Journal of Chemistry

Phytochemistry, Ethnopharmacology, and Bioavailability of Medicinal Plants

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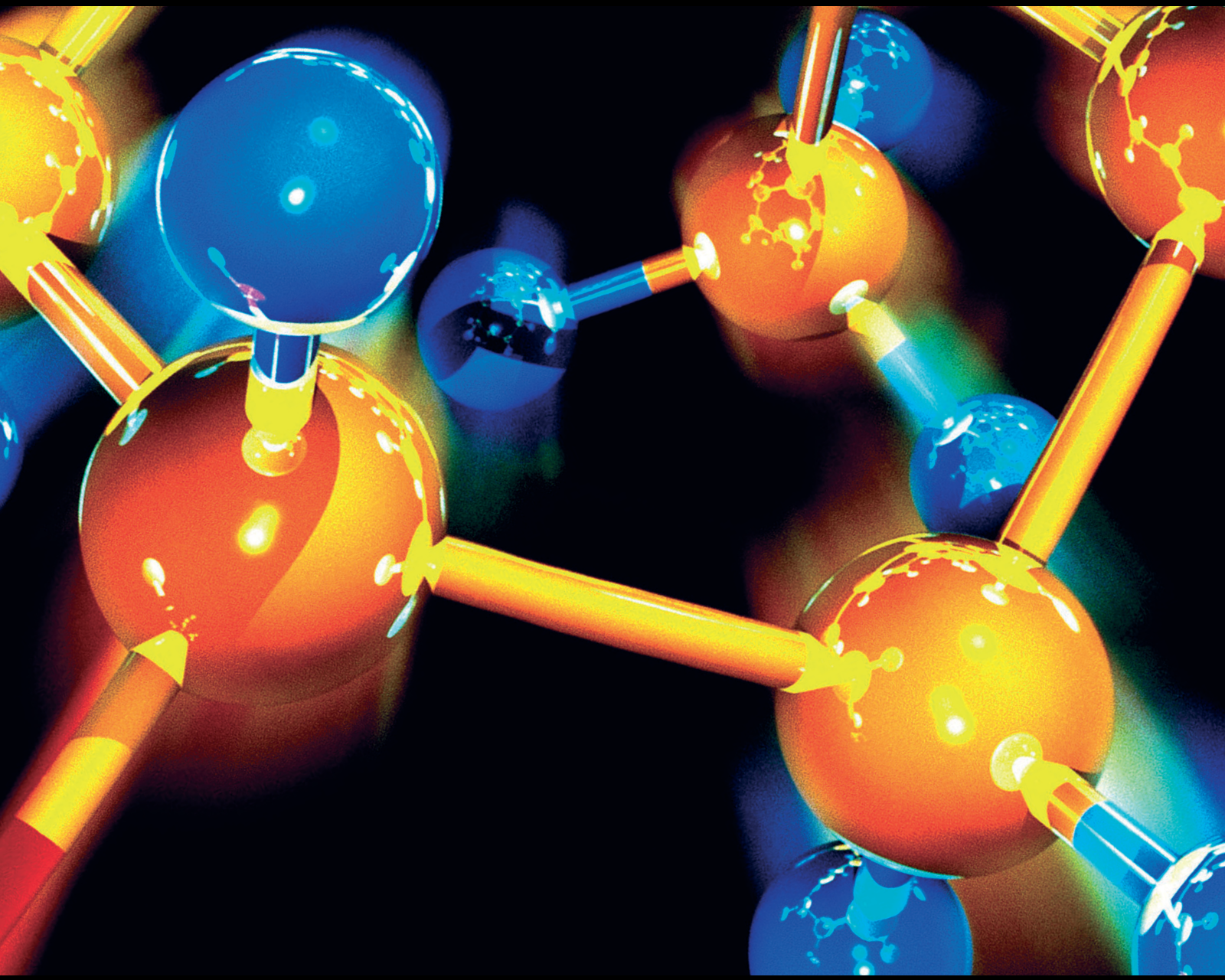
Guest Editors: Erick Gutiérrez-Grijalva and Jayanta Kumar Patra



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Review Article

Study of Traditional Uses, Extraction Procedures, Phytochemical Constituents, and Pharmacological Properties of *Tiliacora triandra*

Gitishree Das,[1] **Sushanto Gouda**,[2] **Rout George Kerry**,[3] **Hernán Cortes**,[4] **María L. Del Prado-Audelo**,[5] **Gerardo Leyva-Gómez**,[6] **Patrick Valere Tsouh Fokou**,[7] **Erick P. Gutiérrez-Grijalva**,[8] **J. Basilio Heredia**,[9] **Han-Seung Shin**,[10] **and Jayanta Kumar Patra**[1]

[1]*Research Institute of Integrative Life Sciences, Dongguk University-Seoul, Goyangsi 10326, Republic of Korea*
[2]*Amity Institute of Forestry and Wildlife, Amity University, Noida, UP, India*
[3]*Department of Biotechnology, Utkal University, Vani Vihar, Bhubaneswar, Odisha 751004, India*
[4]*Laboratorio de Medicina Genomica, Departamento de Genetica, Instituto Nacional de Rehabilitacion Luis Guillermo Ibarra Ibarra, Ciudad de Mexico 14389, Mexico*
[5]*Tecnológico de Monterrey, Escuela de Ingeniería y Ciencias, Campus Ciudad de México, Ciudad de México, Mexico*
[6]*Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad de México 04510, Mexico*
[7]*Department of Biochemistry, Faculty of Science, University of Bamenda, Bambili, Bamenda, Cameroon*
[8]*Catedras CONACYT–Centro de Investigacion en Alimentacion y Desarrollo, A.C. Carretera a Eldorado Km 5.5, Col. Campo el Diez, Culiacan CP 80110, Sinaloa, Mexico*
[9]*Centro de Investigacion en Alimentacion y Desarrollo, A.C. Carretera a Eldorado Km 5.5, Col. Campo el Diez, Culiacan CP 80110, Sinaloa, Mexico*
[10]*Department of Food Science & Biotechnology, Dongguk University-Seoul, Goyangsi 10326, Republic of Korea*

Correspondence should be addressed to Gerardo Leyva-Gómez; leyva@quimica.unam.mx and Jayanta Kumar Patra; jkpatra.cet@gmail.com

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Tiliacora triandra (Colebr.) Diels (Menispermaceae family) is a Southeast Asian angiosperm herb. Traditional medicine in these areas often includes the use of plant parts. Plant extracts are highly effective against various infections including bacterial, fungal, viral, and parasitic. The leaves and root extracts are used to treat gastrointestinal diseases, hypertension, diabetes, skin diseases, and malaria as an antipyretic, detoxification agent, anti-inflammatory, anticancer, and immunomodulator. Bioactive compounds contained in *T. triandra* include phenolic compounds, alkaloids, flavonoids, terpenoids, fatty acids, essential amino acids, peptides, carbohydrates, vitamins, and nucleic acid precursors. Despite the plant species' abundance of bioactive compounds, there is very little in vivo and clinical proof of its pharmacological significance. The present review focuses on the phytochemical configurations, extraction methods for major bioactive compounds, and pharmacology of *T. triandra*, in light of its potent medicinal values.

1. Background

Tiliacora triandra (Colebr.) Diels, locally known as "Yanang," is an angiosperm plant species from the Menispermaceae family, which consists mainly of twining shrubs or small trees and includes 64 genera and 379 species. It is primarily found in tropical countries (https://www.theplantlist.org/browse/A/Menispermaceae/). It is a climbing plant native

to Southeast Asian countries, particularly Thailand and Vietnam. According to the World Flora Online and Plant List databases, the species name (*Tiliacora triandra*) is unresolved, and its status is still uncertain (Figure 1). Besides, the plant list databases also state that it is synonymous with some other species (https://www.theplantlist.org/tpl1.1/record/kew-2517123; https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:581568-1#synonyms). The color of its flowers is yellow. The leaves are commonly used in Thai and Laos cuisine, and the roots and leaves are used in folk medicine in many Southeastern Asian countries [1–3]. Some reports state that leaves and roots from this plant species are used as an antipyretic, detoxification agent, anti-inflammatory agent, anticancer, antibacterial, and immunomodulator. Furthermore, it has been used to treat gastrointestinal diseases, hypertension, diabetes, skin diseases, and malaria [4, 5].

