernonia amygdalina* Delile, *Baccharoides adoensis* (Sch.Bip. ex A. Rich.) Hochr., *Schkuhria pinnata* (Lam.) Kuntze, *Momordica foetida* Schumach. and Thonn., *Entada abyssinica* Steud. ex A. Rich., *Entandrophragma utile* (Dawe and Sprague) Sprague (Obbo et al., 2019), *C. eriophyllum* (Irungu et al., 2007), *Ekebergia capensis* Sparrm (Irungu et al., 2014), *T. robusta* (Irungu et al., 2015) and *F. africana* (Rotich et al., 2015). The toxicity levels of most plant extracts in animal models were found to be minimal, even at dosages above 1000 mg/kg body weight. Aqueous extracts showed no adverse effects even at a dosage of 5000 mg/kg body weight. It is important to note that toxicity/cytotoxicity levels varied considerably, even within the same plant species. This variation could be attributed not only to the extraction solvent but also to differences in study design (*in vivo* or *in vitro*) and the specific plant parts tested.

5 Conclusion

This review has collated valuable foundational data that researchers in the field can utilize for the exploration and development of new antimalarial drug leads. Among the plant species studied, *F. africana* and *L. erecta* were found to have the highest activity, with IC_{50} values below 1 μg/mL against *P. falciparum* (D6), a chloroquine-sensitive strain. These plants also demonstrated significant parasite suppression at an oral dose of 100 mg/kg, with 61.9% and 65.3% for *F. africana* and *L. erecta*, respectively. Their LD_{50} values were above 3000 mg/kg, indicating low toxicity. Additionally, resinone (**39**) a compound isolated from the *Drypetes gerrardii* (Baill.) Hutch showed good activity against *P. falciparum* K1 multidrug-resistant strain, with an IC_{50} below 1 μg/mL. However, no information was provided regarding *in vivo* testing or toxicity assessments of this compound. While the *in vitro* results demonstrated promising activities of some plant extracts and their compounds, there has been limited evaluation of active plants extracts *in vivo*, and no clinical trials have been conducted yet. To address the research gap, preclinical studies should progress beyond *in vitro* and *in vivo* screening for antimalarial properties to include comprehensive studies on efficacy, safety and quality of promising extracts in animal models. Additionally, future studies geared towards product development should factor in intellectual property rights through local bodies such as Kenya Industrial Property Institute to address barriers that may arise and hinder development of lead compounds/phytomedicines from medicinal plants. Furthermore, the study revealed significant variations in the antiplasmodial activities of the plants across different studies. Notably, only a small number of plants had their active compounds identified. Furthermore, it is worth emphasizing the significance of assessing ethnomedical preparation procedures and establishing a correlation with laboratory extraction methods. This correlation is essential as it justifies the process of plant selection and, in turn, contributes to the validation of ethnomedicine. Hence, there is still need for further and extensive research with the aid of a stable strategy in the exploration and advancement of novel antimalarial compounds to tackle the escalating resistance observed in current primary antimalarial drugs across the globe.

Author contributions

BI: Conceptualization, Validation, Writing–original draft, Writing–review and editing. EO: Data curation, Validation, Writing–original draft, Writing–review and editing. MN: Methodology, Validation, Writing–review and editing. SN: Validation, Writing–review and editing. LK: Formal Analysis, Methodology, Validation, Writing–review and editing.

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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.2023.1268924/full#supplementary-material

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Translation of preclinical ethnomedicine data in LMICs: the example of rooibos

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All disease, but especially non-communicable diseases, are related to dysfunction of one or more regulatory systems. In developing countries, long-term management of patients with chronic diseases has many challenges and is generally not financially viable, but Africa in particular, which is rich in diverse ethnomedicines presents a more feasible long-term therapeutic approach in this niche. However, despite comprehensive preclinical investigations on numerous plant-derived candidate medicines, only a small portion of these reach the patient as recognised medicines. In this review, we use the example of rooibos (*Aspalathus linearis* (Burm.f.) R. Dahlgren)–which is globally consumed as aromatic, caffeine-free tea–to illustrate the hurdles that need to be overcome in the low-to middle-income countries, before progression of ethnomedicines to official treatment regimens can be achieved. In terms of methodology, regulatory system focused rooibos papers indexed on PubMed for the past three decades (n = 112) were accessed. Papers reporting duplication of previous results were excluded, as well as review papers. Topics covered includes the high standard of ethnomedicine drug discovery and efficacy testing research performed in Africa (and South Africa in particular in the case of rooibos), the potential bias in terms of preclinical research focus, ethnomedicine ownership and the requirement for independent clinical trial coordination and/or management.

KEYWORDS

rooibos, clinical trial, human, challenges, regulatory systems, gut-brain, inflammation

Introduction

A recent fact sheet by the World Health Organisation reported 37% of deaths in Africa to result from non-communicable diseases, with 63% of deaths classified as premature (i.e., before the age of 70) (Wangou et al., 2022). Even more alarmingly in terms of burden on public health systems, is that the International Diabetes Federation reported a diabetes incidence in Africa of 1 in 22 (IDF, 2021). Furthermore, in countries with increased rates of urbanisation and sedentary lifestyles, such as South Africa, incidence may be as high as 1 in 10 (Erzse et al., 2019). In 2015, the National Institute of Health estimated the cost of diabetes alone in Sub-Saharan Africa at USD 19.45 billion annually–1.2% of gross domestic product (Erzse et al., 2019). From these statistics, it is clear that long-term management of chronic patients using mainstream pharmaceuticals is neither financially viable, nor entirely successful.

Africa is rich in candidate medicines that were initially identified largely via indigenous knowledge systems (Sprent et al., 2010). Thus, Africa should be strategically poised at the frontier of medicine development to address the plight of the chronically ill in Africa and other low-to middle-income countries (LMICs) globally. However, despite African

ethnomedicine riches, only a fraction of *worthy* candidate medicines reaches the point where they are officially recognised and registered as mainstream medicines, which would allow their incorporation into policy documents and widespread application in public sector medical centres.

With this mini-review, using the example of Rooibos (a globally consumed tea, prepared from *Aspalathus linearis* (Burm.f.) R. Dahlgren), we unpack some of the strengths and weaknesses of ethnomedicine research practices such as the research group bias and the relative lack of clinical trials. We also make suggestions on how the more unique challenges faced by African drug discovery and medicines development scientists, on their road to recognition of ethnomedicines as equivalent treatment regimes in disease, may be addressed. These include safety concerns, ethnomedicine ownership, stagnation of medicines development at the preclinical phase.

Rooibos: doomed to fail or on the road to success?

Rooibos is a herbal plant indigenous to South Africa and is globally consumed as tea, for its aromatic taste and health benefits. Rooibos is commercially available in two forms, unfermented (green) and fermented. Although the unfermented rooibos generally exhibits higher potency–likely due to its higher polyphenol content–similar health promoting attributes have been reported for both. Thus, distinction between the two types was not relevant for the purpose of this review.

Rooibos was chosen as example ethnomedicine, as it has been assessed in the context of modulation of many regulatory systems, which are central to the physiological dysregulation resulting in chronic disease. Furthermore, a robust body of literature generated by many different research groups exist on its potential as medicine in both a preventative and therapeutic approaches across various models and diseases, and it has been thoroughly characterised in terms of active constituents.

Evidence of general antioxidant and anti-inflammatory efficacy

Rooibos is widely known and consumed for its antioxidant activity. The antioxidant mechanism of rooibos seems multi-pronged: firstly, it has demonstrated ability to scavenge free radicals in a manner similar to vitamin C and E (Snijman et al., 2009; Lopez et al., 2022), a mechanism ascribed to mainly two active ingredients–aspalathin and nothofagin (Bramati et al., 2003; Joubert et al., 2005; Chen et al., 2013; Canda et al., 2014). In fact, rooibos has such high potency that pro-oxidant outcome have been reported for extracted (i.e., concentrated) rooibos when administered in the absence of disease or conditions of oxidative stress (Joubert et al., 2005). Secondly, rooibos constituents quercetin, aspalathin and catechin inhibit lipid peroxidation (Snijman et al., 2009), a mechanism likely responsible for its reported membrane-protective effects of rooibos (Ulicna et al., 2019; Pretorius and Smith, 2022). A multitude of studies in cell-free systems and in cell culture have also demonstrated improved antioxidant enzyme activities (particularly for catalase and superoxide dismutase (SOD)) (Waisundara and Hoon, 2015) and mitochondrial bioenergetics (Mthembu et al., 2021).

Redox benefits of rooibos administration have also been successfully translated into various *in vivo* settings. In rodent models, these benefits included increased rodent serum SOD and decreased urinary 8-Oxo-dG levels (Baba et al., 2009), attenuation of increases in plasma and hepatic MDA and decreases in whole blood and liver GSH:GSSH ratios (Ajuwon et al., 2014; van der Merwe et al., 2015), restoration of t-BHP- and CCl4-induced reduction of liver antioxidant status (Canda et al., 2014; Ulicna et al., 2019) and improved mitochondrial bioenergetics (elevated mitochondrial enzymes, improved capacity of the electron transport chain and increased energy production) (Ulicna et al., 2019). Also in invertebrate models such as *C. elegans*, rooibos supplementation decreased juglone-induced acute oxidative damage and extended the lifespan of *Caenorhabditis elegans* in a high glucose environment (Chen et al., 2013). Using quantitative real-time PCR, the authors demonstrated that aspalathin targeted stress and aging related genes as a potential mechanism of action.

The antioxidant capacity of rooibos also links closely to an anti-inflammatory outcome. In this context, data from *in vitro* models of inflammation associate rooibos administration with improved tight junction protein expression and interaction status to maintain endothelial cellular structure and barrier integrity (Ku et al., 2015; Lee and Bae, 2015; Pretorius and Smith, 2022), modulation of PGE2 synthesis (Hedbrant et al., 2022), inhibition of basophil activation (Pedretti and Peter, 2020) and an inhibitory effect on concentrations of pro-inflammatory mediators–such as IL-1α, IL-6, IL-8, VCAM-1 and ATF4 gene expression (Lawal et al., 2019b), as well as IFN-γ, IL-12, IL-2, IL-17a, TNF-α, IL-8, IL-6, IL-1β and CXCL10 (Nehme et al., 2023)–and increased anti-inflammatory cytokine, IL-10 (Ichiyama et al., 2007). In addition, the flavonoid actives aspalathin and nothofagin were reported to prevent the expression of cell adhesion molecules and transendothelial migration of neutrophils in LPS- and high glucose-exposed cells (Ku et al., 2015; Lee and Bae, 2015), while other rooibos constituents quercetin, luteolin and chrysoeriol reportedly inhibited antigen- and calcium ionophore-stimulated degranulation in basophils (Morishita et al., 2019).

In the inflammation context, data from *in vivo* studies again corroborates *in vitro* data. Here, rooibos-treated rodents exposed to LPS exhibited blunted TNF-α and IL-6 responses (Ajuwon et al., 2014), while rooibos pre-treatment reversed DEP-induced increases in IL-8, TNF-α, NF-κB and IL-1β and decreases in IL-10 gene expression (Lawal et al., 2019a). Also in response to the hepatotoxic challenge CCl4, a reduced NF-κB expression was reported in rooibos-administered rats (Gabuza et al., 2022). In terms of specific flavonoids, mice treated with aspalathin and nothofagin showed blunted inflammatory responses (TNF-α, IL-6, NF-κB activation, endothelial hyperpermeability and leukocyte migration) to both LPS- and high glucose challenge (Ku et al., 2015; Lee and Bae, 2015).

In addition to general demonstrations of antioxidant and anti-inflammatory actions of rooibos, potential for therapeutic effect in specific disease states have also been reported. A large number of rodent studies have linked antioxidant and anti-inflammatory effects of rooibos to cardiovascular health in particular, including

benefits in terms of both vasculature and myocardial tissue (Katengua-Thamahane et al., 2014; Ku et al., 2015; Lee and Bae, 2015; Obasa et al., 2020; Smit-van Schalkwyk et al., 2020; Smit et al., 2022).

Despite the solid pre-clinical database, we could find only one clinical trial that focused on elucidating benefits of rooibos supplementation in a vulnerable population - in subjects at risk for coronary heart disease, rooibos consumption improved plasma lipid profiles (decreased LDL-cholesterol and triacylglycerols; increased HDL-cholesterol) and redox status (decreases in lipid peroxidation (conjugated dienes); increases in glutathione and GSH: GSSH ratio), suggesting a beneficial effect of rooibos to reduce risk factors implicated in developing cardiovascular disease (Marnewick et al., 2010).

Endocrine (metabolic) efficacy

Given the high incidence of diabetes in developing countries, it is no surprise that a robust body of rooibos research exist in this context. One of the first reports on rooibos illustrated it to ameliorate diet-induced metabolic disturbances (including lipid profile, adipocyte size and hepatic steatosis) in hyperlipidemic mice (Beltran-Debon et al., 2011), suggesting rooibos to have a role as preventative modality in metabolic disease. Aspalathin was named one of the most important active ingredients in rooibos, facilitating a beneficial outcome in terms of glucose levels and insulin sensitivity in *db/db* mice (Kawano et al., 2009).

These positive reports sparked research focused on assessing the anti-diabetic potential of rooibos, which were largely led by researchers in South Africa, where rooibos grows naturally. Research expanded on the first studies, illustrating rooibos to enhance glucose uptake into (C2C12) murine skeletal muscle myotubules and to lower blood glucose with a potency similar to that of metformin (Muller et al., 2012) in streptozotocin (STZ)-induced diabetic rats, with the constituent polyphenols aspalathin and rutin demonstrated to act in synergy to achieve these effects. In addition, the constituent phenylpyruvic acid-2-O-glycoside (PPAG) was illustrated to play a major role in improving glucose metabolism in Chang (human hepatic) cells (Muller et al., 2013). Follow-up studies by the same group expanded on the antidiabetic potential of rooibos by demonstrating its ability to prevent experimentally induced insulin resistance and glucose-associated detrimental effects in several different cell types, including C2C12 murine skeletal muscle myotubules (Mazibuko et al., 2013), H9c2 murine embryonic cardiomyocytes (Dludla et al., 2016), 3T3-L1 murine adipocytes (Mazibuko et al., 2015) and C3A human liver carcinoma cells (Mazibuko-Mbeje et al., 2019) via antioxidant-dependent and independent mechanisms (Mazibuko-Mbeje et al., 2019; Mazibuko-Mbeje et al., 2022). In addition, rooibos was shown to reduce lipid accumulation in 3T3-L1 liver cells (Sanderson et al., 2014), suggesting a further beneficial anti-diabetic mechanism, while aspalathin specifically was demonstrated to protect pancreatic β-cells from *in vitro* glucotoxicity (Moens et al., 2020) and lipotoxicity (Moens et al., 2022).

The robustness of the comprehensive preclinical data set generated by this group, is evident from the similarly positive data by other research groups illustrating benefits of rooibos in other rodent models of diabetes (Son et al., 2013; Kamakura et al., 2015), as well as in L6 myoblasts and pancreatic β-cells (Kamakura et al., 2015; Himpe et al., 2016). New technology was recently used to confirm known effects and mechanisms, e.g., via targeted cellomics screening (Pringle et al., 2021) and network pharmacology- and molecular dynamics simulation-based bioprospecting (Akoonjee et al., 2022). However, despite the robust preclinical data, translation into human models are still lacking.

Rooibos and its constituents have not yet been investigated for other potential endocrine effects to the same extent. Only one group has reported on the effect of rooibos and some constituents on adrenal steroidogenesis. Indeed, an inhibitory effect of rooibos on specific P450 enzymes was demonstrated in fibroblast-like primate kidney (COS-1) cells, as well as a general downregulation of synthesis of corticosteroid and aldosterone precursors in H295r human adrenocarcinoma cells (Schloms et al., 2012; Schloms and Swart, 2014). This data aligns with the "calming" effect anecdotally ascribed to rooibos. Encouragingly, this group also reported on parallel supplementation studies in humans (the same cohort study reported on in Marnewick et al. (2010) and rats. These *in vivo* studies jointly provided evidence of decreased glucocorticoid synthesis after rooibos consumption–most notably a lower corticosterone: testosterone ratio in rodents, and a lower cortisol:cortisone in humans (Schloms and Swart, 2014).

Neglected regulatory systems

Although rooibos is anecdotally acclaimed for its calming effect few studies have investigated the effect of rooibos on the brain and nervous system and associated behavioural outcomes. Somewhat disappointingly, most *in vitro* studies in this context–albeit with positive results–seem largely focused on antioxidant effects (Hong et al., 2014; Fisher et al., 2020; Ma et al., 2020; Lopez et al., 2022; Brasil et al., 2023). Only two of these expanded their investigation and also reported different modulatory mechanisms in the context of serotonin metabolism (Hong et al., 2014; Lopez et al., 2022), which provides some additional mechanistic support for an anxiolytic or calming effect.

However, the limited scope of these studies may be a limitation of neuronal cell models, as *in vivo* studies in rodents reported a larger variety of beneficial functional outcomes for rooibos, such as improved long-term spatial memory that was associated with increased striatal dopamine and 3-methoxytyramine (dopamine metabolite) levels, but not excessive motor activity (Pyrzanowska et al., 2019), as well as reduced anxiety and increased exploratory behaviour (Pyrzanowska et al., 2021). These findings have also been corroborated in zebrafish larvae (Lopez et al., 2022), in which a significant GABA agonist effect of rooibos was elucidated.

Collectively, available literature shows neuroprotective potential mediated by an increase in antioxidant enzyme activity (Akinrinmade et al., 2017) and/or modulation of neurotransmission (Lopez et al., 2022), which translates to improved behavioural outcome (Pyrzanowska et al., 2019; Pyrzanowska et al., 2021; Lopez et al., 2022). However, in this context, literature is still relatively sparse.

Somewhat related to this, when considering the known signalling between the brain and the gut, the tradition route of

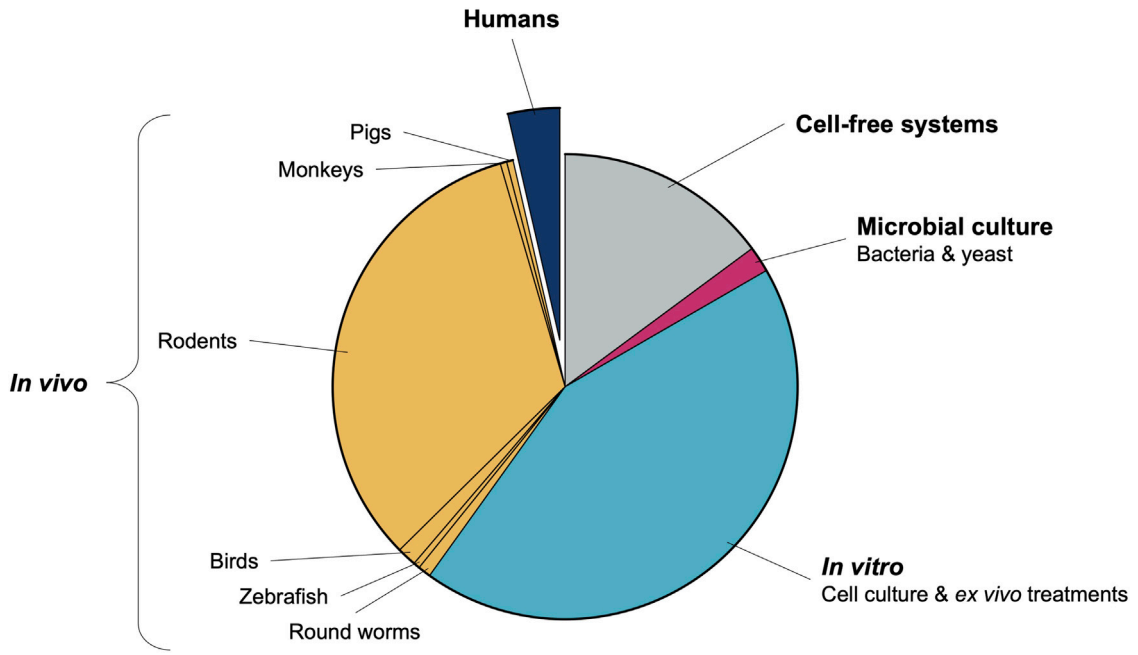

FIGURE 1
Relative popularity of models used in published rooibos-related health research studies.

rooibos consumption (orally as a tea), as well as the recognition of the microbiome as additional regulatory system, it seems logical to also consider effects of rooibos in the gut. Indeed, in the rooibos context, emerging evidence suggests a prebiotic-like modulation of the gut microbiome. In vervet monkeys, rooibos normalised Firmicutes to Bacteroidetes ratio to rescue high-fat high-sugar diet-induced metabolic dysregulation (Mangwana, 2020), and increased relative abundance of beneficial bacterial species (*Faecalibacterium prausnitzii, Prevotella stercorea* and *P. copri, Akkermansia muciniphia, Bacteroides intestinalis, Desulfovibrio piger* and *Bifidobacterium adolescentis*). More recent *in vitro* studies confirmed increased probiotic microbial growth and favourably modulated secretome trace amine content to promote gut health (Pretorius et al., 2022; Pretorius and Smith, 2022). Taken together, changes in the gut microbiome are likely to influence host regulation and homeostasis and as such, the potential benefit conferred by rooibos supplementation necessitates additional robust investigation.

Requirements for transformation from tea to treatment

A few facts are clear from the rooibos context, which may be extrapolated more broadly to ethnomedicine in Africa. Firstly, existing data on rooibos is largely positive and reproducible across various experimental models and research groups, suggesting that rooibos has significant merit as candidate ethnomedicine. Secondly, perhaps due to limited scope of interest or specific expertise of isolated research groups, data on specific systems (such as redox) are almost excessive, while relatively lacking for others. Thirdly, despite almost two decades of preclinical research, translation into human trials is near absent (Figure 1), suggesting absence of a coordinated, systematic approach to medicine development. Moreover, the human studies that have been performed are often limited (few parameters assessed on available samples) and self-serving to the research group interest. We provide some suggestions on how these issues may be addressed going forward.

Addressing safety concerns

A potential obstacle to human testing of ethnomedicines in general, is concerns around safety. As would be the case for any mainstream candidate pharmaceutical, specific pharmacological and pharmacovigilance data on toxicity, risk for drug-interaction, ADME profile, risk of off-target adverse events, etc. would be required for any ethnomedicine to be registered as a mainstream pharmaceutical. Unfortunately, it seems as if this approach is deemed financially unfeasible in the African ethnomedicine environment, where marketing and exporting plant-derived products as food supplements are already generating substantial revenue.

In the case of rooibos, significant safety data has been generated mostly by preclinical researchers using *in vitro* models to address this. In terms of cytochrome p450 (CYP450) and other enzymes related to enzymes related to drug metabolism, data consistently suggest that rooibos may affect drug bioavailability (Marnewick et al., 2003; Matsuda et al., 2007; Fantoukh et al., 2019; Patel et al., 2019). This data has been confirmed in rodents in the context of hypoglycemic and hypolipidemic treatments (including metformin and atorvastatin) (Patel et al., 2019), of which atorvastatin is metabolised by CYP450 enzymes. Importantly, no herb-drug interaction was reported for metformin and rooibos co-treatment (Patel et al., 2019), suggesting this may be an avenue in to pursue in humans (Dludla et al., 2018). All *in vivo* studies seem to indicate potentiation of drug action by rooibos, especially that of

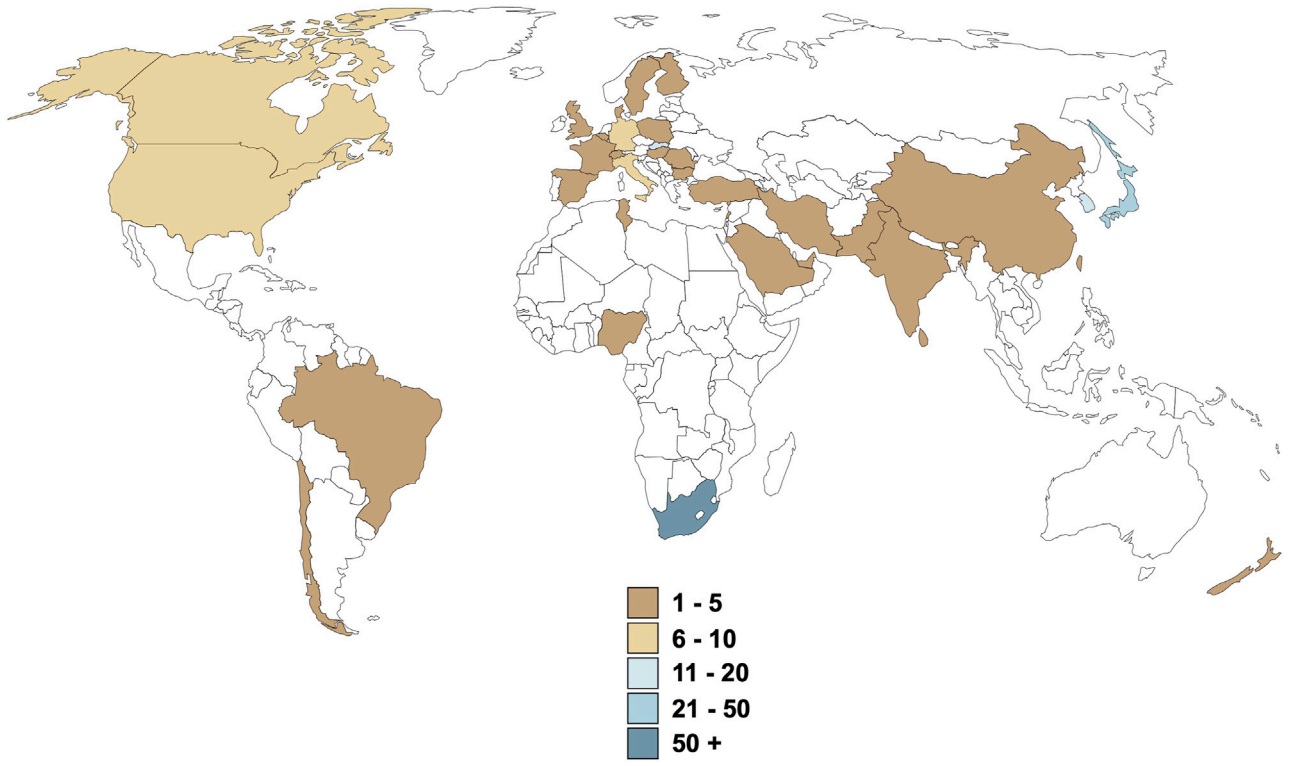

FIGURE 2
Origin of health-related rooibos research over the last 3 decades. Values are representative of the number of publications produced, excluding literature reviews and case reports.

atorvastatin, i.e., lower doses of pharmaceuticals may be required for therapeutic effect when used in combination with rooibos. A compounding approach may therefore limit incidence of adverse drug effects, as well as lowering treatment costs, illustrating both the importance of safety testing and the significant role that ethnomedicines could play in patient management, especially in poor countries. However, pre-clinical researchers rarely have the means or intent of progressing to clinical trials, which halts the transition to clinical trials at this point.

Progression to clinical trials

Although reproducibility of research data should be confirmed, the decision that pre-clinical data is sufficient to warrant clinical trials in humans, should be made timeously to prevent stagnation of medicines development at the preclinical stage. We believe that the cost, in terms of human resources, monetary expense, legislative compliance and administrative resources, is only one reason for the relative lack of throughput. In the case of rooibos, clear research "silos" are evident, although there is clearly global interest in rooibos (Figure 2), suggesting lack of a coordinated effort. Given the fact that identification of commercial ownership of an ethnomedicine is a complex negotiation, especially when no refinement or extraction process is required for therapeutic potency, the manufacturing pharmaceutical industry is understandably not interested in coordinating or funding research on any particular ethnomedicine. Although indigenous ownership has been acknowledged for rooibos via traditional knowledge and access-benefit sharing regulations leading to the Rooibos Benefit Sharing Agreement (UEBT, 2017; Schroeder et al., 2020; Meyer and Naicker, 2023), this resulted from a tedious 6-year administrative process. While the signing of this agreement has positively impacted the future of rooibos as potential medicine, this route is not the norm for the majority of ethnomedicines, where bioprospecting is neglected and ethnomedicines are sold as dietary supplements only. There thus exists a dire need for a knowledgeable and suitably equipped entity to take charge of the ethnomedicine pipeline from bench to bedside (as is currently attempted for rooibos by the South African Rooibos Council).

The World Health Organization (WHO) seems to be taking the lead in recognition of traditional medicines, with the new WHO Global Centre for Traditional Medicine scheduled to open its doors in 2024 (WHO, 2023). While the priority of this centre is not yet clear, the expectation and hope is that this centre may play a role in "talent identification" of ethnomedicines, connecting role players with various expertise and backgrounds to construct multidisciplinary, equipped teams, as well as facilitating the progression of candidate medicines through a clinical trial process.

Conclusion

Clearly, preclinical researchers in Africa are doing high quality research to aid in identification of potential

ethnomedicines and to provide scientific support for traditional practices which may contribute to mainstream medical care. However, given the dire state of long-term chronic patient management in poor countries, we believe that a centralised agency is required to improve the coherence of currently disjointed efforts, in order to achieve affordable medicine and improvement of quality of life for all.

Author contributions

LP: Conceptualization, Validation, Writing–original draft, Writing–review and editing. CS: Conceptualization, Supervision, Validation, Writing–original draft, Writing–review and editing.

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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.

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Stevens' Cure (Umckaloabo)—the vindication of a patent medicine

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Stevens' Cure (Umckaloabo) emerged as a patent medicine claiming to treat tuberculosis in the United Kingdom at the beginning of the 20th century. However, due to its identity being shrouded in secrecy, it was never truly accepted by the medical community. It was "rediscovered" in the 1970s and subsequently developed into a very popular and successful phytopharmaceutical for the treatment of upper respiratory tract infections. Whether Stevens' Cure contained the same ingredient(s) as the modern Umckaloabo has not yet been demonstrated. We attempted to elucidate for the first time the identity of the original ingredient by comparative analysis of historical product samples. Three historical samples of Stevens' Cure were compared with *Pelargonium sidoides* DC. and *P. reniforme* Curt. root per UPLC-MS analysis. We confirm that the ingredient–*P. sidoides* DC.—is indeed the same as used in modern phytotherapy. We also attribute the first ethnopharmacological record of *P. sidoides* DC. being used for the treatment of tuberculosis to C. H. Stevens, the "creator" of Umckaloabo.

KEYWORDS

Umckaloabo, Stevens' Cure, *Pelargonium sidoides* DC., *Pelargonium reniforme* Curt., umckalin, identification

1 Introduction

Stevens' Cure, today better known as Umckaloabo, was introduced to the United Kingdom as a patent medicine at the end of the 19th century. Similar to other early introductions of southern African medicinal herbs, such as devil's claw (*Harpagophytum* spp.), rooibos [*Aspalathus linearis* (Burm.f.) R.Dahlgren], honeybush (*Cyclopia* spp.), buchu (*Agathosma* spp.), Cape aloe (*Aloe ferox* Mill.) and uzara [*Xysmalobium undulatum* (L.) W.T. Aiton] (Stander et al., 2019; Brendler, 2021; Brendler and Abdel-Tawab, 2022; Brendler and Cock, 2022; Brendler et al., 2023), its arrival in Europe was driven by entrepreneurship and, as opposed to later (and less successful) attempts, such as hoodia [*Hoodia gordonii* (Masson) Sweet ex Decne] and sceletium (*Mesembryanthemum tortuosum* L.) (Brendler, 2020; Brendler et al., 2021), uninhibited by regulations. Suffering from tuberculosis, Charles H. Stevens was sent by his doctor for convalescence to South Africa, where he was miraculously cured by a local Sotho healer. He brought the cure, an unidentified botanical, back to England and began to market it to fellow sufferers as a patent medicine branded as Umckaloabo. Aggressive advertising (by mail, in dailies, through anonymously published books and paraphernalia such as bookmarks) made his business flourish. Stevens was open to the medical establishment, promoting his remedy on multiple occasions, however, his secrecy and claims of a cure for tuberculosis did not pair well with the scrutiny of science and he was largely ignored or labelled a quack (British Medical Association, 1909a; British Medical Association, 1909b; American Medical Association, 1910; American Medical Association, 1930). It is Stevens' contentious

nature that we owe the opportunity to–belatedly–uncover his secret. He pressed multiple litigations against the medical establishment (British Medical Association, 1912) but lost due to his unwillingness to disclose the identity of his ingredient, among other reasons. However, every one of those court cases led to attempts to elucidate the composition of his remedy: samples were procured, government officials tasked, and renowned botanical institutes involved (Misc, 1936ff.). Even though none of these attempts were successful, they left a paper trail and the samples ended up in official collections. We were able to locate four samples in the Economic Botany Collection of the Royal Botanical Gardens, Kew, three of which could be authenticated as Stevens' Cure beyond a reasonable doubt (see Section 2.1). These were analyzed and results are presented below. Stevens' success also caught international attention, and the Swiss French physician Adrien Sechehaye started to treat his patients with Umckaloabo and reported his successes to the European medical societies (Sechehaye, 1923; Sechehaye, 1934). Soon, interest was raised also in Germany (Bojanowski, 1937). That may be the reason for why after Stevens' death the "brand" and its secret ended up there. In parallel, however, antibiotics became available for the treatment of tuberculosis, and Umckaloabo fell to the wayside until it was revived by the quest of German scientist Sabine Bladt in the early 1970s (Bladt, 1974). Bladt, curiously, was unaware of the historic samples, or at least nothing in her published work hints at her knowing of their existence. Her ethnobotanical and biochemical approach led to the identification of *Pelargonium reniforme* as the source plant of Umckaloabo (Bladt, 1977). In retrospect, it must be assumed that her collected plant material was not correctly identified (pers. comm. K. P. Latté, July 2020), since she reported the presence of umckalin, which was subsequently found to be the marker compound for *P. sidoides* (Kayser, 1997), while absent in *P. reniforme* (Latté, 1999). In fact, Kolodziej and co-workers (Kolodziej, 2000; Kolodziej et al., 2002; Kolodziej, 2007) have identified several compounds including coumarin and coumarin sulfates (umckalin and umckalin sulfate) in *P. sidoides* that were not detected in *P. reniforme*. The presence of umckalin as a unique chemical marker for *P. sidoides* was also confirmed by Viljoen and colleagues (Viljoen et al., 2015).

The transformation of Umckaloabo from a patent medicine into a modern phytopharmaceutical has been reviewed by us previously (Brendler and van Wyk, 2008; Brendler, 2009). Since then, more than 100 publications have been added to the already impressive body of data on biochemistry, pharmacology, clinical efficacy, and safety of Umckaloabo. The contemporary brand owner (Schwabe Group, Karlsruhe, Germany) has promoted the investigation of Umckaloabo (EPs® 7630[1]) resulting in more than 30 clinical trials over the last 25 years (total study population >10,000) for the treatment of acute respiratory tract infections. A Cochrane review of eight studies (Timmer et al., 2013) found some evidence for efficacy but deemed the overall quality low. More recent reviews (Matthys et al., 2016; Careddu and Pettenazzo, 2018; Seifert et al., 2019) included a larger body of data in their reviews and meta-analyses, and attested efficacy in children, adolescents and adult patients with acute bronchitis, rhinosinusitis or tonsillopharyngitis. An investigation of EPs® 7630 effect on respiratory viruses (Michaelis et al., 2011) and its excellent safety profile (Kamin et al., 2018; Schapowal et al., 2019) led to it being discussed as having potential to affect the human immune response in the context of COVID-19 (Brendler et al., 2020), which has since been confirmed *in vitro* and *in vivo* (Papies et al., 2021; Emanuel et al., 2023).

2 Materials and methods

2.1 Samples and sampling

Commercial samples of dried *Pelargonium sidoides* and *P. reniforme* roots were supplied by Ulrich Feiter of Parceval (Pty) Ltd., Wellington, South Africa. The batch numbers were 20090, 20091, and 20092 for *P. sidoides,* and 2023, 20094, and 20095 for *P. reniforme*. The three historic samples, EBC 45821, EBC 45819 and EBC 77377 were procured from the Economic Botany Collection of the Royal Botanic Gardens, Kew, United Kingdom. The authenticity of those samples could be confirmed with the help of catalogue notes and by relating them to a file labelled "Umckaloaba" [sic!] of various correspondence regarding the British Medical Association's attempts to identify the active ingredient in Stevens' Cure (Misc, 1936ff.) (EBS 45821 and EBS 77377), whereas EBS 45819 was contained in an envelope addressed to the Royal Botanic Gardens from Pharmacie Hahn, Geneva, 1922. Adrien Sechehaye mentioned this pharmacy as his supplier for Umckaloabo (Helmstädter, 1996).

2.2 Extraction

Approximately 0.2 g of dry plant material was extracted with 50% methanol in water containing 1% formic acid (2 mL) in a 15 mL polypropylene centrifuge tube by soaking it overnight, followed by extraction in an ultrasonic bath (0.5 Hz, Integral systems, RSA) for 60 min at room temperature. The extracts were centrifuged (Hermle Z160m, 3,000 × g for 5 min) to remove any particulates and transferred into vials. An additional analysis was performed on the hydrolyzed samples.

2.3 Standards

Standards of umckalin, gallic acid and citric acid were obtained from Sigma-Aldrich. Standard solutions of umckalin were prepared quantitatively ranging from 1 to 100 µg/mL in concentration in methanol.

2.4 LC-MS analysis

A Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity ultra performance liquid chromatograph (UPLC) (Waters, Milford, MA, United States) was used for high resolution UPLC-MS analysis. In short electrospray ionization in the negative mode was used, a Waters HSS T3, 2.1 mm × 100 mm, 1.7 µm column and mobile phase gradient of 0.1% formic acid (Solvent A) and acetonitrile containing 0.1% formic

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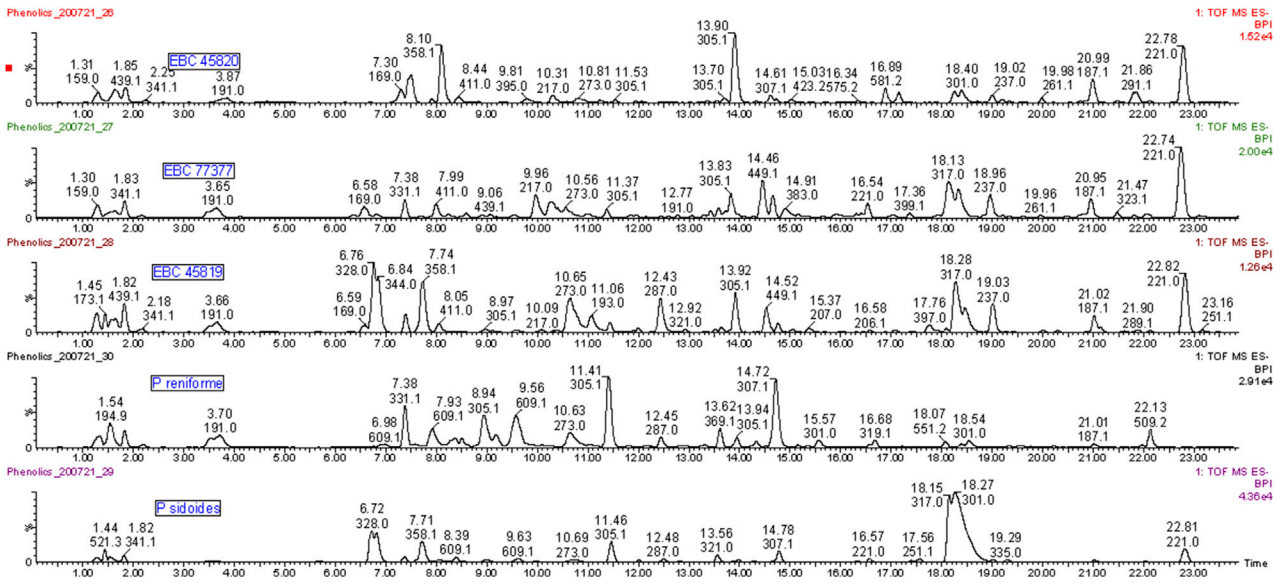

FIGURE 1
Phenolic compounds in extracts from authentic root samples of *Pelargonium reniforme, P. sidoides* and three historical samples of Stevens' Cure (EBC 45819, EBC 77377, EBC 45820). Umckalin (Rt ~22.8 min) is a known marker compound for *P. sidoides*. Umckalin sulfate (Rt ~18.3 min) occurs at high levels in *P. sidoides* and the Kew samples but not in *P. reniforme*. Epigallocatechin (Rt 11.4 min) and gallocatechin (Rt 8.9 min) are major compounds in *P. reniforme* and only minor compounds in *P. sidoides* and the Kew samples. The turgorins (m/z 328 and 358, 6.7 and 8.09 min) are present in the historical samples and *P. sidoides* and not in *P. reniforme*.

acid as solvent B (Stander et al., 2017). The flow rate was 0.25 mL/min, the gradient started with a 1-min hold at 100% (A) followed by a linear gradient to 28% solvent (B) in 22 min and another linear gradient over 50 s to 40%, a wash step at 100% (B) for a minute and re-equilibration to initial conditions over 4 min to give a total run time of 29 min. The column was at 60°C. Data was acquired in MS^E mode where both low energy data and high energy fragmentation data are acquired.