Different chronic diseases continue to increase worldwide with treatments that are not sufficiently effective and demand new medication options. Natural products are an alternative like the Mahanil-Tang-Thong formulation is a Thai herbal formulation used for various medicinal purposes. Chania et al. [6] demonstrated the antimalarial activity of medicinal plants in the Mahanil-Tang-Thong formulation. Recently, Maki et al. [7] investigated the anti-inflammatory activity and liver cancer cytotoxicity of Poh-Pu remedy from traditional Thai medicine. These compounds from natural sources contribute to some ailments and improve patients' quality of life. *T. triandra* is still a valued plant with high nutritional and medical potentials and is in the process of further basic, preclinical, and clinical research. This work aims to present an analysis of recent and relevant information on the pharmacological study of *T. triandra* against infectious, anticancer, antidiabetic, and neuroprotective processes, among others, as an indication of the relevance of this plant for wide traditional uses.

2. Traditional Uses

Some medications' high costs and harmful effects have encouraged the pursuit of effective and low-priced substitutes obtained from nature. *T. triandra*, often known as bamboo grass, has been successfully used by indigenous populations throughout Southeast Asia to treat various illnesses and problems. There are few studies regarding the ethnopharmacological potential of *T. triandra*, which is shown in Figure 2. Leaves and roots of *T. triandra* have been traditionally used in folk medicine in Thailand and Asian countries to treat several illnesses, including malaria, gastrointestinal diseases, alcohol intoxication, skin diseases, hypertension, and fever [8].

These effects are related to their phytochemical content. Additionally, aqueous extracts of *T. triandra* leaves have been used in Thai cuisine [9]. Likewise, ethnobotanical reports on *T. triandra* have led scientists to evaluate its biological properties such as antiproliferative, antioxidant, antidiabetic, anti-inflammatory, and neuroprotective potential. *T. triandra* is quite effective against peptic ulcers and can act as an antidote for food poisoning and environmental toxicants [8, 10, 11]. Moreover, various reports have indicated that *T. triandra* may be beneficial to treat diabetes [4], tuberculosis [12], increased cholesterol [1], cancer [2, 13], and various neurological conditions [5, 14–16]. Their phytochemical composition is considered responsible for these effects. As a result, understanding Ayurveda, ethnobotany, and information on tribal medicinal sources will be critical in producing pharmaceuticals with low or no adverse effects.

3. Extraction and Isolation Procedures of Major Compounds from *T. triandra*

Most procedures for extracting active compounds from *T. triandra* involve ethanol or methanol as the solvent. However, those methods exhibit several differences, depending on the substances of interest to be extracted, the wanted methodological innovations, and the desired pharmaceutical properties (Table 1). In this respect, in a pioneer study, Wiriyachitra and Phuriyakorn [21] extracted alkaloids from 1kg of roots of *T. triandra* with methanol/aqueous chloroform/ammonia (15 : 5 : 1) at room temperature. The authors concentrated their extract at reduced pressure, dissolved it in anhydrous acetic acid, and poured it into water. This blend was clarified and basified with ammonia to precipitate the alkaloids in chloroform. Afterward, the authors evaporated the chloroform extract and obtained the raw alkaloids with an efficiency of 8%. Finally, the authors separated the alkaloids on a silica gel column through chromatography. The alkaloids tiliacorinine, nortiliacorinine A, tiliacorine, and tiliacorinine 2′-N-oxide were obtained with this procedure.

Another study employed a sophisticated design to extract polysaccharide gum from the leaves of *T. triandra* [22]. The authors evaluated the effect of temperature on extraction (25–85°C), time of extraction (60–180 min), and dried leaf: water ratio (1 : 5–1 : 15) through a central composite design with a quadratic model. In all cases, the mixture was extracted with three volumes of 95% ethanol (w/v). The authors found that gum extraction's optimal conditions were an extraction temperature of 85°C, extraction time of 100min, and dried leaf:water ratio of 1 : 6. Likewise, their findings indicated that their experimental design efficiently optimized the gum extraction conditions from the plant. In a similar study, Singthong et al. [24] explored the extraction competencies of bioactive compounds from *T. triandra* using various solvents (water, ethanol, and acetone). Their results revealed that water extraction is the most suitable method to extract phenolic compounds because it produces the highest yield. Besides alkaloids and polyphenols, *T. triandra* contains other bioactive components, which were extracted by Duangjai and Saokaew, [1]. They proposed a method to extract the plants' leaf fatty acids by consecutively macerated with hexane, dichloromethane, methanol, and water. The results indicated that hexane extraction was effective in obtaining fatty acids. Rahman et al. [25] aimed to find the phytochemical components from the stem bark of *T. triandra*. The authors mixed approximately 150g of stem bark powder with methanol (80%), and the mixture was stored for two weeks with occasional shaking. The extracts

Figure 1: Photo of the whole plant and various parts of *Tiliacora triandra* (Reproduced under the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License, https://tropical.theferns.info/viewtropical.php?id=Tiliacora+triandra, accessed on 2020.12.09 [55]).

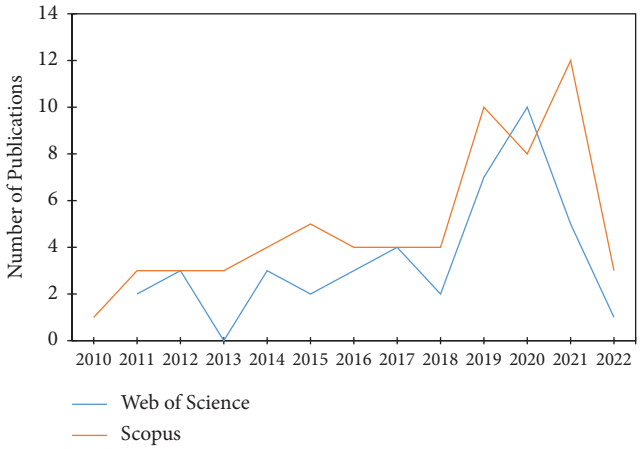

Figure 2: Publications registered in Web of Science and Scopus databases using the term "*T. triandra*" from 2010 to 2022.

were filtered and then dried at 50°C under decreasing pressure. The authors reported the extraction of terpenoids, flavonoids, phenolics, saponins, alkaloids, and cardiac glycosides; however, their exact quantities were not reported.

Finally, two very recent studies reported the isolation of at least 26 compounds that had not been described previously in *T. triandra* [4, 17] (Table 1). In the first research [17], the authors macerated 800 g of leaves and 500 g of twigs from *T. triandra* in 95% ethanol; afterward, the blending was filtered and vaporized to obtain the raw ethanol extract (84.2 g), which was mixed with water. The blend was partitioned between ethyl acetate and hexane, and the soluble fractions were isolated through column chromatography. Then, the subfractions found by chromatography were subjected to additional purification by reverse-phase HPLC. The second study utilized a similar approach coupled with GC-MS analysis to identify new phytochemical components of the plant [4]. The above procedures resulted in separating and identifying 6 [17] and 18 [4] new compounds, respectively. Therefore, the development and perfection of the extraction techniques and the incorporation of more innovative technologies will allow easy identification of new bioactive compounds from the *T. triandra* plant species in the forthcoming years.

Furthermore, several bioactive properties of the identified phytochemicals are presented in Table 2. Besides identifying phytochemicals in the leaves, twigs, and stem bark of this plant, these plant parts also exhibited several bioactive properties like antioxidant, anticancer, antimicrobial, and antiproliferative, among others. Moreover, some bioactive compounds have shown outstanding results against some metabolic syndrome disorders (e.g., lowering lipids and sugars) (Table 2).