3 Results

Direct evidence of the botanical identity of the three historical samples of Stevens' Cure is revealed here for the first time (Figure 1).

Umckalin (*m/z* 221.0454, 22.8 min) is a known chemical marker for *P. sidoides* (Viljoen et al., 2015) and occurred as a major phenolic compound in all three of the Kew samples (363–738 mg/kg in the latter–see Figure 1; Supplementary Material).

Umckalin sulfate (6-hydroxy-5,7-dimethylcoumarin-8-sulfate, *m/z* 301.0009, 17.45 min), is a major compound in *P. sidoides* (Kolodziej, 2000; Kolodziej et al., 2002; Kolodziej, 2007) and in two of the three museum samples and a minor compound in the third. Epigallocatechin (*m/z* 305.0664, 11.1 min) is a major compound in *P. reniforme* and only a minor one in *P. sidoides* and the three historical samples. Note, that there are more than 1 *m/z* 305 peaks in Figure 1—the one at 11.4 min is epigallocatechin, the one at 8.9 min (*P. reniforme* only) is gallocatechin and the one at 13 min an unidentified flavonoid sulfate. Sulfated flavonoids are an uncommon group of compounds but have been found in some plant families. Teles et al. (2018) recently published a review on these compounds. Persicarin (isorhamnetin-3-sulfate) is the first sulfated flavonoid reported, the sulfate is an O-sulfate, whereas with umckalin-sulfate it is a C-sulfate. O-sulfates forms a strong $[HSO_4^-]$ *m/z* 96.96 fragment, whereas C-sulfates forms a $[M\text{-}H\text{-}SO_3]^-$ as a main fragment ion. See Figure 2 for the structures and Supplementary Material for the spectra and fragmentation information of the marker compounds. An overview of compounds tentatively identified in *Pelargonium* extracts in this study is provided in Table 1.

The coumarin sulfates can be distinguished from the flavonoid sulfates in the fragment ions. The coumarin sulfates have a base peak or strong fragment ion at $[M\text{-}H\text{-}SO_3]^-$ where the sulfate is on the coumarin ring whereas if the sulfate is on a flavonoid glycoside on another position, a fragment ion at *m/z* 96.96 is detected for $[HSO_4]^-$. It is challenging to identify the sulfated flavonoid glycosides, because only the elemental composition and the one fragment (*m/z* 96.96) is mostly detected and the compounds are not stable enough to isolate preparatively (Schötz, K., Nöldner, 2006).

Interestingly, sulfated flavonoids have been shown to have antimicrobial affects and they are also some of the main metabolites found in human blood after administration of the aglycones (Teles et al., 2018).

The turgorins (LMF 3, *m/z* 328.0440, 6.77; LMF 2, *m/z* 344.0596, 6.8 min) are present in the historical samples and *P. sidoides* but not in *P. reniforme* (Schildknecht, 1984).

4 Discussion

While the presence of a small amount of *P. reniforme* in the historical samples cannot be ruled out with certainty, it is safe to state that most of the plant material is from *P. sidoides*. The quantitative results are presented in the Supplementary Material. In early attempts to elucidate the composition of Stevens' Cure it was speculated that it may belong to the genus *Rumex* (catalogue note to

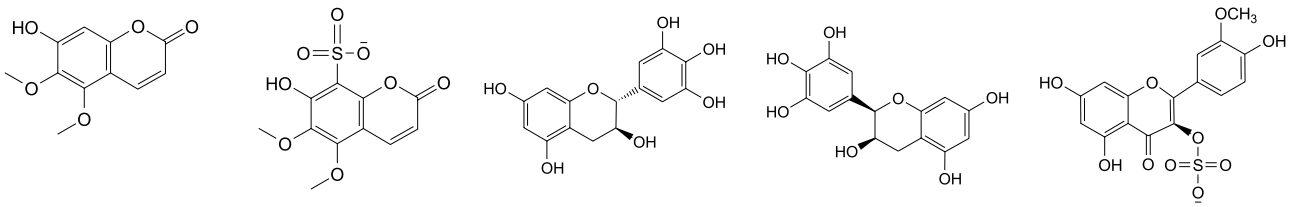

FIGURE 2
Structure of the main markers of *Pelargonium sidoides* (umckalin, umckalin sulfate, gallocatechin and epi-gallocatechin) and persicarin (isorhamnetin-3-sulfate), the first isolated sulfated flavonoid (left to right).

TABLE 1 List of compounds tentatively identified in *Pelargonium* extracts in this study showing, retention time, detected [M-H] ion, elemental composition and MS^E fragments (with the base peaks in bold) as well as literature references to where the compounds were previously detected.

Time	*m/z*	$[M-H]^-$	MS^E fragment ions	Tentative identification	References
2.73	191.0196	$C_6H_7O_7$	**111.0086**, 87.0093, 85.0305, 67.0197	Citric acid	Standard
6.54	169.0132	$C_7H_5O_5$	125.0253	Gallic acid	Standard
6.77	328.044	$C_{10}H_{11}N_5O_6P$	134.0464	K-LMF 3	Schildknecht (1984)
6.84	344.0396	$C_{10}H_{11}N_5O_7P$	**150.0427**, 133.0148, 78.9575	K-LMF 2	Schildknecht (1984)
7.33	331.1025	$C_{14}H_{19}O_9$	139.0399, **169.0504**	Koaburaside	Chung et al. (1997)
7.69	358.0538	$C_{11}H_{13}N_5O_7P$	**164.0568**, 133.0144, 78.9575	Methoxy LMF 2	**New**
7.84	411.0127	$C_{13}H_{15}O_{13}S$	**241.0038**, 169.0153, 125.0251, 96.9602	PLMF 1	Schildknecht (1984)
7.93	609.1234	$C_{30}H_{25}O_{14}$	441.0830, 423.0706, 305.0659, 272.9700, 193.0133, 177.0190, **125.0234**	(epi) Gallocatechin Dimer	Callemien and Collin (2008)
8.36	609.1243	$C_{30}H_{25}O_{14}$	441.0830, 423.0690, 409.0430, 305.0659, 272.9694, 193.0133, 177.0190, **125.0244**	(epi) Gallocatechin Dimer	Callemien and Collin (2008)
8.77	305.065	$C_{15}H_{13}O_7$	221.0451, 219.0695, 167.0366, 139.0405, 137.0251, **125.0242**, 111.0467, 109.0299	Gallocatechin	Kolodziej (2000)
9.24	217.0173	$C_8H_9O_5S$	**96.9583**, 193.0120	Unknown flavonoid sulfate	**New**
9.64	913.1762	$C_{34}H_{41}O_{29}$	609.1199, 441.0815, 423.0703, 305.0645, 177.0192, **125.0241**	(epi) Gallocatechin Trimer	Callemien and Collin (2008)
10.3	365.0172	$C_{12}H_{13}O_{11}S$	347.0041, **210.9911**, 153.0182, 122.9763, 109.0287, 96.9592	Phenolic glycoside sulfate	Pereira et al. (2015)
10.65	272.9704	$C_9H_5O_8S$	**193.0143**, 175.0061, 149.0240, 121.0299, 93.0346, 77.0383	Dihydroxy coumarin sulfate	Gödecke et al. (2005)
11.06	272.9798	$C_9H_5O_8S$	**193.0137**, 177.0175	Dihydroxy coumarin sulfate	Gödecke et al. (2005)
11.4	305.0664	$C_{15}H_{13}O_7$	219.0676, 179.0377, 167.0336, 151.0371, 139.0394, 137.0238, **125.0241**, 109.0293	Epigallocatechin	Kolodziej (2000)
11.99	485.1284	$C_{21}H_{25}O_{13}$	303.0444, **177.0183**, 125.0233	Gallocatechin O-hexoside	New
12.77	191.0337	$C_{10}H_7O_4$	125.0222	Scopoletin	Kayser and Kolodziej (1995)
12.43	286.9853	$C_{10}H_7O_8S$	207.0294, **192.0061**, 164.0107, 108.0211	Hydroxy methoxy coumarin sulfate	**New**
13.8	305.0686	$C_{12}H_{17}O_7S$	225.1135, 165.0938, 147.0842, **96.9598**, 59.0126	Unknown flavonoid sulfate	**New**
13.58	369.0815	$C_{16}H_{17}O_{10}$	**192.0059**, 207.0292, 163.0040	Unknown	
14.34	289.0736	$C_{12}H_{17}O_6S$	125.0246, 176.1043??	Epicatechin	Kolodziej (2000)
14.6	449.1058	$C_{21}H_{21}O_{11}$	**287.0547**, 269.0450, 259.0606, 125.0240	Eriodictoyl-hexoside	**New**
14.7	307.085	$C_{12}H_{17}O_7S$	227.1297, 167.1039, 123.0820, **96.9596**, 59.0128	Unknown flavonoid sulfate	**New**
15.5	301.0004	$C_{11}H_{10}SO_8$	221.0437, 206.0199, **190.9985**, 163.0031, 135.0099, 125.0228	Fraxidin sulfate	Kolodziej et al. (2002)

(Continued on following page)

TABLE 1 (*Continued*) List of compounds tentatively identified in *Pelargonium* extracts in this study showing, retention time, detected [M-H] ion, elemental composition and MS^E fragments (with the base peaks in bold) as well as literature references to where the compounds were previously detected.

Time	*m/z*	$[M-H]^-$	MS^E fragment ions	Tentative identification	References
16.53	221.0445	$C_{11}H_9O_5$	**190.9990**, 163.0049, 91.0186	Fraxidin/fraxinol	Kayser and Kolodziej (1995)
16.56	319.0798	$C_{16}H_{15}O_7$	179.0363, 164.0126, **139.0403**, 125.0236, 111.0448	Unknown	
16.8	581.2255	$C_{28}H_{37}O_{13}$	419.1681, 404.1408, 373.1212, 227.1284, 153.0561	Unknown	
17.15	581.2236	$C_{28}H_{37}O_{13}$	419.1750, 373.1436, 227.1278, 153.0562	Unknown	
18.2	316.9959	$C_{11}H_9O_9S$	237.0402, 222.0173, **206.9928**, 178.9978, 151.0045, 107.0139, 95.0131, 67.0180	8-hydroxy-5,7 dimethoxycoumarin-6-sulfate	Kolodziej et al. (2002)
18.6	301.0009	$C_{11}H_{10}SO_8$	221.0457, 206.0226, **190.9987**, 163.0037, 135.0087, 119.0145, 95.0134, 91.0190	5,6 dimethoxycoumarin-7-sulfate	Kolodziej et al. (2002)
19.03	237.0394	$C_{11}H_9O_6$	222.0145, **206.9935**, 178.9966, 151.0046, 123.0092, 95.0135, 67.0182	6,8-dihydroxy-5,7-dimethoxycoumarin	Kayser and Kolodziej (1995)
21	187.0965	$C_9H_{15}O_4$	Solvent contaminant		
22.1	509.2018	$C_{25}H_{33}O_{11}$	289.1097, 177.0164	Unknown compound	
22.8	221.0454	$C_{11}H_9O_5$	206.0216, **190.9977**, 163.0034, 135.0119, 119.0149, 95.0130, 91.0182	Umckalin (7-hydroxy-5,6-dimethoxycoumarin)	Kayser and Kolodziej (1995)
26.78	415.1773	$C_{20}H_{31}O_7S$	**96.9601**, 79.9564	Unknown flavonoid sulfate	**New**
29	399.1832	$C_{20}H_{31}O_6S$	**96.9597**, 79.9589	Unknown flavonoid sulfate	**New**
29.52	399.1823	$C_{20}H_{31}O_6S$	**96.9585**	Unknown flavonoid sulfate	**New**

EBS 45819). However, no unique peaks corresponding to the presence of anthraquinones (chrysophanol and its glycosides) were detected in the samples that were not present in *P. sidoides* in both positive (data not shown) and negative mode.

Tuberculosis and/or respiratory ailments do not appear in the historical southern African literature prior to the 20th century (Brendler and van Wyk, 2008). The earliest record by Sanderson (ca. 1860) is from the Eastern Free State (adjoining Lesotho), where Khoi people were said to use the plant as a cure for unspecified ailments (Smith, 1966).

The earliest record from the Eastern Cape documented the species as *iYeza lezikhali* and *iKhubalo* in isiXhosa and reported it to be used for "dysentery, attended by inflammation and fever" (Smith, 1895). In Lesotho, *P. sidoides* was known as *khoaara e nyenyane* (in Sesotho) and the roots used to treat colic (Phillips, 1917). Stevens claimed that the plant was known as *Umckaloabo* in Basutoland (now Lesotho) and that the treatment prescribed by a Basotho healer completely cured him of his tuberculosis (Sechehaye, 1934). We now know that the given provenance of the plant, which included the Gold Coast and Liberia (Anonymous, 1931), and the botanical affinity given as Polygonaceae, were deliberate attempts at hiding the geographical origin and identity of the plant material. Nevertheless, the recorded use against tuberculosis is a valuable original ethnobotanical record for the species, that may have been completely forgotten had it not been for Stevens.

Pelargonium sidoides occurs neither in the Western Cape Province, nor in KwaZulu-Natal Province and Eswatini. The report by Watt and Breyer-Brandwijk (Watt and Breyer-Brandwijk, 1962) that the plant was used in the Cape as an "old remedy for delay in the onset of menses" was based on Kling (Kling, 1923), who merely cited the well-established history of this indication for *P. grossularioides* (L.) L'Herit (at the time known as *P. anceps* DC.). The confusion was due to the shared Malay/Afrikaans vernacular names (*rabas* or *rabassam*), first recorded by Pappe (Pappe, 1847; 1850) and later by Kling (1923) and Laidler (Laidler, 1928). Therefore, data in Watt and Breyer-Brandwijk (1962) were probably inaccurate but taken at face value by later authors (Hutchings et al., 1996). These authors added the treatment of severe diarrhea, a prolapsed rectum, severe gonorrhea, and a stomach ailment in babies known as *intisila* to the repertoire of the species, claimed to be used by the Zulu and Swazi.

5 Conclusion

The analysis of historical samples leaves no doubt that Stevens' Cure was prepared from *Pelargonium sidoides*, a Sotho medicine traditionally used in Lesotho against colic and, according to Stevens (Sechehaye, 1934), also against tuberculosis. Recorded uses in South Africa do not include respiratory ailments until the late 20th century. Although Stevens' claim of a cure for tuberculosis was never validated beyond pre-clinical investigations (Seidel and Taylor, 2004; Kim et al., 2009; Qasaymeh et al., 2019), the use of his remedy is vindicated, not only by its botanical identity but also by several clinical studies showing support for the treatment of respiratory infections.

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

TB: Conceptualization, Investigation, Project administration, Resources, Writing–original draft, Writing–review and editing. MS: Data curation, Investigation, Methodology, Writing–original draft, Writing–review and editing. B-EW: Conceptualization, Investigation, Resources, Validation, Writing–original draft, Writing–review and editing.

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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.

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Supplementary material

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An *in vitro* study to elucidate the effects of Product Nkabinde on immune response in peripheral blood mononuclear cells of healthy donors

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Introduction: A significant number of the South African population still rely on traditional medicines (TM) for their primary healthcare. However, little to no scientific data is available on the effects of most TM products on cytokine and cellular biomarkers of the immune response. We evaluated the impact of a TM [Product Nkabinde (PN)] in inducing cellular and cytokine biomarkers of immune response in peripheral blood mononuclear cells (PBMCs).

Methods: PN, a combination of four indigenous South African plants was used in this study. The IC_{50} was established using the cell viability assay over 24 h. Luminex and flow cytometry assays were used to measure cytokine and cellular levels in PBMCs stimulated with PN and/or PHA over 24, 48, and 72 h, respectively. UPLC-HRMS was used to analyze an ethanol: water extract of PN to better understand the possible active compounds.

Results: The IC_{50} concentration of PN in treated PBMCs was established at 325.3 µg/mL. In the cellular activation assay, the percentages of CD38-HLA-DR + on total $CD4^+$ T cells were significantly increased in PBMCs stimulated with PN compared to unstimulated controls after 24 h (p = 0.008). PN significantly induced the production of anti-inflammatory IL-10 (p = < 0.001); proinflammatory cytokines IL-1α and IL-1β (p = < 0.001), TNF-α (p < 0.0001); and chemokine MIP-1β (p = < 0.001) compared to the unstimulated control after 24 h. At 48 h incubation, the production of proinflammatory cytokines IL-1α (p = 0.003) was significantly induced following treatment with PN, and IL-10 was induced (p = 0.006). Based on the UPLC-HRMS analysis, four daphnane diterpenoids viz., yuanhuacine A (1), gniditrin (2), yuanhuajine (3) and yuanhuacine (4) were identified based on their accurate mass and fragmentation pattern.

Conclusion: The results show that PN possesses *in vitro* immunomodulatory properties that may influence immune and inflammatory responses. This study contributes to scientific knowledge about the immune effects of TM. More studies using PN are needed to further understand key parameters mediating induction, expression, and regulation of the immune response in the context of pathogen-associated infections.

KEYWORDS

traditional medicine, normal peripheral blood mononuclear cells, cytokines, T cell activation, immunomodulation

Introduction

South Africa has the largest HIV epidemic in the world, accounting for just over 20% of all people living with HIV (PLWH) (Africa UCFS, 2019; UNAIDS and Geneva, 2019). With over 4 million people on antiretroviral treatment (ART) in 2019, South Africa runs the world's largest public sector ART program (Organization WH, 2019; UNAIDS and Geneva, 2019). The introduction of ART, particularly early initiation of ART, has dramatically reduced mortality and morbidity rates and improved the quality of life of PLWH (Organization WH, 2016; UNAIDS and Geneva, 2019). While the benefits of modern medicine are well documented, their use has not fully replaced the indigenous healthcare system in South Africa (Babb et al., 2007; Ngcobo and Gqaleni, 2016; Sibanda et al., 2016).

Traditional medicine (TM) is an important feature of the everyday life of many South Africans, with approximately 80% of the rural communities consulting traditional healers at some point in their lifetime (Baleta, 1998; Rankoana, 2022; Maluleka and Nkwe, 2022). Some consumers have used TM as complementary or alternative medicine. Several factors such as cultural and religious beliefs, a desire to alleviate ART side effects, as a supplemental dietary intake, improve immune response against the virus, and achieve added efficacy in suppressing the virus and managing the disease have been attributed to this complementary or continued use (Malangu, 2007; Dahab et al., 2008; Peltzer and Mngqundaniso, 2008; Peltzer, 2009; Organization WH, 2013; Appelbaum Belisle et al., 2015; Zuma et al., 2017). In addition, TM is considered a reliable, accessible, and affordable source of dual treatment that helps to maintain health and improve quality of life (F et al., 1992). A limited number of healthcare facilities with practitioners of modern medicine have also been documented as other factors associated with the use of TM and its practitioners in South Africa (Peltzer and Mngqundaniso, 2008; F et al., 1992). Although consumers have widespread access to and use various TM treatments and therapies, information about their therapeutic value and safety are not well characterised (WHO). Also, consumers might be exposed to varying batches and components of herbal extracts, resulting in different responses (including immunity) and efficacy from person to person (Wachtel-Galor and Benzie, 2011).

TM has been purported to have immune boosting and antiviral capabilities (Gqalenia et al., 2012; Khodadadi, 2015; Anywar et al., 2020), showing the likelihood of TM mixtures to neutralize HIV infection using lymphocyte models (Gqalenia et al., 2012). Similarly, secretion of pro-inflammatory cytokines interleukin (IL)-1α, IL-1β, IL-6, IL-10, tumour necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) was induced in human peripheral blood mononuclear cells (PBMCs) stimulated with a commercial traditional immune booster known as *uMakhonya*® (Ngcobo and Gqaleni, 2016). Furthermore, this study showed that *uMakhonya*® induced the secretion of both anti-inflammatory and pro-inflammatory cytokines depending on the concentration used and lipopolysaccharide (LPS) stimulation. Additionally, *uMakhonya*® significantly decreased the sIL-2R levels in Gram-positive pathogen *Staphylococcus aureus* LPS stimulated PBMCs, implying its anti-inflammatory effect. The use of *Allium sativum* (also known as garlic) upregulated IL-10 and suppressed the levels of IL-1α, TNF, IL-8, and IL-6 in PBMCs and whole blood of healthy volunteers and these showed the potential benefit in patients with inflammatory bowel disease (Hodge et al., 2002). *Aloe vera* has been shown to have anti-inflammatory properties due to the ability to downregulate TNF-α and IL-6 levels (Das et al., 2011; Yazdani et al., 2022). Similarly, another study also showed that crude extracts of *Warburgia ugandensis* subsp. *Ugandans,* exert their immunostimulatory effects via the production of interferon-gamma (IFN)-γ and IL-4 in female BALB/c mice (Ngure et al., 2014). The expression of soluble factors is known to trigger the recruitment and activation of immune cells. Studies have shown that traditional medicine may increase the percentages of $CD4^+$ and $CD8^+$ T cells (Djohan et al., 2009; Phetkate et al., 2012; Salehi et al., 2018).

Given the existing evidence that TM use exerts anti-inflammatory properties and is synonymous with immune boosting, there is still a dearth of an updated comprehensive compilation of promising medicinal plants in South Africa. Product Nkabinde (PN), a promising traditional medicine prepared from four different medicinal plants, is thought to modulate the immune system and is active against opportunistic infections. It has been used for the management of symptoms of *Syphilis* and HIV infection. A recent study found that *Gnidia sericocephala*, one of the plants constituting PN, contains phytochemicals which inhibit HIV viral replication and/or reverse HIV latency (Tembeni et al., 2022). However, less is known about the effects of the PN on cytokine and cellular biomarkers of the immune response. Our aim is to evaluate the *in vitro* cytokines and cellular immune response differences in PBMCs treated with PN to provide evidence for its appropriate use in patients. Assessing the in vitro immune properties of PN may likely contribute to the understanding of the product's safety and its potential benefit to the wider population.

Materials and methods

Collection and identification of plant samples

Plant samples used to constitute PN were collected according to good collection practices (Organization WH, 2003) PN is formulated using four (Organization WH, 2016) medicinal plant parts; *Sclerocarya birrea* stems, *Gnidia sericocephala* roots, *Senna italica* roots and *Pentanisia prunelloides* roots. All these medicinal plants were collected from Tugela Ferry in Msinga Local Municipality of KwaZulu- Natal (Coordinates: Latitude: 28°28′6″S, Longitude: 30°28′15″E, Lat/Long (dec), −28.46844, 30.47096) and delivered by Traditional Health Practitioner (THP) Mr. Nkabinde, who disclosed the traditional uses of the plants to the research team. The plants were identified at the H.G.W.J Schweickerdt Plant Herbarium at the University of Pretoria where specimens were deposited as demonstrated in Table 1. Product Nkabinde was prepared according to the instructions of the THP. Briefly, the plant material from the four individual medicinal plants was dried and grounded to powder using a pestle and mortar. The resultant powder was combined at a ratio of 1:1:1:1 and boiled in water for 5–8 min followed by cooling at room temperature. The extract was then filter-sterilized and freeze-dried after 48 h of freezing using a Benchtop Freeze Dryer [(VirTis) SP Scientific, Warminster, PA, USA].

This is a Type C extract consisting of botanical drugs and their extracts derived from lesser-studied species and the drugs derived from them, which are not included in a national or regional pharmacopeia and are not used commercially at an international level. A patent application has been submitted in South Africa with Ref 2023/03587 for Product Nkabinde constituents.

TABLE 1 Identification of the medicinal plants collected to constitute Product Nkabinde, and their voucher specimen numbers at the H.G.W.J Schweickerdt Plant Herbarium at the University of Pretoria.

Plant name	Plant part	Voucher number
Sclerocarya birrea (MGN)	Stems and leaf	126589
Gnidia sericocephala (SDK)	roots	126590
Senna italica (SPN)	roots	126591
Pentanisia prunelloides (CLM)	roots	126592

Blood samples

For this *in vitro* experimental and exploratory study, whole blood samples were donated by 8 healthy female donors enrolled through the Centre for the AIDS Programme of Research in South Africa (CAPRISA) volunteer blood study (BREC REF: BE432/14). The gender of the donors was based on the available volunteers, and it is appropriate that all donors were female as women are disproportionately affected by HIV and other adverse sequelae in our settings. Written informed consent to participate in the study was obtained from each donor. Approximately 45 mL (mL) of whole blood were obtained from each donor using BD Vacutainer® acid citrate dextrose blood collection tubes from which PBMCs were isolated by Ficoll-Paque density gradient centrifugation. This study acquired ethical approval from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC REF: BE665/18).

Effects of PN on cell viability

To assess the effects of PN on cell viability, 1000 µL of PBMCs (1×10^6) was added into a 24-well plate and treated with PN at final concentrations of 100, 250, 500, 1000, and 2000 µg/mL, respectively. The plate was incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. At the end of this incubation period, 100 µL of cells and CellTiter-Glo™ Reagent (Promega, Madison, USA) were added to each well of the 96 well plate according to the manufacturer's protocol, followed by an additional incubation for 10 min in the dark at room temperature. The relative light units (RLU) of the samples in each well were measured using a Glo-max luminometer (Promega, Multi-detection system) following the manufacturer's instructions. A dose-response curve was generated for the ATP levels using the Relative Light Units (RLU) and the dilutions of PN and different control samples (Gqalenia et al., 2012). The IC_{50} of the PN was calculated from the dose response using GraphPad Prism (version 8).

T cell subset analysis

To assess the effects of PN on T cell subsets, 1000 µL of PBMCs (1×10^6) seeded into each well of a 24-well plate were treated with either the IC_{50} concentration (325.3 µg/mL) of PN or 5 µg/mL phytohemagglutinin (PHA) or the combination of PN and PHA or left untreated and incubated for 24, 48, and 72 h at 37°C in a humidified atmosphere containing 5% CO_2. After incubation of treatment, the cells were centrifuged at 1500 rpm for 10 min to pellet the cells, and the cell culture supernatants were stored at −80°C for cytokine quantification. PBMCs were washed with sterile PBS supplemented with 2% FCS and then stained for 45 min at room temperature with an antibody cocktail containing CD3-APC-H7, CD4-PE-CY5.5, CD8-BV711, CD3-APC-H7, HLA-DR-A700, CD38-PE-Cy-7, CCR5 APC, CCR6 BV605, CD14-Pacific Blue (monocyte exclusion), CD19-Pacific Blue (NK cells exclusion/ B cells exclusion), and viability markers (Live/dead ™ Fixable Violet Dead Cells Stain Kit, Invitrogen (Thermo-Fisher), Massachusetts, USA). The cells were washed with Perm/Wash buffer and acquired on the BD LSR Fortessa (BD Biosciences, Franklin Lakes, NJ, USA). At least 500 000 events were acquired from each sample. Data analysis was performed using FlowJo v10.4.1 software (Tree Star, Ashland, OR USA), according to the gating strategy (Figure 1).

Cytokine quantification

The concentrations of 12 cytokines were assessed from stored undiluted cell supernatants using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic bead panel (Merck KGaA, Darmstadt, Germany) as per the manufacturer's instructions. The

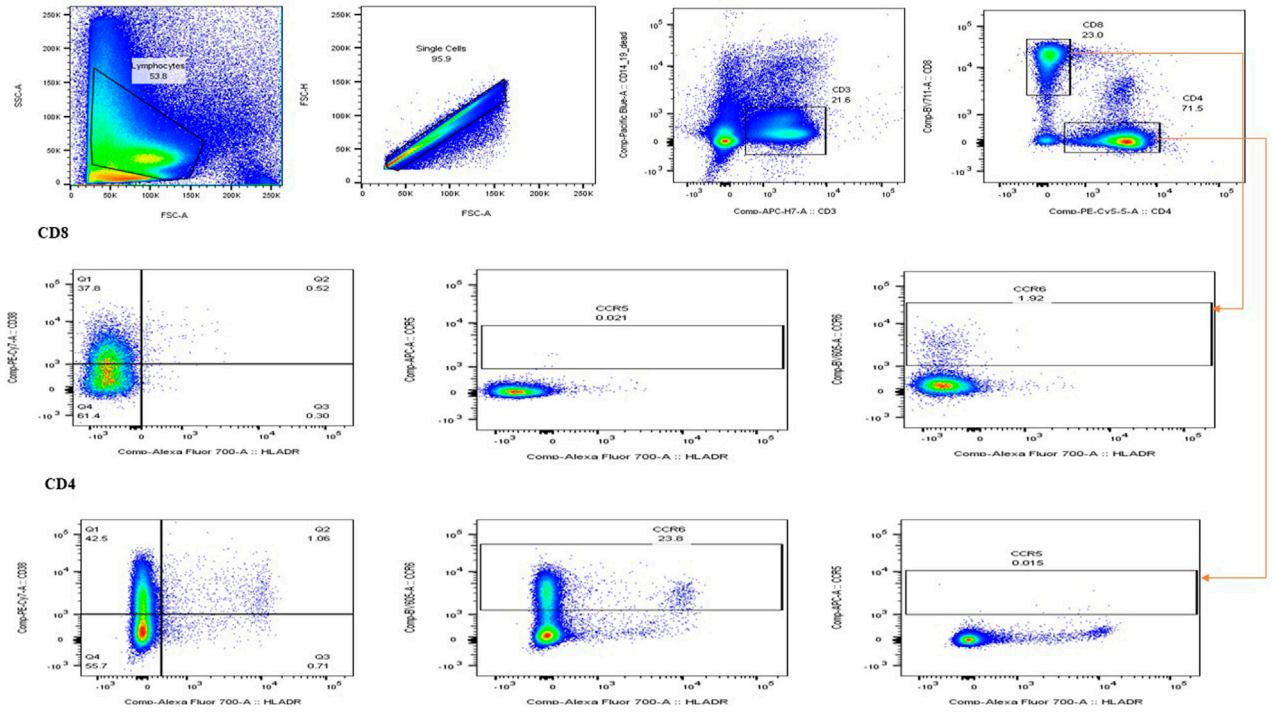

FIGURE 1
Illustration of the gating strategy used for flow cytometry. The forward scatter and side scatter were used to distinguish between different cell types. Lymphocytes and T cells, including the expression of CD38$^+$, HLA-DR + activation markers, and CCR5+ and CCR6+ co-receptors, were of interest.

cytokines panel included pro-inflammatory cytokines: IL-1α, IL-β, IL-6, TNF–α, GM–CSF; chemokines (interleukin (IL)-8, interferon gamma-induced protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)–1α and macrophage inflammatory protein (MIP)-1β); Hematopoietic cytokines (IL-7) and regulatory cytokines (IL-10. Data were acquired on the Bio-Plex R 200 system (Bio-Rad Laboratories, Hercules, CA, USA). Optimisation of standard curves was performed automatically using the Bio-Plex Manager software version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA). Values with coefficients of variation <20% and with observed recoveries between 70% and 130% were considered reliable. Values that were below the detectable limit were assigned half of the lowest limit of detection value (LLOD), while values that were above the detectable limit were assigned double the highest limit of detection (HLOD) value.

Preparation of an ethanol: water (1:1) extract for UPLC-HRMS analysis

An ethanol: water (EtOH: H_2O) (1:1) extract of PN was made for UPLC-HRMS analysis. The extract was prepared by using 50 g (combined weight) of a 1:1:1:1 mixture containing *Pentanisia prunelloides* (Rubiaceae), *Sclerocarya birrea* (Anacardiaceae), *Gnidia sericocephala* (Thymelaeaceae) and *Senna italic* (Fabaceae), weighed into a 1000 mL conical flask. The plant material was fully submerged in 500 mL of the 50% EtOH solution. The flask was covered with parafilm and shaken for 1 h at 180 rpm on a shaker bed. The solvent was carefully decanted from the solid plant material and filtered using a Büchner funnel and Whatman No. 1 filter paper. The solution was concentrated using a rotary evaporator. Immediately after concentration, extracts were subjected to spray drying using a Buchi Mini Spray Dryer B-290 (Buchi, Flawil, Switzerland) at an inlet temperature of 150°C. Free-flowing fine powder of the extract was subsequently collected from the collecting vessel.

UPLC-HRMS chromatographic conditions

UPLC-HRMS analysis was conducted using a Waters Acquity UPLC system (Waters Corp., MA, USA), equipped with both a binary solvent delivery system and an auto-sampler. The dried EtOH: H_2O (1:1) extract was prepared by dissolving it in a methanol (MeOH): H_2O (1:1) solution before filtering through a 0.22 µm nylon syringe filter to remove particulate matter. Compound separation was performed on an ACQUITY UPLC® BEH (2.1 × 100 mm, 1.7 µm) column (Waters Inc., Milford, MA, USA). A gradient elution method was employed using H_2O (0.1% formic acid) and MeOH (0.1% formic acid) (Romil-SpS™, Microsep, South Africa) as solvent A and solvent B, respectively. The elution method was optimised as follows: 97% solvent A, held for 0.1 min, followed by a linear gradient increase to 100% solvent B at 14 min. A 3-min washing hold was used (14–17 min) before reconditioning the column with the starting conditions (17.5–20 min). The column

temperature was held constant at 40°C to ensure repeatable results with a fixed flow rate of 0.3 mL/min and an injection volume of 5 µL.

UPLC-HRMS instrumentation and conditions

The system is setup with a Waters ACQUITY UPLC® hyphenated to a quadrupole mass filter with a high-resolution time-of-flight (TOF) mass analyser. A Waters® Xevo G2 high-definition mass spectrometer (HDMS) system (Waters Inc., Milford, MA, USA) was used for compound separation and detection. The instrument was operated using MassLynx™ v. 4.1 (Waters Inc., Milford, MA, USA) software. Sodium iodide clusters were used to calibrate the MS using the Intellistart software function over a mass range of 50–1200 Da. The MS source parameters were optimised for ESI positive mode and were set as follows: source temperature of 120°C, extraction cone voltage of 4.0 V, sampling cone of 30.0 V, cone gas flow of 20.0 L/h, desolvation temperature of 350°C, desolvation gas flow of 600.0 L/h, and a capillary voltage of 2.8 kV for the positive mode. An internal lock mass control standard comprised a 2 ng/µL solution of leucine enkephalin (*m/z* 555.2693). To account for experimental drift in mass, a lock mass solution was infused directly into the source at a rate of 3 µL/min. The lock mass infusion was done intermittently every 10 s.

UPLC-HRMS dat acquisition and data processing

Mass spectral scans were collected every 0.3 s with the raw data collected in a continuous fashion, with mass-to-charge ratios (*m/z*) of 50–1200 Da recorded. Data was collected in data-independent acquisition (DIA) mode using two functions with a low and high collision energy (MS^E). The collision energies were maintained at 10 V for the low MS transfer collision energy and 30 V for the high MS transfer collision energy.

Statistical analysis

The half maximum concentration (IC_{50}) was determined using Prism 8.0 (GraphPad, Inc., La Jolla, CA, United States). We used a linear mixed model that fitted the log10 expressions of the cytokines to assess cytokine production after exposing PBMCs for 24, 48, and 72 h with PN water extract and PHA and a generalised linear mixed model (with logit link and beta distribution) that fitted the proportions of T cells values. The models adjusted for the effect of the treatment group together with the time point at 24, 48, and 72 h. Bonferroni multiple comparisons were used to compare pairwise treatment groups. Our analysis did not adjust for multiple endpoints since this was an exploratory analysis. Differences with $p < 0.05$ were considered statistically significant. All statistical analysis was performed using GraphPad Prism 8.0 (GraphPad, Inc., La Jolla, CA, United States) and Statistical Analysis Software (SAS) version 9.4 (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, United States).

Results

The cytotoxic effects of the water extract of PN were determined against PBMCs isolated from eight healthy donors at concentrations ranging from 100 to 2000 µg/mL. A significant decrease in cell viability was observed in treated PBMCs in a dose-dependent manner. PN water extracts showed low cytotoxic effects (80%–90%) toward PBMCs at concentrations between 100–250 µg/mL. However, this was followed by a sharp decrease in cell viability when concentrations ranging from 500 to 2,000 µg/mL were used. The IC_{50} value that represents the concentration of PN extracts able to reduce cell viability by 50% was established at 325.3 µg/mL (Figure 2) and it was used for further experiments.

Effects of PN water extracts on T cell frequencies and phenotypes

In the assessment of PN water extract effects on T cell frequencies and phenotypes, flow cytometry (Flow jo) analysis revealed nuanced alterations in T cell subsets. The treatment with PN water extracts did not yield significant changes in the overall percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in PBMCs treated with PN water extracts or PHA (Figure 3; Supplementary Table S1). This finding suggests a non-discriminatory effect of PN water extracts on T cell populations and, therefore, evaluated the activation markers for $CD4^+$ and $CD8^+$ T cells. PHA stimulation significantly induced $CD4^+$ T cell activation compared to the unstimulated PBMCs, irrespective of the incubation period. PBMCs treated with PHA had a significant increase in the percentages of CD38+HLA-DR+ [mean 2.124 vs. mean 0.7850; OR: 2.721, CI: (1.022; 7.239), $p = 0.042$] on total $CD4^+$ T cells compared to the unstimulated PBMCs after 24 h stimulation. The effect size here is considerable, as reflected by the OR, and the CI suggests a moderate degree of variability, which is not unexpected given biological systems' inherent complexity. Similarly, PHA induced significant increases in the percentages of CD38+HLA-DR+ on total $CD4^+$ T cells compared to PN water extracts after 24 h [mean 2.124 vs. mean 0.6513; OR: 0.305, CI (0.107; 0.870), $p = 0.018$)] and 72 h (mean 1.155 vs. mean 0.1305; OR: 0.119, CI: (0.015; 0.969), $p = 0.045$). The narrow CIs indicate a strong effect and less variability while the *p*-value further supports the statistical significance of these findings.