4. Phytochemical Constituents of *Tiliacora triandra*

Numerous research groups have explored the active phytochemical compounds responsible for the therapeutic effects of this herb. According to these investigations, *T. triandra* and its components include a variety of bioactive

Table 1: Phytochemical constituents of *Tiliacora triandra*.

Plant species name Compound	Chemical structure	Reference Part of the plant	Reference
Decamethyltetrasiloxane Dibenzylhydroxylamine Dihydroxydimethylsilane Eicosanoic acid Hexadecamethylheptasiloxane Hexamethylcyclotrisiloxane Methyl-N-hydroxybenzenecarboximidoate Methyl tetradic-5-ynoate Pentadecanoic acid		Twigs and leaves	[4]
1-Cyclododecylethanone	0		
1,1,3,3,5,5,7,7,9,9,11,11,13,13-Tetradecamethylheptasiloxane	1		
2-Heptadecanone	2		
Tris-(trimethylsilyl)borate	3		
Ethyl linoleate	4		
Ethyl linolenate	5		
Pheophorbide A ethyl ester	6		
Pheophorbide A	7	Twigs and leaves	[17]
5,7-Dihydroxy-6-oxoheptadecanoic acid	8		
5-Hydroxymethyl-2-furancarboxaldehyde Neophytadiene	0		
Oleamide	1		
Oleic acid	2		
Phytol	3	Leaves	[9]
Vitamin E	4		
2-(Cyclohexen-1-yl)acetic acid	5		
2,6-Dimethyl-3-(methoxymethyl)-benzoquinone	6		
Palmitic acid	7		
Petroselinic acid Stearic acid	8	Leaves	[2]
Nortiliacorinine A	0	Root	[12, 18–21]
Tiliacorine	1		
Tiliacorinine 2′-N-oxide	2	Root	[12, 18, 20]
Oxoanolobine	3	Leaves	[13]
L-Arabinose	4		
D-Galactose	5		
L-Rhamnose	6	Leaves	[22]
Catechin	7		
Chlorophyll A	8		
Chlorophyll B Isoquercetin	0		
Rutin	1		
Tannic acid	2		[23]
Quercetin	3		

Table 2: Studies on the bioactive potential of *Tiliacora triandra* plant parts and their respective identified compounds.

Plant part	Identified chemicals	Bioactive effect	Type of extract	Dose range	Test type (in vivo/in vitro)	Model use	References
Mature leaves	Vitamin E, phytol, and 1-cyclohexenylacetic acid	Antioxidant (DPPH method), cytotoxicity and genotoxicity	Methanol	8.4 mg/ml (DPPH), 10 mg/ml (lymphocyte), and 0.41 mg/ml (HeLa cells)	In vitro	Lymphocytes and HeLa cells	[9]
Leaves	Hexadecenoic, octadecanoic, and (z)-6-octadecanoic acids	Cytotoxicity against lung cancer	Hexane	125 μg/mL	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay	A549RT-eto cells	[2]
Leaves and twigs	n-Palmitic acid, dibenzylhydroxylamine, oleic acid, and stearic acid	Antidiabetic	Ethanol	400 mg/kg	Hypoglycemic activity	HOMA-IR and HOMA-β induced rats	[4]
Leaves and twigs	Pheophorbide A, ethyl pheophorbide, ethyl linoleate, ethyl-5,7-dihydroxy-6-oxooctadecanoate, and 5,7-dihydroxy-6-oxoheptadecanoic acid	α-Glucosidase and α-amylase	Ethyl acetate	α-Glucosidase activity at 11.58–424.06μM and α-amylase at 26.27μM	α-Glucosidase and α-amylase	—	[17]
Aerial parts	Gallic acid, cyanidin, and quercetin	CAT, GSH-Px, and SOD activities	Water	Spatial memory (400 mg·kg^{-1}BW), AChE activity in the hippocampus (200mg·kg^{-1}BW), oxidative stress (100, 200, and 400 mg·kg^{-1}BW)	Acute toxicity and antioxidant enzyme expression	Male Wistar rats	[5]
Stem barks	Alkaloids, cardiac glycosides, flavonoids, phenolic compounds, saponins, and terpenoids	Antimicrobial and antifungal activities	Methanol	62.5 ug/mL	Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)	—	[25]
Leaves	Oxoanolobine	Anticancer activities against human cancer cell lines	Methanol and aqueous	Cytotoxic activity against lung cancer (NCI-H187) cell line (27.60±4.30 μg/mL).	Resazurin microplate assay (REMA), tested with 3 cell lines	Oral cavity cancer (KB), lung cancer (NCI-H187), and breast cancer (MCF-7) cell lines	[13]
Edible plant	Tiliacorinine, tiliacorine, and 2′-nortiliacorinine bisbenzylisoquinoline	Anti-TB	Dichloromethane	3.1μg/ml	MTT assay	MRC-5 cells	[12]

components such as alkaloids, polysaccharides, polyphenolic compounds, fatty acids, and minerals (Table 1).

It was reported that Paris and Sasorith [19] extracted and identified a phytochemical component of *T. triandra*, an alkaloid named tiliacorinine; nevertheless, its chemical structure was not reported. Subsequently, Wiriyachitra and Phuriyakorn [21] also examined root extracts from the plant by thin-layer chromatography and identified four bisbenzylisoquinoline alkaloids, tiliacorinine, nortiliacorinine A, tiliacorine, and tiliacorinine 2′-N-oxide. According to the authors, tiliacorinine and tiliacorine were the main compounds obtained. Likewise, tiliacorinine was the minor polar component, whereas tiliacorinine 2′-N-oxide was the most polar alkaloid. Moreover, the authors determined the absolute configuration of the four compounds through nuclear magnetic resonance (NMR) and biosynthesis experiments. Subsequent studies corroborated that tiliacorinine, nortiliacorinine A, and tiliacorine are the major alkaloids of *T. triandra* roots [12, 18, 20].

Despite the numerous types of research studies on the alkaloids found in *T. triandra* roots, other potentially relevant phytochemical components from the leaves have not received enough attention. Thus, Singthong et al. [22] performed gum extraction from the plant leaves and characterized their physicochemical properties. Their results indicated that the gum contained considerable quantities of proteins, lipids, xylose, glucose, galactose, arabinose, and rhamnose, which might have numerous industrial applications. Likewise, other studies revealed that extracts from *T. triandra* leaves contain saponins, flavonoids, triterpenes, and condensed tannins, which would be associated with the plant's antioxidant activity and other beneficial effects [25–27]. Interestingly, the study conducted by Phadungkit [26] exposed that the leaves extract of *T. triandra* possesses an antimutagenic effect. Thus, Kaewpiboon et al. [2] employed diverse chromatographic techniques like NMR and gas chromatography-mass spectroscopy (GC-MS) to separate and detect the compounds responsible for the anticancer activity. Stearic acid, petroselinic acid, and palmitic acid were identified in the *T. triandra* leaf extract [2]. Remarkably, those fatty acids exhibited chemosensitizer activity. A more comprehensive work indicated that the plant leaf also contains significant amounts of vitamin E, phytol, oleic acid, oleamide, 2-(cyclohexene-1-yl) acetic acid, neophytadiene, 5-hydroxymethyl-2-furancarboxaldehyde, and 2,6-dimethyl-3-(methoxymethyl)-benzoquinone [9]. Moreover, another study accomplished by Rattana et al. [13] identified the alkaloid oxoanolobine as a significant component in the extracts from *T. triandra* leaves. Interestingly, oxoanolobine exhibited potent anticancer activity in a lung cancer cell line (NCI-H187). Similarly, a study conducted by Weerawatanakorn et al. [23] aimed to analyze the polyphenols content of *T. triandra* leaves by HPLC/DAD/MS. Their results indicated that the leaves contain substantial quantities of catechin, chlorophyll A, chlorophyll B, isoquercetin, rutin, tannic acid, and quercetin.