Of note, the opposite trend was observed on CD38+HLA-DR- on total $CD4^+$ T cells, with percentages being significantly lower in PBMCs stimulated with PN water extract compared to PHA after 24 h [mean 26.31 vs. mean 41.54; OR: 0.503; CI: (0.261; 0.970), $p = 0.035$], the *p*-value corroborates the significance of this observation. The percentages of CD38+HLA-DR- on total $CD4^+$ T cells were significantly higher in PBMCs stimulated with PHA compared to unstimulated controls [mean 38.94 vs. mean 22.22; OR: 2.232; CI: (1.129; 4.415), $p = 0.013$] after 72 h. Compared to unstimulated controls after 24 h, the percentages of CD38-HLA-DR+ on total

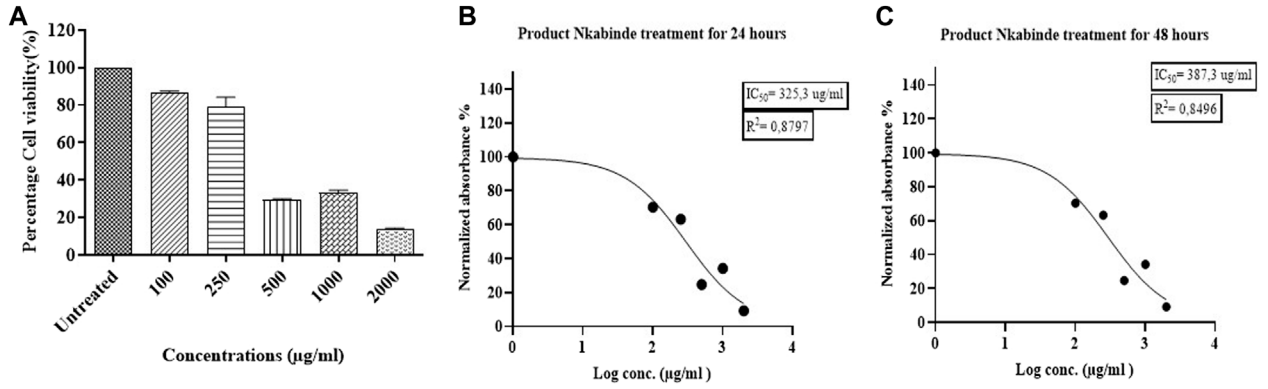

FIGURE 2
(A) The cytotoxic effects of PN water extracts on PBMCs at a concentration of 100–2000 µg/mL. **(B–C)** Determination of the IC50 values for the PN at 24 and 48 h, respectively. The IC50 of the treated PBMCs was established at a concentration of 325.3 µg/mL. At concentrations (400–2000 µg/mL) above the IC50, PN became cytotoxic to the cells. Experiments were conducted in triplicate.

$CD4^+$ T cells were significantly increased in PBMCs stimulated with PHA [mean 0.7413 vs. mean 2.468; OR: 2.158; CI: (0.738; 6.309), p = 0.001], the combination of PHA+PN water extract [mean 0.7413 vs. mean 2.074; OR; 2.811, CI: (1.220; 6.482), p = 0.008], and PN water extract alone [mean 0.7413 vs. mean 2.063; OR: 2.796; CI: (1.212; 6.450), p = 0.008]. Similar increases were also observed after 48 h stimulation, for PN [mean 1.865 vs. mean 0.4225; OR: 0.4225; CI: (1.547; 12.506), p = 0.002] and for PHA [mean 2.039 vs. mean 0.4225; OR: 4.815; CI (1.708; 13.576), p = 0.001] for both the stimulants the odds ratios were four times higher than unstimulated, although the CI was out of range, this may be due to the sample size chosen. At 72 h, in PBMCs treated with PHA, we observed a significant increase in the percentages of CCR5 on total $CD4^+$ T cells compared to the unstimulated PBMCs [mean 0.1328 vs. mean 0.04528; OR: 2.585; CI: (1.122; 5.952), p = 0.018] or PN water extracts [mean 0.1328 vs. mean 0.03809; OR: 0.337; CI: (0.140; 0.810), p = 0.008]. PHA and PN water extracts had similar expression levels of CCR6 on total $CD4^+$ T cells, irrespective of the incubation period. Of note, PBMCs stimulated with PHA [mean 0.008735 vs. mean 0.09550; OR: 0.177; CI: (0.049; 0.641), p = 0.003] or PN water extracts [mean 0.008735 vs. mean 0.006528; OR: 0.157; CI: (0.040; 0.607), p = 0.003] after 48 h expressed significantly lower percentages of CCR5 on total $CD8^+$ T cells compared to unstimulated controls.

It is imperative to acknowledge the limitations imposed by the small sample size in this study. While the observed mean differences and corresponding ORs provide valuable insights into the effects of PN water extracts on T cell subset expression, the small sample size may affect the generalizability of these results. The CIs, although informative of the effect size variability, should be interpreted with caution, as they may not accurately reflect the population parameters due to the limited number of observations.

In conclusion, the differential effects of PN water extracts and PHA on various T cell subsets underscore the complexity of immune modulation by these agents. The significant changes in activated and non-activated $CD4^+$ and $CD8^+$ T cell subsets highlight the potential immunomodulatory properties of PN water extracts. However, further research with larger sample sizes is warranted to validate these findings and elucidate the underlying mechanisms.

Effects of PN water extracts on cytokine production

There was cytokine production after exposing PBMCs for 24, 48, and 72 h with PN water extract (Figure 4B; Supplementary Table S2). Compared to unstimulated control, treatment with PN water extracts was significantly increased for IL-10 production after 24 h [median 2.154 (IQR 1.731–2.334) vs. median 3.846 (IQR 3.110–3.936); mean difference (MD) of the log values: 1.240; CI: (1.478; 4.983), p = <0.001], the same pattern was also seen after 48 h [median 2.182 (IQR 2.037–3.062) vs. median 3.807 (IQR 3.357–3.964); MD: 1.237; CI: (0.677; 5.779), p = 0.006] and 72 h with [median 3.071 (2.047–3.512) vs. median 3.603 (IQR 3.333–3.890); MD: 1.154; CI: (−0.308; 3.932), p = 0.138], after 72 h the p-value showed no significance (Figure 4B; Supplementary Table S2). A significant increase in IL-1α production occurred in PBMCs treated with PN water extracts compared to unstimulated controls at 24 h [median 2.884 (IQR 2.652–3.101) vs. median 0.8183 (IQR 0.4838–1.336); MD: 1.518; CI: (2.805; 6.225), p = <0.001] and 48 h [median 2.892 (IQR 2.727–3.031) vs. median 0.9965 (IQR 0.7836–2.011); MD: 1.341; CI: (0.852; 5.646), p = 0.003]. Although IL-1β was produced after 24 h exposure to PN, the was decline after 48 and 72 h, and the MD were less than to one and negative respectively. Chemokines MIP-1α and MIP-1β production was significantly higher following stimulation with PN water extracts in comparison with unstimulated controls at 24 h, MIP-1α production median 3.631 (IQR 3.578–4.166) vs. median 2.149 (IQR 1.997–2.378); MD: 1.143; CI: (1.554; 5.335), p = <0.001] MIP-1β production [median 4.921 (IQR 4.632–5.466) vs. median 3.069 (IQR 2.726–3.439); MD: 1.247; CI: (2.048; 6.417), p = <0.001] respectively. Chemokines MIP-1α and MIP-1β production are associated with downregulation of CCR5. TNF-α was most evident at 24 h [median 3.958 (IQR 3.573–4.037)

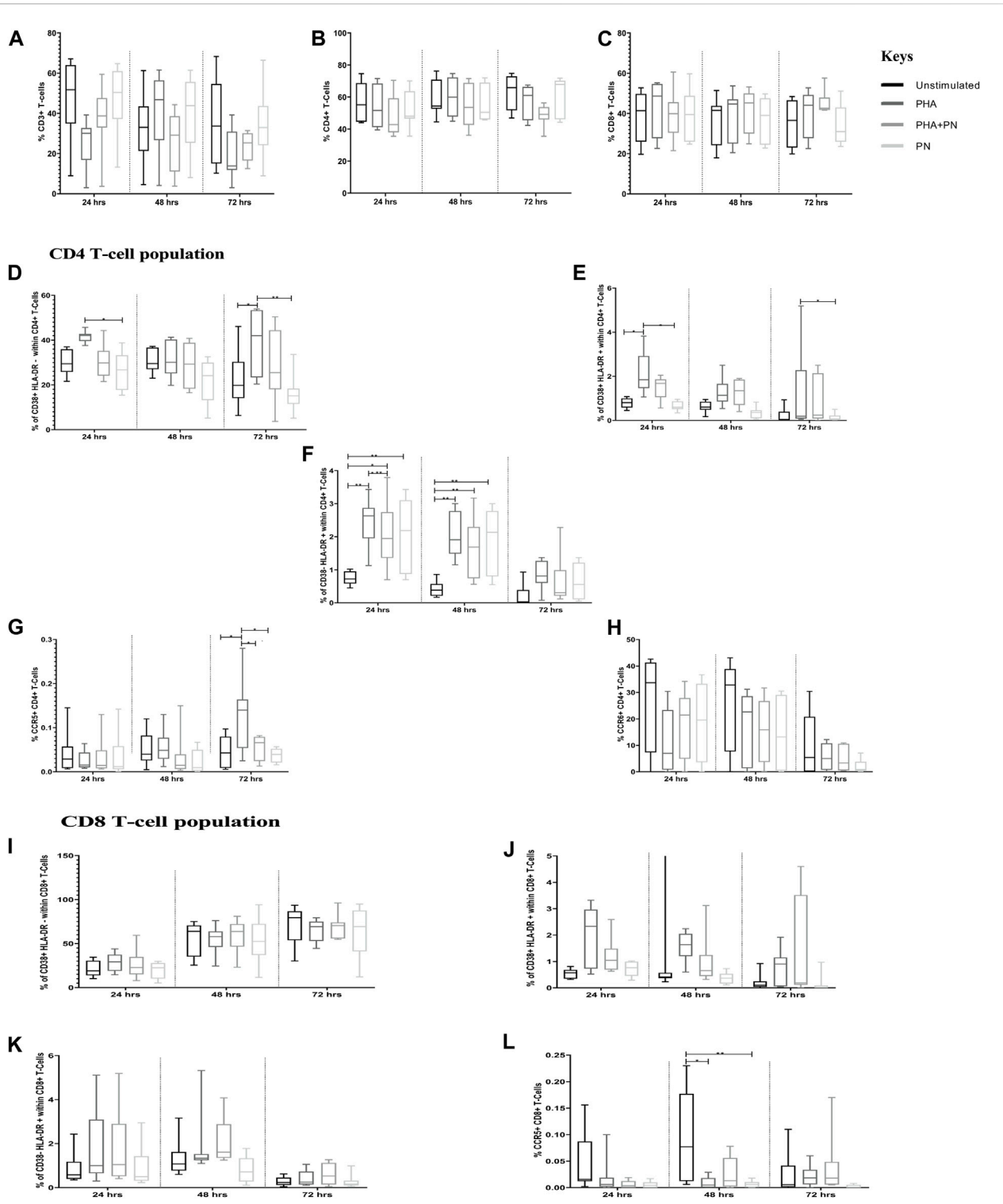

FIGURE 3
Differences in percentages of total T-cell subsets in PBMCs treated with PN water extract, PHA, PHA-PN combination, and unstimulated control. **(A-C)** shows the total percentages of CD3, CD4, and CD8. **(D-L)** illustrates the expression of activation markers on $CD4^+$ T cells and $CD8^+$ T cells after 24, 48, and 72 h of stimulation. PHA was used at a final concentration of 5 μg/mL, and PN was used at a concentration of 325.3 μg/mL. Bonferroni multiple comparison tests were used to compare pairwise treatment groups using Statistical Analysis Software (SAS) version 9.4., significance is represented as $*p < 0.05$ and $**p < 0.01$ compared to different stimulants.

vs. median 2.239 (IQR 1.922–2.553); MD: 1.777; CI: (2.083; 4.934), $p < 0.0001$) and 48 h [median 3.915 (IQR 3.836–3.960) vs. median 2.571 (IQR 2.114–3.184); MD: 1.478; CI: (0.365; 4.684), $p = 0.014$] (Figure 4B; Supplementary Table S2). Similarly, IL-10 [median 3.801(IQR 3.178–4.056) vs. median 2.154 (IQR 1.731–2.334), MD: 1.392; CI: (1.444; 4.949), $p = <0.001$], IL-1α [median 2.884

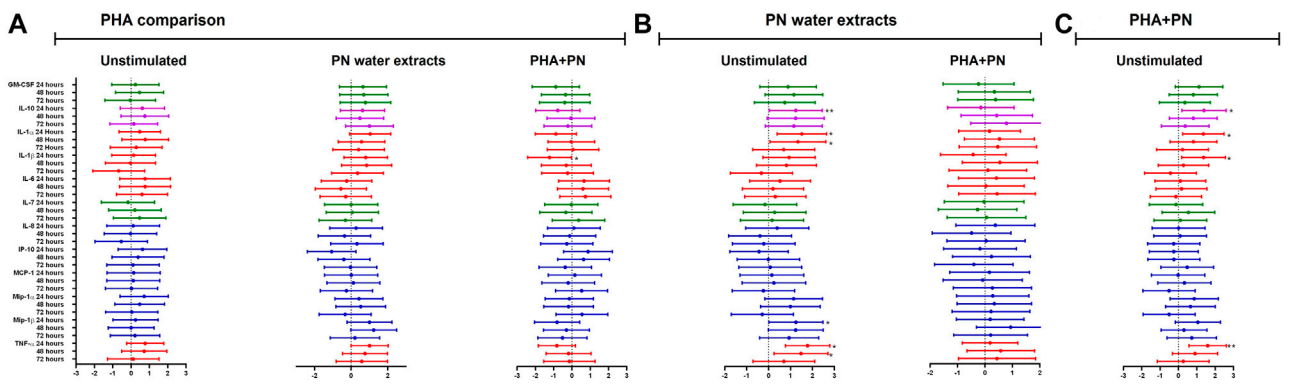

FIGURE 4
Estimate values (including minimum and maximum values) from linear mixed models to determine the effect of **(A)** PHA, **(B)** PN water extract, or **(C)** PHA-PN combination on the cytokine milieu compared to unstimulated control. Individual associations are shown between stimulants and pro-inflammatory cytokines (red), chemokines (blue), growth factors (green), and anti-inflammatory (blue), with error bars depicting standard error. Stars denote the degree of significance, $p < 0.05$: *, $p < 0.01$: **, and $p < 0.001$: ***.

(IQR 2.588–3.136) vs. median 0.8183 (IQR 0.4838–1.336); MD: 1.355; CI: (2.309; 5.729), p = <0.001] production tended to be significantly induced in PBMCs treated with a combination of PHA and PN water extract compared to unstimulated controls by 24 h, but the levels dropped by 48 and 72 h (Figure 4C; Supplementary Table S2). The pro-inflammatory cytokine TNF-α [median 3.839 (IQR 3.780–4.007) vs. median 2.239 (IQR 1.922–2.553); MD: 1.593; CI: (1.841; 4.692), p < 0.001] was expressed at 24 h, however, there was a lower secretion after 48 and 72 h, the MD was lower than one (Figure 4C; Supplementary Table S2). IL-1β production was significantly increased at 24 h in PBMCs treated with a combination of PHA and PN water extract in comparison with unstimulated controls [median 3.360 (IQR 2.423–3.759) vs. median 1.520 (IQR 0.8473–2.092); MD: 1.369; CI: (1.496; 5.527), p= <0.001] with MD of 1.520 (Figure 4C; Supplementary Table S2). The medicine has been shown to modulate the immune system by secretion of various cytokines.

Extraction (50% EtOH) of PN and UPLC-HRMS analysis

A 12.3% yield was obtained for the 50% ethanol extract, and subsequent UPLC-HRMS analysis revealed the presence of daphnane diterpenes in the 13–14 min retention time region in ESI positive ionisation conditions (Figure 5; Figure 6).

Based on prior work conducted by (31) compound 1 (peak 1) was identified as yuanhuacine A (UNAIDS and Geneva, 2019). Based on the UPLC-HRMS analysis, yuanhuacine A (UNAIDS and Geneva, 2019) was observed at a retention time (RT) of 13.53 min and m/z 621.2711 $[M + H]^+$ with a corresponding molecular formula of $C_{35}H_{40}O_{10}$ (calc. 621.0360). The compound was observed to have 16 degrees of unsaturation and a corresponding mass error of 1.1 mDa. Compound 2 was identified as gniditrin (Africa UCFS, 2019) and observed at RT 13.56 min and m/z 647.2868 $[M + H]^+$ corresponding to molecular formula of $C_{37}H_{42}O_{10}$ (calc. 647.2856) with 17 degrees of unsaturation and an associated mass error of 1.2 mDa. Compound 3 was identified as yuanhuajine (Organization WH, 2019) and observed at RT 13.83 min and m/z 647.2856 $[M + H]^+$ corresponding to molecular formula of $C_{37}H_{42}O_{10}$ (calc. 647.2856) with 17 degrees of unsaturation and an associated mass error of 0.8 mDa. Compound 4 was identified as yuanhuacine (Organization WH, 2016) and observed at RT 14.00 min and m/z 649.3026 $[M + H]^+$ corresponding molecular formula of $C_{37}H_{45}O_{10}$ (calc. 649.3267) and an associated mass error of 1.3 mDa. The associated chemical structures of compounds one to four are displayed in Figure 6.

Discussion

Traditional medicine remains the alternate source of basic healthcare to prevent and treat numerous infectious diseases in resource-limited countries. Studies have suggested that TM also possesses beneficial therapeutic properties, including antioxidant, anti-inflammatory, anti-microbial, and immunomodulatory effects (Mahomoodally, 2013; Parham et al., 2020; Salehi et al., 2020). However, it remains unclear how TM influences the cytokine and cellular immune response. Here, we compared immunological differences in T cell subset expression profile of PBMCs treated with PN to PHA and unstimulated controls. The proportion of HLA-DR+ $CD4^+$ T cells was two-fold higher in the PBMCs treated with PN water extracts at 24 and 48 h. CCR5+ on total $CD8^+$ T cells was reduced in PBMCs stimulated with PN water extract in comparison to 48 h. Furthermore, the PN water extract strongly induced anti-inflammatory cytokine, a pro-inflammatory cytokine, and chemokine production at 24 and 48 h, but not at 72 h.

Several studies have demonstrated an effect on immune cell activation following treatment with South African TM products, and this immunomodulatory effect was time-dependent (Denzler et al., 2010; Yin et al., 2010; Nahas and Balla, 2011; Gomez-Cadena et al., 2020; Nugraha et al., 2020; Alhazmi et al., 2021). Although the PN water extract did not exert hyperactivation of $CD4^+$ T cells, as

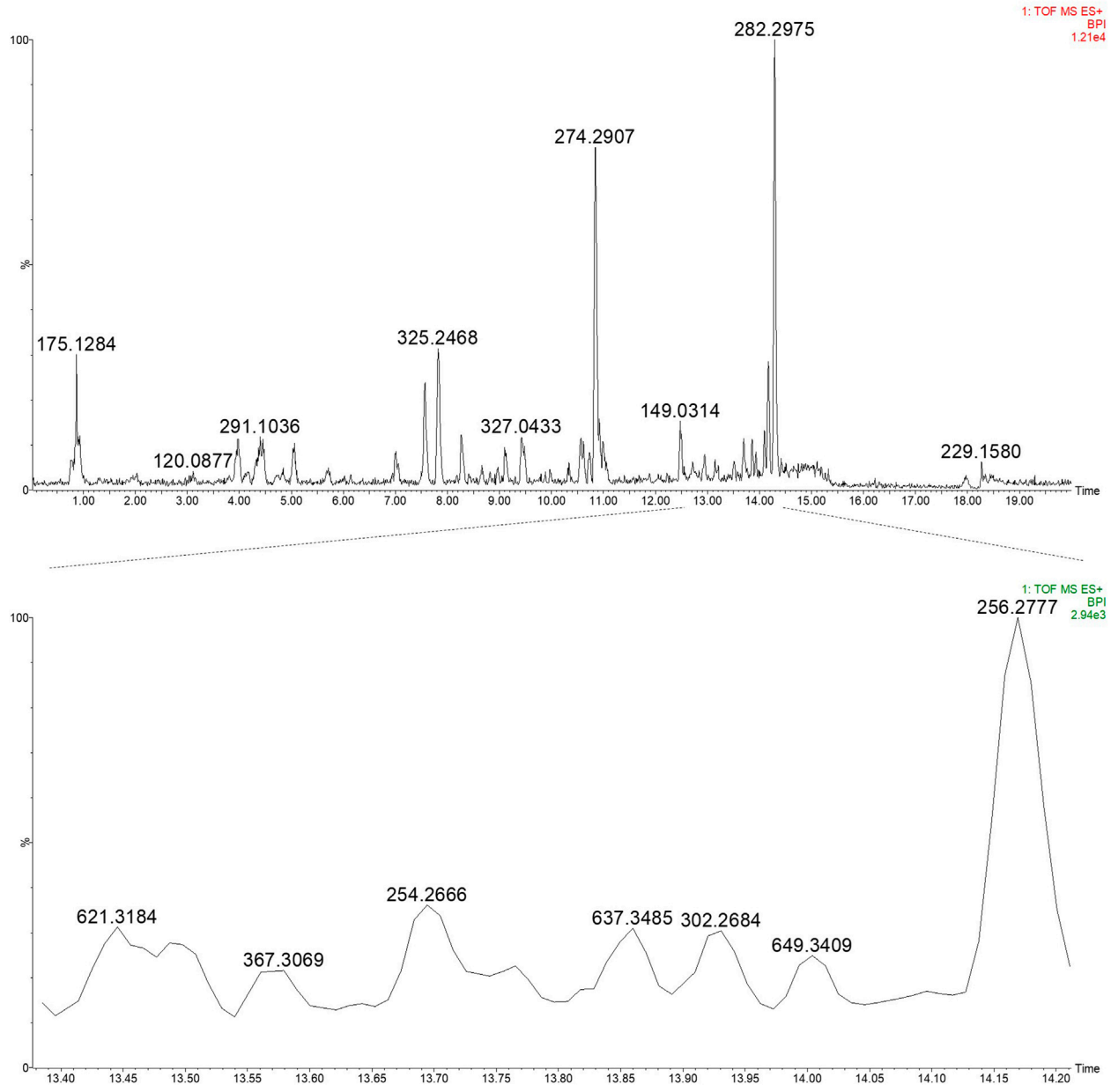

FIGURE 5
UPLC-HRMS profile of the EtOH:H2O (1:1) extract of PN (top) and an expansion of region 13.40–14.20 min highlighting the daphnane diterpenes (peak 1–4) (bottom) in ESI positive ionisation mode.

defined by CD38+HLA-DR + phenotype; CD38-HLA-DR + phenotype was induced after 24 h stimulation. HLA-DR molecules are normally produced by antigen-presenting cells and play a role in presenting antigens to $CD4^+$ T cells (Ten Broeke et al., 2013). In addition, HLA-DR+ is also produced by T cells and has been associated with T cell activation and peripheral tolerance, and induction of apoptosis (Gotsman et al., 2008). Furthermore, HLA-DR + expression by CD4 T cells is associated with an effector T cell phenotype that directly destroys infected cells or indirectly modulates the immune response through the expression of cytokines (Ahmed et al., 2018; Tippalagama et al., 2021). Based on the results obtained in this study, PN has immunomodulatory effects on treated PBMCs, and it is reasonable to suggest that PN exerts this effect through the activation of T cells to fight against invading pathogens and possibly induce apoptosis. However, more *in vivo* and *in vitro* studies are needed to better understand the role of PN in modulating immune response and disease progression.

This study found that the PN water extract exerted an inhibitory effect on the expression of CCR5 on total $CD8^+$ T cells. Chen et al. (2003) showed similar effects when researching the Chinese herbal medicine Shikonin, which is extracted from dried roots of *Lithospermum erythrorhizon Siebold and Zucc* (commonly called zicao), by demonstrating that it suppressed the expression of CCR5 on macrophages and HEK cells (Chen et al., 2003).

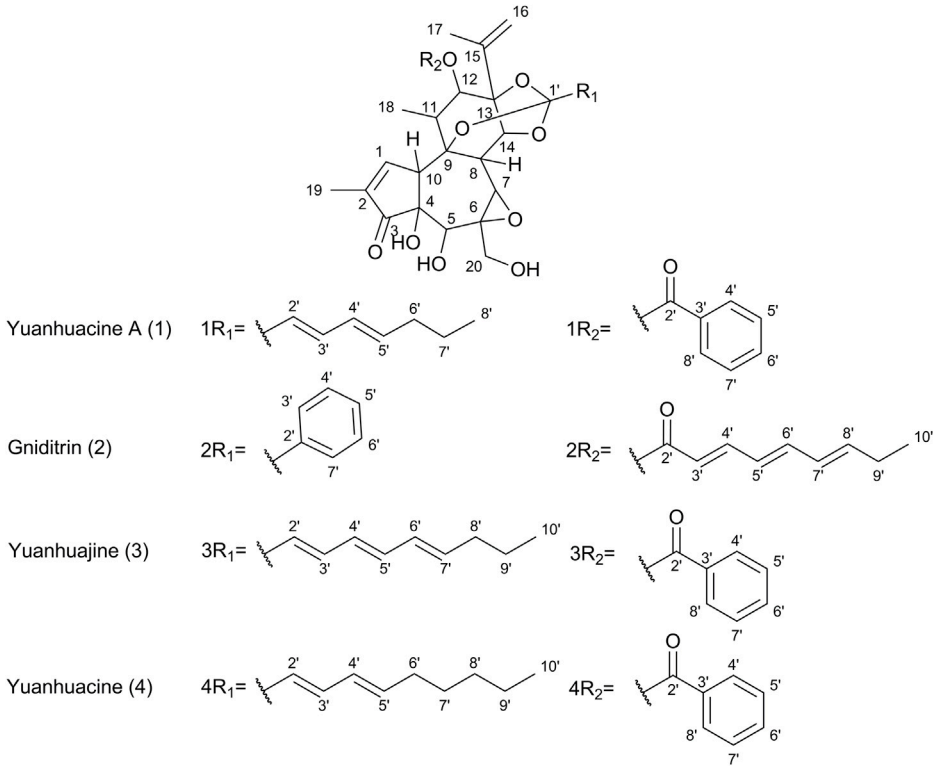

FIGURE 6
Chemical structures of select daphnane diterpenes observed in the 50% EtOH extract of PN, identified by UPLC-HRMS analysis.

Chemokine receptor CCR5 is expressed in several cells and plays an important role in the differentiation, activation, and recruitment of T cells (Littman, 1998; Berger et al., 1999; Murphy et al., 2012; Wang et al., 2019). It is also the main HIV co-receptor involved in virus entry into $CD4^+$ T cells and the cell-to-cell spread of R5-tropic viruses (Jasinska et al., 2022). Furthermore, previous studies have demonstrated that CCR5 induces activation and recruitment of $CD8^+$ T cells to inflamed tissues (Palendira et al., 2008; Kohlmeier et al., 2011) or following HIV, coronavirus, hepatitis C virus, Epstein-Barr virus, or cytomegalovirus infections (Fukada et al., 2002; Glass and Lane, 2003a; Glass and Lane, 2003b; Shacklett et al., 2003; Lederman et al., 2006; Hugues et al., 2007). Any interference with CCR5 may inhibit cell proliferation and block viral entry. Thus, our findings suggest that PN promotes the immunomodulatory effect on the cellular response by interfering with the chemokine receptor signalling and possibly receptor gene transcription in $CD8^+$ T cells.

Several studies show that TM formulations have a stimulatory effect on the production of inflammatory and anti-inflammatory cytokines (Barak et al., 2002; Denzler et al., 2010; Elanchezhiyan, 2012; Ngcobo and Gqaleni, 2016; Ngcobo et al., 2017; Kiyomi et al., 2021). In this study, the increase of pro-inflammatory cytokines (IL-1α, TNF-α), and chemokine (MIP-1α and MIP-1β) production in PBMCs treated with PN were observed as early as 24 h after treatment. In addition, PN water extracts increased the production of the anti-inflammatory cytokine IL-10. Similar findings of increased anti-inflammatory cytokine (IL-10), pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α), and chemokine production were observed in PBMCs treated with the combination of PHA and PN water extracts. This study strengthens evidence that PN can illicit increased production of MIP1-β, IL-1α, and IL-1β after treatment with traditional medicine as previous shown with other traditional medicines (Barak et al., 2002; Denzler et al., 2010; Hoosen and Pool, 2018). Pro-inflammatory cytokines like TNF-α, IL-1α, IL-1β, and chemokine MIP1-β have been implicated in multiple immunopathological conditions (Sandeep Varma et al., 2011). IL-1α and IL-1β are known to induce an inflammatory response and acute immune response, including the proliferation and recruitment of macrophages and neutrophils to the infection site (Sims and Smith, 2010; McKinnon et al., 2018; Palazon Riquelme and Lopez Castejon, 2018; Kaneko et al., 2019; Cavalli et al., 2021). The Th1-specific cytokines, TNF-α, produced by macrophages or monocytes during acute inflammation, are responsible for a diverse range of signaling events within cells, leading to the cellular response to inflammation, necrosis or apoptosis, and homeostasis of the immune system (Idriss and Naismith, 2000; Urschel and Cicha, 2015; Jang et al., 2021). In contrast to pro-inflammatory cytokines, IL-10 is an anti-inflammatory Th2-specific cytokine that modulates the activation of T-cells, monocytes, and macrophages to limit or stop inflammation and minimize the degree of host damage (Murphy et al., 2012; Saraiva and O Garra, 2010; Mion et al., 2014; Steen et al., 2020). Our data suggest that PN promotes Th1 and Th2 responses for the resolution of infection through coordinating a variety of T cell responses and the development of humoral immune responses.

In a bid to identify the possible bioactive compounds found in PN, an EtOH: H_2O (1:1) extract was prepared and analysed using UPLC-HRMS. The extract solvent was carefully selected to ensure the extraction of both polar and non-polar compounds, which would likely be extracted by conventional boiling, as done when preparing the decoction traditionally. The extraction solvent selected is also essential as the traditional decoction used in the bioassays cannot be analysed directly on UPLC-HRMS. The UPLC-HRMS analysis revealed the presence of a few daphnane diterpenoids (genkwanine-type) known to occur within PN. The compounds *viz.*, yuanhuacine A (UNAIDS and Geneva, 2019), gniditrin (Africa UCFS, 2019), yuanhuajine (Organization WH, 2019) and yuanhuacine (Organization WH, 2016) were identified based on their accurate mass and fragmentation pattern (Supplementary Figures S1–S4), which aligns with data previously published by (31). These compounds were previously shown to exhibit potent anti-HIV activity (Tembeni et al., 2022)Of special note, yuanhuacine A (UNAIDS and Geneva, 2019), and a mixture containing yuanhuacine (Organization WH, 2016), were found to induce HIV latency reversal (65.5% ± 6.3% and 58.2% ± 4.7%, respectively) at 0.15 μM and were found to induce T cell activation by functioning as Protein C Kinase activators (Tembeni et al., 2022) Based on the previously published biological data for this compound class, it is hypothesised that the compounds identified likely contributed to the observed biological activity.

We conclude that PN possesses *in vitro* immunomodulatory properties that may impact immune cell activation and chemokine receptor signaling. This *in vitro* study indicates that PN induces pro-inflammatory and anti-inflammatory effects that are needed for an enhanced immune response that protects the host from pathogens. This study is the first to evaluate the above using the combination of the four plants that are filed for a patent. Future *in vitro* and animal studies using PN are needed to further understand key parameters mediating induction, expression, and regulation of the immune response. In future studies, we aim to investigate the *in vitro* anti-HIV properties of PN.

Limitations of this study

The limitation of the study is that the sample size was not large enough, additionally this is an experimental exploratory *in vitro* study design that still requires further *in vivo* and clinical studies to further validate the results. In addition, the limitation of the study is the use of a combination of four medicinal plants which precludes the identification of specific biological compounds responsible for the observed biological activity. Even though this may be a limitation, the approach is important for validating the use of PN by the public who are patients of the traditional health practitioner. Furthermore, the study did not account for the seasonal variations, which have been well described in the literature to affect the phytochemistry of plants. Depending on the season at which the plants are harvested may very well influence the phytochemistry profile and, hence, the potency and biological activity thereof (Ncube et al., 2011). Additionally, variations in extraction solvents would lead to variations in the plant constituents extracted.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC REF: BE432/14) and (BREC REF: BE665/18). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Writing–original draft, Writing–review and editing. ML: Formal Analysis, Methodology, Software, Writing–review and editing. SN: Resources, Writing–review and editing. MN: Resources, Writing–review and editing. GM: Formal Analysis, Methodology, Writing–review and editing. AM: Formal Analysis, Methodology, Writing–review and editing. SP: Formal Analysis, Methodology, Writing–review and editing. SN: Formal Analysis, Methodology, Writing–review and editing. LI: Formal Analysis, Methodology, Writing–review and editing. VM: Formal Analysis, Methodology, Writing–review and editing. MN: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing–review and editing. NG: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing–review and editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1308913/full#supplementary-material

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Exploring nature's antidote: unveiling the inhibitory potential of selected medicinal plants from Kisumu, Kenya against venom from some snakes of medical significance in sub-Saharan Africa

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Background: The present study investigated the efficacy of *Conyza bonariensis, Commiphora africana, Senna obtusifolia, Warburgia ugandensis, Vernonia glabra,* and *Zanthoxylum usambarense* against *Bitis arietans* venom (BAV), *Naja ashei* venom (NAV), and *Naja subfulva* venom (NSV).

Methods: 40 extracts and fractions were prepared using n-hexane, dichloromethane, ethyl acetate, and methanol. *In vitro* efficacy against snake venom phospholipase A_2 (svPLA$_2$) was determined in 96-well microtiter and agarose-egg yolk coagulation assays. *in vivo* efficacy against venom-induced cytotoxicity was determined using *Artemia salina*. Two commercial antivenoms were used for comparison.

Results: The 96-well microtiter assay revealed poor svPLA$_2$ inhibition of BAV by antivenom (range: 20.76% ± 13.29% to 51.29% ± 3.26%) but strong inhibition (>90%) by dichloromethane and hexane fractions of *C. africana*, hexane and ethyl acetate extracts and fraction of *W. ugandensis*, dichloromethane fraction of *V. glabra*, and the methanol extract of *S. obtusifolia*. The methanol extract and fraction of *C. africana*, and the hexane extract of *Z. usambarense* strongly inhibited (>90%) svPLA$_2$ activity in NAV. The hexane and ethyl acetate fractions of *V. glabra* and the dichloromethane, ethyl acetate, and methanol extracts of *C. africana* strongly inhibited (>90%) svPLA$_2$ in NSV. The agarose egg yolk coagulation assay showed significant inhibition of BAV by the dichloromethane fraction of *C. africana* (EC_{50} = 3.51 ± 2.58 µg/mL), significant

Abbreviations: $CaCl_2$, Calcium Chloride; CHCl3, Chloroform; ELISA, Enzyme Linked Immunosorbent Assay; EC_{50}, Effective concentration of extract/antivenom that spares 50% of *A. salina* from death; $FECl_3$, Ferric Chloride; H_2SO_4, Sulphuric acid, HCl, Hydrochloric acid; LC_{50}, Lethal concentration that kills 50% of *A. salina*, Na_2CO_3, Sodium Carbonate, UV-VIS, Ultraviolet and visible, mg. GAE.g^{-1}, milligrams of gallic acid equivalents per Gram, mg. CE. g^{-1}, milligrams of catechin equivalents per Gram; MPC, Minimum phospholipase concentration; µL, Microliter; µg/mL, Microgram per millilitre; mM, millimoles; NaOH: sodium hydroxide; Na_2WO_4, Sodium tungstate, H_3PO_4, Phosphoric acid; SvPLA$_2$, Snake venom phospholipase A_2; WHO, World Health Organization.

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inhibition of NAV by the methanol fraction of *C. africana* (EC_{50} = 7.35 ± 1.800 µg/mL), and significant inhibition of NSV by the hexane extract of *V. glabra* (EC_{50} = 7.94 ± 1.50 µg/mL). All antivenoms were non-cytotoxic in *A. salina* but the methanol extract of *C. africana* and the hexane extracts of *V. glabra* and *Z. usambarense* were cytotoxic. The dichloromethane fraction of *C. africana* significantly neutralized BAV-induced cytotoxicity, the methanol fraction and extract of *C. africana* neutralized NAV-induced cytotoxicity, while the ethyl acetate extract of *V. glabra* significantly neutralized NSV-induced cytotoxicity. Glycosides, flavonoids, phenolics, and tannins were identified in the non-cytotoxic extracts/fractions.

Conclusion: These findings validate the local use of *C. africana* and *V. glabra* in snakebite but not *C. bonariensis, S. obtusifolia, W. ugandensis*, and *Z. usambarense*. Further work is needed to isolate pure compounds from the effective plants and identify their mechanisms of action.

KEYWORDS

***Bitis arietans*, medicinal plants, Naja ashei, preclinical efficacy evaluation, Naja subfulva, snake venom, *Artemia salina* bioassay**

Introduction

An estimated 5 million people are bitten by snakes every year, about half of whom experience clinical illness, and up to 140,000 die from complications related to envenomation (Chippaux, 1998; Kasturiratne et al., 2008). Snakebites are prevalent among low-income individuals residing in rural, tropical areas with limited access to healthcare (Oliveira et al., 2023). Consequently, local people frequently rely on folk medicine, which includes the use of medicinal plants. Several such plants, including *C. bonariensis, C. africana, S. obtusifolia, Warburgia ugandensis, Vernonia glabra,* and *Zanthoxylum usambarense* have gained notoriety among the Luo people in Kisumu, Kenya, due to their putative anti-snake venom properties (Owuor et al., 2005; Owuor and Kisangau, 2006). These plants are known by the locals as "yadh asere" (*C. bonariensis*), "arupiny" (*C. africana*), "olusia" (*V. glabra*), "sogo" (*W. ugandensis*), and "roko" (*Z. usambarense*). They have widespread ethnomedicinal use locally including in snakebite and share phylogenetic relationships with plants previously reported as anti-snake bite remedies, e.g., *Senna siamea*, *Conyza sumatrensis*, and *Zanthoxylum chalybeum* (Owuor and Kisangau, 2006). Treatments include the use of cut, suck, and bind techniques, followed by the application of plant leaf and root poultices secured with bark or cloth strips (Owuor et al., 2005). However, there is a general concern about the efficacy and safety of alternative remedies in managing diseases (Puzari et al., 2022). Rigorous scientific scrutiny of these remedies is essential to determine the validity of the ethnomedicinal claims and to ensure the development of safe and efficacious interventions for snakebite victims (Puzari et al., 2022).

B. arietans, N. ashei, and *N. subfulva* are snakes of medical importance in sub-Saharan Africa (Calvete et al., 2007; Currier, 2012; Tasoulis and Isbister, 2017; Onyango, 2018; Okumu et al., 2020; Dyba et al., 2021) (Figure 1). Antivenom is the mainstay of treatment for envenomation by these snakes but is expensive, has limited availability, and does not sufficiently neutralize some key venom toxins, e.g., cytotoxins which cause dermonecrosis in snake bite victims. Medicinal plants are used to plug this gap, but they lack scientific validity. This study employed a combination of *in vitro* and *in vivo* methods to evaluate the antivenom properties of *C. bonariensis, C. africana, S. obtusifolia, W. ugandensis, V. glabra,* and *Z. usambarense* against *B. arietans*, *N. ashei*, and *N. subfulva* venoms.

Materials and methods

Collection and identification of medicinal plants

Plant materials were collected in November 2016 in Kisumu County. The East African Herbarium in Nairobi, Kenya identified

FIGURE 1
Photos of *Bitis arietans* **(A)**, *Naja ashei* **(B)**, and *Naja subfulva* **(C)**. Photos by Mitchel Okumu.

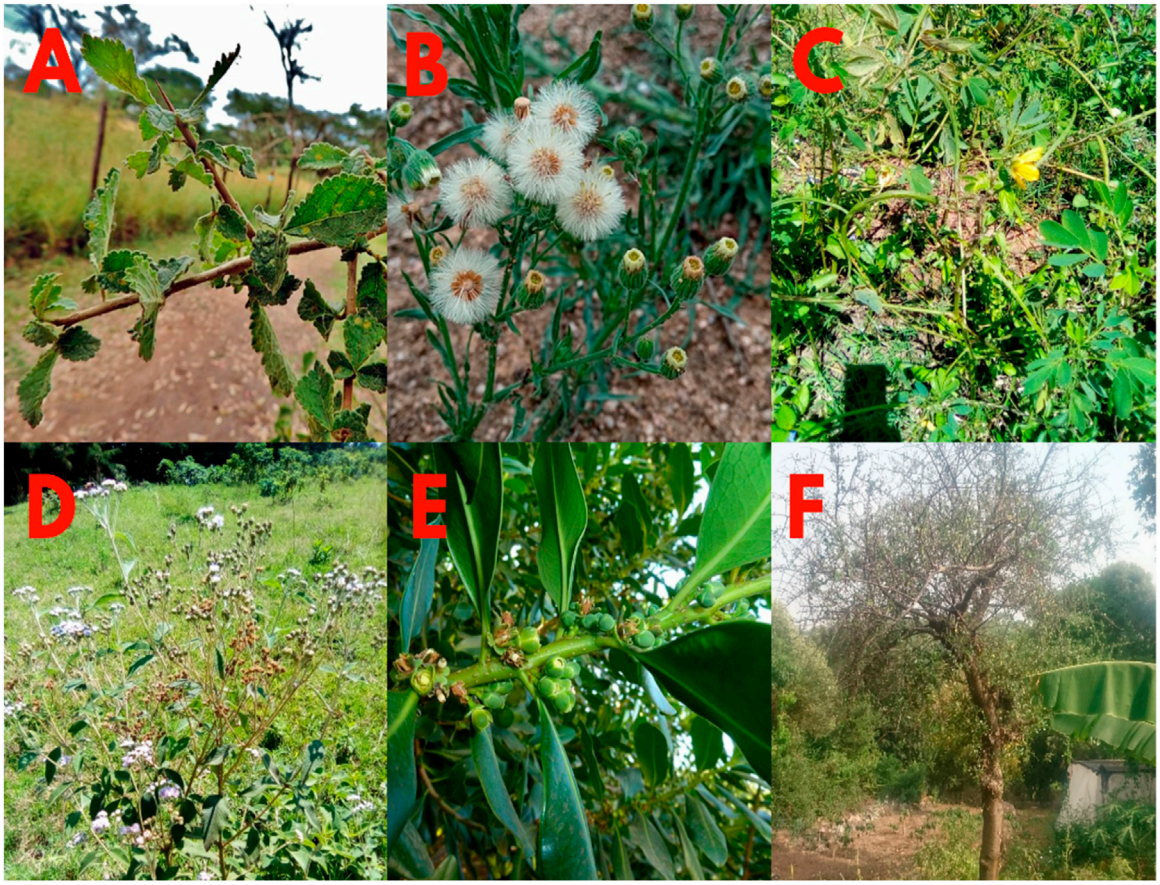

FIGURE 2
Photos of *Commiphora africana* (A. Rich) Engl **(A)**, *Conyza bonariensis* (L.)., Cronquist **(B)**, *Senna obtusifolia* (L.) Irwin and Barneby **(C)**, *Vernonia glabra* (Streetz) **(D)**, *Warburgia ugandensis* (Sprague) **(E)**, and *Zanthoxylum usambarense* (Engl.) Kokwaro **(F)** used in this study.

and verified the plant specimens, as shown in (Supplementary Figure S1) (Supplementary Section). **REF NMK/BOT/CTX/1/2/1.** The selection of the plants was based on five factors: 1) their extensive local ethnopharmacological use in treating snakebites; 2) their evolutionary link to other plants used for the same purpose; 3) the findings of an Owuor and Kisangau survey on the use and practice of herbal medicine (Owuor and Kisangau, 2006), 4) the lack of published research outlining the plants' bioactive ingredients, and 5) their availability for evaluation. An overview of the plants used in this study is as shown in (Figure 2).

Preparation of plant material

After being cleaned to get rid of any dust that stuck to them, the plant materials were shade-dried and then ground into a powder using an electric mill (Retsch Grindomax, Germany).

Chemical and reagents

n-hexane, dichloromethane, ethyl acetate, and methanol were purchased from Loba Chemie (India). Phosphate buffered saline (PBS) tablets, Calcium chloride, Fuchsin acid (Carbol Fuchsin), gallic acid, catechin, agarose, and rutin were bought from Sigma Aldrich (USA). Sodium carbonate, Folin-phenol reagent, Folin-Denis reagent, aluminum chloride, sodium hydroxide pellets, lead acetate, Sodium hydrogen phosphate, and picric acid were bought from FINAR (India). The antivenoms used in this study were manufactured in India and Mexico.

Soxhlet extraction of the medicinal plants

Powdered plant materials were sequentially extracted by Soxhlet extraction using n-hexane, dichloromethane, ethyl acetate, and methanol and concentrated under reduced pressure at 40°C on a rotary evaporator (Stuart, Cole-Parmer-UK) (Janardhan et al., 2014). The percentage yield of the extracts was calculated as %w/w.

Extraction of medicinal plants using a modified maceration technique

Powdered plant materials were separately mixed with methanol, macerated for 72 h, and concentrated at 40°C under reduced pressure on a rotary evaporator (Stuart, Cole-Parmer-

TABLE 1 The *in vitro* neutralization capacity of antivenom, extracts, and fractions of *Commiphora africana*, *Conyza bonariensis, Senna obtusifolia, Vernonia glabra, Warburgia ugandensis,* and *Zanthoxylum usambarense* against snake venom phospholipase A_2.

Plant species	Plant part	Solvent used	*Bitis arietans*		*Naja ashei*		*Naja subfulva*	
			Soxhlet	Maceration	Soxhlet	Maceration	Soxhlet	Maceration
Commiphora africana (A. Rich.) Engl.	**Bark**	Hexane	No activity	24.24 ± 3.65	37.36 ± 6.29	No activity	8.33 ± 2.74	8.92 ± 1.58
		Dichloromethane	No activity	10.61 ± 2.98	39.06 ± 7.54	5.10 ± 1.34	21.58 ± 5.41	7.26 ± 2.34
		Ethyl acetate	No activity	62.12 ± 9.54	48.30 ± 8.76	No activity	27.14 ± 6.87	1.03 ± 0.89
		Methanol	No activity	50.00 ± 8.17	93.64 ± 2.55	82.80 ± 9.54	79.06 ± 9.23	54.98 ± 8.21
	Stem bark	Hexane	73.17 ± 5.28	96.18 ± 0.93	37.54 ± 6.87	5.21 ± 1.67	8.76 ± 2.34	5.18 ± 1.67
		Dichloromethane	14.63 ± 3.17	94.25 ± 3.21	33.96 ± 6.12	46.88 ± 8.32	7.48 ± 2.11	22.65 ± 5.89
		Ethyl acetate	21.95 ± 4.43	No activity	48.87 ± 8.43	79.17 ± 9.01	11.54 ± 3.42	23.3 ± 6.12
		Methanol	No activity	No activity	95.61 ± 0.28	95.55 ± 1.69	91.67 ± 5.32	83.17 ± 9.78
	Roots	Hexane	No activity	No activity	34.77 ± 7.32	No activity	20.85 ± 3.55	No activity
		Dichloromethane	No activity	No activity	33.20 ± 6.89	No activity	10.99 ± 2.87	16.59 ± 4.56
		Ethyl acetate	25.40 ± 6.72	No activity	44.20 ± 8.14	No activity	12.78 ± 2.94	No activity
		Methanol	7.94 ± 2.86	No activity	45.77 ± 8.26	21.82 ± 4.76	21.52 ± 5.98	No activity
Conyza bonariensis (L.) Cronquist	**Leaves**	Hexane	No activity	No activity	38.46 ± 7.68	35.76 ± 7.93	14.41 ± 7.63	No activity
		Dichloromethane	33.96 ± 7.14	10.34 ± 2.36	15.96 ± 4.23	No activity	8.30 ± 2.22	6.28 ± 2.01
		Ethyl acetate	41.51 ± 8.93	37.93 ± 7.84	35.77 ± 7.43	No activity	13.97 ± 2.28	No activity
		Methanol	37.74 ± 7.29	20.69 ± 4.71	23.08 ± 5.78	No activity	No activity	1.35 ± 0.78
Senna obtusifolia (L.) Irwin & Barneby	**Leaves**	Hexane	60.71 ± 10.47	5.00 ± 1.23	5.22 ± 1.78	13.56 ± 3.87	No activity	47.94 ± 8.09
		Dichloromethane	7.14 ± 2.14	2.50 ± 0.65	26.10 ± 5.56	No activity	28.19 ± 6.44	40.48 ± 7.56
		Ethyl acetate	No activity	No activity	36.14 ± 6.98	19.49 ± 4.98	67.55 ± 9.12	64.36 ± 9.32
		Methanol	94.33 ± 0.87	No activity	44.58 ± 8.32	7.06 ± 2.21	64.36 ± 9.99	50.75 ± 8.87
Vernonia glabra (Streetz) Vatke	**Leaves**	Hexane	76.19 ± 5.69	54.29 ± 8.45	17.36 ± 4.89	9.03 ± 2.87	97.39 ± 0.18	94.49 ± 3.76
		Dichloromethane	16.19 ± 3.81	93.33 ± 3.67	25.69 ± 5.21	No activity	99.42 ± 0.06	86.96 ± 9.23
		Ethyl acetate	78.10 ± 6.39	35.23 ± 6.89	No activity	34.03 ± 7.21	92.46 ± 1.76	91.88 ± 4.47
		Methanol	35.24 ± 7.57	37.14 ± 7.86	19.44 ± 4.76	No activity	96.81 ± 5.47	No activity
Warburgia ugandensis Sprague	**Leaves**	Hexane	40.74 ± 8.26	47.22 ± 8.63	43.97 ± 1.27	No activity	28.80 ± 6.67	14.89 ± 4.23
		Dichloromethane	28.70 ± 5.81	65.74 ± 9.86	33.62 ± 5.44	No activity	30.42 ± 7.21	24.60 ± 5.96
		Ethyl acetate	42.59 ± 9.72	40.74 ± 7.58	No activity	No activity	51.13 ± 8.65	27.18 ± 6.43
		Methanol	32.41 ± 6.25	50.00 ± 8.11	No activity	No activity	21.04 ± 5.69	38.51 ± 7.98
	Leaf stalk	Hexane	96.41 ± 0.22	5.00 ± 1.56	5.32 ± 1.45	14.41 ± 3.65	No activity	36.94 ± 7.23
		Dichloromethane	No activity	80.00 ± 9.47	30.04 ± 6.21	No activity	No activity	28.73 ± 6.12
		Ethyl acetate	92.69 ± 1.17	91.79 ± 4.15	8.37 ± 2.34	No activity	No activity	35.07 ± 7.34
		Methanol	37.50 ± 5.39	17.50 ± 3.14	13.31 ± 3.89	8.47 ± 2.54	No activity	25.37 ± 5.56
Zanthoxylum usambarense (Engl.) Kokwaro	**Leaves**	Hexane	No activity	No activity	58.23 ± 9.12	No activity	No activity	No activity
		Dichloromethane	No activity	No activity	19.28 ± 4.67	No activity	No activity	No activity
		Ethyl acetate	No activity	No activity	50.60 ± 8.78	No activity	No activity	No activity
		Methanol	No activity	No activity	29.72 ± 6.67	No activity	45.21 ± 8.78	No activity

(Continued on following page)

TABLE 1 (*Continued*) The *in vitro* neutralization capacity of antivenom, extracts, and fractions of *Commiphora africana*, *Conyza bonariensis*, *Senna obtusifolia*, *Vernonia glabra*, *Warburgia ugandensis*, and *Zanthoxylum usambarense* against snake venom phospholipase A_2.

Plant species	Plant part	Solvent used	*Bitis arietans*		*Naja ashei*		*Naja subfulva*	
			Soxhlet	Maceration	Soxhlet	Maceration	Soxhlet	Maceration
	Roots	Hexane	No activity	No activity	93.25 ± 9.54	24.01 ± 5.43	No activity	15.50 ± 4.23
		Dichloromethane	No activity	No activity	85.23 ± 9.32	85.59 ± 9.23	No activity	22.48 ± 4.65
		Ethyl acetate	No activity	No activity	29.96 ± 6.43	31.36 ± 6.78	No activity	16.67 ± 5.89
		Methanol	No activity	No activity	33.76 ± 6.78	44.63 ± 8.41	No activity	25.78 ± 5.77
Vins bioproducts antivenom	-	-	51.29 ± 3.26		38.13 ± 4.99		20.76 ± 13.29	
Inoserp biopharma antivenom			38.96 ± 2.65		27.35 ± 10.70		25.76 ± 11.22	

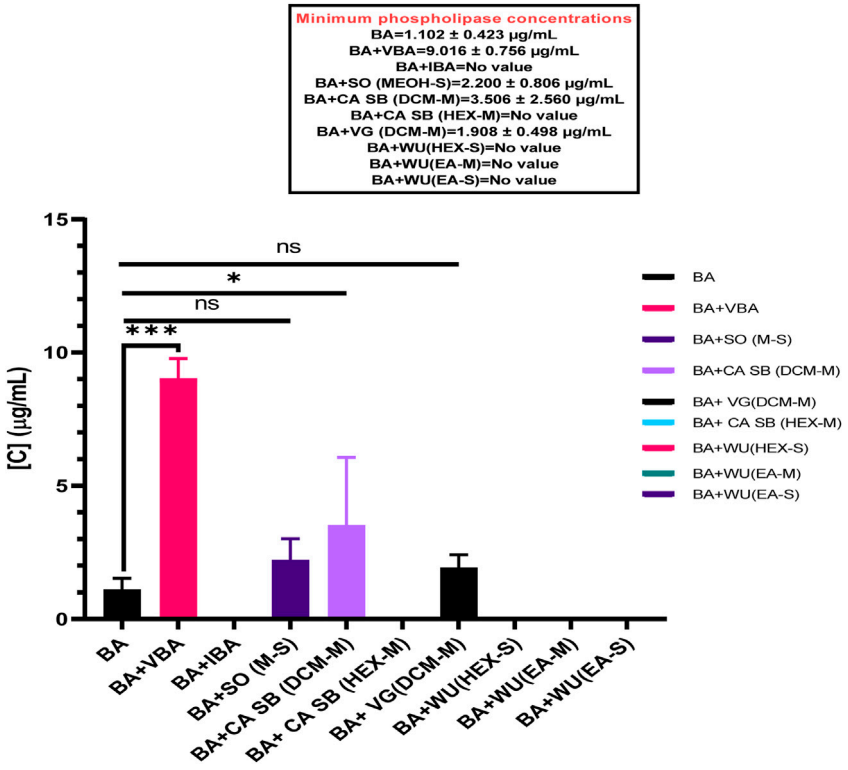

FIGURE 3
The effects of extracts, fractions, and antivenoms on the minimum phospholipase concentration of *Bitis arietans* venom. BA: *Bitis arietans*, VG (DCM-M): Dichloromethane fraction of *Vernonia glabra* leaves, CA SB (HEX-M): Hexane fraction of *Commiphora africana* stem bark, CA SB (DCM-M): Dichloromethane fraction of *Commiphora africana* stem bark. SO (MEOH-S): Methanol extract of *Senna obtusifolia* leaves, WU (HEX-S): Hexane extract of *Warburgia ugandensis* leaf stalk, WU (EA-M): Ethyl acetate fraction of *Warburgia ugandensis* leaf stalk, VBA: Vins bioproducts antivenom, IBA: Inoserp biopharma antivenom.

UK). The methanol extracts were separated into four parts, distributed in de-ionized water, partitioned sequentially with n-hexane, dichloromethane, ethyl acetate, and concentrated under reduced pressure at 40°C on a rotary evaporator (Stuart, Cole-Parmer-UK) (Alsayari et al., 2018). The percentage yield of the extracts was calculated as %w/w.

Ethics

The biosafety, animal care, and use committee of the University of Nairobi was consulted before the authors handled any experimental animal, as shown in Supplementary Figure S2 (Supplementary section) (REF BAUEC/2019/220).

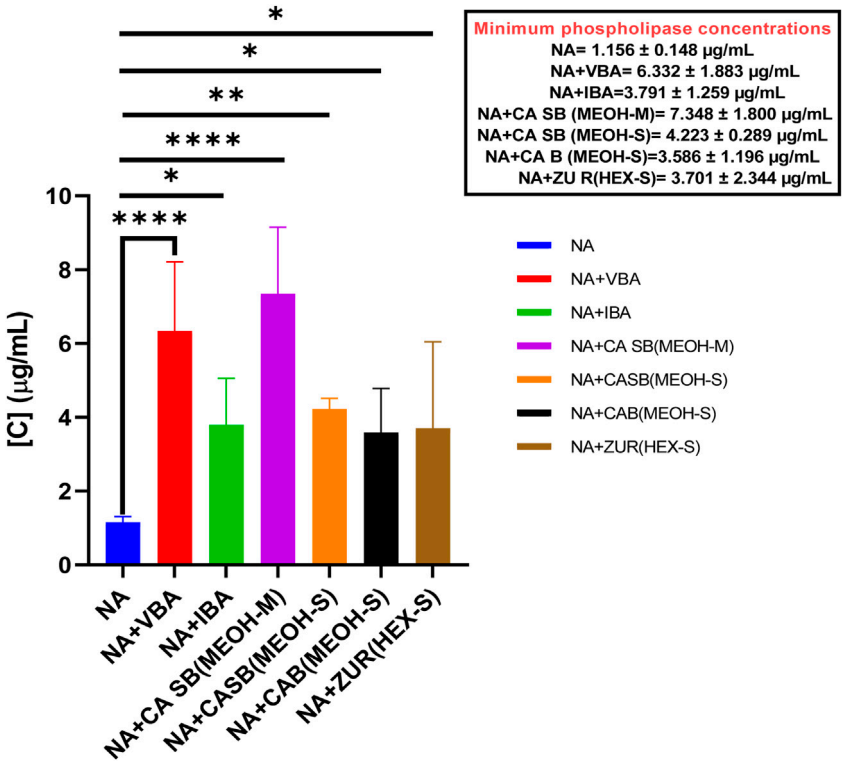

FIGURE 4
The effects of extracts, fractions, and antivenoms on the minimum phospholipase concentration of *Naja ashei* venom. NA: *Naja ashei*, CA B (MEOH-S): Methanol extract of *Commiphora africana* bark, ZU R (HEX-S): Hexane extract of *Zanthoxylum usambarense* root, CA SB (MEOH-S): Methanol extract of *Commiphora africana* stem bark, CA SB (MEOH-M): Methanol extract of *Commiphora africana* stem bark, VBA: Vins bioproducts antivenom, IBA: Inoserp biopharma antivenom.

Snake venom

Nine specimens of the large brown spitting cobras (*N. ashei*), Eastern Forest cobras (*N. subfulva*) and puff adders (*B. arietans*) were collected in the wild and identified by a herpetologist at Bioken snake farm, Kenya. Venom was collected from these snakes using the beaker method, snap frozen, lyophilized (Labconco, USA), and kept as a powder at −20°C until it was reconstituted in phosphate buffered saline.

Determination of the *in vitro* anti-snake venom phospholipase A_2 activity of the prepared extracts

The 96-well microtiter plate assay

The methods of Iwanaga and Suzuki (Iwanaga and Suzuki, 1979) and Molander and colleagues (Molander et al., 2014) were used. 10 µL of a 10 µg/mL concentration of each of the venoms (in 0.1 M phosphate buffered saline) and 20 µL of a 100 µg/mL concentration of each of the prepared extracts were micro pipetted (Finnpipette, Thermo Fisher Scientific, USA) into 96-well microtiter plates (Costar®3590, USA) before 200 µL of a 1.1% egg yolk suspension in 0.1 M PBS adjusted to pH 8.1 and 0.2 mM $CaCl_2$ was added to each well, and the absorbance of the mixtures was taken at 620 nm on a multi plate reader (Thermo Fisher Multiskan, USA). The plates were incubated (Memmert, Germany) at 37°C for 20 min and the absorbance measured again at 620 nm. $svPLA_2$ activity was measured as the decrease in turbidity of the egg yolk suspension from 0 to 20 min. The inhibition of $svPLA_2$ activity by the extract was expressed as percentage inhibition of enzymatic activity taking the absorbance of a well to which no venom was added as 100%. Extracts were tested in triplicate and antivenom was used as a positive reference.

The agarose-egg yolk coagulation assay

Extracts with >90% inhibition of the $svPLA_2$ activity in the aforementioned assay were further evaluated in the agarose egg yolk coagulation assay described by Habermann and Hardt (Habermann and Hardt, 1972) as follows.

1. **Group I (Venom only group):** 10 µL of graded (0.5 µg/mL to 10.0 µg/mL) dilutions of venom only.
2. **Group II (Venom + extract/fraction mixture group):** Pre-incubated mixture of 10 µL of venom (0.5 µg/mL to 10.0 µg/mL) + 20 µL of a 100 µg/mL concentration of each of the extracts/fractions.
3. **Group III (Venom + antivenom):** Pre-incubated mixture of 10 µL of venom (0.5 µg/mL to 10.0 µg/mL) + 20 µL of a 100 µg/mL concentration of each of the antivenoms.

These mixtures were micro pipetted into 0.5 mm wells on an agarose-egg yolk medium and incubated (Memmert, Germany) at 50°C for 24 h. 10% Carbol Fuchsin was used to visualize the

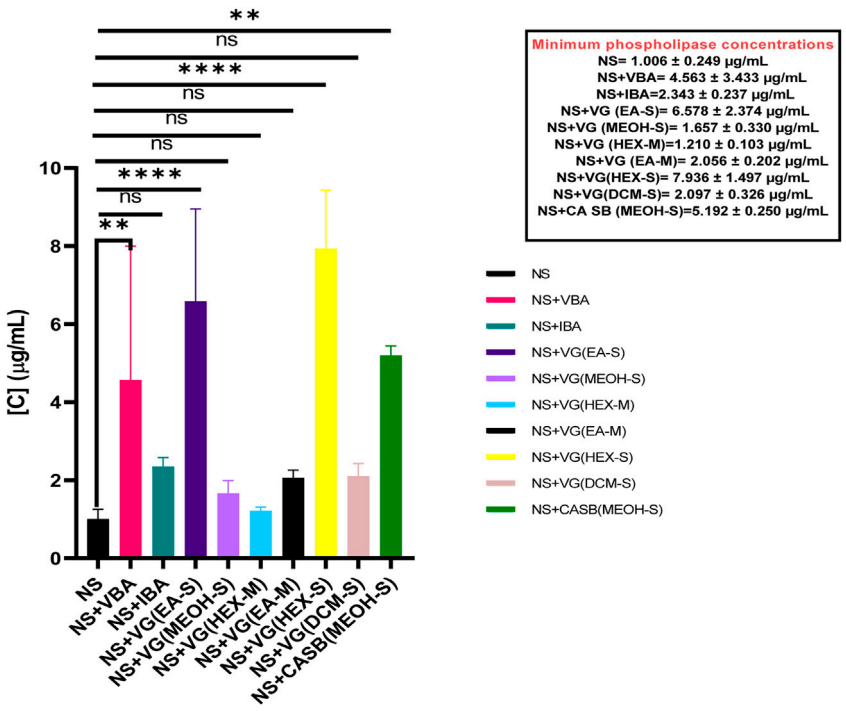

FIGURE 5
The effects of extracts, fractions, and antivenoms on the minimum phospholipase concentration of *Naja subfulva* venom in the snake venom phospholipase A_2 agarose-egg yolk assay. NS: *Naja subfulva*, VG (DCM-S): Dichloromethane extract of Vernonia glabra leaves, VG (HEX-S): Hexane extract of Vernonia glabra leaves, VG (EA-M), Ethyl acetate fraction of *Vernonia glabra*, VG (HEX-M): Hexane fraction of *Vernonia glabra*, VG (MEOH-S): Methanol extract of *Vernonia glabra* leaves, VG (EA-S): Ethyl acetate extract of *Vernonia glabra* leaves, CA SB (MEOH-S): Methanol extract of *Commiphora africana* stem bark, VBA: Vins bioproducts antivenom, IA: Inoserp antivenom.

enzymatic halos in each group and the diameter of the enzymatic halos was measured using a digital vernier calliper (Rolson, United Kingdom) and expressed as the minimum phospholipase concentration (MPC) i.e., the least dose of venom which is responsible for an enzymatic halo of 10 mm in the case of BAV and 15 mm in the case of NAV and NSV.

Cytotoxicity of the venoms, extracts, and antivenoms in *Artemia salina*

The *in vivo* toxicities of the extracts, venoms, and antivenoms were evaluated in *Artemia salina* according to the method described by Meyer *et al.* (1982) with modifications as described by Nguta *et al.* (2014). This was replicated in 5 different sample tubes for each venom, extract, or antivenom concentration. Physiological buffer saline (1 mL) was used as the negative control and vincristine sulphate was used as the positive control.

Neutralization of *Artemia salina* venom-induced cytotoxicity by the extracts and antivenom

The WHO pre-incubation neutralization protocol was used and adjusted to *A. salina* (WHO, 2016). Varying doses of the extracts or antivenom (50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, and 800 µg/mL) were incubated (Memmert, Germany) with a $2LC_{50}$ dose of each of the venoms at 37°C for 30 min. The resulting mixtures were added to vials containing *A. salina* and the survivors were counted after 24, 48, and 72 h of exposure. The median effective concentration of the extracts was defined as the minimum amount of extract (in µL) required to neutralize 1 mg of venom.

Initial screening of the extracts for phytochemicals

Standard methods were used for preliminary phytochemical screening of the extracts and fractions (Kokate et al., 2006; Evans, 2009; Kumar et al., 2013). The presence of alkaloids (dragendorff's test), anthraquinones, carboxylic acids, cardiac glycosides (keller-killiani test), flavonoids (alkaline reagent test), phenolics (Ferric chloride test), phytosterols, resins, saponins (foam test), tannins (Ferric chloride test), and terpenoids (Salkowski test) were investigated.

Quantitative phytochemical composition

Total phenolics, flavonoids, glycosides, and tannins were estimated using a UV-VIS spectrophotometer (Spectronic 21-D, USA). Analytical grade gallic acid, catechin, and rutin were used as standards.

TABLE 2 The cytotoxicity of antivenom, extracts, and fractions of *Commiphora africana, Vernonia glabra,* and *Zanthoxylum usambarense* in *Artemia salina*.

Description of the plant, solvent used, and the method of extraction				Number of dead *Artemia salina* per tested dose (n = 10)			LC_{50} (µg/mL)	Implication
Plant	Plant part	Solvent used	Method of extraction	10 µg/mL	100 µg/mL	1000 µg/mL		
Commiphora africana (A. Rich.) Engl.	Bark	Methanol	Soxhlet	00	00	06	3377.52 (No CI)	Non cytotoxic
	Stem bark	Dichloromethane	Maceration	00	00	03	6133.87 (No CI)	Non cytotoxic
	Stem bark	Methanol	Soxhlet	06	19	26	611.72 (251.06-3437.50)	Cytotoxic
	Stem bark	Methanol	Maceration	00	00	00	No death	Non cytotoxic
Vernonia glabra (Streetz) Vatke	Leaves	Hexane	Soxhlet	39	40	46	0.04 (No CI)	Cytotoxic
	Leaves	Ethyl acetate	Soxhlet	06	10	29	4049.78 (No CI)	Non cytotoxic
Zanthoxylum usambarense (Engl.) Kokwaro	Leaf stalk	Hexane	Soxhlet	07	43	50	31.54 (22.50-44.03)	Cytotoxic
Vins bio products antivenom	-	-	-	00	00	00	No death	Non cytotoxic
Inoserp biopharma antivenom	-	-	-	00	00	00	No death	Non cytotoxic
Vincristine	-	-	-	0	30	46	102.62 (No CI)	Cytotoxic
Sulphate (standard)								

LC_{50}, Lethal concentration of the test substance responsible for the death of 50% of *Artemia salina* larvae; µg/mL; Micrograms per millilitre; CI, confidence interval.

Determination of total phenolic content (TPC)

The method of Harnafi et al. was used (Harnafi et al., 2008). The extracts/fractions were mixed with 7.5% w/v Na_2CO_3 solution and 2.5 mL of Folin-Ciocalteau reagent (FINAR, India), and the absorbance was read at 765 nm on a UV-VIS spectrophotometer (Spectronic 21-D, USA) and a gallic acid standard curve was generated. The assay was performed in triplicate and the results were expressed as milligrams of Gallic acid equivalents per Gram of the dry plant material (mg.GAE.g^{-1}).

Determination of total flavonoid content (TFC)

The method of Atanassova et al. was used (Atanassova et al., 2011). The extract/fractions were mixed with distilled water, 5% w/v sodium nitrite ($NaNO_2$), 10% w/v aluminum chloride ($AlCl_3$), and 1 M sodium hydroxide (NaOH), and the absorbance was read on a UV-VIS spectrophotometer (Spectronic 21-D, USA) at 510 nm. The flavonoid content was determined from a catechin standard curve. The assay was performed in triplicate and the results were calculated as milligrams of Catechin equivalents per Gram of the dry plant material (mg. CE. g^{-1}).

Tannin content

The method of Amadi et al. was used (Amadi et al., 2004). The extracts/fractions were boiled gently for 1 h and mixed with 2.5 mL of Folin-Denis reagent, 5 mL of saturated Na_2CO_3 solution, and 25 mL of distilled water. The mixture was left to stand for 30 min in a water bath (Memmert, Germany) at 25°C and the absorbance was read on a UV-VIS spectrophotometer (Spectronic 21-D, USA) at 700 nm. The tannin content was determined from a tannic acid standard curve. The assay was performed in triplicate and the results were calculated as below:

$$\text{Tannic acid } (\text{mg}/100\text{g}) = \frac{\text{C} \times \text{extract volume} \times 100}{\text{Aliquot volume} \times \text{weight of sample}}$$

Where C is concentration of tannic acid read off the graph.

Cardiac glycoside content

The method described by Muhamad and Abubakar (Muhammad and Abubakar, 2016) was used. The extracts/fractions were mixed with distilled water, 12.5% lead acetate, 47% w/v Na_2HPO_4, and Baljet reagent (95 mL of 1% picric acid+5 mL of 10% NaOH). A blank titration was carried out using 10 mL distilled water and 10 mL Baljet reagent (95 mL of 1% picric acid+5 mL of 10% NaOH). This mixture was allowed to stand for 1 hour and the absorbance was read on a UV-VIS spectrophotometer (Spectronic 21-D, USA) at 495 nm. The percentage (%) of total glycosides present in extracts/fractions was calculated as % of total glycosides= (A×100)/77 g %. Where A = absorbance of samples.

Data analysis

The effect of each of the extracts/fractions/antivenoms on the minimum phospholipase concentration of venom (s) was compared using one way-ANOVA and Dunnet's multiple comparison test.

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TABLE 3 Qualitative phytochemical composition of the extracts and fractions of *Commiphora africana* and *Vernonia glabra*.

Plant name	Solvent	Extraction method	Alkaloids	Anthraquinones	Carboxylic acid	Cardiac glycosides	Flavonoids	Phenolics	Phyto sterols	Resins	Saponins	Tannins	Terpenoids
Commiphora africana (A. Rich.) Engl. Stem bark	Dichloro methane	Maceration	-	+	-	+	+	+	-	-	-	+	-
	Methanol	Maceration	-	+	-	+	+	+	-	+	+	+	-
Bark	Methanol	Soxhlet	-	+	-	+	+	+	-	-	+	+	-
Vernonia glabra (Streetz) Vatke (leaves)	Ethyl Acetate	Soxhlet	-	-	-	+	+	+	-	-	+	+	-

+, Present; -: Absent.

The lethality of venoms, extracts, fractions, and antivenoms in *A. salina* and their capacity to neutralize venom-induced cytotoxicity in the same model was analyzed using probit regression analysis. Results on the phytochemical composition of the extracts/fractions were summarized in a table. $p < 0.05$ was considered significant.

Results

The percentage yield of extracts

The percentage yield of the hexane root extract of *C. africana* prepared by the Soxhlet method was the lowest (0.23%), while the percentage yield of the dichloromethane leaf extract of *V. glabra* prepared by the maceration method was the highest (54.65%), as observed in (Supplementary Table S1).

Information on the snakes whose venom was used in the study

Most of the snakes used in this study were sourced from the Watamu area in Kenya. (Supplementary Table S2) ***in vitro* microtiter well svPLA$_2$ neutralization assay**.

The microtiter well assay revealed poor (<90%) anti-svPLA$_2$ inhibition of BAV by the tested antivenoms (range: 20.76% ± 13.29% to 51.29% ± 3.26%) but potent (>90%) anti-svPLA$_2$ inhibition of the venom by dichloromethane and hexane fractions of *C. africana* stem bark, hexane and ethyl acetate extracts and fraction of *W. ugandensis* leaves, dichloromethane fraction of *V. glabra* leaves, and the methanol extract of *S. obtusifolia* leaves.

>90% anti-svPLA$_2$ inhibition was observed against NAV with the methanol extract and fraction of *C. africana* stem bark, the methanol extract from the *C. africana* bark, and the hexane extract of *Z. usambarense* leaves.

>90% anti-svPLA$_2$ inhibition was noted against NSV with hexane and ethyl acetate fractions of *V. glabra* leaves and dichloromethane, ethyl acetate, and methanol extracts of *C. africana* bark (Table 1).

In vitro agarose-egg yolk svPLA$_2$ neutralization assay

BAV had a minimum phospholipase concentration (MPC) of 1.102 ± 0.423 µg/mL. When separately incubated with various extracts, fractions, and antivenom, the MPC of the venom ranged from 1.908 ± 0.498 µg/mL to 9.016 ± 0.756 µg/mL. However, the only test substances that significantly inhibited *B. arietans* venom were Vins bioproducts antivenom, MPC = 9.016 ± 0.756 µg/mL ($p < 0.0001$) and the dichloromethane fraction of *C. africana* stem bark, MPC = 3.506 ± 2.560 µg/mL ($p = 0.0007$) (Figure 3).

NAV had an MPC of 1.156 ± 0.148 µg/mL. When separately incubated with various extracts, fractions, and antivenom, the MPC of the venom ranged from 3.586 ± 1.196 µg/mL to 7.348 ± 1.800 µg/mL. All the tested extracts, fractions, and antivenom significantly inhibited the phospholipase A$_2$ activity of *N. ashei* including the methanol extract of *C. africana* bark, MPC = 3.586 ± 1.196 µg/mL ($p = 0.0343$), the hexane extract of *Z. usambarense* roots, MPC = 3.701 ± 2.344 µg/mL ($p = 0.0248$), Inoserp biopharma antivenom,

TABLE 4 Quantitative phytochemical composition of the extracts and fractions of *Commiphora africana* and *Vernonia glabra*.

Plant name	Solvent	Extraction method	Glycoside content (%)	Total phenolic content (mg/g of gallic acid equivalents)	Total flavonoid content (mg/g of catechin equivalents)	Tannic acid content (%)
Commiphora africana (A. Rich.) Engl. (stem bark)	Dichloromethane	Maceration	0.001	0.540	2.430	0.005
	Methanol	Maceration	0.001	1.100	0.600	0.008
	Methanol	Soxhlet	0.002	2.180	0.330	0.007
Vernonia glabra (Streetz) Vatke (leaves)	Ethyl Acetate	Soxhlet	0.003	0.490	2.990	0.010

TABLE 5 Neutralization of snake venom-induced cytotoxicity in *Artemia salina* by antivenom, extracts, and fractions of *Commiphora africana* and *Vernonia glabra*.

Venom	Inhibitor	Mortality per treatment						Neutralization efficacy of inhibitor
		$2LC_{50}$ only	$2LC_{50}$ + 50 µg/mL inhibitor	$2LC_{50}$ + 100 µg/mL inhibitor	$2LC_{50}$ + 200 µg/mL inhibitor	$2LC_{50}$ + 400 µg/mL inhibitor	$2LC_{50}$ + 800 µg/mL inhibitor	EC_{50}
Bitis arietans	CA SB (DCM-M)	50	50	50	48	14	11	336.12 ± 59.97
	VBA	50	50	50	50	50	50	Ineffective
	IA	50	50	50	50	50	50	Ineffective
Naja ashei	CA SB (MEOH-M)	50	44	45	42	31	10	532.79 ± 169.04
	CA B (MEOH-S)	50	49	34	23	11	07	221.37 ± 30.33
	VBA	50	50	50	50	50	50	Ineffective
	IA	50	50	50	50	50	50	Ineffective
Naja subfulva	VG (EA-S)	50	50	49	40	18	08	329.39 ± 15.92
	VBA	50	50	50	50	50	50	Ineffective
	IA	50	50	50	50	50	50	Ineffective

LC_{50}, Concentration of venom responsible for 50% mortality of *Artemia salina*; EC_{50}, Concentration of extract/fraction or antivenom responsible for sparing 50% of *Artemia salina* from venom-induced death; CA SB (DCM-M), the dichloromethane fraction of *commiphora africana* stem bark; CA SB (MEOH-M), the methanol fraction of *Commiphora africana* stem bark; CA B (MEOH-S), the methanol extract of *Commiphora africana* bark; VG (EA-S), the ethyl acetate extract of *Vernonia glabra* leaves; VBA, vins bioproducts antivenom; IA, inoserp antivenom.

MPC = 3.791 ± 1.259 µg/mL ($p = 0.0191$), the methanol extract of *C. africana* stem bark, MPC = 4.223 ± 0.289 µg/mL ($p = 0.0051$), Vins bioproducts antivenom, MPC = 6.332 ± 1.883 µg/mL ($p < 0.001$), and the methanol fraction of *C. africana* stem bark, MPC = 7.348 ± 1.800 µg/mL ($p < 0.0001$). (Figure 4).

NSV venom had an MPC of 1.006 ± 0.249 µg/mL. When separately incubated with various extracts, fractions, and antivenom, the MPC of the venom ranged from 1.210 ± 0.103 µg/mL to 7.936 ± 1.497 µg/mL. However, the only test substances that significantly inhibited *Naja subfulva* venom were Vins bioproducts antivenom, MPC = 4.563 ± 3.433 µg/mL ($p = 0.0049$), the ethyl acetate extract of *V. glabra* leaves, MPC = 6.578 ± 2.374 µg/mL, the hexane extract of *V. glabra* leaves, MPC = 7.936 ± 1.497 µg/mL ($p < 0.0001$), and the methanol extract of *C. africana* stem bark, MPC = 5.192 ± 0.25 µg/mL ($p = 0.0022$) (Figure 5).