Finally, two very recent studies identified several phytochemical components that had not been reported previously [4, 17]. Makinde et al. [17] analyzed the phytochemical compounds of leaves and twigs of *T. triandra*. Their findings demonstrated six compounds extracted from the plant for the first time (Table 1), including two new fatty acid byproducts. Similarly, the same research group reported the presence of (at least) 18 new compounds [4], which included hexamethylcyclotrisiloxane, pentadecanoic acid, eicosanoic acid, and dibenzyl hydroxylamine, among others (Table 1). Therefore, all these studies highlighted the compounds with numerous biological activities present in the *T. triandra* plant species and warranted further studies to identify more new components for medical and industrial purposes.

5. Pharmacological Properties of *Tiliacora triandra*

T. triandra has been investigated for its pharmacological properties against noncommunicable diseases (e.g., diabetes, cancer, metabolic syndrome, hyperlipidemia, obesity, inflammation, and oxidative disorders) and communicable or infective diseases (those produced by bacteria, parasites, and viruses).

5.1. Effect of Tiliacora triandra on Communicable or Infectious Diseases

5.1.1. Antimicrobial Effects. Multidrug-resistant bacterial strains are the primary causes of infectious diseases worldwide, and it has become a public health problem. Hence, numerous natural products have attracted increasing interest due to their antimicrobial activity and fewer side effects, and research groups focused on identifying bioactive phytochemicals responsible for those properties.

Many studies have explored the antimicrobial properties of various plant extracts, and several herbal formulations are already available against infectious diseases produced by bacteria, fungi, or viruses worldwide. An example of this is the traditional herbal formulation called "*Benchalokawichian*" remedy containing *T. triandra*, popular in Thailand to treat the common cold, fever, and influenza [28]. In this regard, *T. triandra* extracts obtained from *Benchalokawichian* and plant stem barks exhibited effective antimicrobial activity against bacteria such as *Escherichia coli*, *Bacillus cereus*, *Shigella sonnei*, *Acinetobacter baumannii*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Bacillus subtilis*, and *Agrobacterium* spp. in *in vitro* assays [25, 28]. The authors reported that the inhibition zones ranged from 11 to 13 mm at the 250 μg/disc concentration, whereas these oscillated from 16 to 21 at 500 μg/disc mm against Gram-positive bacteria. Similarly, the inhibition zones ranged from 13 to 15mm at 250 μg/disc and 17 to 21mm at 500 μg/disc for Gram-negative bacteria. The minimum inhibitory concentration (MIC) fluctuated from 62.5 to 125 μg/mL. Moreover, the extract inhibited the fungus *Aspergillus niger*, *Candida albicans, Trichoderma viride*, *Trichoderma harzianum*, and *Microphamina phaseolina* (inhibition zones from 15 to 22mm, MIC = 62.5–125 μg/mL) [25]. Bioactive compounds such as alkaloids, cardiac glycosides, flavonoids, phenolic compounds, saponins, and

terpenoids were noted to be responsible for the antimicrobial and antifungal properties of the plant extract, i.e., stem of *T. triandra* (Table 2).

Likewise, Sureram et al. [12] reported the antimicrobial potential (by microplate Alamar blue assay) of aerial extracts isolated from *T. triandra* and a chemical derivative against multidrug-resistant *Mycobacterium tuberculosis* strains isolated from extrapulmonary and pulmonary patients (Table 2). The study included numerous isolates with variable resistance to the antibiotics isoniazid, rifampin, ethambutol, streptomycin, and ofloxacin. Their results showed that the bisbenzylisoquinoline alkaloids 2′-nortiliacorinine, tiliacorinine, and tiliacorine (isolated from *T. triandra*) and the derivative 13′-bromo-tiliacorinine exhibited potential antimicrobial effects against 59 clinical strains of multidrug-resistant *M. tuberculosis* with MIC values ranging from 0.7 to 6.2 μg/mL (Table 2) tested in MRC-5 cells using MTT assay. Interestingly, 2′-nortiliacorinine, tiliacorine, and 13′-bromo-tiliacorinine showed more potent activity against the multidrug-resistant isolates than the standard antimicrobial drugs like isoniazid, rifampin, ethambutol, streptomycin, and ofloxacin, suggesting that these compounds would serve as potential new chemical scaffolds for antimycobacterial activity. However, further analyses will be necessary to ensure their specificity and safety profile in human cells.

On the other hand, a similar study by Makinde et al. [3] demonstrated that the ethyl acetate fraction of the leaves, n-hexane, and the ethyl acetate fraction of the twigs exhibited antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* F2365, *Escherichia coli* O157:H7, and *Bacillus cereus* (MIC values from 1.5 to 12 mg/mL) [3] with MIC values ranging from 0.39 to 6.25 mg/mL and MBC values ranging from 1.5 to 12 mg/mL compared to antimicrobials like vancomycin, ceftazidime, and penicillin G. Despite the antimicrobial potential of evaluated extracts, no specific phytochemicals were identified in this study; thus, further analyses are required to select the best compounds for optimization.

Concerning the possible antimicrobial mechanisms of action of *T. triandra*, several phytochemicals contained by the plant have shown biological activity against numerous microorganisms. For example, terpenoids, flavonoids, phenolics, saponins, alkaloids, and cardiac glycosides possess antibacterial activity. That activity is possibly due to their capability to complex with bacterial cell walls and to their ability to provoke leakage of proteins and enzymes from cells.

Finally, it is noteworthy that in vitro antimicrobial activities do not always correlate with efficacy in human beings; thus, it will be needed to analyze their effect in animal models before their utilization in humans.

5.1.2. Antiplasmodial Effects. Traditional herbal formulations have been used to treat malaria for a long time; even the most effective allopathic medicines are obtained from plants (quinine and artemisinin). Since other medicinal plants could have antiplasmodial effects, Nutmakul et al. [29] evaluated the potential usefulness of the multiherbal formulation *Benchalokawichian* (BLW), an antipyretic formulation used against malaria like fever that consists of *T. triandra* along with other herbal components. For this purpose, the authors separately extracted constituent plants and compared their efficacy against chloroquine-resistant (W2) and sensitive (D7) strains of *Plasmodium falciparum* by flow cytometry. Furthermore, in order to calculate the selectivity index (SI), they evaluated the toxicity against peripheral blood mononuclear cells through the WST assay.

The BLW exhibited antiplasmodial activity and good SI values ranging from 3.55 to 19.74. In particular, extracts from *T. triandra* showed IC_{50} <5 μg/mL against *Plasmodium falciparum* W2 and 3D7 strains, indicating high antiplasmodial activity compared to camptothecin which was used as a positive control. Likewise, these extracts exhibited SI values >10, suggesting their selectivity and good safety profile.

Subsequently, the authors selected the *T. triandra* extract that presented the higher antiplasmodial effect and SI to isolate their active constituents. They isolated and purified tiliacorinine and yanangcorinine and then explored their antiplasmodial potential and SI. Their results demonstrated that both compounds had good antiplasmodial activity but a low SI separately, indicating that their combination produces a synergistic effect that increases their efficacy and allows their toxicity. Despite promising results, it should be mentioned that these are preliminary data; thus, further preclinical studies are required to unveil the antimalarial potential of *T. triandra*.

5.2. Effect of Tiliacora triandra on Noncommunicable Diseases

5.2.1. Anticancer Effects. The use of carcinoma-derived cell lines in toxicity tests is a method widely accepted to analyze plant extracts' biological and toxic effects. The cytotoxic potential of the water and ethanol extracts of leaves of *T. triandra* against lung cancer cell line (NCI-H187), oral cavity cancer cells (KB), lymphocytes, and HeLa cells has been reported in several studies [9, 13]. One of those studies demonstrated that *T. triandra* extracts exert a dose-dependent cytotoxic effect (0.41 mg/m) on HeLa cells (human cervical carcinoma) viability (50% (IC50)) and lymphocytes isolated from human blood samples [9] (Table 2). The results suggest that *T. triandra* leaf extracts are reasonably safe at the cellular level when equated with untreated cells (negative control) and positive control cells (DMSO treated cells) and incubated with 100 μM of H_2O_2 for 15 min. Compounds, namely, phytol, oleic acid, 1-cyclohexenylacetic acid, oleamide, and vitamin E (α-Tocopherol) were identified as the main phytochemicals responsible for the cytotoxicity and genotoxicity activities of the plant extracts (Table 2).

On the other hand, Rattana et al. [13] evaluated the anticancer activity of *T. triandra* leaves extracts on several human carcinoma cell lines (NCI-H187, oral cavity cancer (KB), and breast cancer cells MCF-7) through in vitro anticancer activity tests, resazurin microplate assay (REMA). Methanol and water extracts exhibited strong activity against NCI-H187 (IC_{50} ranging from 11.93 to 12.27 μg/mL) and KB

(IC_{50} ranging from 12.06 to 32.15 μg/mL) cells (Table 2). The authors concluded that oxoanolobine was the main bioactive compound responsible for these effects and that *T. triandra* might be used to treat and prevent cancer in different cell lines such as oral cavity cancer (KB), lung cancer (NCI-H187), and breast cancer (MCF-7) (Table 2) in comparison to the standard available anticancer drugs like doxorubicin and ellipticine that were used as positive controls in the assays.

On the other hand, it is known that cancer cells possess the capability to develop resistance to drugs, which dramatically reduces the effectiveness of cancer treatments. A key mechanism of this resistance is the increased expression of the P-glycoprotein, a plasma membrane protein that transports numerous anticancer drugs out of the cell. Thus, inhibitors of the P-glycoprotein are of particular medical interest. In this respect, a study suggested that certain constituents of *T. triandra* may act as chemosensitizers on the P-glycoprotein function in the multidrug-resistant A549RT-eto cell lines [2] (Table 2). The subsequent analysis and isolation of components demonstrated that a mixture of three fatty acids, namely, hexadecenoic, octadecanoic, and (z)-6-octadecanoic acids contained by the plant leaves is responsible for the cytotoxic effect against lung cancer even at a concentration of 125 μg/mL determined through MTT assay (Table 2). Etoposide was used as the specific positive control for the A549RT-eto-resistant cells. The M3FA at 125 mg/mL was found to have a clear ability to restore etoposide sensitivity to the A549RT-eto cell line and showed a broadly similar RF value (1.24) to that for F22 at the same concentration (RF=1.30), although this was just over two-fold lower than that for the verapamil control.

5.2.2. Antioxidant Effects. A study on rats has indicated that the extracts of *T. triandra* may enhance the memory deficit in alcoholic rats partly *via* reduced oxidative stress and suppression of acetylcholinesterase (AChE) [5]. The study showed that gallic acid, cyaniding, and quercetin enhance spatial memory at a concentration of 400 mg·kg^{-1}BW, AChE activity in the hippocampus at a concentration of 200mg·kg^{-1}BW, and oxidative stress at 100, 200, and 400mg·kg^{-1}BW determined through CAT, GSH-Px, and SOD activities in male Wistar rats in 7 and 14 days, respectively (Table 2). The study used donepezil (a standard drug for treating memory deficit patients) as a positive control at a concentration of 1mg/kg^{-1}BW. Besides this, another study confirmed the potential antioxidant properties of the *T. triandra* extracts encapsulated with gum Arabic at 10% (w/v) as coating agents [24]. The roughage at a concentrated ratio of 30:70 with *T. triandra* pellets has been reported to serve as a dietary enhancer in male swamp buffaloes [30]. Furthermore, it is known that *T. triandra* is employed in the food industry for its nutritional value [31, 32]. It has also been confirmed that the supplementation of *T. triandra* pellets could enhance feed intake, decrease the protozoa, increase the bacterial population, and increase rumen fermentation efficiency. In contrast, it decreases methane production, thus acting as a good rumen enhancer in beef cattle and buffalos [30, 33].

In 2022, it was reported that *T. triandra* presented protective effects in rats under cisplatin-induced hepatorenal and testicular insults by modulating oxidative inflammation. After four weeks of intragastric administration of *T. triandra* (250 mg/kg) and two weeks of intraperitoneal cisplatin injection (2.5 mg/kg/week), the rats were euthanized to analyze the kidney, liver, testes, and the serum of blood samples. The authors reported that the rats' group treated just with cisplatin presented a higher body weight and lower kidney, liver, and testes weight compared with the control (normal saline) and with the group of *T. triandra* treatment. Meanwhile, the biochemical serum analysis revealed that the *T. triandra* treatment suppressed the increment in some biomarkers related to kidney, liver, and testicular damage such as creatinine, aspartate aminotransferase, luteinizing hormone (LH), and testosterone [34]. The mechanism involves improved sperm count, motility, and viability and ameliorated the reduced serum levels of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). It was reported that the phytochemicals phenolic and flavonoids from leaves were able to attenuate cisplatin triggered hepatorenal in cisplatin-injected male Wistar rats within four weeks' time [34] (Table 3). It is crucial to note that the extraction conditions could modify the antioxidant properties of *T. triandra,* and it is vital to evaluate the adequate conditions in terms of the concentration of the molecules [44]. Wungsintaweekul et al. (2018) reported that the root part of Ya-Nang (*T. triandra*) along with other herbs has been used in Thai traditional medicine such as Ya-Ha-Rak or Ben-Ja-Loke-Vi-Chian for relief fever.

5.2.3. Antidiabetic Effects. Makinde et al. [4] recently reported that extracts rich in fatty acids from *T. triandra* leaves and twigs reduced the α-glucosidase activity with IC_{50} values of 11.58–424.06 μM. Dual inhibition of α-amylase and α-glucosidase is linked to gastrointestinal toxic effects like abdominal discomfort, meteorism, flatulence, and diarrhea [45]. Moreover, the *T. triandra* ethyl acetate fraction of leaves and twigs and the n-hexane fractions of twigs strongly inhibited α-glucosidase activity. However, all extract fractions poorly inhibited α-amylase as desired [3]. Additionally, an in vivo study by Makinde et al. [4] indicated that the ethanol extracts of the aerial parts (leaves and twigs) of *T. triandra* at concentrations of 100 and 400 mg/kg markedly increased the insulin level and reduced the fasting blood glucose in streptozotocin-induced diabetic rats after 30 days of inoculation (Table 2). Moreover, *T. triandra* increased the food and water consumption of the treated animals while the body weight decreased. Besides, these extracts decreased total cholesterol and LDL-cholesterol levels, glycosylated hemoglobin (Hb1Ac), transaminases, and alkaline phosphatase. Finally, the extracts improved kidney and liver function; thus, the authors concluded that the plant might be used to treat diabetes and its complications.

In addition, Thong-asa et al. [35] reported that *T. triandra* leaf extracts at 300 and 600mg/kg doses,

Table 3: Premedical and clinical effectiveness of *Tiliacora triandra*.