Cytotoxicity of the extracts, fractions, and antivenom in *Artemia salina*

The methanol extract of *C. africana* stem bark, the hexane extracts of *V. glabra* leaves and *Z. usambarense* leaf stalk were cytotoxic to *A. salina* with LC_{50} values of 611.72 (251.06-3437.50)

μg/mL, 0.04 μg/mL, and 31.54 (22.50-44.03) μg/mL respectively whereas the methanol stem bark fraction of *C. africana*, Vins bioproducts antivenom, and Inoserp antivenom were the least cytotoxic (Table 2).

Qualitative phytochemical composition of extracts and fractions

Flavonoids, phenolics, glycosides, and tannins were found to be present in the dichloromethane and methanol fractions of *C. africana* stem bark, the methanol extract of *C. africana* bark, and the ethyl acetate extract of *V. glabra* leaves. However, alkaloids, carboxylic acids, phytosterols, and terpenoids were absent in the extracts and fractions (Table 3).

Quantitative phytochemical composition of the non-cytotoxic extracts and fractions

The ethyl acetate extract of *V. glabra* leaves had the highest glycoside (0.003%), total flavonoid (2.990 mg/g catechin equivalents), and tannic acid content (0.010%) while the methanol extract of *C. africana* stem bark had the highest phenolic content (2.180 mg/g gallic acid equivalents) (Table 4).

Neutralization of venom-induced cytotoxicity by extracts, fractions, and antivenom

The dichloromethane fraction of *C. africana* stem bark had an effective concentration of 336.12 ± 59.97 μg/mL against BAV-induced cytotoxicity in *A. salina*. The methanol extract of *C. africana* bark was the most effective against NAV-induced cytotoxicity in *A. salina* with an EC_{50} of 221.37 ± 30.33 μg/mL. The ethyl acetate extract of *V. glabra* leaves had an effective concentration of 329.39 ± 15.92 against NSV-induced cytotoxicity in *A. salina*. However, the test antivenoms were ineffective in neutralizing BAV, NAV and NSV-induced cytotoxicity in *A. salina* (Table 5).

Discussion

Snake venom phospholipases A_2 (svPLA_2) are enzymes which hydrolyze phospholipids and induce several pharmacological effects including edema, modulation of platelet aggregation, neurotoxicity, and myotoxicity (Six and Dennis, 2000; Kini, 2003; Pereanez et al., 2011). The present study observed that extracts and fractions of *C. africana, S. obtusifolia, V. glabra,* and *W. ugandensis* effectively neutralized *sv*PLA_2s in BAV, NAV, and NSV. A similar study by Molander and colleagues evaluated the neutralization capacity of 226 extracts from 94 different plant species where it was reported that 11 water extracts and 28 ethanol extracts showed more than 90% inhibition against svPLA_2 in *Bitis arietans* and *Naja nigricollis* venoms (Molander et al., 2014). These plants included *Lanea acida, Spondias mombin,* and *Capparis tometosa* (Molander et al., 2014).

Phytochemical analysis revealed that the extracts were rich in phenolics, tannins, saponins, and cardiac glycosides. Previous authors have demonstrated that phenolics, tannins, and saponins have antivenom properties (da Silva et al., 2007; Sia et al., 2011; de Moura et al., 2016; Salama et al., 2018; Liu et al., 2024). These antivenom properties were observed when *Saxifraga stolonifera, Rosmarinus officinalis, Plathymenia reticulata, Mimosa pudica,* and *Pentaclethra macroloba* were tested against venom from *Bothrops atrox, Cerastes,* and *Naja kaouthia* (da Silva et al., 2007; Sia et al., 2011; de Moura et al., 2016; Salama et al., 2018; Liu et al., 2024).

Cytotoxicity studies in *A. salina* revealed that some extracts of *V. glabra* leaves, *W. ugandensis* leaf stalk, and *C. africana* stem bark were cytotoxic to *A. salina*. Previous studies by Wanna, Karani, Anywar, Mwangi and their colleagues have shown that *V. glabra* was cytotoxic in *A. salina* (LC_{50} = 658 μg/mL) (Wanna et al., 2023), *W. ugandensis* was non-cytotoxic in Vero cells (CC_{50} of >250 μg/mL) (Karani et al., 2013) but cytotoxic to human glioblastoma cells (IC_{50} = 7.6 μg/mL) (Anywar et al., 2022) and *C. africana* was cytotoxic to Vero cells (CC_{50} > 20 μg/mL) (Mwangi et al., 2020). The compounds responsible for the toxicity of *V. glabra* and *Z. usambarense* have not been studied in detail but a study by Wairagu and colleagues established that cedrol, 9-octadecanoic acid-ethyl-ester, octadecadien-1-ol, citronellyl formate, n-hexadecenoic acid, and 1,2-dihydro-6-methoxy-naphthalene isolated from the dichloromethane crude fraction of *C. africana* resin were toxic to bedbugs (*Cimex lectularius*) (Wairagu et al., 2022). Moreover, E-resveratol 3-O-rutinoside isolated from the methanol fraction of *C. africana* stem bark was highly cytotoxic to breast (MCF-7), liver (HepG2), lung (A549), and prostate (PC3) cancer cell lines (Segun et al., 2019). In the case of *W. ugandensis*, compounds such as polygodial, warbuganal, ugandensolide, and mukaadial have been identified to be toxic against the maize weevil (*Sitophilus zeamais Motchulsky*) and the larger grain borer (*Prostephanus truncates Horn*) while compounds such as muzigadial have been found to be highly toxic to brine shrimp (*A. salina*) and *in vitro* trypanocidal activity against both drug-resistant and drug-sensitive trypanosome strains (Olila and Opuda-Asibo, 2001; Opiyo, 2020).

The *A. salina* model has been used to evaluate the cytotoxicity of medicinal plants (Nguta et al., 2011; Mwangi et al., 2015), environmental contaminants (Barahona and Sanchez-Fortun, 1999; Sanchez-Fortun and Barahona, 2009), and venom (Damotharan et al., 2015; Okumu et al., 2021). The present work was a continuation of our previous work where we investigated the capacity of two antivenoms to neutralize NAV-induced cytotoxicity in *A. salina* (Okumu et al., 2020). Moreover, we showed in another study that the *A. salina* model was a good surrogate for dermonecrosis in mice (Okumu et al., 2021). The present study established that some extracts and fractions of *C. africana* were effective in prolonging the survival of *A. salina* exposed to NAV. Isa and colleagues in a previous research reported that the crude methanol extract and fraction of *C. africana* dose-dependently neutralized *N. nigricollis* envenomation in mice (Isa et al., 2022). Abdullahi et al. reported the anti-snake venom properties of a *C. africana* related plant, i.e., *Commiphora pedunculata* against *N. nigricollis* venom (Abdullahi et al., 2017). While this study has highlighted the capacity of the prepared extracts to neutralize key effects of medically important sub-Saharan snakes, it did not

evaluate the capacity of the extracts/fractions to neutralize other key toxins in the studied snake venoms including protease, hyaluronidase, and neurotoxins (3FTx's). Moreover, further work is needed to understand the identity of the compounds responsible for the observed extract/fraction induced cytotoxicity in *A. salina.*

Conclusion

These findings validate the local use of *C. africana* and *V. glabra* in snakebite envenomation and provide a basis for further work aimed at isolating pure compounds from these plants and identifying their mechanism of action. However, *C. bonariensis, S. obtusifolia, W. ugandensis*, and *Z. usambarense* use in snakebite is limited by poor efficacy and cytotoxicity.

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 animal study was approved by The Biosafety, Animal Use and Ethics Committee of the University of Nairobi. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MO: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing–original draft, Writing–review and editing. JM: Investigation, Project administration, Resources, Supervision, Validation, Writing–original draft, Writing–review and editing. JG: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. PM: Data curation, Investigation, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. VM: Data curation, Investigation, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. FO: Conceptualization, Investigation, Methodology, Project administration, Resources, Writing–original draft, Writing–review and editing.

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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.2024.1369768/full#supplementary-material

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The use and potential abuse of psychoactive plants in southern Africa: an overview of evidence and future potential

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Background: Most Bantu ethnic groups in southern Africa utilize indigenous herbal medicines, some of which have psychoactive properties. Traditional medical practitioners (TMPs) commonly use them not only for divinatory purposes but to treat and manage mental and other illnesses. Unfortunately, the research on their results, risks, and benefits do not align. Little is known about their potential abuse among TMPs and community members in southern Africa. Herbal medicines are complex because whole plants are sometimes used, unlike in other treatments which use only one active ingredient. However, if the key mechanisms of action of these ethnomedicinal plants can be identified through socio-pharmacological research, useful botanical agents can be developed. A review of socio-pharmacological studies to evaluate the consequences of exposure to ethnomedicinal plants with psychoactive properties was conducted with the aim of identifying harm reduction strategies and investigating how the plants could be developed into useful botanicals.

Method: The search methods involved retrieval of records from PubMed/MEDLINE, Embase, Web of Science, Dissertations and Theses Global, and OpenGrey. The English language and human subjects were used as filters. In addition, some information was obtained from TMPs and community members.

Results: The following psychoactive plants were found to be commonly used or abused: *Boophone disticha, Cannabis sativa, Datura stramonium, Leonotis leonurus, Psilocybe cubensis*, and *Sceletium tortuosum*. The commercialization of *Cannabis, L. leonurus, S. tortuosum,* and *Aspalathus* is growing fast. The abuse liability of *B. disticha, D. stramonium, and P. cubensis* appears not to be appreciated. Five countries were found to have TMP policies and three with TMP Councils.

Conclusion: TMPs in the region are aware of the CNS effects of the identified psychoactive plants which can be explored further to develop therapeutic agents. There is a need to work closely with TMPs to reduce harm from the abuse of these plants.

KEYWORDS

psychoactive ethnomedicinal plants, use and potential abuse, harm reduction, traditional medical practitioners, socio-pharmacological study

Introduction

In southern Africa and in the rest of the continent, the COVID-19 pandemic has intensified the struggle around scarce health resources. Previously, more than 80% of the Bantu population in southern Africa had always relied on the use of indigenous herbal medicines, some of which have psychoactive properties. Generally, plants with psychoactive or psychotropic properties, have always been used by traditional medical practitioners (TMPs) for divinatory purposes, treatment, and to manage illness.

TMPs consult the spiritual realm by invoking and conferring with deceased family members. Appropriate action including the prescription of plant medicines and rituals is the prerogative of diviners following the discovery of the cause of any misfortune. In southern African divination, the diviner goes into a trance and appears to be in an altered state of consciousness to assist in the healing. In addition, the diviner is said in that state to be able to communicate with "spirits" and undertake "soul journeys" while dreaming or in a trance. This involves either ceremonially ingested, sniffed, or smoked psychotropic substances (Hewson, 1998). According to the World Health Organization (WHO) definition of traditional medicine, this is to a certain extent not explicable.

WHO (WHO, 2023) defines "traditional medicine" as follows:

The sum total of the knowledge, skill, and practices based on theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2023).

It can be argued that the pharmacology of some animal, plant, and soil products used in TM can be explained scientifically. This includes plants with psychoactive properties, including those which cross the blood–brain barrier that result in psychotropic effects of interference with brain functions, mood awareness, thoughts, and feelings. These are classified as psychotropic drugs.

The therapies used in traditional medical practice have complex interactions. Treatment with traditional medicine does not involve a single specific therapeutic ingredient but often includes specific diagnostic settings and interaction processes (Hewson, 1998; Mhame et al., 2014). Unfortunately, the research evidence and the possible harm and advantages of these therapies following the use of psychoactive substances among TMPs do not align. For example, little has been reported about potential psychoactive plant abuse liability by TMPs and others in southern Africa. Herbal medicines are complex, and their complexity comes from the fact that whole plants are sometimes used, unlike other treatments that use only one active ingredient . However, if the key mechanisms of action of these ethnomedicinal plants can be identified through socio-pharmacological research, useful botanical agents can be developed.

Social pharmacology, or socio-pharmacology, is a relatively new field in clinical pharmacology. In other words, it describes the relationships between society and drugs whether of ethno-origin or not. The discipline studies the life cycle of any drug used in society. The discipline continues evolving and is currently underappreciated. According to Papadopulos et al. (2021), the societal aspects of therapeutics are more than imperative today, given determinants such as the healthcare system, the political setting, unemployment, and exposure to chronic disease.

There has been very little research into the use of psychoactive plants with psychotropic compounds, whether for divinatory or therapeutic purposes, in southern Africa and Africa in general (Jean-Francois, 2020). Existing reports have always been part of other ethnobotanical investigations. In fact, most studies since 1983 have concentrated on medicinal plants. This can be explained by the bias of 19th century researchers regarding psychoactive plants as part of divinatory and healing practices. At the time, the practices were regarded as witchcraft or primitive, resulting in the neglect of the importance of psychoactive medicinal plants. There has additionally been a loss of the oral transmission of information regarding the use of plants because people have forgotten traditional practices. Nevertheless, there has been some interest in psychoactive plant use in southern Africa (Mitchell and Hudson, 2004; Sobiecki, 2008).

This study reviews the relevant literature on the relationship between society and commonly used plants of ethno-origin that have psychoactive properties that result in psychotropic action. It aims to identify harm reduction strategies and investigate how the plants could be developed into useful botanicals for treating and managing mental illnesses.

Methods

Studies on the use of psychoactive plants in traditional medical practice in southern Africa were searched from electric databases including PubMed/MEDLINE, Embase, Web of Science, Dissertations and Theses Global, and OpenGrey. The studies were retrieved mainly by applying the English language and, where necessary, the most spoken vernacular Bantu language in the southern African country. The following search terms were used: "divination," "traditional medical practice," "psychoactive plants," "psychotropic plants," and "witchcraft." In order to construct a global and relevant picture for a regional perspective, some searches included studies at a sub-Saharan Africa level. Finally, a snowball search was expanded to include references cited in the search publications in order to expand the relevant literature.

The inclusion criteria for the search were anything regarding divination, diagnosis and treatment in traditional medical practice using plants, and the use of these plants in the general community for other purposes. Anything else was excluded in the search.

All plants that were eventually described in the study were authenticated by a botanist and curator at a university in South Africa.

The literature study was then followed by interviews with 15 purposively selected TMPs from five Southern African Development Community (SADC) countries who were willing to provide information on their practices. Some community members were also interviewed about their knowledge of psychoactive plants.

Results and discussion

Information obtained from the interviewed TMPs seemed to suggest that only five countries in southern Africa had traditional medicine practice policies and three had TMP councils. A close analysis of the functions of these councils showed that they were not

TABLE 1 Plant species, part of plant used, psychotropic constituents, and classification of pharmacological classification.

Plant species	Part of plant used	Psychotropic constituent(s)	Classification of constituents
Boophone disticha	Bulb	Amaryllidaceae alkaloids	Hallucinogens
Cannabis sativa, L	Flower and leaves	THC	Stimulant/sedative
Datura stramonium L	Seeds	Scopolamine and hyoscyamine	Deliriant/hallucinogen
Helichrysum odoratissimum L	Whole plant	Decaffeocylquinic acid	Sedative and relaxant
Leonotis leonurus (L) R.Br	Leaves and seeds	Adrenocyl–EA and anandamide	Sedative and calamative
Psilocybe cubensis	Whole plant	Pscylocybin and pscilocin	Psychedelic and entheogenic
Mesebryanthemum tortuosum (L) NE Brown	Leaves and flowers	Mesembrenone and mesembrine	Mood altering
Silene capensis	Whole plant	Phytoecdysteroid	Hallucinogen
Dioscorea dregeana (Kunth) Durand & Schinz	Tuber	Dioscorine and crinamine	Sedative

that effective in regulating how psychoactive plants were generally used (Table 1).

However, one of the psychoactive plants, *Cannabis sativa,* is strictly speaking not indigenous but has, over the years since its introduction into southern Africa, found its place in traditional medical practice. *C. sativa* is in the statute books of SADC countries, and efforts to control its abuse are ongoing. With its increased decriminalization and legalization, worldwide harm reduction strategies are being developed. These are very similar to the well-known strategies developed for *Nicotiana tabacum*. *C. sativa*, like the other the psychoactive plants, can be used both for recreational and medicinal purposes. In modern medicine, many chemical substances such as morphine, pseudoephedrine, and alcohol are abused, and those responsible for making them accessible are held accountable for the drug abuse. This, unfortunately, is not currently possible with TMPs whose councils are not that powerful. Moreover, the fact that the psychoactive plants with psychotropic constituents that have been phytochemically identified are not cultivated but can be obtained from the wild by anyone makes it difficult to regulate their abuse.

Perhaps this is where social pharmacology, the new discipline in clinical pharmacology, can be useful in helping with ways to deal with any harm that result from abuse.

Social pharmacology

The term "social pharmacology" was coined in 1960 as a methodology for describing addiction, effect on mood, and the behaviour of individuals in society. Over time, its methodology has improved, and it has now expanded to the knowledge and appreciation of how psychotropic drugs are accessed, used, and abused in the social life cycle and how to monitor their impact on public health (Morgan, 2016). Social pharmacology can also be applied to ethno-psychoactive plants such as those identified in the present study.

There will clearly be changes in the demography of the subgroups who will use these indigenous psychoactive plants. A booming industry, such as with cannabis, will certainly evolve to meet the demands of various types of consumer, as will the development of new products derived from ethno-psychoactive plants. Furthermore, these products will also have unknown health effects. Therefore, laws and attitudes need to change; data will be required as evidence for both the therapeutic and adverse effects of these ethno-psychoactive plants. Such data should be used as evidence in the prevention of hazardous use and in the maximization of potential medical benefits.

Given the historical controversies on *Cannabis*-based medicines and psychedelic plants and substances, rigorous attention is necessary to identify the psychotropic components of these African species and to investigate whether biphasic dose-response or other idiosyncratic properties are operative. Given possible damage to the cognitive functions of the brain, their primary psychoactive and intoxicating components are not known. Some adverse effects have been seen with THC in *Cannabis* (Copper and Adinoff, 2019) which could also be the case with the constituents of ethno-psychoactive plants. Observation of how these adverse effects of THC on cognitive function of the brain may translate to humans underline the importance of considering patterns of usage that may be motivated by abuse. This appreciation will guide hypothesis formulation related to the neuro-cognitive impact of exposure.

There are, however, lessons that can be learnt from *Nicotiana tabacum*, currently from *Cannabis*, and perhaps from *Sceletium tortuosum*. There is also a need to translate our understanding of the positive and negative consequences of ethno-psychoactive plants. This is important from a policy perspective, especially when the pros and cons of implementing regulatory measures are clearly understood. Figure 1 summarizes the interrelationship between society, the public, and ethno-pharmacology that needs to be understood.

It cannot be over emphasized that an understanding of neurocognitive effects, whether positive or negative, is critical in the context of medical use to optimize any products that are developed. This is also important when it comes to clinical utility and mitigating undesirable outcomes such as intoxication and abuse.

During the interviews with TMPs, interesting terminology common to most Bantu vernacular languages meaning "to see", was used to describe the effects of psychoactive plants. The plant would then be described as a plant that makes "one see," analogous with metaphysical "seeing," transcendental enlightenment, revelation, and the ability to arouse ancestral spirits. The veil of

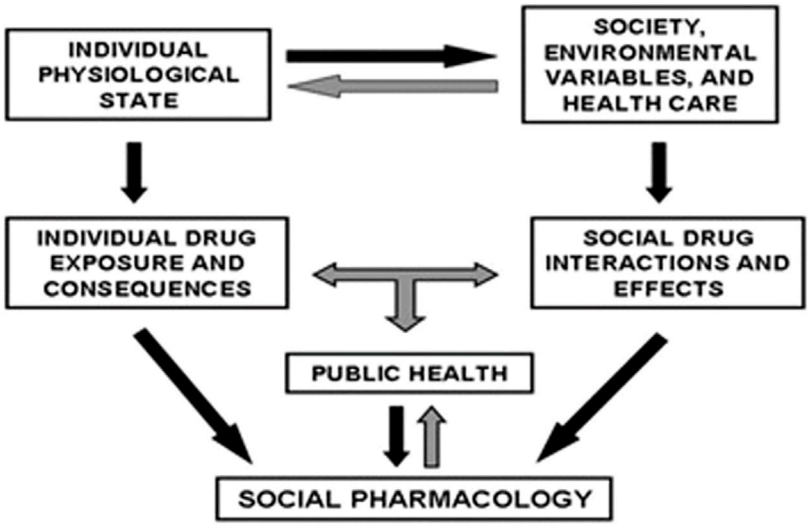

FIGURE 1
Inter-relationships important for social pharmacological methodology (Morgan, 2016).

FIGURE 2
Leafy *B. disticha* showing a scaly exposed bulb.

secrecy about these plants has now been lifted, and the general community is aware of these plants with such properties; hence, the pervasive desire to alter their consciousness.

The most commonly used plants

The following psychoactive plants were found to be commonly used and had the potential for abuse.

Boophone disticha (L.f) Herb, *Amaryllis disticha* Amaryllidaceae

B. disticha (Figure 2) is regarded as a visionary plant called *leshoma* in Sesotho (Pasquali, 2021). It is used as a divinatory "bioscope", giving the ability to see things that ordinary people cannot see during consultation, by Zulu, Xhosa, Shona, and San TMPs. Each ethic group has a particular name for it.

During consultation with a traditional healer, a preparation of *B. disticha* is administered to the enquirer. This causes a visionary cataleptic state during which they can "see" the person responsible for their misfortune". Over the years, such activities have been kept secret by TMPs, but now it is common knowledge in some communities. As a result, careless consumption by adolescents of a *B. disticha* decoction made from the bulb, both for therapeutic and intoxicating purposes—a herbal high—is now reported to be common in some communities (Laing, 1979).

The route of administration is as a liquid preparation of the bulb, usually administered orally.

The decoction from the bulb must be carefully prepared by boiling it at least thrice and discarding the boiled water containing toxic constituents. The last boil is then left to stand over night before being used (Nyazema, 1984).

To date, the presence of 11 alkaloids—including buphanidrine 13 (19.4%), undulatine 14 (18.6%), buphanisine 15 (16.9%), buphanamine 11 (14.1%), nerbowdine 12 (11.1%), crinine 3 (7.2%), distichamine 16 (5.4%), crinamidine 17 (1.2%), acetylnerbowdine 18 (0.6%), lycorine 2 (0.4%), and buphacetine (0.3%)—has been identified and described (Nair and Staden, 2014) with their respective contribution percentage. There is uncertainty over which of the 11 alkaloids is affected by the boiling of the bulb, which thereby influences the "entourage effect" described by observed symptoms including unconsciousness, dilated pupils, tachycardia, raised blood pressure, slightly raised temperature, laboured respiration, psychosis, drunkenness, and visual disturbances (Laing, 1979).

Stafford et al. (2008) reported that *B. disticha* can be used for phytotherapy for mental disorders such as anxiety, depression, epilepsy, and age-related dementia.

All the alkaloids mentioned above produced by *B. disticha* are of the crinane series. As shown in Figure 3, nerbowdine, previously called "haemathamine" (Gelfand and Mitchell, 1952), was thought

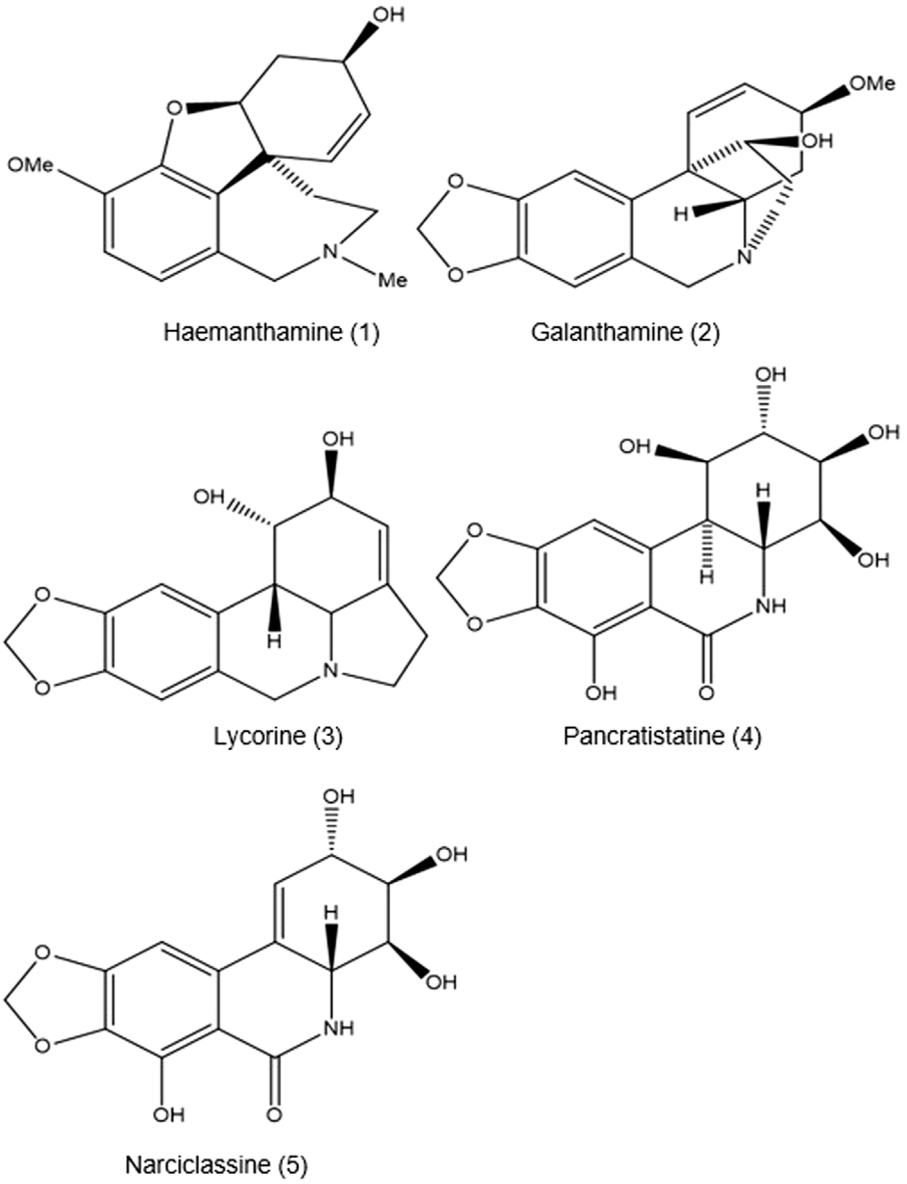

FIGURE 3
Amaryllidaceae alkaloids.

to be responsible for effects similar to those of anti-cholinergic scopolamine.

Several areas of *B. disticha* use in traditional medical practice still require pharmacological validation because the plant is regarded as poisonous. There is a need for rigorous investigation to reduce harm that may be caused by the use and abuse of *B. disticha* (Stafford et al., 2008).

There are no legal sanctions in southern African countries, which opens the drug to possible abuse.

Cannabis sativa, L Cannabaceae

C. sativa is not indigenous to southern Africa, as believed by many people. A more in-depth analysis of the issue of cannabis diffusion in Africa versus its possible status as a native plant has been extensively reviewed in a brief agricultural history of Africa by Duvall (2019). Linguistic evidence indicates that it was introduced into the sub-continent from Asia (Crampton, 2015; du Toit, 1996). Be that as it may, *Cannabis* is now reported to be used by many TMPs (Sobiecki, 2008). Illegal use by non-healers for recreation and ritual is widespread throughout southern Africa. However, South Africa is moving towards the decriminalization of its cultivation for possession personal use. Figure 4 shows a female and a male plant in a household backyard, which is now permitted.

The major route of administration is smoking as a cigarette. It is believed that cannabis may be mixed with *N. tabascum* and other plants to aid divination and its psychotropic effects. Vaporization is another commonly used route of administration. Inhalation is believed to be quickest for onset of action and shortest duration (Iversen, 2001).

FIGURE 4
Backyard female (l) and male (r) *Cannabis*.

C. sativa is used for religious purposes as a sacred herb entheogen by southern African Rastafarians who call it *ganja (ganga)*, a word derived from Indian plantation workers in Jamaica (Booth, 2005; Crampton, 2015). It is mainly used for its THC (tetrahydrocannabinol) content, which is responsible for its intoxicating effects. More than 100 other cannabinoids including, CBD (cannabidiol), are non-intoxicating (Figure 5) (Russo, 2017).

Cannabis grown in different conditions can produce different phytochemical profiles which may not be known by its users. Studies have shown that handling of the plant can cause the oxidation and degradation of cannabinoids, resulting in adverse or unknown biological effects. This is critical information of primary importance for its medical and recreational use (Zandkarimi, Decatur, Casali, Gordon, Skibola, et al., 2023).

There are cannabinoid-like substances in the brain that can interfere with or modulate neurotransmission. This is because cannabinoid-like substances bind to CB1 receptors on the pre-synaptic neuron found in the brain. Depending on the neurotransmitter released as a result of the binding, this can result in different psychotropic effects, depending on the part of the central nervous system (Mouhamed et al., 2018).

There is continued research into the psychological effects of cannabis . An interesting recent study has shown that the volume of the grey matter of the brain increased following a couple of uses of marijuana by some adolescents, resulting in increased risk of anxiety, and decreased ability to think and memory (Orr, Spechler, Cao, Albaugh, Chaarani et al., 2019). The genetic basis for cannabis use and its psychiatric disorders has been published in *Lancet Psychiatry*, indicating that a subset of the population is at high risk (Cheng, Parker, Karadag Koch, Hindley et al., 2023).

Its pharmacological classification is as a stimulant and sedative

The legal status of *C. sativa* in southern Africa is that it is generally illegal for adult (recreational) use in most countries, but there is a decriminalization drive.

Datura stramonium, L Solanaceae

The early Sanskrit word *dustura* or *dahatura* means “divine” inebriation, from which *D. stramonium* derives its name. This explains why, for approximately 400 years, solanaceous plants were regarded as “diabolic incarnations”. Consequently, *Datura* has been referred to as “devil’s apple”, “mad apple”, and “devils work” due to its ability to cause visionary dreams and assist in the foretelling of the future and revealing the causes of disease (Busia and Heckles, 2006).

In southern Africa *D. stramonium* has been reported to be used in traditional medical practice (Figure 6) (Sobiecki, 2008). It is thought that the use of its leaves can relieve headache, and vapours from a leaf infusion can relieve the pain of rheumatism and gout. The smoke of the leaf can be inhaled to relieve asthma and bronchitis.

Route of administration: inhalation

Hysterical and psychotic patients are sedated by using its seeds and leaves. *D. stramonium* is also thought to help in insomnia. The

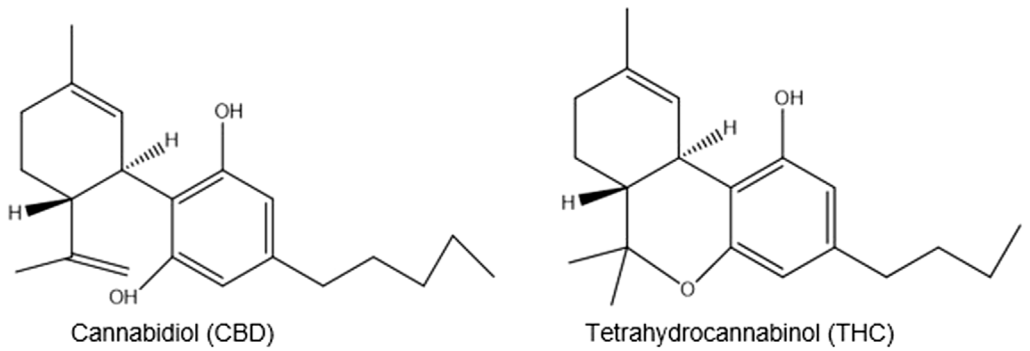

FIGURE 5
Chemical structures of CBD and THC.

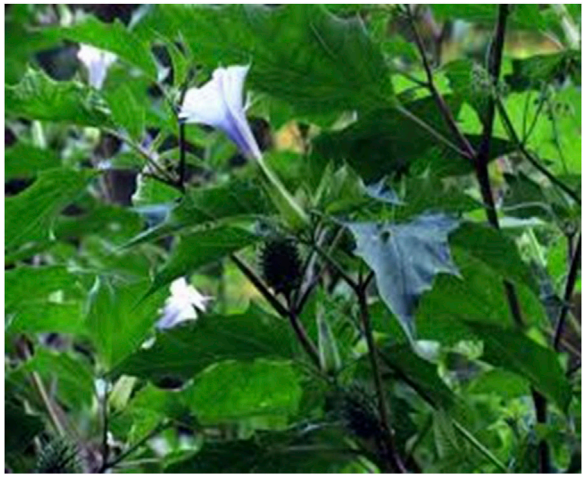

FIGURE 6
Datura with flower-like trumpet.

seeds are reported to be its most medicinally active part (Soni, Siddiqui, Dwivedi and Soni, 2012).

Conscious perception only occurs when the associative cortex of the brain is active. Different structures of the brain have been reported to be responsible for different levels of consciousness. Different neurotransmitters are involved in these, which means that interference with these neurotransmitters at the synapses will affect the consciousness of an individual. The cholinergic system, whose neurotransmitter is acetylcholine, is also responsible for major neuromodulatory systems involved in relaying information between brain structures (Ach) (Roth, 2004).

All parts of *Datura* are reported to contain dangerous levels of tropane alkaloids, atropine, hyoscynamine, and hyoscine (scopolamine) (Figure 7) which can stimulate no-cost highs. The effects of *D. stramonium* have been postulated to derive from the major alkaloid responsible for the principal effects of hyoscine. The tropane alkaloids work additively to trigger hallucination (Hall, Pfefferbaum, Gardner, Stickney and Perl, 1978).

legal status: no legal restrictions despite potential harmful and deadly effects.

Helichrysum odoratissimum L. asteraceae sweet. african incense, aromatic shrub

The name of *H. odoratissimum* is derived from the Greek word *heliscryson*, a compound of the word for "sun" plus *chryos*, which means "gold," referring to the golden flowers which are characteristic of the genus (Figure 8). "*Odoratissimum*" refers to the strong odour of the plant. The plant is commonly termed "African incense", and perhaps this is why it is used as a calmative for insomnia and ritual incense (Maroyi, 2019; Serabele, Chen and Combrinck, 2023). Recently, it has been reported that another species in the *Helichrysum* genus, *H. umbraculigerum*, which is not commonly used, produces phytocannabinoids such as cannabigerol and CBG but not THC and CBD (Benson, 2023). TMPs in southern Africa have their own ways of distinguishing the different *Helichrysum* species, in spite of their florescence being almost identical (Figure 8) (Viljoen et al., 2022).

The principal route of administration is the inhalation of smoke from burnt parts of the plant or direct smoking. It can, however, also be taken as herbal tea.

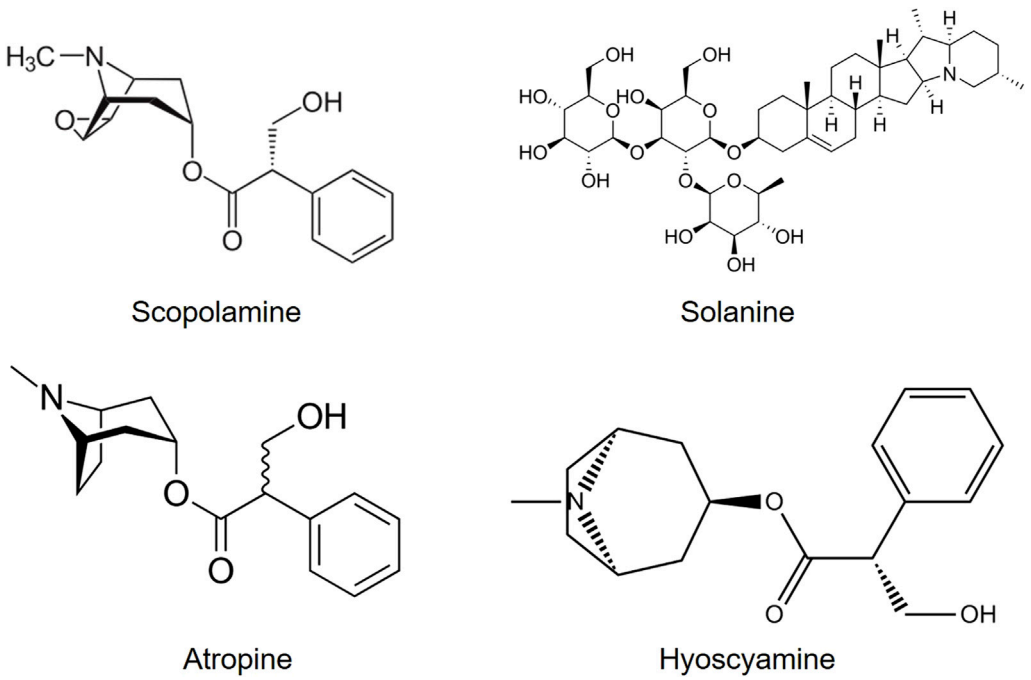

FIGURE 7
Datura alkaloids.

FIGURE 8
H. odoratissimum in the wild.

FIGURE 10
L. leonurus.

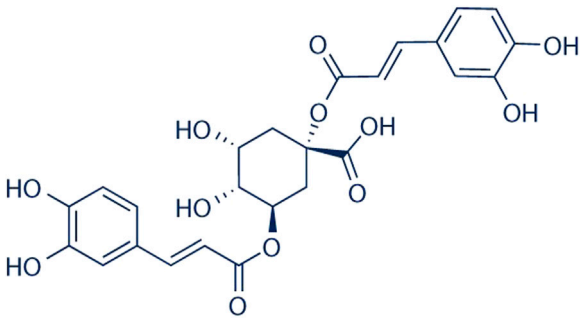

FIGURE 9
Dicaffeoylquinic acid.

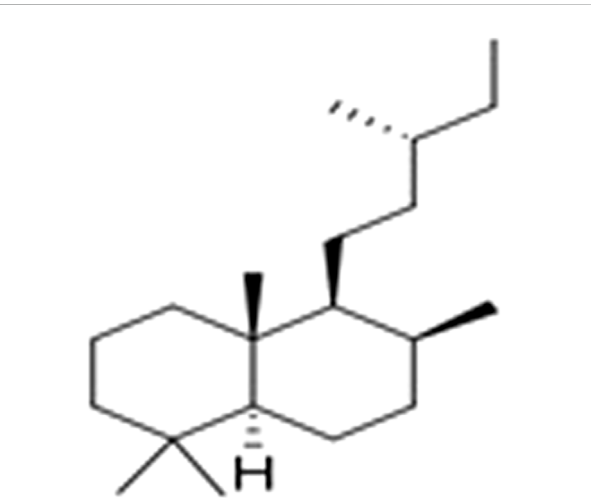

FIGURE 11
Labdane.

According to the phytochemical studies of Viljoen et al. (2022), 4,5-dicaffeoylquinic acid and dicaffeoylquinic acid, also found in coffee beans (Mondolot et al., 2006) (Figure 9), consistently appear to be possible markers for the species. This has also been confirmed by Serabele, Chen and Combrinck (2023), who used high-performance-thin-layer-chromatograph (HPTLC) coupled with mass spectrometry (UPLC-MS) analysis. What is interesting, however, is that essential oils such as α-pinene-containing oils and neryl acetate also may be possible markers of the species, using the method of analysis of Tundis, Statti, Conforti, Bianchi, Agrimonti et al. (2005). Their study of *Helichrysum italicum* showed that environmental factors influenced the phytochemistry (volatile constituents) and biological effects (antibacterial activity) of the plant. Interestingly, *H. italicum* is commonly used as a herbal medicine in Mediterranean regions (Appendino et al., 2015).