Plant parts used	Bioactive compound	Positive control/ standard used	Duration of test	Biomedical application	Model tested on/method uses	Possible mechanism	References
Aerial parts	Gallic acid, cyanidin, and quercetin	1,3,3-Tetra ethoxy propane (TEP), H_2O_2	14 days	Brain dysfunction and neurodegeneration	Male Wistar rats	Decreased oxidative stress and the suppression of AChE	[5]
Leaf	Flavonoids	10% Tween 80	4 weeks	Nurture glycemic control and helps against diabetes	High sugar intake mice	Improvement of glucose clearance, inhibition of glycogen breakdown, promotion of glycogen synthesis, inhibition of glucose intestinal absorption, and increased peripheral insulin sensitivity	[35]
Leaves	Phenolic, chlorophyll, alkaloids, and flavonoids	Ascorbic acid, absolute ethanol, tyrosinase, and theophylline	24–72h	Antioxidant and melanogenesis stimulating activities for anti-grey hair treatment	B16F10 melanoma cells	High potential for melanogenesis stimulating activity of the bioactive compounds	[36]
Leaves juice	Calcium	Pandan leaf juice	85h	Rich sources of calcium and antioxidant	30 general consumers	Higher amounts of beta-carotene	[37]
Leaves	p-Hydroxybenzoic acid, minecoside, flavones, glycoside, and cinnamic acids	10% Tween 80	7 days	Enhance memory and hippocampal choline acetyltransferase activity in mice	Male ICR mice (*Mus musculus*) in Morris water maze	Significantly increasing the number of viable cells and mediating the stress-induced neuronal damage through the sympathy-adrenomedullary system and the hypothalamic-pituitary-adrenal system	[16]
Leaves	Phenols and flavonoids	Bilateral common carotid artery occlusion (BLCCAO) +10% Tween 80	37 days	Prevents dentate gyrus neuronal damage in mice	Mice, cognitive tests in the Morris water maze	Enhanced spatial learning and learning flexibility and prevented neuronal death in the DG of mice following ischemia/reperfusion	[15]
Leaves	Tiliacorinine, 20-nortiliacorinine, and tiliacorine	Isoniazid, rifampin, ethambutol, streptomycin, and ofloxacin	8 days	Exhibits antimicrobial activity against multidrug-resistant *Mycobacterium tuberculosis* through bisbenzylisoquinoline	Multidrug-resistant isolates of *M.* tuberculosis through microplate alamarBlue assay and human fetal lung fibroblast cell line	Inhibition of RNA and protein synthesis	[12]
Roots	—	Gentamicin and amphotericin B	48h	Antimicrobial activity against pathogenic strains	*Candida albicans* and methicillin-resistant *Staphylococcus aureus*	—	[28]
Gums	Propionate and butyrate	10% maltodextrin	72h	Probiotics' impact on *Lactobacillus casei* and *L. acidophilus*	*Lactobacillus casei* and *Lactobacillus acidophilus* in vitro colon experiment	Enhanced the accumulation of lactic acid, short-chain fatty acids (SCFA), and beneficial colon bacteria (i.e., lactobacilli and bifidobacteria)	[38]
Stem barks	Phenolics, flavonoids terpenoids, alkaloids, saponins, and cardiac glycosides	Kanamycin and clotrimazole	24–42h	Possess antimicrobial and antifungal activities against multiple strains	*Escherichia coli, Shigella sonnei*, *S. dysenteriae*, *Agrobacterium* spp., and *Aspergillus niger*	Forms complexes with extracellular and soluble proteins as well as bacterial cell walls	[25]

Table 3: Continued.

Plant parts used	Bioactive compound	Positive control/ standard used	Duration of test	Biomedical application	Model tested on/method uses	Possible mechanism	References
Leaves	Thiocyanine, anthocyanins, chlorophyll, and carotenoids	Orlistat	24h	Inhibited pancreatic lipase activity and act as cholesterol-lowering agents	Radioactive cholesterol-treated Caco-2 cells	Inhibition of intestinal cholesterol absorption and lipid digestion and the suppression of cholesterol micellar solubility	[1]
Leaves	Alkaloids and polyphenols	Glibenclamide	8 weeks	Hypoglycemic activities in normal and streptozotocin-induced diabetic rats	Normal and streptozotocin-induced diabetic rats	Stimulating insulin secretion from the pancreas	[39]
Leaves	Tannic acid, gallic acid, and rutin	Dimethylsulfoxide (DMSO)	15min–3h	Anti-inflammatory activity	Murine macrophages RAW 264.7 cells	Downregulated the induction of inflammatory iNOS and COX-2 proteins in LPS-stimulated macrophages	[23]
Leaves	Phenolic and flavonoid	Distilled water	12 weeks	Antihyperglycemic, hyperlipidemia, oxidative stress, and inflammatory conditions in HFD-induced obese mice	High-fat diet (HFD)-induced obese mice	Reduce the malondialdehyde in serum and liver tissue and decrease circulating nonesterified fatty acid and inhibition of hepatic triglyceride synthesis	[40]
Leaves	Hexadecanoic: octadecanoic acid: Z-6-octadecenoic acids	Etoposide	88h	Act as chemosensitizer on polycoprotein function in multidrug-resistant A549RT-eto cell line	MDR human nonsmall-cell lung carcinoma cell line with a high P-gp expression level (A549RT-eto)	Enhanced the relative rate of rhodamine-123 accumulation and reduced P-gp activity	[2]
Leaves	Oxoanolobine	Doxorubicin and ellipticine	48h	Cytotoxic activity against lung cancer (NCI-H187) cell line and oral cavity cancer (KB)	Oral cavity cancer (KB), lung cancer (NCI-H187), and breast cancer (MCF-7) cell lines	--	[13]
Leaves	Vitamin E, phytol, and 1-cyclohexenylacetic acid.	Distilled water and DMSO	48h	Cytotoxicity toward human peripheral blood mononuclear cells (PBMCs) and HeLa cells	Lymphocytes and HeLa cells	--	[9]
Whole plant	Phenolic compounds	DMSO (1%) with PRRSV	76h	Antiporcine reproductive and respiratory syndrome (PRRS) activity	PRRSV propagated MARC-145 tissue	Inhibit PRRSV infection in vitro and replication in MARC-145 cells	[41]
Leaves	Phenolic and flavonoids	Cisplatin and saline water	4 weeks	Attenuation of cisplatin triggered hepatorenal and testicular toxicity	Cisplatin-injected male Wistar rats	Modulating oxidative inflammation, apoptosis, and endocrine deficit	[34]
Leaves	Flavonoids and phenolics	Cisplatin and saline water	5 weeks	Inhibition of CDDP-induced redox-mediated neurotoxicity and behavioral deficit in rats.	Cisplatin-injected male Wistar rats	Abated neurobehavioral deficits, MDA, and cytokine levels and restored CAT, GPx, GSH, SOD, and AChE activities	[42]

Table 3: Continued.

Plant parts used	Bioactive compound	Positive control/ standard used	Duration of test	Biomedical application	Model tested on/method uses	Possible mechanism	References
Leaves and twigs	Gallic acid, cyanidin, quercetin, condense tannin, triterpene, and saponins	Cisplatin and saline water	5 weeks	Antiallodynic and antihyperalgesia activities against cisplatin-induced peripheral neuropathy	Cisplatin-injected male Wistar rats	Significantly restored motor coordination deficits induced by CISP and rats showed marked improvement in thermal/chemical hyperalgesia and mechanical allodynia	[43]

respectively, increased glycogen, whereas reduced blood glucose and serum insulin rates in muscle and liver in mice after administration for four weeks (Table 3). These effects involved an enhancement in glucose clearance, a decrease in glucose intestinal absorption, and an increase in peripheral insulin sensitivity in comparison to high sugar intake mice that were used as a positive control for 4 weeks. The n-hexane extracts of twigs of *T. triandra* were reported to exhibit promising nitric oxide, α-amylase, and α-glucosidase inhibition activities [3]. Furthermore, the leaf extract of this plant also demonstrated hypoglycemia in the mouse model that was fed with a high sugar intake followed by increased glycogen storage in both the liver and muscle and reduced serum insulin level [35]. The therapeutic effect of 5,7-dihydroxy-6-oxoheptadecanoic acid (DHA) extracted from *T. triandra* on rat models of type 2 diabetes mellitus (T2DM) accounted to display a significant increase in fasting blood glucose (FBG), serum lipid profiles, and a reduction in liver antioxidant enzymes such as catalase CAT, superoxide dismutase SOD, and glutathione peroxidase GSH-Px [46]. Besides, Pasachan et al. (56) reported that the aqueous leaf extract of the *T. triandra* inhibited the hepatic glucose production in HepG2 cells and type-2 diabetic rats. Similarly, Song et al. [47] reported the administration of crude ethanol extract of *T. triandra* and its major bioactive compound, DHA, in streptozotocin-induced diabetic Sprague Dawley rats using standard diet fed rats as a positive control (25 and 400mg/kg, respectively) for 30 consecutive days. Authors reported the decrement of urinary protein and albumin levels and the suppression of TNF-α, IL-6 and IL-β after four weeks of both treatments, which secretion is related to NF-κB promotion of oxidative stress in diabetes [47]. The authors used the ethanolic extract of leaves and twigs of *T. triandra* (TTE) and subsequently isolated the best bioactive compound, 5,7-dihydroxy-6-oxoheptadecanoic acid (DHA). Although, in the same extract, they were also able to identify ethyl-5,7-dihydroxy-6-oxooctadecanoate, ethyl pheophorbide A, and pheophorbide A. Interestingly, the authors evaluated TTE at doses of 100 and 400mg/kg, in addition to DHA at a dose of 25mg/kg, with the respective negative controls orally for 30 days. The authors determined that TTE at 400mg/kg and DHA improved diabetic nephropathy, renal and testicular oxidative stress and proinflammation, and reproductive imbalance induced by diabetes, through an improvement in the endogenous antioxidant system, a decrease of lipid peroxidation, and suppression of proinflammation. Although preliminary results are promising, further studies related to the mode of antidiabetic action and the toxicity profile of *T. triandra* extracts are essential. A sufficient amount of information has been gathered for the preclinical antidiabetic potency of *T. triandra.* However, only a clinical trial will guarantee its usefulness in controlling diabetes.

5.2.4. Neuroprotective Effect. Some studies have suggested that *T. triandra* may have a neuroprotective effect, mainly attributed to the antioxidant capacity of the chemicals found in this species. Phunchago et al. [5] evaluated the neuroprotective and cognitive enhancing effects of extracts of the aerial parts of *T. triandra* in ethanol-dependent male Wistar rats. The authors suggested that the *T. triandra* extracts improved the oxidative stress, thus increasing neuron density in the hippocampus, which might be related to the improved memory impairment in ethanol-dependent rats upon 14 days of extract administration at a dose of 100, 200, and 400mg·kg^{-1}BW (Table 3). The results were significantly better than that of the control uses, i.e., the control (without any form of treatment) and the positive controls (ethanol+Aricept/donepezil at a dose of 1mg/kg^{-1}BW and ethanol+vitamin C at dose of 250mg/kg^{-1}BW). The registered components of the extract corresponded to gallic acid, cyanidin, and quercetin in concentrations of 4.81, 307, and 9028μ/100mg of extract. However, the authors also showed other important signals in the chromatograms of the aqueous extract of *T. triandra* that were not considered. Additionally, the global effect observed in memory improvement, increased neuronal density, and oxidative stress could not be differentiated to an extract component, but the authors hypothesized that it could be attributed mainly to quercetin. Subsequent studies could be carried out with the treatment of *T. triandra* extract preventively or after induced ethanol dependence. Similarly, Thong-asa and Bullangpoti [14] tested the neuroprotective potential of the ethanolic extract of *T. triandra* leaves in mice with cerebral ischemic reperfusion injury. Mice exhibited reduced calcium and malondialdehyde, increased antioxidant enzyme activity, and reduced glutathione, superoxide dismutase, and catalase after thirty minutes of BCCAO followed by 45min of reperfusion in mice pretreated with the *T. triandra* extract (300 and 600mg/kg) in contrast to the other combinations tested, such as Sham+10% Tween 80, bilateral common carotid artery occlusion (BCCAO)+10% Tween 80, and Sham+10% Tween 80, and BCCAO+10% Tween 80. The effect of *T. triandra* extracts thus prevents brain oxidative stress, brain infarction, and neurodegeneration in the cerebral cortex and dorsal hippocampus. Furthermore, Thong-asa et al. [15] revealed that leaf extracts of *T. triandra* at a concentration of 300 and 600mg/kg could improve learning flexibility and spatial learning and prevent neuronal death in the mouse model of cerebral ischemia/reperfusion injury (Table 3). The leaf extracts of *T. triandra* are also reported to reduce the etoposide resistance of the A549RT-eto cell lines, which proves the chemosensitizer potential of the *T. triandra* extracts [2]. Several authors have reported the effects of leaf extracts of *T. triandra* on cerebral ischemia/reperfusion injury in mice [15,16]. Huang et al. studied the effect of the antioxidant and anti-inflammatory properties mediated neuroprotective effects of a hydroethanolic extract of *T. triandra* against cisplatin-induced neurotoxicity. They have concluded that the extracts of the *T. triandra*, can reduce the neurotoxicity caused by cisplatin via the improvement of cognitive function and by the integrity linked with the enhanced antioxidant defense mechanism and the antiapoptotic and anti-inflammatory pathways. Furthermore, Thong-asa and Bullangpoti reported the neuroprotective effect of leaves against ischemia-reperfusion in mice by lessening neuronal death and brain infarction in the

hippocampus and cerebral cortex [14]. These findings were further confirmed in another study that verified that *T. triandra* leaf extract at a dose of 300 and 600mg/kg body weight could considerably exhibit neuroprotective effects on hippocampal neurons by increasing the hippocampal choline acetyltransferase (ChAT) activity and total hippocampal cell number in the brain area [16].

In 2021, Liu et al. [43] evaluated the mitigation of peripheral neuropathy triggered by cisplatin in rats using *T. triandra*. The authors found that rats under the *T. triandra* powder treatment restored motor coordination deficits and recovery in thermal and chemical hyperalgesia and mechanical allodynia. Notably, rats presented a significant alteration in hematological parameters generated by cisplatin. However, rats under *T. triandra* treatment (250 and 500mg/kg, po) for 5 weeks showed an increase in white blood cells, platelets, and red blood cells levels, demonstrating the effectiveness of the treatment in amelioration of cisplatin-induced peripheral neuropathic pain in male Wistar rats. These results suggested that the potent antioxidant properties and the ability to reduce inflammation presented by the *T. triandra* power are related to the compounds presented in the employed extract, such as 5,7-dihydroxy-6-oxoheptadecanoic acid, ethyl-5,7-dihydroxy-6-oxooctadecanoate, ethyl linoleate, ethyl linoleate, ethyl pheophorbide A, and pheophorbide A compared to the CISP-treated group for the same duration (2.5mg/kg/week for 4 weeks) and the normal saline-treated rats group [43]. Globally, the literature search supports the neuroprotective efficacy of *T. triandra* in in vitro and animal models. Thus, human trials will likely open a new avenue for its use as a neuroprotective agent.

5.2.5. Antiobesogenic Effect. Some other studies have also evaluated the hypocholesterolemic and antiobesogenic potential of *T. triandra*. A study by Duangjai and Saokaew [1] indicated that methanolic extracts of *T. triandra* leaves at different concentrations (100, 200, 400, and 500 μg/mL) decreased cholesterol uptake by 48% in a Caco-2 cell assay; this effect might be related to the inhibitory capacity of the extracts on pancreatic lipase (IC_{50} = 273.5 μg/mL) (Table 3). Thus, the authors suggested that *T. triandra* might be a potential source of cholesterol-lowering chemicals. *T. triandra* plant extracts at various concentrations (100, 200, 400, and 500 μg/mL) were reported to show promising cholesterol-lowering effects by inhibiting the pancreatic lipase activity (IC_{50} = 273.5 μg/mL) and decreasing the cholesterol micellar solubility [1] when compared with ezetimibe, a cholesterol absorption inhibitor, at 100 μM, which was used as a positive control of cholesterol uptake in *in vitro* experiments (Table 3). It was also reported that the inhibition potential of *T. triandra* extracts (273.5 μg/mL) for pancreatic lipase activity is less potent than orlistat (IC_{50} at 1.52 ng/mL), a well-known pancreatic lipase inhibitor, which prevents dietary fat from being absorbed in the intestine. A possible limitation of this study is that there is no detailed description of the pharmacological effect according to the composition of the extract. The authors hypothesize that anthocyanins decrease the micellar cholesterol solubility and then suppress cholesterol uptake in Caco-2 cells. Also, the authors suggest that phenolic compounds modulate the size and solubility of cholesterol micelles and inhibitory cholesterol uptake. However, this phenomenon would seem to be dose-dependent and related to the type of chemical composition. The antiobesogenic potential of *T. triandra* in terms of inhibiting pancreatic lipase, enrichment of lipolysis, and reduction of lipid accretion was reported by Ruangaram and Kato [48]. A compound in *T. triandra* (tiliacorinine 12′-O-acetate) was studied for its vasorelaxation properties and its mechanism in isolated rat aorta [49]. Tiliacorinine is one of the main constituents of *T. triandra* with a moderate vasorelaxant effect. However, the acetylation of tiliacorinine and tiliacorinine 12′-O-acetate exhibited higher vasorelaxant activity. The mechanism elucidated by the authors confirms that tiliacorinine 12′-O-acetate induced endothelium-dependent vasorelaxation through the eNOS/NO/sGC pathway in rat aorta and also induced endothelium-independent vasorelaxation involving the modulation of sGC activity, Kv channels, and Ca^{2+} influx through Ca^{2+} channels and intracellular Ca^{2+} release on smooth muscle cells. Last, *T. triandra* alleviates hyperglycemia, hyperlipidemia, oxidative stress, and inflammation properties in obese conditions induced by a high-fat diet in rats [40]. The studies regarding the effect of *T. triandra* on noncommunicable diseases are still not definitive. Many studies are still needed, among them are the toxicological and pharmacokinetic studies identifying and isolating the compound or compounds responsible for the potential clinical effect and testing their administration mode. Moreover, the mechanism of action of *T. triandra* extracts is still not elucidated.