There are no legal restrictions on its use.

Leonotis leonurus L. R. Br Lamiaceae

L. leonurus, although often referred to as "wild dagga," an Afrikaans word for cannabis, is not related to true dagga (*C. sativa*). *Leonotis* is a Greek word meaning "lion ear".

In traditional medical practice, its dried leaves and pink or brilliant orange flowers, (Figure 10) are used to treat many ailments, including epilepsy. Non-healers use it for its calming effect.

Route of administration: The leaves and flowers are smoked for mild euphoric effect less potent than cannabis.

Terpenoids with the main compounds (mono-, sesqui-, and diterpenoids) have been reported to be biologically active. Labdane diterpenes (Figure 11) were found to be more abundant compounds by Nsuala, Enslin and Viljeon (2015).

However, a phytocannabinoid-like compound, adrenoyl-EA (adronyl-ethanolamine), (Figure 12) has recently been identified in the flowers. The compound has the same structure as endocannabinoid anandamide (N-acylethanolamine) (Figure 13) which is also found in cocoa beans. Anandamide is as an agonist of the CB1 and TRPV1 receptor proteins in humans, whose other

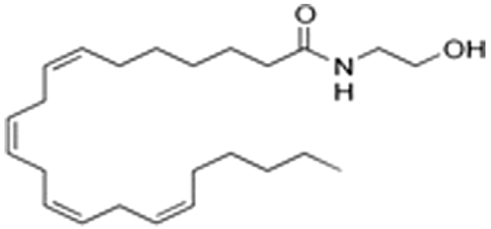

FIGURE 12
Adronylethanolamine (Adrenyl-EA).

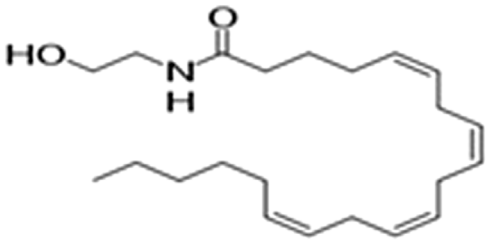

FIGURE 13
Arachidonylethanolamine (anandamide).

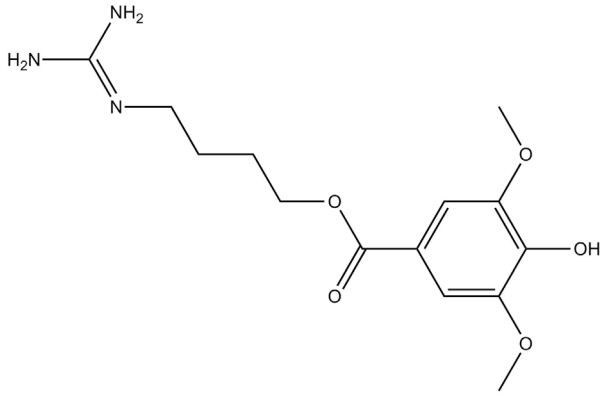

FIGURE 14
Leonurine.

agonists are ideal candidates for the development of anti-inflammatory, neuroprotective, and anticancer drugs (Hunter, Stander, Kossman, Chakraborty, Prince et al., 2020).

There are other compounds that have been isolated from the plant. However, according to Nsuala, Enslin and Viljeon (2015), leonurine (Figure 14) has been reported in the popular literature. Interestingly there has been no scientific analysis of extracts of *L. leonurus* that have reported leonurine.

Legal status: no restrictions on its use.

Psilocybe cubensis (Erale) Singer. Hymenogastraceae

P. cubensis is a fungus with neurotropic (hallucinogenic or psychotropic) properties, also referred to as narcotic, magic,

FIGURE 15
P. cubensis.

sacred, psychedelic entheogenic mushrooms; they have been reported as found all over the world, including southern Africa (Matsushimaet al., 2009). Because of the legal status of mushrooms use, coupled with continued secrecy surrounding some traditional medical practices, it was difficult to obtain information on how *P. cubensis* (Figure 15) is actually employed in culturally sanctioned visionary experiences in ritual or religious contexts.

Its route of administration is oral.

This is also the case in West Africa (Osemwegjie, Okhuoya and Dania, 2014). Ethnomycological studies in some parts of Africa have been found to be varied, incoherent, skewed, and not balanced in terms of information on mushrooms of unknown use.

However, information obtained about the collection and uses of mushrooms indicate that there is community knowledge about which mushrooms can be eaten, which can be used for medicinal purposes, and which are poisonous. There are beliefs such as the following: 1) any wild mushroom attacked by insects, rodents, and other animals where they are growing can be classified as edible; 2) any mushroom given to a live chicken, pig, dog, and which does not die is regarded as edible; 3) brightly coloured and pleasantly smelling mushrooms could be edible and associated with spirituality. Such human experiments provide more knowledge about the better use of wild mushrooms in general, leading to their acceptance as food, medicine, or poison.

P. cubensis, which is reported to grow on cow and wildlife dung in Zimbabwe (van der Horst, 2018), is similar to that which grows on Asian elephant dung in India. Psilocybin, psilocin (Supplementary Figure S1), and baeocystin are reported to be the biologically active substances in the mushroom. Of these biologically active substances, psilocybin is the most stable. It is found in some of the mushrooms and is converted to psilocin, which produces the psychoactive effect (Stamets, 1996). The activity produced by the mushrooms depends on the species and variety and where it grows and is subsequently harvested for use. The same is true with any of the psychoactive plants.

There are currently several clinical trials with psilocybin (University of San Francisco, 2023). Previous studies at the Johns Hopkins Centre for Psychedelic and Consciousness Research showed that psilocybin could be effective for therapeutic purposes, such as for depression, drug abuse, and end-of-life mental disorders (Ziff et al., 2022). Despite this, "magic mushrooms" are easily accessible to the general public.

Mesembryanthemum tortuosum, Sceletium tortuosum. L. N.E.brown Aizoaceae (kanna) mesembryanthemaceae

S. tortuosum L.) N.E. Br. and *S. expansum* L. Bolus were formerly known as *Mesembryanthemum tortuosum* L. and *Mesembryanthemum expansum* L. (Supplementary Figure S3). *Sceletus* is a Latin word that describes the plant's prominent leaf veins (Supplementary Figure S4).

A number of habitats have been found suitable for the growth of different genera of its family. It is found in south-west Africa, including Angola, South Africa, Zimbabwe, Botswana, and Namibia (Supplementary Figure S4) (Faber, Laubscher and Jimoh, 2021).

Traditionally, *S. tortuosum* was reported to be useful for toothache and abdominal pain. Furthermore, it is thought to elevate mood, suppress hunger and thirst, induce analgesia, aid hypnosis, reduce anxiety, is used as an intoxicating/euphoric substance (Olatunji et al., 2021), and is sometimes used together with cannabis to enhance the latter's intoxicating effect (Michell, 2004).

The route of administration is mainly oral by mastication, and by inhalation through smoking the dried plant material or snorting the root powder.

The plant contains different types of mesembrine alkaloids (Supplementary Figure S5) which are found in a few plant genera, *Sceletium* being one (Faber, Laubscher and Jimoh, 2020).

Mesembrine is a serotonin re-uptake inhibitor and is also thought to act as a monoamine-releasing agent (Coetzee et al., 2016). Historical use of different extracts of the plant by the San and Khoi people has been shown to have various biological properties (Manganyi et al., 2021).

It can therefore be argued that *S. tortuosum* has both pharmaceutical and economic significance because it contains mesembrenone and mesembrine, which can be developed as useful products that promote health and/or to treat some psychological disorders.

Silene capensis Caryophyllacae, African Dream root

According to anthropologist Manton Hirst, *S. capensis* has been used for hundreds of years. It is a stringy, leafy green plant found near rivers that produces a fragrant white flower that only blooms at night, hence perhaps its name as the "African dream herb" (Hirst, 2005). It is believed that *S. capensis* is used to heighten one's intuitive capacity to extract wisdom from the dream realm and apply it the waking state. Details of how *S. capensis* is used depend on the Bantu ethnic group, including how the plant material preparation is consumed.

The plant's route of administration is oral, by chewing the root bark, or scooping or sipping the foam that it produces during preparation.

S. capensis is used in ritual ceremonies; hence, the potential for abuse because information on accessibility and availability is now public knowledge. Perceptions regarding its value and potential harm have been reported to be transformed by those participating in ceremonies.

Most *Silene* species are hermaphrodite, and more than 400 bioactive compounds have been isolated, including phytoecdysteroids, the predominant constituents (Mamadalieva, 2012). Supplementary Figure S6 shows the general chemical structure of phytoecdysteroids, and Supplementary Figure S7 gives the structure of 20-hydroxyecdysone.

Ecdysteroids can be used for chemotaxonomy in this genus because many *Silene* species contain complex ecdysteroid cocktails (Zibareva et al., 2009). Despite what is now known about the responsibility of 20-hydroxyecdysone for genomic biological effects manifested in different animal models (Dinan, Dioh, Veillet, and Lafont, 2021), ecdysteroids are thought not to be responsible for CNS effects.

According to Sparg, Light, & van Staden, (2004), the frothing and foam formation associated with preparation for use could be explained by the presence of saponins— oleanane-type aglycone moieties. Supplementary Figure S8 shows the basic structure of a saponin said to be commonly present in the Caryophyllacae order. Since the foam is generally consumed during the ceremonies, it is therefore assumed that its psychoactive effects are due to the saponin content rather than the phytoecdysteroids.

Observations made by Hostettmann & Marston (1995) confirm that cleavage of the saponins aglycone moiety can serve as a useful substance for the synthesis of hormones such as progesterone in industry. The amphiphilic nature of saponins is useful in soap manufacture and surfactants for the cosmetic industry (Guclu-Ustundag and Mazza, 2007).

Ground root is now commercially available in gel capsules which contain approximately 500 mg of ground powder of the root. It is promoted for lucid dreams as well as enhancing the quality of sleep, with a maximum daily dose of 30 g (Sumpter, 2021).

There are no legal restrictions on its use

Dioscorea dregeana (Kunth) T. Durand & Schinz

D. dregeana (wild yam) (Supplementary Figure S9) grows naturally in Eswatini, Mozambique, and South Africa. It is mainly used as a sedative, depending on the mental disorder (Maroyi, 2022).

Its route of administration is oral as a weak decoction which is a more vigorous extraction of the active ingredient from the plant's tuber.

The tubers of *D. dregegeana* contain the following compounds: sitosterol, stigmasterol, dodecanosyl 3-[4′-hydroxy, 3′-methoxyphenyl] propenoate, 3, 4′,5-trihydroxybibenzyl, crinamine, and dioscorine. Of these phytochemicals, dioscorine (Supplementary Figure S10) has intoxicative and soporific effects. On the other hand, crinamine (Supplementary Figure S11) is a selective monoamine B inhibitor (Naidoo et al., 2020).

An animal study found that a combination of the psychoactive principles in the tuber produced central nervous system depressant/sedative and anxiolytic effects (Patel and Galani, 2017). Bio-prospecting of *D. dregeana* and wild tubers in the food and pharmaceutical industries can benefit from the observed effects.

There are no legal restrictions on its use.

Potential pharmaceutical development with ethno-psychoactive plants

Over the years, the commercialization of tradition African medicinal plants has increased as a result of interest in and knowledge of their use. For example, the economic value of indigenous medicinal plants in South Africa now represents 5.69% of the national health budget, and the plant trade is a key rural industry and business stimulant (Rispel and Setswe, 2007). It is therefore necessary to investigate ethno-psychoactive plant use for their psychotropic actions.

These plants contain secondary metabolites including terpenoids, flavonoids, and alkaloids. These metabolites feature various chemical structures and produce a variety of beneficial biological which are a valuable source of compounds that the pharmaceutical, nutraceutical, cosmetic, and fine chemical industries can use. The present study shows that ethno-psychoactive plants are a major source of many drugs that can be developed for therapeutic purposes. Many of these plants contain useful substances, either in the whole plant or in some parts that can used be for therapeutic purposes in disease, diagnosis, and prevention. It has been estimated that 84% of drugs or their structures now used for mental disorders have been obtained from natural sources (Bharate et al., 2018). According to Halilu (2022), medicinal plants can be a significant source of income for research in pharmaceutical science and socio-economic development. *B. disticha* is certainly a plant from which neuro-protective products can be developed for Alzheimer's and Parkinson's diseases, epilepsy, depression, and anxiety. The crinamine contained in *B. disticha* has been demonstrated to be a selective inhibitor of monoamine oxidase B, which is useful in the treatment of Parkinson's disease. Another plant which contains crinamine, *Crossyne guttata,* is used by Rastafarian bush doctors for alcoholism, which is accentuated by MAO (Naidoo et al., 2020).

Research on treatments for depression, post-traumatic stress disorder, and other psychiatric conditions using psychedelics has been brought closer to legalization (Krediet et al., 2020).

Another group of psychoactive plants, the *Silene* genus, has been exploited as an industrial source of phytoecdysteroids that can be used to develop anabolic steroids. *S. capensis*, the dream root, is another source of hallucinogens that can be developed for specific mental disorders and to improve the quality of sleep. *C. sativa* (THC), *D. stramonium, N. tabacum*, and *P. cubensis* can be exploited just as *C. sinensis* and *N. tabascum* have been by industry. The recent discovery that non-cannabis plants such as *H. umbraculigerum* have cannabis qualities opens new avenues for the development of products of medical use, particularly psychiatrics (Berman et al., 2023). This species has been shown to have a parallel evolution of cannabinoid biosynthesis which can be exploited to produce botanicals for medicinal use.

On the other end of the spectrum, psychosis—a collection of symptoms affecting the mind, where there has been some loss of contact with reality—can be treated and managed in traditional medical practice. Psychosis in traditional medical practice is regarded as a disequilibrium that results from psychological or spiritual factors or both. It is also referred to as a collection of symptoms that affect the brain, causing a loss of contact with reality. The healing of such patients emphasizes correcting this disequilibrium by using *C. sativa*, *D. dregeana, S. tortuosum, L. leonurus,* and *H. odoratissimum*. Unfortunately, little is being done to authenticate their effects within a scientific paradigm because they do not contain a simple ingredient, making them more complex than conventional pharmaceutical products like prochlormazine. The many substances that may be contained in a plant responsible for beneficial effects may act synergistically and produce what is known as "entourage effect" (Ferber, Namdar, Hen-Shoval, Eger, Hinanit Koltai, et al., 2020). This synergistic interaction may be a strength for medicinal plants, but unfortunately it is also an obstacle to standardizing research, which is a concern for those trying to develop pharmaceutical products from medicinal plants. It should be a concern for TMPs, conventional healthcare providers, and consumers. Such complexity requires psycho-pharmacological and social pharmacological information on the value of botanicals used for mental problems, diagnosed in traditional medical practice, in the assessment of a risk-effective balance.

Conclusion

TMPs in southern Africa commonly use indigenous herbal medicines for divination and also for to treat and manage mental and other illnesses. Unfortunately, the results of research, risks, and benefits from their use do not align. Psychoactive plants could be explored further to develop therapeutic agents. Although the CNS effects of the psychoactive plants identified are known, little is known about their potential for abuse among TMPs and in some communities in southern Africa. There is therefore a need to work closely with TMPs to reduce harm from the abuse of these plants while also promoting pharmaceutical development. This continues to be an under-investigated area that deserves continued scientific enquiry.

Author contributions

NN: conceptualization, writing–original draft, and writing–review and editing. JC: writing–review and editing. BE: writing–original draft and writing–review and editing.

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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.2024.1269247/full#supplementary-material

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Ethnobotanical study of traditional medicinal plants used to treat human ailments in West Shewa community, Oromia, Ethiopia

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Introduction: Plants have formed the basis of traditional medicine (TM) systems, which have been used for thousands of years. According to reports, one-quarter of the commonly used medicines contain compounds isolated from plants. This study aims to identify and document the plants for ethno-pharmacological use by the indigenous communities of West Shoa Zone, Oromia region, Ethiopia.

Methods: The cross-sectional study was conducted from November 2020 to November 2021 in West Shewa Zone, Oromia Region, Ethiopia. The ethnobotanical data was collected from Ejere District, Ada Berga District, Dandi District, Ambo District, Ambo Town, Toke Kutaye District, and Bako Tibe District. A descriptive statistical method (percentage and/or frequency) was employed to summarize ethnobotanical data. Moreover, the informant consensus factor was computed. Microsoft Excel spreadsheet software (Microsoft Corporation, 2016) and SPSS (version 25) were used to organize and analyze the data.

Result: In the study area, a total of 51 families of medicinal plants with 108 Species were identified. Fabaceae 8 species, Asteraceae, Solanaceae and Lamiaceae each with 6 species and Cucurubitacieae 5 species were the frequently reported medicinal plants. The leaf (57.2%) was the most widely used medicinal plant parts, and oral administration (56.5%) was the most cited route of administration. In the present study, most of the medicinal plants were used fresh, which was (75%) and the most common disease the healers treated was gastrointestinal disease, followed by skin disease and febrile illness. The major threat to medicinal plants in the study area was agricultural expansion, which was reported by 30.6% of the respondents. The study area was rich in medicinal plants, Fabaceae which commonly used family.

Conclusion: Most of the medication prepared by the traditional healers was taken orally and derived from the leaf part of the medicinal plant. Since this research is a preliminary study which will be used as a base for further study. The efficacy and safety of the medicinal plant claim should be studied in the future.

KEYWORDS

ethnobotanical study, traditional medicine, medicinal plants, West shewa, Ethiopia

Introduction

The use of natural products as medicinal agents dates back to prehistory. According to a World Health Organization (WHO) report, 60%–79% of the population in developing countries depends on traditional medicine from plants for health requirements (WHO, 2010). The use of indigenous traditional medicine in Ethiopia is also estimated between 60% and 79% (World Health Organization, 2019). The Ethiopian people have been using medicinal plants to treat different diseases for many centuries. Religious and secular pharmacopeia has been compiled since the 15th century. Medicinal plants are an integral part of the variety of cultures in Ethiopia, which has resulted in medical system pluralism (Pankhurst, 1965).

According to reports, one-quarter of the commonly used medicines contain compounds isolated from plants (Rates, 2001). The search for new drugs is a priority of WHO, drug companies, and research institutes due to the emergence and spread of drug-resistant pathogens that have acquired new resistance mechanisms, leading to antimicrobial resistance, which continues to threaten our ability to treat common infections (WHO Pathogens Priority List Working Group et al., 2018). Especially alarming is the rapid global spread of multi- and pan-resistant bacteria (also known as "superbugs") that cause infections that are not treatable with existing antimicrobial drugs. Emerging and reemerging infectious diseases continue to impose a constant threat on the human population (Howard and Fletcher, 2012).

Even though the national policy of Ethiopia indicates identifying and encouraging the utilization of beneficial TM components (EFDA, 2020), little has been done to enhance and develop the beneficial aspects of TM, including relevant research to explore possibilities for its gradual integration into modern medicine (Kassaye et al., 2006). Indigenous knowledge is usually kept secret, only to be passed orally to the healer's older son at their oldest age (Battiste and Youngblood, 2000). The expansion of modern education has also impacted the transfer culture, and knowledge on medicinal plants is being lost at a faster rate (IBCR, 2001). Additionally, the loss of medicinal plants is due to population pressure, agricultural expansion, and deforestation (Abebe et al., 2001; Berhan and Dessie, 2002).

Due to changing lifestyles, extreme secrecy of traditional healers, and negligence of youngsters, the practice and dependence of ethnic societies on folk medicines are in rapid decline globally (Kumar et al., 2012). Therefore, documentation of the traditional uses of medicinal plants needs immediate attention (Bussmann and Sharon, 2006; Jeyaprakash et al., 2017). Furthermore, documentation of indigenous and traditional knowledge is very important for future critical studies leading to sustainable utilization of natural resources and facing the challenges of biopiracy and patenting indigenous and traditional knowledge by others. Therefore, it is urgent to explore and document this unique indigenous and traditional knowledge of the tribal community before it diminishes with the knowledgeable persons. This study aimed to identify and document the plants for ethno-pharmacological use by the indigenous communities of the West Shoa, located in the Oromia regional state, Ethiopia.

Methods

Study area description

Geographical location

The study was conducted in the West Shoa Zone which is found in Oromia Region, Ethiopia. West Shewa is bordered on the South by the Southwest Shewa Zone, on the Southwest by Jimma Zone, on the West by East Wolega, on the Northwest by Horo Gudru Welega, on the North by the Amhara Region, on the Northeast by North Shewa, and from the East by Sheggar City (Figure 1). The district is located between 8°49′26″to 8°55′22″N and 37°49′50″ to 38°8′08″E (Ogato et al., 2020). Based on the agro-climatic classification of Ethiopia, it lies within the three agro-climatic zones, Highland 27%, mid-altitude 56% and 17% lowlands. The zone has a bimodal rainfall pattern; summer is the main rainy season with its peak in July (June to August), and the short rainy season from February to April. Rainfall varies from 813.2 mm to 1669.1 mm. The maximum temperature ranges from 24°C to 29°C while the minimum temperature ranges from 11°C to 13°C (Abate, 2008).

Study design and period

A cross-sectional survey was conducted from November 2020 to November 2021.

Site and informant selection procedure

Seven Woredas and one town were purposively selected. Accordingly, Ambo town, Ejere, Ambo Woreda, Ada Berga, Bako Tibe, Toke Kutaye, and Dandi Woredas were selected (Figure 1). From each woredas traditional healers or knowledgeable persons were selected based on the information gathered from each selected kebele administration office, health office, agricultural office, and other people in the study area.

Selection of informants

The selection of key informants was conducted by snowball method with the help of local authorities, elders, and knowledgeable persons. All registered traditional healers and unregistered but famous elders and knowledgeable persons within each woreda were included. Prior to the administration of the questionnaire, conversations with the informants were done with the assistance of local leaders of the selected study area to elaborate the objectives of the study and to build on trust with the common goal of documenting and preserving knowledge on medicinal plants.

Ethno-medicinal data collection and tools

Fourteen data collectors participated after 5 days of training. Semi-structured questionnaires, Focus group discussions, and field observation were used for data collection. The tool for data collection was developed by reviewing previous research (Yirga and Zeraburk, 2011; Chekole, 2017), and modified to fit the local

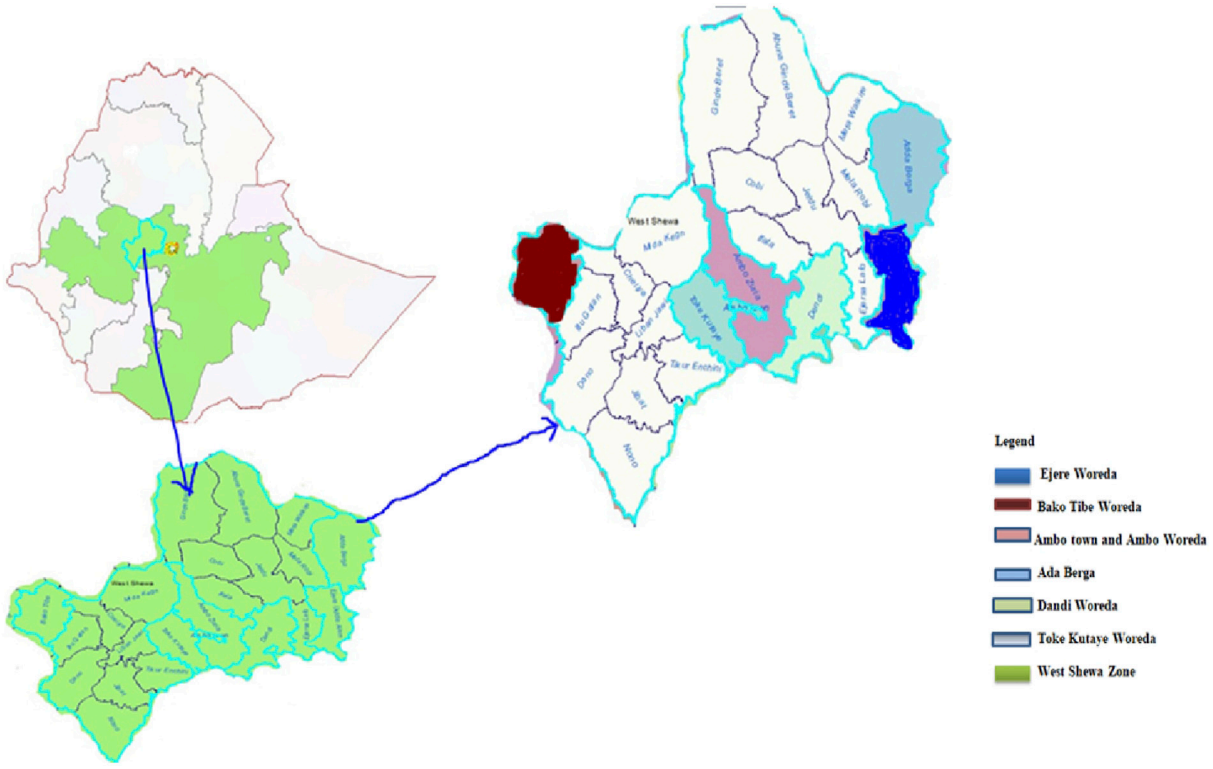

FIGURE 1
Map of the study area.

population. The questionnaire was designed in English and translated by professionals to the Afan Oromo. Questionnaire content validation was done by pretest. Content Information regarding local names of medicinal plants, methods of preparation, part(s) used, dosage used and route of administration of medicinal plants, types of disease, and duration of administrations were recorded.

Field observation

Field observation was performed with the help of local guides and interviewed informants in the study area. Based on the information provided by informants, specimens were collected, numbered, pressed, and dried for identification.

Focus group discussion

Focus group discussions were conducted for the selected district with the community members who have used medicinal plants from well-known traditional healers in the community.

Plant identification

Voucher specimens were collected for each plant species. The specimens were dried, pressed, and identified in the National Herbarium (ETH), Addis Ababa University. Finally, the identified voucher specimens were deposited at the National Herbarium (AAU).

Data quality management

The quality of data was maintained by providing training for data collectors, regarding the purpose of the study and data handling. The collected data was checked for completeness and consistency before analysis of data.

Data analysis

A descriptive statistical method (percentage and/or frequency) was employed to summarize ethnobotanical data. Moreover, the informant consensus factor was computed. Microsoft Excel spreadsheet software (Microsoft Corporation, 2016) and SPSS (version 22) were used to organize and analyze the data.

Informant consensus factor (ICF) was calculated for categories of ailments to identify the agreements of the informants on the reported cures using the formula used by Gazzaneo, de Lucena (Gazzaneo et al., 2005) and Teklehaymanot and Giday (Teklehaymanot and Giday, 2007). ICF was calculated as follows:

$$\text{IFC} = \frac{Nuc - Ns}{Nuc - 1}$$

TABLE 1 Sociodemocratic characteristics of respondents of Southwest Shewa zone.

Variables	Categories	Number (N = 126)	Percent (%)
Sex	Male	100	79.4
	Female	26	20.6
Resident of participant	Baco Woreda	20	15.8
	Ejere Woreda	20	15.8
	Toke Kutaye Woreda	20	15.8
	Ambo town	12	9.5
	Ada Berga Woreda	16	12.7
	Ambo Woreda	20	15.9
	Dandi Woreda	18	14.3
Religion	Protestant	34	26.9
	Ortodox	70	55.5
	Muslim	18	14.3
	Waqefata	4	3.2
Ethnicity	Oromo	118	93.7
	Amhara	8	6.3
Educational Status	Could not read and write	60	47.6
	Can read and write	6	4.8
	Primary School (1–8)	51	40.5
	Secondary school (9–12)	9	7.1
Job	Farmer	96	76.2
	Gov. employer	22	17.5
	Private work	8	6.3
Source of Knowledge	Family	112	88.9
	Friend	10	7.9
	Traditional healer	4	3.2
Pattern of work	Pert-time	118	93.7
	Full time	8	6.3
Age	<50	48	38.1
	≥50	78	61.9
Number of patient/week	<10	114	90.5
	>10	12	9.5
Experience in years	<10	40	31.7
	>10	86	68.3

Where Nuc is the number of use citations in each illness category and Ns = is the number of species used by all informants for this illness category. The ICF values range from 0 to 1, with high values (i.e., close to 1) indicating that relatively few plants are used by a large proportion of informants, while low values (˂ 0.5) indicate that informants do not agree on the plant species to be used to treat a category of ailments.

Ethical consideration

The ethical clearance was obtained by the Ethical Review Committee of the College of Medicine and Health Sciences of Ambo University. Then the letter written from Ambo University was given to the West Shewa Zone health office. The West Shewa zone health office wrote a supportive letter to each Woreda Health office. In addition, oral informed

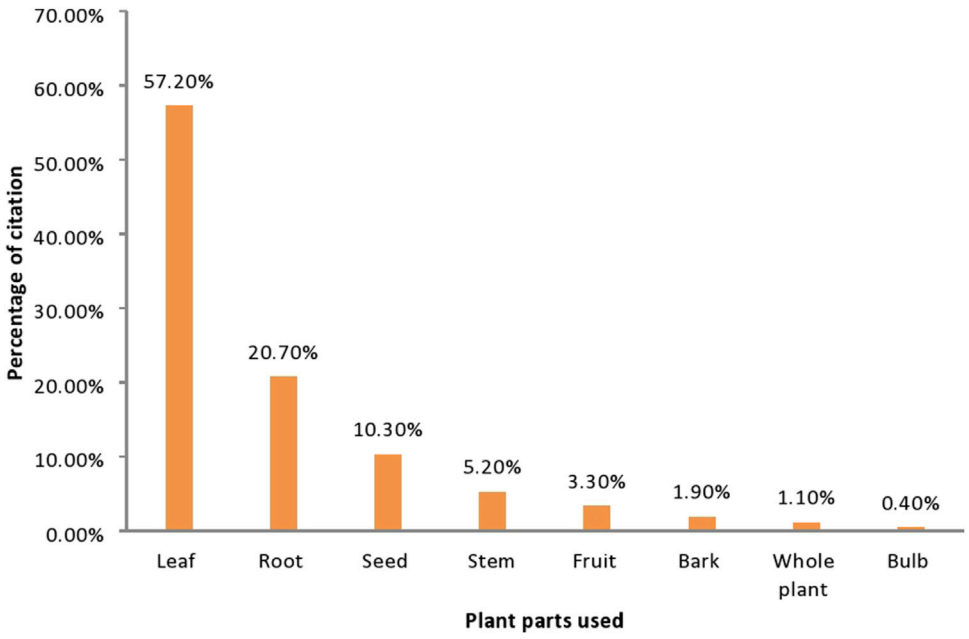

FIGURE 2
Part the medicinal plants used for medicine by traditional healers in West Shewa, Oromia, Ethiopia.

consent was obtained and those who voluntarily participated was continued the interview. The confidentiality was assured by excluding their names and the right not to participate or to discontinue the interview wherever they wanted in the study was respected.

Results

Socio-demographic characteristics

From six woredas and one administrative town a total of 126 participants responded to the interview. Out of the participants 79.4% were male and the majority of the participants were Orthodox and Oromo. The more than two-third of the participants (78%) were greater than 50 years. More than 80% of the participants had the experience of giving traditional medicine greater than 10 years. More than eighty percent of the participant got the knowledge about traditional medicine from the family members (Table 1).

Medicinal plant family and species

In the study area a total of 51 families of medicinal plants with 108 species were identified. Fabaceae (8 species), Asteraceae (6 species), Solanaceae (6 species), Lamiaceae (6 species), and Cucurubitacieae (5 species) were the most frequently reported medicinal plant in the study area (Supplementary Table S1).

Plant parts used

In the present the leaf (57.2%) was the most widely used medicinal plant parts followed by the root which was 20.7% (Figure 2).

Route of administration of the remedies prepared from medicinal plant and condition of medicinal plant

The oral administration was the most (56.5%) cited route of administration followed by the topical (34.3%), inhalation (6.3%) and nasal (2.9%) route of administration. On the other hand most of the medicinal plants were used as fresh which was (75%) and 22% of the medicinal plants were used after drying the rest (3%) can be utilized both in the form of dry and fresh.

Method of preparations

In the present study the majority of the medicine were prepared by squeezing (25.8%) followed by powder (19.6%) (Figure 3).

Additive during medicine preparation

Out of 126 participants 120 (95.2%) prepare the medicine in combination with other medicinal plant or non-medicine ingredients such as water, honey, milk, and food. Out of the additives used 102 (80.9%) of the participants were used medicinal ingredient and 18 (14.3%) were ingredient with non-medicinal value but used as excipient.

Saying during plant collection

During plant collection majority 84 (66.67%) of the participants did not talk and 42 (33.33%) pray to their God before collection.

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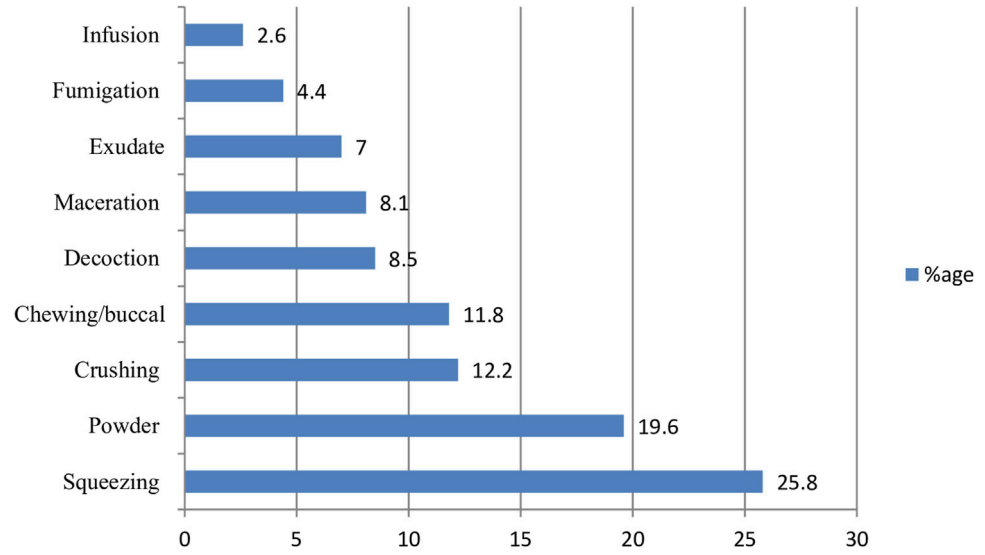

FIGURE 3
The method medicine preparations used by the traditional healers in West Shewa, Oromia, Ethiopia.

TABLE 2 Disease category and ICF of Medicinal plants used by traditional healers in West Shewa, Oromia, Ethiopia.

Disease category	No species	No of reports	ICF
Tumour	2	5	0.75
Rabies	6	19	0.7
Febrile illness	12	31	0.63
STI (Gonorrhoea, hepatitis, herpes simplex, Syphilis	5	11	0.6
Skin disease (Allergic reaction, wound healing, skin disorder, tinea versicolar, ring worm, scabies, Eczema, leprosy, Broken bone, dislocation, wart, itching, desert wound, burn)	18	38	0.54
Haemorrhoid	6	11	0.5
Tonsillitis	10	18	0.47
Abdominal cramp/distension/stomacache/tape worm/diarrhoea/dyspepsia/indigestion, ascariasis, constipation	29	50	0.43
Toothache	9	15	0.43
Common cold/Cough, Asthma, respiratory disease	7	11	0.4
Leshmaniasis, measles, herpes simplex	11	17	0.4
Anthrax	11	14	0.23
Others (Ear pain, Eye pain, emergency, evil eye, evil spirit, impotency)	10	12	0.18
Cardiovascular (Diabetes mellitus, edema, hypertension, kidney disease, Swelling, stop bleeding, urinary retention)	14	14	0
Poisoning (snake and Spider)	4	4	0

Storage and packaging material for medicine

The majority of the participants used plastic bag (81 reports) followed by bottle (60 reports), fasten with clothes (27 reports), putting in cabinet (6 reports) and few of them use sack (3 reports) for storage and packaging of medicine after preparation.

Disease category and informant consensus factor

The most common disease the healers treat were gastrointestinal disease followed by skin disease and febrile illness (Table 2).

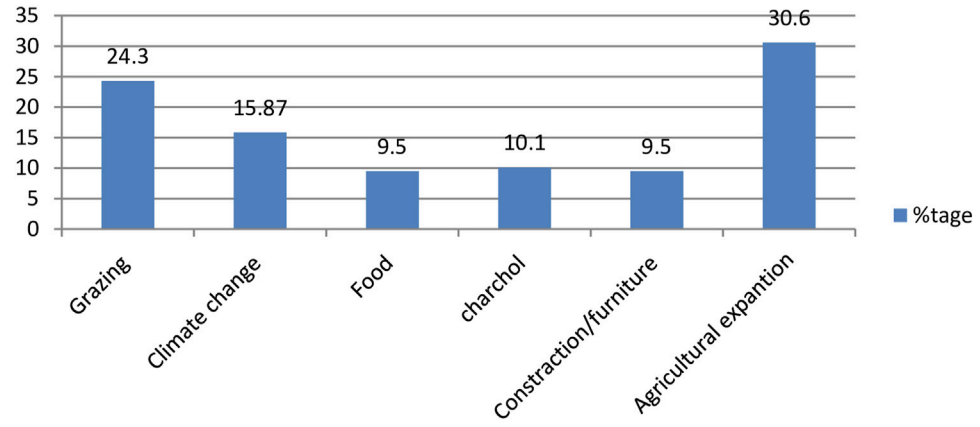

FIGURE 4
Treats to medicinal plant by traditional healers in West Shewa, Oromia, Ethiopia.

Duration of the medication storage after preparation and action taken for treatment failure

The mean of the expired date of the medication after preparation was 6.67 months with a range of 1 month–24 years. The majority of the participants (118, 89.6%) reported that their medicine is effective and would treat the patients. The rest of the participants 6.8% reported that would advise the patient to visit modern health facilities if the treatment failed and other said they will increase a dose. The figures cited here may not accurately represent reality, as it is based on self-reported data, and their validity could not be verified due to its nature.

Threats to medicinal plants

The major threat to medicinal plant in the study area was agricultural expansion which was reported by 30.6% of the respondents (Figure 4).

Discussions

More than two-thirds of the participants were male, and the majority of the participants were greater than 50 years old. The study conducted in Jimma Zone, Ethiopia, showed males were the majority who participated as traditional healers (Yineger and Yewhalaw, 2007). This may be due to the traditional knowledge in the community being passed from male parents to the firstborn (Bishaw, 1990). Moreover, the elders were the community members who had knowledge of medicinal plants, which was also in line with the study conducted by Chekole (Chekole, 2017). It was also reported in the study conducted in southern Brazil by Meretika et al. (Meretika et al., 2010) that the elders have greater knowledge of medicinal plants. In the present study, the majority of the participants obtained their knowledge of traditional medicine from family members. This was in line with the study conducted in the central zone of Tigray, which revealed that indigenous knowledge was only transferred to the selected family members (Gidey, 2010).

The study area was rich in medicinal plants (108 medicinal plants species) were identified and this showed still large number of the community member depends on the traditional medicinal plants for their healthcare needs. In the study area, Fabaceae was the most frequently cited medicinal plant family, followed by Asteraceae, Solanaceae, Lamiaceae, and Cucurbitaceae. This was in line with the study conducted in different parts of Ethiopia (Bekalo et al., 2009; Megersa et al., 2013; Alebie and Mehamed, 2016; Kebebew, 2016) and in other countries in Africa (Kambizi and Afolayan, 2001).

The leaves were the most frequently cited plant parts used, followed by the root. This agrees with other findings conducted in Ethiopia (Awas and Demissew, 2009; Eshete et al., 2016; Shimels et al., 2017; Weckmüller et al., 2019), as leaves were frequently plant parts used in the traditional remedy preparations. Because of the numerous potential dangers that surround them, plants have evolved defense mechanisms to fend off herbivores, pathogens (such as bacteria, viruses, fungus, nematodes, mites, and insects), and diverse abiotic stresses (Teklay et al., 2013). As a result, plants generate a variety of organic substances known as secondary metabolites, which are inherently unrelated to the growth and development of the plants (Piasecka et al., 2015). It may be argued that since the leaves are more exposed to the enemy, these compounds serve as a form of defense (Bartwal et al., 2013). Therefore, the use of leaves as a primary component of a plant in a medicine preparation could be regarded as a sign of scientific validity. It is also important to note that the discovery of several aliphatic medicines was historically attributed to the zoopharmacognostic approach, which involves the monitoring of animal self-medication behavior. This further supports the preference for using leaves as a source of TMs (Shurkin, 2014; Tuasha et al., 2018). The preference for leaves over other plant components may be attributed to the ease with which they can be prepared in comparison to medicine formulations from roots, stem barks, whole plants, and seeds.

The oral route of administration was the most commonly used way of administrating of medicinal plants in the study area, followed by topical administration. The finding regarding administration routes corresponds to the finding made by Mesfin et al. (Mesfin et al., 2013) and Tolossa et al. (Tolossa, 2007). The oral route of administration was significantly higher than other methods in the study community, most likely because the most prevalent disease in the area is associated with internal disorders such as stomachaches, intestinal parasites, tonsillitis, and others, for which oral administration was more efficient. Furthermore, oral administration may be connected with a significant contribution to a quick physiological response to the causative agents, enhancing the therapeutic potential of traditional medicinal herbs.

The fresh 74.9% was the most commonly used condition of medicinal plant. This was in line with the study conducted in different parts of Ethiopia (Gidey et al., 2011; Mengesha, 2016; Hordofa and DGNRA, 2017; Eshete and Molla, 2021).

Squeezing (25.8%) was the most commonly used method of preparation, followed by powder which accounts 19.6%. The finding was in line with the study conducted by (Getnet et al., 2016). However, unlike the present study, Mengesha et al. (Mengesha, 2016) and Fenetahun et al. (Fenetahun et al., 2017) reported crushing as the most commonly reported method of medicinal plant preparation.

The majority of the participants, 95.2%, prepare the medicine in combination with other medicinal plants or non-medicine ingredients. Out of the additives, 80.9% were added for its medicinal purposes. Non medicinal ingredients like water, honey, milk, and food were utilized in formulating the medicine. The findings of the present study was in line with the study conducted in Jimma Zone which reported most of the additives reported were medicinal plant which increase the effectiveness (Abera, 2003; Yineger and Yewhalaw, 2007).

The majority of the dried medicinal plants were stored in a plastic bag, followed by a bottle. This finding was in line with the study conducted by Chekole (Chekole, 2017) revealed that plastic bags and cloths were mainly used for the storage of dried medicinal plants. The other studies conducted in different districts in Ethiopia also revealed that plastic bags were used as a preservation method (Abera, 2003; Tesfaye and Erena, 2020; Kindie et al., 2021).

The most common diseases treated by medicinal plants reported by the participants were gastrointestinal diseases followed by skin diseases. This was in line with the study conducted by Teklehaymanot and Giday (Teklehaymanot and Giday, 2007) that showed the largest number of remedies were used to treat gastrointestinal disorder followed by external injuries (skin disorder). The other study conducted in Debrelibanos district also revealed that the highest number of plant species were used to treat gastrointestinal disorders like abdominal pain, intestinal parasites, and diarrhea (Seyoum and Zerihun, 2014).

The informant consensus factor indicates the agreements of the informants on the reported cures for the group of diseases (Teklehaymanot, 2009). The highest informant consensus factor reported for the present study was for tumors by *Cucumis dipsaceus and Jasminum abyssinicum*, which was 0.75, followed by rabies, which was 0.7. The ICF in the current study was relatively low as compared to the previous studies (Mesfin et al., 2009; Enyew et al., 2014). This may be because in the present study, the six districts have been included, which causes the variation in the agreement of medicinal plants used for specific diseases. The knowledge of medicinal plants varies from place to place because the knowledge is localized to a specific area.

The major threats to medicinal plants in the study area were agricultural expansion followed by animal grazing. The present study was in line with the study conducted in Horro Guduru that revealed agricultural expansion was the most common medicinal plants threats (Birhanu et al., 2015). The other studies conducted in different parts of Ethiopia also mentioned agricultural expansion as the main threat to medicinal plants (Mesfin et al., 2009; Bekele and Reddy, 2015; Wubetu et al., 2017). This is due to the rapid population growth in the country and decreasing productivity of the existing land for production. The loss of biodiversity diminishes the supply of raw materials for drug discovery and affects the spread of disease as a source of primary healthcare for 80 percent of the world's population (Alves and Rosa, 2007). Threats to traditional medicinal plant also cause disappearance of indigenous knowledge about the plants (Calixto, 2005).

In general, the present study helps to preserve the traditional knowledge of medicinal plants in the West Shewa Community. For the scientific community, it could be the basis for picking plant, which could be a candidate for drug discovery and development since many modern drugs originated from traditional medicines. It could also have contributed to the preservation of a culture by documenting the value of the medicinal plants in the studied communities. This study also provides economic opportunity for the communities of West Shewa and the healers those depends on the plants for their livelihood, and the community at large since they use them at an affordable cost in their area of residence.

Conclusion

The study area was rich in medicinal plants. A total of 51 families of medicinal plants with 108 species were identified. Fabaceae were the most common family used in the West Shewa zone. The traditional healers reported that leaves were commonly used as plant parts for the preparation of the medicine, and most of the traditional medicines prepared were given through oral route of administration. The traditional healers commonly used fresh plants for the preparation of medicine, and squeezing was the most common method of preparation. The major threat to the medicinal plant in the study area was agricultural expansion. Since this is the preliminary study, another study needs to be conducted on the efficacy and safety of the claimed medicinal plant. Additionally, standardization and determination of the dose of medicinal plant needs to be conducted.

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 humans were approved by Ethical Review Committee of the College of Medicine and Health Sciences of Ambo University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because the participants were both traditional healers and knowledgable people and some of them could not write and read. Therefore, we used verbal informed consent to tell us information about traditional medicine. If they are not volunteers they will not give any information.

Author contributions

TB: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing–original draft, Writing–review and editing. DG: Writing–original draft, Writing–review and editing, Conceptualization, Methodology, Resources, Software. SM: Investigation, Writing–original draft, Writing–review and editing, Supervision. GU: Writing–original draft, Writing–review and editing, Data curation, Investigation. LM: Data curation, Writing–original draft, Writing–review and editing. BG: Data curation, Writing–original draft, Writing–review and editing. GT: Writing–original draft, Writing–review and editing, Conceptualization, Investigation, Methodology, Resources, Software.

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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.

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Supplementary material

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Mimosa pudica L. aqueous extract protects mice against pilocarpine–picrotoxin kindling-induced temporal lobe epilepsy, oxidative stress, and alteration in GABAergic/cholinergic pathways and BDNF expression

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Ethnopharmacological studies revealed that the leaves and stems of *Mimosa pudica* L. (Fabaceae) are widely used for the treatment of epilepsy. This study sought to investigate the effects of the aqueous extract of *Mimosa pudica* leaves and stems against pilocarpine–picrotoxin kindling-induced temporal lobe epilepsy in mice and its implication on oxidative/nitrosative stress, GABAergic/cholinergic signalling, and brain-derived neurotrophic factor (BDNF) expression. The animals were treated for seven consecutive days as follows: one normal group and one negative control group that received orally distilled water; four test groups that received orally four doses of *Mimosa pudica* (20, 40, 80, and 160 mg/kg), respectively; and one positive control group that received 300 mg/kg sodium valproate intraperitoneally. One hour after the first treatment (first day), *status epilepticus* was induced by intraperitoneal injection of a single dose of pilocarpine (360 mg/kg). Then, 23 hours after the injection of pilocarpine to the mice, once again, they received their different treatments. Sixty minutes later, they were injected with a sub-convulsive dose of picrotoxin (1 mg/kg), and the anticonvulsant property of the extract was determined. On day 7, open-field, rotarod, and catalepsy tests were performed. Finally, the mice were sacrificed, and the hippocampi were isolated to quantify some biochemical markers of oxidative/nitrosative stress, GABAergic/cholinergic signalling, and BDNF levels in the hippocampus. *Mimosa pudica* extracts (160 mg/kg) significantly increased the latency time to *status epilepticus* by 70.91%. It significantly decreased the number of clonic and tonic seizures to 9.33 ±

1.03 and 5.00 ± 0.89, and their duration to 11.50 ± 2.07 and 6.83 ± 0.75 s, respectively. Exploratory behaviour, motor coordination, and catalepsy were significantly ameliorated, respectively, in the open-field, rotarod, and catalepsy tests. Pilocarpine–picrotoxin-induced alteration of oxidant–antioxidant balance, GABA-transaminase stability, acetylcholinesterase/butyrylcholinesterase activity, and neurogenesis were attenuated by the extract (80–160 mg/kg). This study showed that the aqueous extract of *Mimosa pudica* leaves and stems ameliorated epileptogenesis of temporal lobe epilepsy and could be used for the treatment of temporal lobe epilepsy.

KEYWORDS

***Mimosa pudica*, temporal lobe epilepsy, oxidative stress, GABAergic status, cholinergic status, brain-derived neurotrophic factors**

1 Introduction

Epileptic patients witness chronic neurological disorder characterized by recurrent unprovoked and spontaneous seizures, which is triggered by an abnormal electrical phenomenon in the brain (Kanner and Balabanov, 2002). Globally, an estimated 70 million individuals are affected by epilepsy (Tang et al., 2017), with sub-Saharan Africa (0.9%) and central Africa (6.0%) documenting the highest prevalence rates of epilepsy in Africa (Ba-Diop et al., 2014). The rate in Cameroon is 25–66/1,000 and might be the highest in the world, with a typical prevalence of 6.6% (Boullé et al., 2019).

During epileptic convulsions, the activation of voltage-gated glutamate receptors causes a significant augmentation in the level of Ca^{2+} and Na^{+} influxes, accompanied by the transport of Cl^{-} and H_2O across the neuronal membranes. Activation of glutamate receptors followed by excitotoxicity, including suppression of axon sprouting by inhibitory GABAergic interneurons, can generate an epiphenomenon related to spontaneous seizures (Kanaani et al., 2010; Morimoto et al., 2004). These processes lead to epileptogenesis causing development of oxidative/nitrosative stress, neuroinflammation, and brain-derived neurotropic factor (BDNF) expression (Grosso and Geronzi, 2014; Ignacio-Mejía et al., 2023; Wang et al., 2021).

Due to the complexity of epileptogenesis and epilepsy physiopathologies taken individually and the substitute of mechanisms implicated when these pathologies are progressing, their management is more than challenging. During the last few decades, increasing efforts have been made to overcome this complex syndrome. Antiepileptic drugs act by reducing the excitability of neuronal membranes by interacting with a number of neuromediators, receptors, and ion channels (Rossetti and Lowenstein, 2011). Unfortunately, drug management of temporal lobe epilepsy faces a significant therapeutic challenge despite the substantial progress made (Kwan et al., 2010; Simonato et al., 2014). Current antiepileptic drugs used against temporal lobe epilepsy neither provide a cure nor prevent relapse. In addition, antiepileptic drugs can neither stop neurodegeneration nor reverse the apoptosis and necrosis of neurons (Perucca and Gilliam, 2012). Hence, the development of a newer, safer, more effective and affordable pharmacological agent has become a major goal in epilepsy research.

Drugs/medicine stemmed from plants are a good alternative as they constitute a patent source of new metabolites, which are important to antagonise epileptogenesis, break the progression of epileptic seizures, and optimize therapeutic efficacy, with fewer side effects. The plant kingdom is a major target in the search for new drugs and lead compounds. In addition, herbal medicine remains the hope of about 80% of the population worldwide, mainly in developing countries (Taiwe et al., 2015; Taiwe et al., 2016a). Ethnopharmacological studies have revealed that the *Mimosa pudica* L. (Fabaceae) extract is used in traditional medicine for the treatment of epilepsy, anxiety, and infantile convulsions (Azmi et al., 2011; Bum et al., 2004; Majeed et al., 2021; Tripathi et al., 2022). Previous studies indicated that the decoction prepared from the leaves of *Mimosa pudica* administered orally to mice strongly protected against pentylenetetrazol- and strychnine-induced generalised clonic–tonic convulsions. It also had a potent anticonvulsant property against the turning behaviour and *exitus* induced by *N*-methyl-D-aspartate. Based on its traditional uses and previous findings, it should be interesting to investigate whether the *Mimosa pudica* aqueous extract could protect animals against epileptogenesis of epilepsy and, eventually, temporal lobe epilepsy (Bum et al., 2004). In addition, previous phytochemical studies showed that the *Mimosa pudica* aqueous extract contains some bioactive components such as terpenoids, flavonoids (quercetin-7-rhamnoside, luteolin 3, acacetin-7-rutinoside, and quercetin-3-glucoside-7-rhamnoside), glycosides, alkaloids, quinines, phenols, tannins, flavonoids, and saponins. Other studies demonstrated the presence of mimosine, crocetin dimethyl ester, fatty acids, and green oil (Joseph et al., 2013; Kiruba et al., 2011). The *Mimosa pudica* extract exerts anti-inflammatory, antinociceptive, and analgesic activities in rodents (Joseph et al., 2013; Khalid et al., 2011). Other studies have established antihyperglycemic, antivenomous, immunomodulatory, antihepatotoxic, diuretic, and antimalarial activities (Azmi et al., 2011; Baghel et al., 2013; Ganguly et al., 2007). However, no scientific evidence has been reported about the antiepileptogenic and anticonvulsant properties of *Mimosa pudica* extracts in the mouse model of temporal lobe epilepsy. Therefore, we hypothesised that the *Mimosa pudica* aqueous extract could antagonise *status epilepticus*, epileptogenesis, and epileptic seizures in this pharmacoresistant model. The overall objective of this research was to evaluate the anticonvulsant and antiepileptogenic properties of an aqueous extract of *Mimosa pudica* in the pilocarpine–picrotoxin model of temporal lobe epilepsy and investigate the effects of the aqueous extract of *Mimosa pudica* leaves and stems on some parameters of *status*

epilepticus and epileptic convulsions, oxidative and nitrosative stresses, GABAergic and cholinergic transmissions, and the expression of BDNF.

2 Materials and methods

2.1 Plant material and preparation of the *Mimosa pudica* aqueous extract

The leaves and stems of *Mimosa pudica* used in our study were harvested in Buea (July 2021), Fako division (the Southwest Region of Cameroon; harvesting coordinates 4°15′06″N and 9°29′03″E). The field studies did not involve protected species. The plant sample was authenticated by the National Herbarium of Yaoundé (Cameroon), where a voucher was deposited (sample number 54102/HNC).

The aqueous extract of *Mimosa pudica* leaves and stems was prepared by using a method similar to that of Cameroonian traditional healers. The leaves and stems of *Mimosa pudica* are the preferred part of the plant used in Cameroonian traditional medicine for treating epilepsy and infantile convulsions. According to traditional healers, the leaves and stems are usually harvested, sun dried, and pulverized to obtain powder. Approximately 100 g of the powdered material is macerated in 500 mL of water and boiled. The decoction obtained is administered orally to epileptic patients at the dose range of 40–160 mg/kg during or before the occurrence of epileptic seizures. Therefore, in our experiments, the leaves and stems of *Mimosa pudica* were cut into pieces and allowed to dry at room temperature (25°C). The dried leaves and stems were then reduced to fine particles. The powder (500 g) was boiled in 5,000 mL of distilled water for 20 min. After it cooled, the concoction was filtered with Whatman No. 1 filter paper. The collected filtrate (aqueous extract) was dried using a rotary evaporator. The aqueous extract of *Mimosa pudica* leaves and stems was prepared using distilled water at a concentration of 16 mg/mL and considered an initial concentration. The extract was prepared daily, 45 min to 1 h before its oral administration to mice using a non-flexible gavage needle with round end, and fixed at the extremity of a 1-mL syringe. The aqueous extract of *Mimosa pudica* leaves and stems was given 1 h before each pharmacological testing at a volume of 10 mL/kg, and the concentrations 2, 4, 8, and 16 mg/mL were used, respectively.

2.2 High-performance liquid chromatography analysis of the aqueous extract of *Mimosa pudica*

An amount of 10 mg of the sample (*Mimosa pudica* aqueous extract) was dissolved in 10 mL methanol. The solution was filtered through a membrane filter prior to high-performance liquid chromatography analysis. The sample was screened by means of an HPLC system (AKTA™ Purifier, Amersham Biosciences). The aqueous extract of *Mimosa pudica* was analysed by HPLC using a Vydac C18 column (4.6 × 250 mm, 5 µm particle size), and for elution of the compounds, a gradient of two solvents denoted A and B was employed. The mobile phases were 90% acetonitrile mixed with water (called A) and 0.1% trifluoroacetic acid mixed with water (called B). The flow rate used for this experiment was set up at 1.0 mL/min and used a volume of 10 µL as the injection volume. The retention time and UV spectrum of major peaks were analysed and compared with standard compounds. The eluant was monitored at 215 nm. Finally, the fractions eluted from the HPLC system were then combined in tubes and then lyophilized to afford the products as powders.

2.3 Chemicals and reagents

Acetylthiocholine iodide, Ellman's reagent, pilocarpine hydrochloride, picrotoxin, sodium valproate, vitamin C, NaCl, Tris-HCl, trichloroacetic acid, thiobarbituric acid and all the other reagents used for biochemical determination were purchased from Sigma-Aldrich, St Louis, United States.

2.4 Experimental animals and ethical consideration

Adult male mice, *Mus musculus* Swiss, weighing 25 ± 2 g, 2–3 months old, were used in this experiment. They were obtained from the National Veterinary Laboratory, Garoua, Cameroon, and acclimatized for 3 days (72 h) in the Life Science Laboratory of the University of Buea, Cameroon. The animals were housed in conventional cages (45 cm long, 45 cm wide, 25 cm high) at 25°C, on a 12/12-h light–dark cycle, with lights on at 06:00 h and off at 18:00 h. They were supplied with food and water *ad libitum*. All experiments were performed according to the Guide for the Care and Use of Laboratory Animal published by the United States National Institutes of Health (NIH publication No. 85-23, revised 1996). In addition, all the experiments were approved by the University of Buea–Institutional Animal Care and Use Committee (UB-IACUC) with the following permit number: UB-IACUC N 07/2022. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.5 Pharmacological testing

2.5.1 Acute pilocarpine-induced *status epilepticus* test

Mice were randomly grouped in different lots of six mice each and administered the following treatment: groups 1 (normal control group) and 2 (negative control group) received orally 10 mL/kg of distilled water; groups 3–6 received orally different doses of the *Mimosa pudica* aqueous extract (test groups; 20, 40, 80, and 160 mg/kg); and group 7 received sodium valproate (positive control group 1; 300 mg/kg). Forty minutes after the first administration of the different treatments to mice, they were injected intraperitoneally with N-methylscopolamine at a dose of 1 mg/kg to lower the peripheral effects of pilocarpine. Twenty minutes after the injection of N-methyl-scopolamine, *status epilepticus* was induced in each mouse by an acute intraperitoneal injection of 360 mg/kg pilocarpine (Moto et al., 2018; Nguezeye et al., 2023). Mice from the normal control group

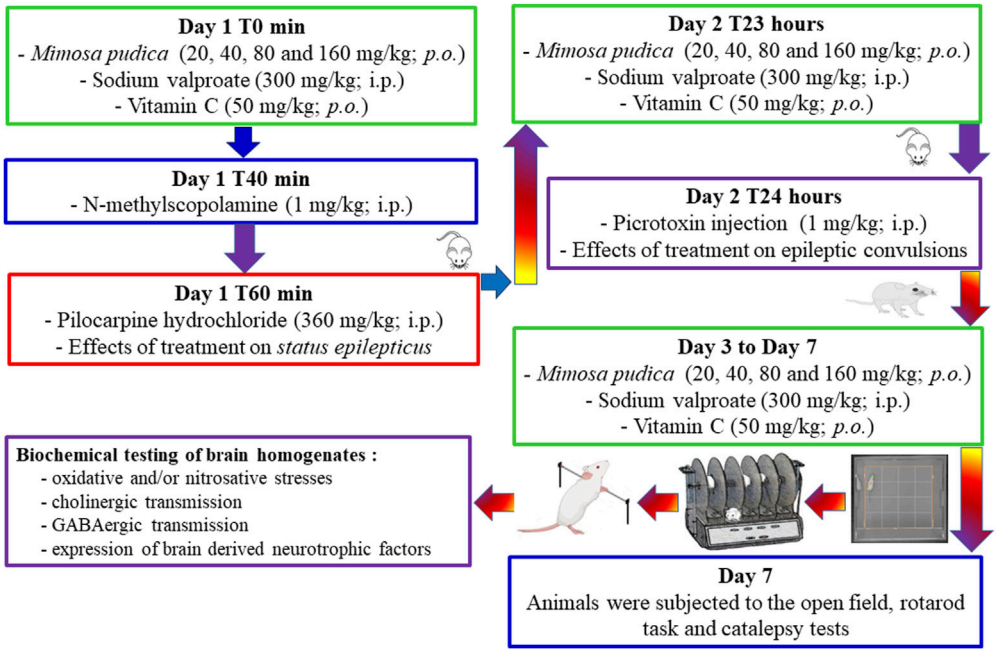

FIGURE 1
Schematic representation of the experimental design.

named group 1 did not receive N-methylscopolamine and pilocarpine; however they received saline. Immediately after the intraperitoneal injection of pilocarpine, each animal was returned to its cage, and their behaviour was observed for a 6-h duration. Severity and duration of acute epileptic seizures were categorised based on the Racine scale (Phelan et al., 2015). Hypoactivity, followed by behavioural changes (orofacial modifications, twitching of vibrissae, yawning, salivation, eye blinking, and tonic–clonic seizures), was observed in the studied animals. During the experiment, animals were video-monitored for the appearance of *status epilepticus*. The severity of seizures was assessed using the Racine scale: stage 0: no response; stage 1: hyperactivity and clonus of vibrissae; stage 2: shaking of the head and myoclonic jerks; stage 3: unilateral clonus of the forelimbs; stage 4: rearing and bilateral clonus of the forelimbs; stage 5: tonic–clonic seizures with the loss of the righting reflex. The animals were selected for further studies (day-second) based on the development of stage 5 attack of the Racine scale for two consecutive periods (Figure 1).

2.5.2 Anticonvulsant properties of *Mimosa pudica* aqueous extracts during the acute phase of temporal lobe epilepsy induced by picrotoxin in pilocarpine-treated mice

Excitus and convulsions were induced by picrotoxin in mice 24 h after the acute pilocarpine-induced *status epilepticus* test. This spontaneous neuropathology was facilitated by intraperitoneal injection of a sub-convulsive dose of picrotoxin (1 mg/kg) to mice. In brief, animals were treated for the second day, or 23 hours after the injection of pilocarpine, with distilled water for groups 1 and 2, the respective doses of the extracts for groups 3–6, and sodium valproate for group 7, respectively. One hour later, a sub-convulsive dose of picrotoxin (1 mg/kg) was injected intraperitoneally to mice (groups 2–7), except group 1 that was injected intraperitoneally with saline. Each animal was observed immediately for a period of 30 min, and the incidence of seizures (the latency time to first clonic seizure, latency time to first tonic seizure, the number of clonic seizures, duration of clonic seizures, the number of tonic seizures, and the duration of tonic seizures) was noted (Hamani and Mello, 1997; Nguezeye et al., 2023). Tonic–clonic seizures involve both tonic (a sudden stiffness or tension in the muscles of the arms, legs, or trunk) and clonic (twitching or hock-like jerks of a muscle or a group of muscles) phases of muscle activity. The latency of tonic–clonic seizures was used to determine the seizure score. This score was calculated according to the following formula: Score = 1- negative control group latency/test group or positive control group latency (Taiwe et al., 2016b).

2.5.3 Behavioural assessment

On day 7, mice were grouped into different lots of six mice each and administered the following treatment: groups 1 (normal control group) and 2 (negative control group) received 10 mL/kg of distilled water orally; groups 3–6 received different doses of the *Mimosa pudica* aqueous extract orally (test groups; 20, 40, 80, and 160 mg/kg); and group 7 received sodium valproate (positive control group 1; 300 mg/kg). One group of naïve mice (not pilocarpine-challenged mice) was added and treated orally with 50 mg/kg vitamin C, which served as a reference drug (positive control group) for the behavioural tests (exploration, locomotion, and motor coordination) (Martinc et al., 2014). One hour after the last daily administration of the different doses of the plant extracts or the standard drugs, mice were observed for 5 min in the open-field paradigm (day 7), 1 min on the rotating rod (day 7), and 5 min on a horizontal bar (day 7) (Figure 1).

2.5.3.1 Open-field test

Locomotor activity, exploration, and ambulatory behaviour were quantified by using the open-field paradigm (a wooden square box: 40 cm × 40 cm × 45 cm; with 16 smaller squares: 10 cm × 10 cm). Each animal was placed individually in the centre of the arena and allowed to explore the open arena freely (Taïwe et al., 2012). Each animal was observed in the open field to score crossing (the number of square floor units entered), rearing (the number of times the animal stood on its hind legs), grooming, defecation, and centre time, 1 h after administration of *Mimosa pudica* aqueous extracts (20, 40, 80, and 160 mg/kg), sodium valproate (300 mg/kg, i.p.), vitamin C (50 mg/kg, orally), or vehicle (10 mL/kg, p.o.). Mice were video-tracked and recorded using ANY-maze version 6.03 (Stoelting Company, Wood Dale, IL, United States). At the end of each experiment, faecal boli defecated was collected, and the entire apparatus was wiped with 95% ethanol prior to use and before subsequent tests to remove any scent clues left by the previous subject mouse.

2.5.3.2 Rotarod test

On day 7, 15 min after the open-field paradigm, the motor coordination test was used to measure any sign of neurotoxicity and locomotion of treated mice using the rotating rod method. An initial selection of animals was performed on the previous day of experiment excluding those that did not remain on the rotarod bar during a 1-min session each. The bar with a diameter of 2.50 cm was rotated at a constant speed of 12 revolutions per minute (Figure 1). The integrity of motor coordination was measured on the basis of the number of falls from the revolving bar in 1 min. During the test session itself, that is, after the oral administration of the different treatments, both the latency to fall from the rotating rod and the number of falls were determined (Taiwe et al., 2016a).

2.5.3.3 Catalepsy test

On day 7, 15 min after the rotarod test, catalepsy was evaluated according to the standard bar hanging procedure by placing each animal with both forelegs over a horizontal bar, elevated 4.5 cm from the floor (Waku et al., 2021). Catalepsy was considered finished when the forepaw touched the floor or when the animal climbed the bar. Measurement was done for a duration of 30 and 60 min 15 min after the rotarod test. The time during which the animal maintained the cataleptic position was determined for up to 5 min, with three attempts allowed to replace the mouse over the bar.

2.5.4 Biochemical analysis

Immediately after the last behavioural test, each animal was sacrificed; the brain was dissected and cleaned using the ice-cold saline solution (0.9%, w/v). The hippocampus was removed and weighed. Homogenates (10%, w/v) were prepared with ice-cold 0.1 M phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm for 15 min duration. The aliquots of the obtained supernatants were then collected and used to determine, according to different protocols, the level of each studied biochemical parameters.

2.5.4.1 Evaluation of the brain GABA concentration and determination of GABA-transaminase activity

Concentration of GABA in the brain homogenate was quantified as described previously (Sandoval-Salazar et al., 2016; Vega Rasgado et al., 2018), and the final GABA level in each homogenate was expressed in μg/g of wet tissue (Taïwe et al., 2010). The GABA-transaminase (GABA-T) activity was measured in the homogenates using the spectrophotometric method (Szyndler et al., 2006; Vega Rasgado et al., 2018).

2.5.4.2 Quantification of nitrosative and oxidant stress markers

Malondialdehyde (MDA), an indicator of lipid peroxidation, was determined with a spectrophotometer using thiobarbituric acid assay (Liu et al., 2012). Reduced glutathione (GSH, endogenous antioxidant) was quantified by its reaction with 5, 5′-dithiobis (2- nitrobenzoic acid) (Ellman's reagent) to yield a yellow chromophore (Rahman et al., 2006). The activity of superoxide dismutase (SOD) was assayed according to the method described by Sun et al. (Mesa-Herrera et al., 2019), while the nitrite level (NO) (indicator of nitric oxide production) was estimated using the method of Cortas and Wakid (Filgueiras et al., 2021).

2.5.4.3 Determination of the brain cholinergic status

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were quantified in the hippocampal homogenates, according to the method of Ellman (Ni et al., 2016). The kinetic profile of enzyme cholinesterase activities was determined using a spectrophotometer at 412 nm for 3 min at 30-s intervals. One unit of acetylcholinesterase or butyrylcholinesterase activity was defined as the number of micromoles (μmol) of acetylthiocholine iodide or butyrylthiocholine iodide hydrolysed per min per mg of protein.

2.5.4.4 Measurement of BDNF

The concentration of BDNF was determined using the Promega BDNF Emax® ImmunoAssay System kit (Madison, United States), as indicated by the manufacturer's protocol. In brief, the hippocampus was weighed, homogenized in lysis buffer, and centrifuged (12,000 × g, 4°C) for 5 min; then, the supernatants were collected. The relative concentration of BDNF was expressed as per milligram total protein in the tissue. Protein was measured as described previously (Augustyniak et al., 2015), using bovine serum albumin as the standard and measured in the range of 0.01–0.10 mg/mL.

2.6 Acute toxicity studies of the *Mimosa pudica* aqueous extract in naïve mice

The acute toxicity of the *Mimosa pudica* aqueous extract was determined using an established guideline (Abubakar et al., 2022; Erhirhie et al., 2018). The extract was given orally, at a dose of 5,000 mg/kg, to one female mouse under fasting for 8 h. Thereafter, the same dose of extract was administered to four female mice, giving a total of five animals at intervals of 48 h. One normal group of animals, which constituted of five female mice, received distilled water (10 mL/kg). Each animal was observed for 24 h immediately after the administration of the treatment, and then, they were further observed for up to

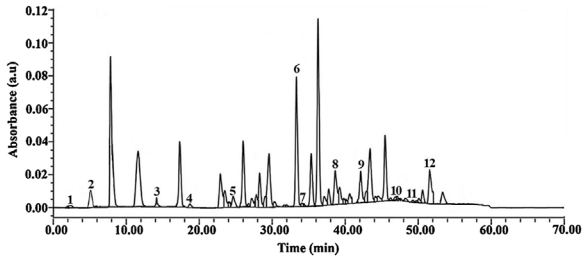

FIGURE 2
HPLC chromatogram of the *Mimosa pudica* aqueous extract showing peaks with similar retention time to the eluted compounds. 1: Gallic acid (2.15 min), 2: chlorogenic acid (5.09 min), 3: ferulic acid (14.05 min), 4: hyperoside (18.34 min), 5: luteolin (26.89 min), 6: fisetin (33.47 min) 7: apigenin7-glucoside (34.01 min), 8: naringenin (38.22 min), 9: benzene-triol (42.15 min), 10: apigenin (47.09 min), 11: chrysin (49.36 min), and 12: mimosine (51.83 min).

14 days for any signs of toxicity and deaths, as well as for the latency of death. Finally, the global harmonised system was used for the estimation of the lethal dose 50 (DL_{50}) of the *Mimosa pudica* aqueous extract.

2.7 Statistical analysis

Eight groups of animals were used in this study (seven groups for the anticonvulsant test and one group of animals were added during behavioural testing). Results were expressed as means ± standard error of mean (SEM), for six animals per group; statistical differences between controls and treated groups were tested by a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The differences were considered significant at $P < 0.05$. Statistical analyses were performed using GraphPad prism 9.0.0 (GraphPad Software, San Diego, CA, United States).

3 Results

3.1 Yield of extraction of the *Mimosa pudica* aqueous extract

After it cooled, the decoction prepared from *Mimosa pudica* leaves and stems was filtered with Whatman No. 1 filter paper. The collected filtrate (aqueous extract) was dried using a rotary evaporator, and a dry extract was obtained [yield of the extraction was 10.20% (w/w)].

3.2 High-performance liquid chromatography profile of the *Mimosa pudica* aqueous extract

The HPLC chromatograms of the *Mimosa pudica* aqueous extract showed the presence of some active compounds, and the majority of the components were eluted between 2.15 and 51.83 min, corresponding to 0.01–0.12 AU (Figure 2). The number of HPLC peaks (fractions) collected from the aqueous extract of *Mimosa pudica* was 12, corresponding respectively to 12 chemical compounds. When compared with standard compounds, some of them were elucidated: gallic acid (1), chlorogenic acid (2), ferulic acid (3), hyperoside (4), luteolin (5), fisetin (6) apigenin7-glucoside (7), naringenin (8), benzene-triol (9), apigenin (10) chrysin (11), and mimosine (12) (Figure 2).

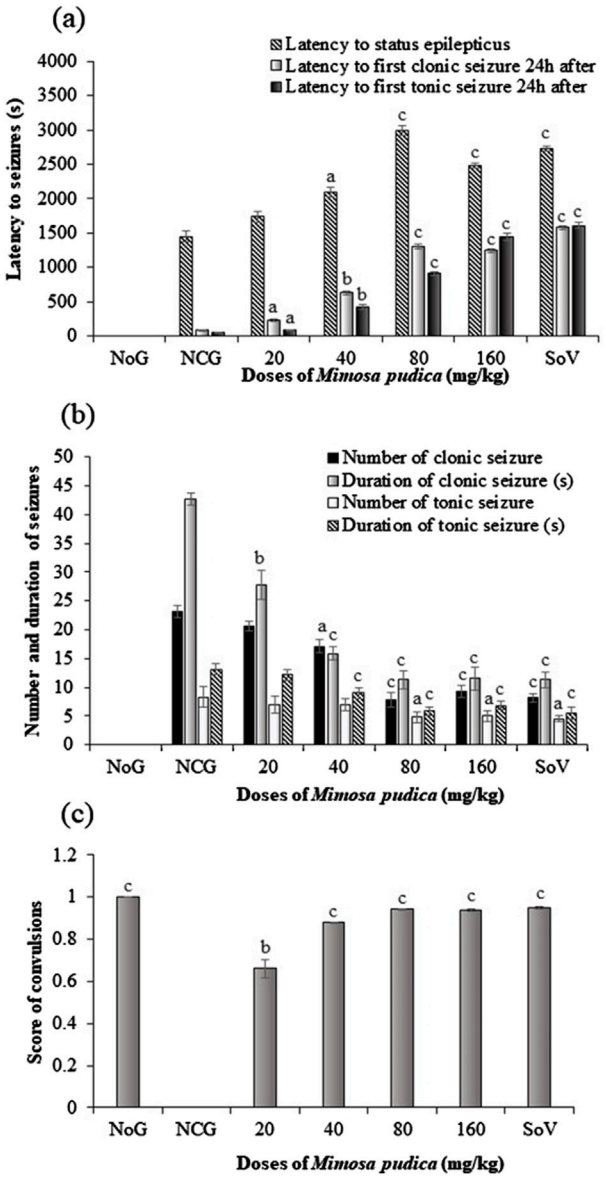

FIGURE 3
Effects of the *Mimosa pudica* aqueous extract on *status epilepticus* and convulsions induced by pilocarpine and picrotoxin (latency to *status epilepticus*, first clonic and tonic–clonic seizures 24 h after *status epilepticus* **(A)**, number and duration of clonic, tonic–clonic seizures **(B)**, and the score of generalized tonic–clonic seizures **(C)**. Results are expressed as mean ± S.E.M. n = 6 animals. Statistical differences were tested using one-way ANOVA, followed by Tukey's test (HSD) multiple comparison test. Significant difference set at $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$, versus the negative control group. NoG, normal group treated with distilled water; NCG, negative control group treated with distilled water; SoV, sodium valproate (300 mg/kg); s, second. All mice were subjected to epileptogenesis induced by pilocarpine (360 mg/kg) except the normal group.

3.3 Effects of the *Mimosa pudica* aqueous extract on *status epilepticus* and epileptic convulsions induced by pilocarpine and picrotoxin

Oral administration of the *Mimosa pudica* aqueous extract produced a significant difference [F (6, 35) = 14.01, p < 0.001] in the latency time to *status epilepticus* in mice (Figure 3A). The extract significantly increased the latency to *status epilepticus* from 1,451.00 ± 83.61 s in the distilled water-treated pilocarpine–picrotoxin group to 2,997.40 ± 72.45 s (P < 0.001) and 2,479.95 ± 34.83 (P < 0.001) s, in the tests administered 80 and 160 mg/kg, respectively. Similarly, 300 mg/kg sodium valproate significantly (P < 0.001) increased the latency to *status epilepticus* from 1,451.00 ± 83.61 s to 2,732.00 ± 35.81 s (P < 0.001) in the positive control group of mice.

One-way ANOVA revealed a significant difference in the effect of *Mimosa pudica* and pilocarpine–picrotoxin treatments [F (6, 35) = 52.59 p < 0.001] in the latency to first clonic seizure 24 h after *status epilepticus*. *Mimosa pudica* administration significantly (P < 0.001) delayed the latency to first clonic seizures to 1,301.54 ± 29.42 s (P < 0.001) and 1,243.04 ± 23.31 s for the groups of animals treated with the doses of 80 and 160 mg/kg, respectively, when compared with the negative control group (83.33 ± 7.45 s) (Figure 3A).

The standard antiepileptic drug like the *Mimosa pudica* extract significantly (p < 0.001) increased this latency time, as compared to the vehicle-treated pilocarpine–picrotoxin mice (Figure 3A). As shown in Figure 3A, one-way ANOVA revealed a significant effect of treatments [F (6, 35) = 47.91, P < 0.01] in the latency to the first clonic seizure 24 h after *status epilepticus*. Moreover, pilocarpine–picrotoxin injection significantly reduced this latency time to 50 ± 3.29 s in comparison with the normal control group of mice. However, pre-treatment of mice with the 40–160 mg/kg *Mimosa pudica* aqueous extract significantly increased the latency to first clonic seizure to 906.50 ± 31.78 s and 1,443.67 ± 50.87 s, respectively when compared with vehicle-treated pilocarpine–picrotoxin (Figure 3A).

One-way ANOVA revealed a significant effect of treatment in the number of clonic seizures [F (6, 35) = 477 p < 0.001] and duration [F (6, 35) = 466 p < 0.001] (Figure 3B). The number of clonic seizures decreased from 23.16 ± 0.98 in the negative control group to 7.83 ± 1.16 (P < 0.001) and 9.33 ± 1.03 (P < 0.001) in the groups of mice administered 80 and 160 mg/kg extract, respectively (Figure 3B). The duration of clonic seizure also decreased from 42.66 ± 1.03 s in the negative control group to 11.33 ± 1.63 s (P < 0.001) and to 11.55 ± 2.07 s (P < 0.001) in the group of mice administered with the doses of 80 and 160 mg/kg aqueous extract, respectively. Likewise, sodium valproate (300 mg/kg) decreased the number of clonic seizure and duration to 8.16 ± 0.75 (P < 0.001) s and 11.33 ± 1.36 s (P < 0.001), respectively, when compared to the vehicle-treated pilocarpine–picrotoxin mice (Figure 3B).

As shown in Figure 3B, one-way ANOVA revealed a significant effect of treatments for the number [F (6, 35) = 36.95 p < 0.001] and duration [F (6, 35) = 168.6 p < 0.001] of clonic seizures. The *Mimosa pudica* aqueous extract significantly reduced the number of clonic seizures form 8.33 ± 1.75 in the pilocarpine–picrotoxin model to 4.83 ± 0.98 (P < 0.001) and 5.00 ± 0.89 in the groups of mice administered 80 and 160 mg/kg aqueous extract, respectively. Interestingly, the extract also reduced the duration of clonic seizures from 13.16 ± 1.98 s in the pilocarpine–picrotoxin model to 5.83 ± 0.75 s (P < 0.001) and 6.83 ± 0.75 s (P < 0.001) at the dose 80 and 160 mg/kg *Mimosa pudica* extract, respectively. Obviously, the standard antiepileptic drug, sodium valproate (300 mg/kg), reduced the number of clonic seizure from 8.33 ± 1.75 s in the negative control group to 4.50 ± 0.54 s (P < 0.001) and its corresponding duration from 13.16 ± 0.98 s in the pilocarpine model to 5.50 ± 1.04 s (P < 0.001) (Figure 3B).

One-way ANOVA indicated a significant effect of treatments [F (6, 35) = 2,840 p < 0.001] in the score of clonic seizure. The score of clonic seizure increased from 0 in the distilled water-treated pilocarpine–picrotoxin mice to 0.92 ± 0.01 (P < 0.001) and 0.94 ± 0.01, in the groups administered 80 and 160 mg/kg *Mimosa pudica* respectively. Similarly, the antiepileptic drug sodium valproate (300 mg/kg) significantly increased (P < 0.001) the score of clonic seizures when compared to the negative control group (Figure 3C).

3.4 Effects of the *Mimosa pudica* aqueous extract on locomotion and exploratory behaviour

3.4.1 Effects of the *Mimosa pudica* aqueous extract on exploratory behaviour in the open-field test

As reported below (Table 1), administration of pilocarpine–picrotoxin in the negative control group of mice caused a reduction in the number of crossing, grooming, and time spent at the centre of the open field in comparison with those of the normal group. The *Mimosa pudica* aqueous extract increased the crossing (P < 0.001), grooming (P < 0.01), and time spent at the centre of the open field (P < 0.001) in the test groups treated with the plant extract. Sodium valproate (300 mg/kg, i.p.) and vitamin C (50 mg/kg, p.o.) also induced an increase in the crossing, grooming, and time spent in the centre. The number of rearing (P < 0.001) and faecal boli (P < 0.001) decreased when compared with the negative control groups, respectively. Likewise, mice treated with sodium valproate (300 mg/kg, i.p.) and vitamin C (50 mg/kg, p.o.) also expressed significant performance comparable to that of *Mimosa pudica*-treated pilocarpine–picrotoxin mice (Table 1).

3.4.2 Effects of the *Mimosa pudica* aqueous extract on locomotion in the rotarod test

A significant difference in the motor coordination of different groups of treated mice is shown in Table 2 with respect to time, that is, at 0 min [F (7, 40) = 19.88; P < 0.05], 30 min [F (7, 40) = 62.14; P < 0.01], and 60 min [F (7, 40) = 142.1; P < 0.001]. The time spent on the rotating bar in the normal group of mice was significantly higher than that seen in the negative control group at 0 min, 30 min, and 60 min. Similarly, the aqueous extracts of *Mimosa pudica* administered at the doses of 80 and 160 mg/kg significantly prolonged the time spent on the rotating bar when compared to the time registered in the negative control group (Table 2). In addition, the plant extract significantly reduced the number of falls at 0 min (P < 0.001), 30 min (P < 0.001), and 60 min (P <

TABLE 1 Effects of the *Mimosa pudica* aqueous extract on rearing, crossing, grooming, centre time, and quantity of faecal boli in the mouse activity of pilocarpine–picrotoxin-treated animals subjected to the open-field test.

Parameter	NoG	NCG	*Mimosa pudica* (mg/kg)				SoV (mg/kg)	VitC (mg/kg)
			20	40	80	160	300	50
Rearing	6.33 ± 1.00^{a}	10.83 ± 0.88	4.16 ± 0.83^{b}	3.66 ± 0.77^{b}	2.33 ± 0.66^{c}	1.83 ± 0.55^{c}	1.33 ± 0.44^{c}	1.50 ± 0.66^{c}
Crossing	9.16 ± 0.88^{b}	4.16 ± 0.83	14.16 ± 1.88^{c}	19.83 ± 1.16^{c}	26.83 ± 1.88^{c}	32.33 ± 1.33^{c}	34.16 ± 1.55^{c}	32.16 ± 5.83^{c}
Grooming	2.66 ± 0.44^{a}	1.16 ± 0.27	2.33 ± 0.44^{a}	3.16 ± 0.55^{b}	3.83 ± 0.88^{b}	4.16 ± 0.88^{b}	4.16 ± 0.83^{b}	4.33 ± 0.66^{b}
Faecal boli (g)	0.21 ± 0.06^{b}	0.64 ± 0.13	0.19 ± 0.02^{b}	0.06 ± 0.06^{c}	0.04 ± 0.05^{c}	0.03 ± 0.05^{c}	0.03 ± 0.04^{c}	0.03 ± 0.04^{c}
Centre time (s)	8.33 ± 1.11^{a}	3.83 ± 0.27	6.33 ± 1.55^{a}	21.16 ± 5.55^{c}	23.66 ± 1.55^{c}	27.66 ± 1.22^{c}	30.33 ± 1.33^{c}	35.66 ± 5.22^{c}

Results are expressed as mean ± S.E.M. n = 6 animals. Statistical differences were tested using one-way ANOVA, followed by Tukey's test (HSD). Significant difference set at $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$, versus the negative control group. NoG, normal group; NCG, negative control group treated with distilled water (10 mL/kg) and pilocarpine (360 mg/kg); SoV, sodium valproate (300 mg/kg); VitC, vitamin C (50 mg/kg).

TABLE 2 Effects of the *Mimosa pudica* aqueous extract on the locomotor activity of pilocarpine–picrotoxin-treated animals subjected to the rotarod test.

Treatment (mg/kg)	NoG	NCG	*Mimosa pudica* (mg/kg)				SoV (mg/kg)	VitC (mg/kg)
	--		20	40	80	160	300	50
Time on the bar								
0 min	52.55 ± 1.88^{b}	22.16 ± 4.83	35.66 ± 5.22	39.16 ± 3.16	46.33 ± 4.66^{a}	48.66 ± 2.66^{a}	49.33 ± 7.44^{a}	52.83 ± 2.88^{b}
30 min	55.33 ± 2.33^{b}	20.16 ± 2.83	40.16 ± 2.16^{a}	46.16 ± 3.22^{a}	48.83 ± 3.50^{a}	52.83 ± 3.16^{b}	54.16 ± 2.22^{b}	55.66 ± 2.33^{b}
60 min	60.00 ± 0.00^{c}	15.16 ± 0.83	49.16 ± 6.16^{c}	56.33 ± 4.22^{c}	59.83 ± 0.27^{c}	60.00 ± 0.00^{c}	60.00 ± 0.00^{c}	60.00 ± 0.00^{c}
Number of falls								
0 min	0.33 ± 0.44^{c}	3.83 ± 0.55	1.66 ± 0.44^{b}	1.83 ± 0.55^{b}	1.33 ± 0.44^{b}	1.16 ± 0.27^{c}	1.16 ± 0.27^{c}	1.16 ± 0.27^{c}
30 min	1.16 ± 0.27^{c}	3.66 ± 0.77	1.61 ± 0.66^{b}	1.33 ± 0.55b	1.16 ± 0.27^{c}	1.16 ± 0.61^{c}	0.83 ± 0.27^{c}	0.83 ± 0.27^{c}
60 min	0.00 ± 0.00^{c}	2.50 ± 0.50	1.33 ± 0.44^{b}	0.50 ± 0.50^{c}	0.16 ± 0.27^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}

Results are expressed as mean ± S.E.M. n = 6 animals. Statistical differences were tested by a one-way ANOVA, followed by Tukey's (HSD) multiple comparison test; the differences were considered significant at $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$, versus the negative control group. NoG, normal group; NCG, negative control group treated with distilled water (10 mL/kg) and pilocarpine (360 mg/kg); SoV, sodium valproate (300 mg/kg); VitC, vitamin C (50 mg/kg).

0.001), respectively, when compared with the negative control. The level of the number of falls in the distilled water-treated pilocarpine–picrotoxin mice was significantly higher than that observed in the distilled water-treated mice. The doses of 80 and 160 mg/kg reduced the fall at 30 and 60 min when compared to the negative control group. Likewise, mice treated with sodium valproate (300 mg/kg, i.p.) and vitamin C (50 mg/kg, *p.o.*) also expressed remarkable performance comparable to that of *Mimosa pudica*-treated pilocarpine–picrotoxin mice (Table 2).

3.4.3 Effects of the *Mimosa pudica* aqueous extract on motor coordination in the catalepsy test

Analysis of variance depicted a significant effect of oral administration of *Mimosa pudica* in pilocarpine-treated mice subjected to the catalepsy test at 30 min [F (7, 56) = 239.9; P < 0.001] and 60 min [F (7, 56) = 350.7; P < 0.001]. There was a significant reduction of catalepsy between the normal groups and the negative control group. *Mimosa pudica*, when administered at the respective doses 80 and 160 mg/kg, triggered catalepsy at 30 min (63.75 ± 2.25 and 74.25 ± 3.31 s) and 60 min (105.74 ± 6.00 and 111.87 ± 10.81 s) in animals (Figure 4). The effects of *Mimosa pudica* were similar to those of 300 mg/kg sodium valproate (158.75 ± 12.81 and 177.62 ± 14.37 s, respectively, at 30 and 60 min) and vitamin C (50 mg/kg, given orally; 124.12 ± 2.12 and 146.87 ± 3.62 s, respectively, at 30 and 60 min) (Figure 4).

3.5 Effects of the *Mimosa pudica* aqueous extract on biochemical parameters

3.5.1 Effects of the *Mimosa pudica* aqueous extract on the level of GABA and the activities of GABA-transaminase, acetylcholinesterase, and butyrylcholinesterase

A significant difference was observed in the levels of GABA in the hippocampus of all groups of mice [F (7, 40) = 273.3; P < 0.01] (Table 3). GABA levels were significantly lower in the negative control group than those observed in the normal group. The plant extract significantly increased this concentration by 40.15% and 47.23% at the doses of 80 and 160 mg/kg, respectively. Obviously, sodium valproate and vitamin C also significantly increased the level of GABA by 46.74% and 47.62% in the hippocampus, at the doses of 300 and 50 mg/kg, respectively.

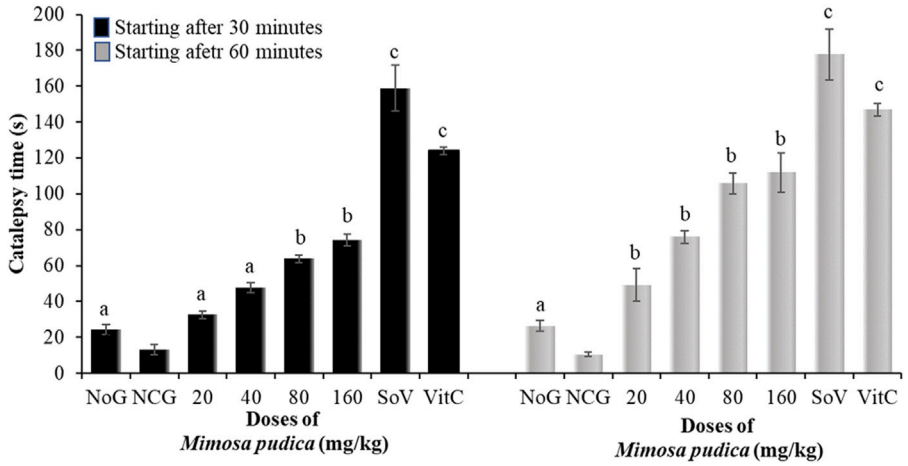

FIGURE 4
Effects of the *Mimosa pudica* aqueous extract on the motor coordination of pilocarpine–picrotoxin-treated mice subjected to the catalepsy test. Results are expressed as mean ± S.E.M. n = 6 animals. Statistical differences were tested by a one-way ANOVA, followed by Tukey's (HSD) multiple comparison test; the differences were considered significant at $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$, versus the negative control group. NoG, normal group treated with distilled water; NCG, negative control group treated with distilled water; SoV, sodium valproate (300 mg/kg); VitC, vitamin C (50 mg/kg); s, second. All mice were subjected to epileptogenesis induced by pilocarpine (360 mg/kg) except the normal group.

TABLE 3 Effects of the *Mimosa pudica* aqueous extract on GABAergic and cholinergic neurotransmission in the hippocampus of pilocarpine–picrotoxin-treated mice.

Treatment (mg/kg)	NoG	NCG	*Mimosa pudica* (mg/kg)				SoV (mg/kg)	VitC (mg/kg)
	--		20	40	80	160	300	50
GABA (µg/g de tissue)	394.67 ± 1.33^{b}	265.67 ± 6.67	273.17 ± 4.17	304.83 ± 4.11^{a}	372.33 ± 10.67^{b}	391.17 ± 4.83^{b}	389.83 ± 5.56^{b}	392.17 ± 5.06^{b}
GABA-T (pg/min/mg de tissue)	42.33 ± 0.44^{b}	105.83 ± 5.17	79.67 ± 4.89^{a}	68.83 ± 7.17^{a}	54.83 ± 3.5^{b}	42.33 ± 3.89^{b}	40.17 ± 1.83^{b}	45.17 ± 1.89^{b}
AchE (µmol/min/mg de tissue)	12.51 ± 1.08^{a}	16.61 ± 0.68	15.42 ± 0.58	13.37 ± 0.93^{a}	12.33 ± 1.03^{a}	12.37 ± 0.93^{a}	12.87 ± 0.88^{a}	12.05 ± 0.80^{a}
BchE (µmol/min/mg of tissue)	11.87 ± 0.87^{a}	17.54 ± 0.75	15.87 ± 0.91	13.89 ± 1.01^{a}	11.638 ± 0.63^{a}	11.93 ± 0.78^{a}	11.92 ± 1.03^{a}	11.01 ± 0.53^{a}

Results are expressed as mean ± S.E.M. n = 6 animals. Statistical differences were tested using one-way ANOVA, followed by Tukey's test (HSD). Significant difference set at $^{a}P < 0.05$, $^{b}P<0.01$, and $^{c}P < 0.001$, versus the negative control group. NoG, normal group; NCG, negative control group treated with distilled water (10 mL/kg) and pilocarpine (360 mg/kg); SoV, sodium valproate (300 mg/kg); VitC, vitamin C (50 mg/kg); GABA, gamma amino butyric acid; GABA-T, gamma amino butyric acid transaminase; AchE, acetylcholinesterase; BchE, butyrylcholinesterase.

Oral administration of the *Mimosa pudica* aqueous extract in mice significantly decreased the activity of GABA-transaminase [$F(7, 40) = 118.1$; $P < 0.01$] in the hippocampus. The plant extract significantly inhibited this activity by 48.19% and 60.01% at the doses of 80 and 160 mg/kg, respectively. The effects of the *Mimosa pudica* aqueous extract were similar to those of sodium valproate (300 mg/kg; 62.05% inhibition) and vitamin C (50 mg/kg; 57.32% inhibition).

During the early phases of pilocarpine–picrotoxin-induced seizures, cholinergic neurotransmission is further stimulated and excitotoxicity triggered. Oral administration of the *Mimosa pudica* aqueous extract significantly inhibited ($p < 0.05$) the activity of acetylcholinesterase, which was inhibited by 25.77% and 25.44% at the doses of 80 and 160 mg/kg, respectively (Table 3). This inhibitory action was similar to that of sodium valproate (300 mg/kg; 22.42% inhibition) and vitamin C (50 mg/kg; 27.39% inhibition) (Table 3). The plant extract significantly inhibited ($p < 0.05$) the activity of butyrylcholinesterase by 33.67% and 31.96% at the doses of 80 and 160 mg/kg, respectively. This inhibitory activity was similar to that of 300 mg/kg sodium valproate (32.03% inhibition) or 50 mg/kg vitamin C (37.23% inhibition) (Table 3).

3.5.2 Effects of the *Mimosa pudica* aqueous extract on the levels of MDA, GSH, and NO and the activities of CAT and SOD

As shown in Table 4, the levels of MDA in the brain were strongly increased in the negative control group of mice compared to the normal group. *Mimosa pudica* induced a sustained and dose-related antioxidant effect by inhibiting the MDA production [$F(7, 40) = 81.71$; $P < 0.001$] (Table 4). The calculated results revealed that the brain MDA level in the negative control group was 0.48 ± 0.01 µmol/g. The brain MDA levels were significantly decreased to 0.24 ± 0.01 µmol/g ($P < 0.05$) and 0.18 ± 0.03 µmol/g, ($P < 0.01$),

TABLE 4 Effects of the *Mimosa pudica* aqueous extract on the concentrations of MDA, GSH, and NO and the activities of CAT and SOD of pilocarpine–picrotoxin-treated mice.

Parameter	NoG	NCG	*Mimosa pudica* (mg/kg)				SoV (mg/kg)	VitC (mg/kg)
			20	40	80	160	300	50
MDA (μmol/g)	0.18 ± 0.02^{b}	0.48 ± 0.01	0.34 ± 0.02	0.27 ± 0.02^{a}	0.24 ± 0.01^{a}	0.18 ± 0.03^{b}	0.19 ± 0.01^{b}	0.16 ± 0.01^{b}
NO (μmol/mg protein)	1.28 ± 0.36^{b}	2.97 ± 0.12	2.73 ± 0.47	2.48 ± 0.42	1.50 ± 0.14^{a}	1.26 ± 0.36^{b}	1.25 ± 0.42^{b}	1.21 ± 0.33^{b}
GSH (μmol/mg protein)	7.86 ± 0.46^{c}	2.72 ± 0.62	3.86 ± 0.58^{a}	4.33 ± 0.78^{b}	7.04 ± 1.23^{c}	7.99 ± 0.65^{c}	8.22 ± 1.17^{c}	8.03 ± 0.69^{c}
SOD (U/mg protein)	16.71 ± 1.49^{a}	12.95 ± 0.97	13.36 ± 1.64	14.79 ± 0.77	15.82 ± 1.35^{a}	15.82 ± 1.20^{a}	15.26 ± 1.43^{a}	17.64 ± 2.00^{a}
CAT (U/mg protein)	0.28 ± 0.06	0.17 ± 0.03	0.27 ± 0.00	0.26 ± 0.03	0.30 ± 0.05^{a}	0.31 ± 0.03^{a}	0.30 ± 0.06^{a}	0.31 ± 0.06^{a}

Results are expressed as mean ± S.E.M. n = 6 animals. Statistical differences were tested using one-way ANOVA, followed by Tukey's (HSD) multiple comparison test. Significant difference set at $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ versus the negative control group. NCG, negative control group received distilled water. NoG, normal group received distilled water; SoV, sodium valproate; VitC, vitamin C; MDA, malondialdehyde; NO, nitric oxide; SOD, superoxide dismutase; GSH, glutathione; CAT, catalase. All mice were subjected to epileptogenesis induced by pilocarpine (360 mg/kg) except the normal group. *M. pudica*, *Mimosa pudica*.

respectively, for 80 and 160 mg/kg extract-treated groups. Similarly, sodium valproate at 300 mg/kg decreased MDA levels from 0.48 ± 0.01 μmol/g in the negative control group to 0.19 ± 0.01 μmol/g same as with vitamin C at 50 mg/kg, where it decreased from 0.48 ± 0.01 to 0.16 ± 0.01 μmol/g, ($P < 0.01$) (Table 4).

As shown in Table 4, the brain of both the positive control group of mice and animals treated with the *Mimosa pudica* aqueous extract presented a constant decrease in NO activity [$F(7, 40) = 16.09$; $P < 0.001$]. The brain of the animal receiving the *Mimosa pudica* aqueous extract at doses of 80 mg/kg (1.50 ± 0.14) and 160 mg/kg (1.26 ± 0.36) μmol/mg protein ($P < 0.01$) showed a significant decrease in the NO concentration at the end of the test when compared to control mice, which is 2.97 ± 0.12 μmol/mg protein. Similarly, sodium valproate and vitamin C administered at doses of 300 mg/kg and 50 mg/kg decreased the brain NO activity from 2.97 ± 0.12 μmol/mg protein in the negative control group to 1.25 ± 0.42 and 1.21 ± 0.33 μmol/mg protein ($P < 0.01$), respectively.

Table 4 presents the level of brain GSH. The brain GSH was significantly lower ($p < 0.001$) in the negative control group administered with pilocarpine–picrotoxin (2.80 ± 0.62 μmol/mg protein) when compared to that of the normal group (7.87 ± 0.47 μmol/mg protein). The estimation of glutathione activity revealed that at doses of 80 and 160 mg/kg, the *Mimosa pudica* aqueous extract significantly optimised the production of brain GSH to 7.04 ± 1.23 and 7.99 ± 0.65 μmol/mg protein ($P < 0.001$), respectively, in comparison with the low production of 2.72 ± 0.62 μmol/mg protein in the parasitized treated group of mice. Likewise, sodium valproate at a dose of 300 mg/kg alone also caused a significant increase of 8.22 ± 1.17 μmol/mg protein ($P < 0.001$), similar to that of vitamin C of 8.03 ± 0.69 μmol/mg protein ($P < 0.001$).

The report of the SOD activity shows that the aqueous extract *Mimosa pudica* acted as a spontaneous stimulant of antioxidant activity [$F(7, 40) = 4.49$; $P < 0.01$]. Pre-treatment of mice with graded doses of the aqueous extracts significantly triggered an increase in SOD activity in 80 mg/kg (15.82 ± 1.35) and 160 mg/kg (15.82 ± 1.20) U/mg protein, ($P < 0.05$) in opposition to the control animals, where the production was instead retarded with a maximum at 12.95 ± 0.97. In accordance with the extract-treated groups, sodium valproate at 300 mg/kg alone and vitamin C also triggered a significant increase to 15.26 ± 1.43 and 17.64 ± 2.00 U/mg protein ($P < 0.05$), respectively (Table 4).

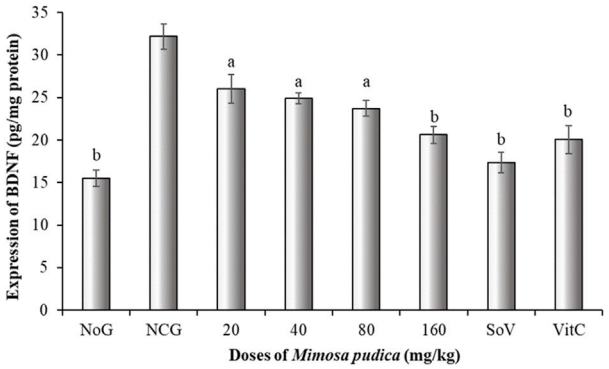

FIGURE 5
Effects of the *Mimosa pudica* aqueous extract on brain-derived neuro factor expression of pilocarpine–picrotoxin-treated mice. Results are expressed as mean ± S.E.M. n = 6 animals. Statistical differences were tested by a one-way ANOVA, followed by Tukey's (HSD) multiple comparison test; the differences were considered significant at $^{a}P < 0.05$ and $^{b}P < 0.01$, versus the negative control group. NoG, normal group treated with distilled water; NCG, negative control group treated with distilled water; SoV, sodium valproate (300 mg/kg); VitC: vitamin C (50 mg/kg). All mice were subjected to epileptogenesis induced by pilocarpine (360 mg/kg) except the normal group.

A significant augmentation in the brain CAT activity [$F(7, 40) = 4,08$; $P < 0.01$] in the animals treated with the aqueous extract of *Mimosa pudica* (80 mg/kg, p.o.) [0.30 ± 0.05 U/mg protein, $P < 0.05$], (160 mg/kg, p.o.) [0.31 ± 0.03 U/mg protein, $P < 0.05$], vitamin C (50 mg/kg, p.o.) [0.30 ± 0.06 U/mg protein, $P < 0.05$], and sodium valproate (300 mg/kg, i.p.) [0.31 ± 0.06 U/mg protein, $P < 0.05$] was observed 1 h after oral administration (Table 4).

3.5.3 Effects of the *Mimosa pudica* aqueous extract on the brain-derived neuro factor

The negative control group of mice expressed an increment of BDNF expression. Surprisingly, administration of *Mimosa pudica* extracts exerted a significant inhibitory effect by slowing BDNF production in the brain [$F(7, 40) = 67.54$; $P < 0.001$] (Figure 5). The

TABLE 5 Acute toxicity of the *Mimosa pudica* aqueous extract administered orally to different groups of female and male mice.

Treatment	Dose (mg/kg)	Toxicity signs	Mortality latency (h)	D/T mice	
				Male	Female
Distilled water		None	—	0/3	0/3
Mimosa pudica	500	None	—	0/3	0/3
	1,000	None	—	0/3	0/3
	2,000	None	—	0/3	0/3
	5,000	None	—	0/3	0/3

D/T, dead/treated mice; None, no toxic symptoms during the observation period; mortality latency, time to death after the oral administration. Control group received distilled water (10 mL/kg, *per os*). D/T, death/treated mice; *M. pudica*, *Mimosa pudica*.

estimation of BDNF levels revealed that at doses of 80 and 160 mg/kg, the *Mimosa pudica* aqueous extract significantly slowed BDNF production to 23.72 ± 0.90 and 20.61 ± 1.01 pg/mg protein, respectively, in comparison to the high-production 32.17 ± 1.47 pg/mg protein in the negative control group of mice. Likewise, sodium valproate at 300 mg/kg alone also induced a significant reduction of this concentration to 17.33 ± 1.18 pg/mg protein ($P < 0.01$), as well as vitamin C, where the concentration was 20.05 ± 1.66 pg/mg protein ($P < 0.01$) (Figure 5).

3.6 Acute oral toxicity study of the *Mimosa pudica* aqueous extract in naive mice

The *Mimosa pudica* aqueous extract was administered to two groups of mice (female and male). Mice in each group were carefully examined for any signs of toxicity (behavioural changes and mortality) for 14 days (Table 5). There were no deaths or any signs of toxicity observed after oral administration of single doses of extract at any dose level up to the highest dose tested (5,000 mg/kg). This did not allow us to determine LD_{50}.

4 Discussion

Epilepsy strongly alters the living condition of individuals worldwide and particularly in the emerging state (Tang et al., 2017). Epilepsy was induced artificially in the laboratory by intraperitoneal injection with pilocarpine in mice at 360 mg/kg, a dose that triggers seizures. The derived metabolites existing in natural products are greatly valorised for their prophylactic and therapeutic actions against epilepsy. This study was elaborated to assess the anticonvulsant and antiepileptogenic properties of an aqueous extract of *Mimosa pudica* in mouse model epilepsy: oxidative stress metabolism pathways, cholinergic, GABAergic, and BDNF signalling. The outcome of the anticonvulsant and antiepileptogenic test performed on mice pre-treated orally with the *Mimosa pudica* aqueous extract (80 and 160 mg/kg) and the intraperitoneal injection with pilocarpine–picrotoxin depicted that the extract did not alter the occurrence of seizures; nevertheless, the extract considerably attenuated seizure development in mice through the increment of latency to the *status epilepticus,* 60 min post-treatment. Small quantities of picrotoxin were administered to mice 24 h after pilocarpine injection to reactivate pilocarpine and reinforce convulsion. Once again, the treatment raised the time of onset of first clonic seizures and generalized clonic–tonic seizures, as well as the decrement of the number and duration of clonic seizures and generalized tonic–clonic seizure. Moreover, the extract significantly increased the score of generalized tonic–clonic seizures. This effect is analogous to that of sodium valproate, the typical antiepileptic remedy. Sodium valproate is capable to raise the brain GABA content via the obstruction of GABA reuptake, the blockage of GABA transaminase, and the stimulation of GABA decarboxylase (Davis et al., 2000; Hoffmann et al., 2008). Medication that suppresses pilocarpine–picrotoxin-induced convulsions usually relieve temporal lobe epilepsy (Arshad and Naegele, 2020). It can be suggested that the antiepileptogenic and anticonvulsant properties of the plant extract could be mediated by the GABA receptor complex and by enhancing GABA receptor neurotransmission (Greenfield, 2013; Silverman, 2018). Furthermore, the presence of anticonvulsant metabolites such as flavonoids in the extract could explain this effect. With respect to neurological disorders, including infantile convulsions, temporal lobe epilepsy, generalised tonic–clonic epilepsy, many flavonoids are known to provide anticonvulsant and antiepileptogenic activities (Kwon et al., 2019). Emerging evidence suggests that the beneficial effects of flavonoids on epileptic seizures may be associated with modulation of the GABA complex receptors (Hanrahan et al., 2015; Wasowski and Marder, 2012).

The open-field test displayed that the aqueous extract triggered an intensification of crossing, grooming, and the motionless interval at the middle of the open field and declined the number of rearing and faecal boli defecated, establishing an upgrading locomotory and exploratory behaviour in mice improving the locomotor activity (Augustsson, 2004; Bum et al., 2009; Moto et al., 2013).

The rotarod test was conducted in mice, and the results showed that the administration of the *Mimosa pudica* aqueous extract (80–160 mg/kg) impressively prolonged the period of immobilization on the rotarod bar and diminished the recurrences of falls. This behavioural adaptation reveals the capacity of the plant extract to prevent animals from any motor dysfunction (Botton et al., 2010).

The outcome of the catalepsy test revealed that the latency to the initiation of voluntary movement was greatly enhanced, extending the duration of akinesia and other major tranquilising processes. These observations corroborated that the daily administration of the

Mimosa pudica aqueous extract induced a sedative effect, as observed in the incapacity and inability of the mice to initiate motor action (Taiwe et al., 2016a).

The major hostile effect of pilocarpine-induced seizures is the decline of GABA levels and proliferation of GABA-transaminase activity (Jobin and Paulose, 2010). Epilepsy propagation is shut down by GABA, an inhibitory neurotransmitter used as an index of physiological and pathological statuses in the brain. However, this inhibitory effect of GABA is antagonised by GABA-transaminase, which hydrolyses GABA in the synapse (Ding et al., 2011; Zhang et al., 2006). Oral administration of the *Mimosa pudica* aqueous extract significantly improved the brain GABA levels and limited the activity of GABA-transaminase, which suggested an anticonvulsant activity of the extracts (Hosking and Roff Hilton, 2002; Mason et al., 2001). This effect of *Mimosa pudica* could be interrelated to the existence of secondary metabolites in the extract stimulating the $GABA_A$ receptor complex to alleviate GABA-transaminase activity and activate glutamic acid decarboxylase to synthetise GABA, or inhibition of GABA reuptake, thereby promoting GABAergic neurotransmission in the brain (Hashimoto et al., 2006; Kanaani et al., 2010).

Motor and locomotion incoordination, memory deficit, and exploratory disorders originate from a decrease in the number of brain cells in the hippocampal CA1 region (Matilla et al., 1998). In cholinergic neurotransmission, acetylcholinesterase (AchE) constitutes a neurochemical implicated in decrease retention, and its overload is associated with memory impairment (Bachurin et al., 2017). The AchE mechanism of action consists of enzymatic degradation of acetylcholine (Ach) into acetic acid and choline, which are less active in the memory process and exploratory behaviour, resulting in cognitive deficiency (Blake et al., 2014). In the pilocarpine-induced motor and locomotion incoordination, the acetylcholinesterase and butyrylcholinesterase levels rise considerably. Interestingly, the results obtained from this study indicated that *Mimosa pudica* aqueous extracts inhibited the activities of AchE and BchE. In this regard, we could assume that the aqueous extract possesses a compound that interferes with the cholinergic and noradrenergic systems (Botton et al., 2010; Koutseff, 2011), as well as the serotonergic and glutamatergic systems (Horiguchi et al., 2011). Thus, inhibiting the acetylcholinesterase activity slows the hydrolysis of acetylcholine and increases its availability in the synapse, thereby multiplying its binding on the post-synaptic membrane receptors at the level of the cerebral cortex and the hippocampus, prolonging and amplifying its cholinergic neurotransmission to facilitate learning, memory, and exploratory behaviour observed in the open-field paradigm (Bordet, 2003; Viaules, 2016).

Epilepsy is a disproportionate electrical firing of neurons associated with unstable atom production, leading to oxidative stress (Sejima et al., 1997; Waldbaum and Patel, 2010). Pilocarpine–picrotoxin-induced convulsions boost the oxidative stress biomarker activity such as MDA levels and NO in the hippocampus of mice. Lipid peroxidation results in MDA release, which the overload of this marker serves as a sign of oxidative stress and cell denaturation by degradation of biomolecules such as lipids, proteins, and nucleic acids (Ding et al., 2011; Shin et al., 2011), while a decrease serves as a sign of antioxidant effects of the extract. A study on quercetin demonstrated that it possesses antioxidant effects by slowing ROS formation during oxidation of polyunsaturated fatty acids, thus limiting cell apoptosis and their adverse effects (Suematsu et al., 2011). Our finding disclosed that the *Mimosa pudica* aqueous extract powerfully detoxified MDA by reducing its level in plasma, suggesting that the antioxidant effect of the plant extract could be mediated by the quercetin compounds.

Nitric oxide is a free radical in living organisms used as an index of oxidative stress. Inductors of epilepsy stimulate NMDA receptors to liberation calcium, which, in turn, enhances neuronal NOS expression via the stimulation of calcium–calmodulin pathways. Under the influence of monooxygenase contained in neuronal nitric oxide synthetase (nNOS) and in the absence of retro-control, L-arginine will be oxidised into L-citruline, which, in turn, participates in the formation of nitric oxide (NO) radicals (Förstermann and Sessa, 2012; Meffert, 1997). Pilocarpine strongly boosted the production of NO radicals; pre-treatment of mice with the *Mimosa pudica* aqueous extract drastically slowed the formation of NO radicals. This attenuating action could be an indicator that the extract is partially capable of scavenging NOS or suppressing their formation (Puttachary et al., 2015; Yao et al., 2022).

The cytosolic thiol glutathione (GSH), a non-enzymatic antioxidant, when it gets converted to the oxidized form, catalyses the reduction of hydrogen peroxide and improves its viability and, therefore, prevents the formation of hydroxyl radicals (Lushchak, 2012). The decreased concentration of GSH is an indicator of lipid peroxidation. Our plant extracts significantly accelerate the production of GSH in mice. From this finding, it can be suggested that the antioxidant effects of the *Mimosa pudica* aqueous extract are due to the existence of bioactive compounds that mimic the sodium valproate mechanism of action.

SOD is an enzymatic antioxidant which interacts with free radicals, especially superoxide, by catalysing its conversion into hydrogen peroxide to limit its accumulation, thus preventing their detrimental effect and the damage of cells (Ono et al., 2000; Schulz et al., 2000). The pilocarpine-injected mice showed a drastic decrease in the SOD activity. The outcome of the treatment with the plant extract is the restoration of the initial level of SOD activity in the brain tissues of pilocarpine-injected mice, thus enhancing the protection of cell membranes (Freitas, 2009; Freitas et al., 2005; Wang et al., 2009).

The brain, with its high oxygen consumption and the high level of lipid peroxidation, is extremely susceptible to oxidative stress. Catalase stands out as a paramount enzymatic antioxidant. It effectively catalyses the breakdown of hydrogen peroxide into water and oxygen, a potentially harmful byproduct of cellular metabolism. This reaction detoxifies hydrogen peroxide and prevents oxidative damage (Anwar et al., 2024; Kang et al., 2013). Pilocarpine–picrotoxin-induced epileptic convulsions generated a significant reduction in the catalase activity. Interestingly, the collapse in catalase activity was significantly improved by treatment with the *Mimosa pudica* extract at the doses 80–160 mg/kg, suggesting that the extract may partially contain some compounds that could activate catalase or scavenge superoxide anions, hydroxyl radicals, and hydrogen peroxide.

The fundamental mechanisms of connections in neurons and neuroproliferation are strongly influenced by neurotrophins (Lim et al., 2015). The ascent of BDNF signalling initiated by pilocarpine-

induced seizure promotes a compensatory neuroplastic response to neuron damage accompanied with neurodegeneration (Wang et al., 2009). In contrast, treatment of mice with the *Mimosa pudica* aqueous extract significantly decreased BDNF expressions in pilocarpine–picrotoxin-injected mice. These results suggest that the plant extract can suppress the compensatory adaptative response of epileptogenesis (Lim et al., 2015).

In the acute toxicity test, no deaths or any signs of toxicity were observed after the oral administration of a single dose of the *Mimosa pudica* extract at any dose level up to the highest dose tested in mice, thus suggesting that DL_{50} is above 5,000 mg/kg. An extract with a DL_{50} value higher than 5,000 mg/kg *p.o.* is considered non-toxic or relatively non-toxic. This result corroborated with that of Nghonjuyi et al. (2016), who reported that the extract of *Mimosa pudica* had a DL_{50} value higher than 5,000 mg/kg when administered orally.

As a limitation to this work, the effects of the *Mimosa pudica* extract in mouse models of temporal lobe epilepsy induced by pilocarpine–picrotoxin without generalisation of seizures in the hippocampus and cortex but with nonconvulsive focal seizures were not evaluated since the primary aim of our study is to identify only the effects of the *Mimosa pudica* extract aqueous extract on pilocarpine–picrotoxin models of temporal lobe epilepsy, oxidative/nitrosative stress, and alteration in GABAergic/cholinergic pathways and BDNF expression.

5 Conclusion

In summary, this study showed that the aqueous extract of *Mimosa pudica* leaves and stems possesses potent antiepileptogenic and anticonvulsant activities against pilocarpine–picrotoxin-induced temporal lobe epilepsy that may be directly related to the increase in protection against *status epilepticus* and generalized tonic–clonic seizures, through the amelioration of GABAergic and cholinergic transmission pathways, attenuation of oxidative/nitrosative stress, and the normalisation of brain-derived neurotrophic factor expression.

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 approved by the University of Buea–Institutional Animal Care and Use Committee (UB-IACUC) with the following permit number: UB-IACUC N 07/2022. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HMAYM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, and Writing–original draft. SP: Formal analysis, Investigation, Methodology, and Writing–original draft. OSMB: Methodology, Validation, and Writing–review and editing. VTJ: Data curation, Formal analysis, Investigation, Writing–review and editing, and Writing–original draft. NSOM: Formal analysis, Investigation, Methodology, and Writing–original draft. TAO: Data curation, Formal analysis, Investigation, and Writing–original draft. BYW: Formal analysis, Investigation, Writing–original draft, and Data curation. RBB: Data curation, Formal analysis, Investigation, Writing–original draft, and Validation. RNH: Data curation, Formal Analysis, Funding acquisition, Methodology, Validation, Resources, and Writing–review and editing. AAS: Data curation, Formal Analysis, Funding acquisition, Methodology, Validation, Resources, and Writing–review and editing. GST: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing–original draft, and Writing–review and editing.

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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.

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