5.2.6. Miscellaneous Properties. While numerous works of literature established *T. triandra* as an important traditional plant with multiple beneficial effects, research using animal models has further confirmed their roles against different forms of health ailments. Studies support the antiallergic potency of *Benchalokawichian* (BCW) on itching and treatment of other skin allergic disorders [50]. *T. triandra* extracts significantly reduced porcine reproductive and respiratory syndrome, PRRS (derived high burden on the swine industry in the world) virus infectivity in MARC-145 cells, and virus titer 3.5 median, tissue culture infective dose (TCID50)/ml (log10) at 24h postinfection. Authors suggested that *T. triandra* could suitably prevent porcine reproductive and respiratory syndrome (PRRS) caused by the PRRS virus in pigs under the in vivo condition [41]. Their results showed that the extract from the *T. triandra* significantly inhibited the infection of the PRRS virus in the MARC-145 cells at an effective dose of 2.5 $TCID_{50}$/ml (log 10) after 72h of postinfection [41]. However, its use is pending on further validation of *T. triandra* efficacy in pigs infected with PRRS. In another study, Duangjai and Saokaew [1] indicated that the leaf extracts of *T. triandra* decreased cholesterol uptake by up to 48% in a Caco-2 cell model. Another study revealed that leaves act in mice with ischemia/reperfusion injury to support memory and behavioral flexibility [15]. Lupeol in the extracts of *Ha-Rak* (a

combination of plants including that of *T. triandra*) showing cytotoxicity against SW620 cell lines with the IC_{50} values of 30.10–212.24 μg/ml was reported by Somwong and Chuchote [51].

Usage of supplements of bamboo grass (*T. triandra*) is known to improve feed intake, digestibility of nutrients, especially roughage intake, and digestibility of dry matter and neutral dietary fiber in dairy cows without any adverse effects on milk production or quality. Such activities were mainly due to bioactive compounds such as condensed tannins and propionic acid [52]. The authors supported their explanation with the effect produced by condensed tannins (CT). CT binds with dietary proteins and delays protein degradation, increasing protein utilization for microbial protein synthesis and feeding digestion. However, the authors did not determine the structural composition of the *T. triandra* pellet, so further studies would be attractive to establish a more precise correlation. Furthermore, the *T. triandra* supplementation significantly increased the concentration of certain unsaturated fatty acids, such as linoleic conjugates. Overall, *T. triandra* possesses numerous potential bioactive compounds that can be safely used in humans soon through modern-day clinical research. Some of the critical biomedical applications of *T. triandra* are listed (Table 3).

6. Future Perspective of *T. triandra* in Medication and Nutrition

From the above discussion, it can be speculated that the extracts of *T. triandra* possess several nutritional values and potential for treatment against infectious diseases. While several researchers have only focused on the isolation of novel bioactive compounds with multiple applications in the biomedical field, the nutritional potential of the plant has dramatically been overlooked [53]. Although the plant possesses numerous biological activities and may be effective as a source of different drugs, the in-depth in vivo analysis, mechanism of action, and elucidation of the potential pharmacological properties require further investigation. Its pharmacological efficacy could be enhanced by applying modern scientific tools, including nanotechnology and advanced drug delivery systems. Since the scanty information on the in vivo and clinical experimental data, further detailed research must be undertaken to analyze and quantify the benefits of *T. triandra* extracts as a potential source of modern-day drugs and as a nutritional supplement for humans and animals. Recent advancements and developments in drug discovery and delivery systems have indicated that nanoparticles synthesized from diverse sources of plant-based materials tend to display improved biomedical or pharmacological activity in comparison to the crude source of plant material [17,36]. Therefore, future research must focus on the green synthesis of various metallic, zero-valent nanoparticles using the extracts of *T. triandra* as the source of reducing agents in the synthesis process and study its biomedical applications.

7. Conclusions

T. triandra plant species are rich sources of bioactive compounds like alkaloids, flavonoids, terpenoids, proteins, and carbohydrates. Based on its numerous ethnomedicinal potentials, this plant species could also be used as a source of nutraceuticals. Moreover, it possesses antimicrobial, antiparasitic, antidiabetic, anticancer, antioxidant, and anti-obesogenic activities. It is an underappreciated species with several potential uses in traditional herbal therapy. The vast nutritional properties of these plant species need further in-depth investigation for their effective applications in pharmaceutical and biomedical fields. Therefore, further advanced research must be directed toward the in vitro and in vivo investigations of the *T. triandra* plant species for verifying its medicinal properties against several infectious and noninfectious diseases and its utility as a novel nutritional supplement.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

In Silico Elucidation of Potent Inhibitors from Natural Products for Nonstructural Proteins of Dengue Virus

Bibek Raj Bhattarai,[1] **Bikash Adhikari**,[1] **Saroj Basnet**,[2] **Asmita Shrestha**,[1] **Rishab Marahatha**,[1] **Babita Aryal**,[1] **Binod Rayamajhee**,[3] **Pramod Poudel**,[4] **and Niranjan Parajuli**[1]

[1]*Biological Chemistry Lab, Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal*
[2]*Center for Drug Design and Molecular Simulation Division, Cancer Care and Research Center, Kathmandu, Nepal*
[3]*School of Optometry and Vision Science, Faculty of Science, UNSW, Sydney, NSW 2052, Australia*
[4]*Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal*

Correspondence should be addressed to Niranjan Parajuli; niranjan.parajuli@cdc.tu.edu.np

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Medicinal plants have been used from the beginning of human civilization against various health complications. Dengue virus (DENV) has emerged as one of the most widespread viruses in tropical and subtropical countries. Yet no clinically approved antiviral drug is available to combat DENV infection. Consequently, the search for novel antidengue agents from medicinal plants has assumed more insistence than in previous days. This study has focused on 31 potential antidengue molecules from secondary metabolites to examine their inhibitory activity against DENV nonstructural proteins through molecular docking and pharmacokinetics studies. In this research, the wet lab experiments were tested on a computational platform. Agathisflavone and pectolinarin are the top-scored inhibitors of DENV NS2B/NS3 protease and NS5 polymerase, respectively. Epigallocatechin gallate, Pinostrobin, Panduratin A, and Pectolinarin could be potential lead compounds against NS2B/NS3 protease, while acacetin-7-O-rutinoside against NS5 polymerase. Moreover, agathisflavone (LD_{50}= 1430 mg/kg) and pectolinarin (LD_{50}= 5000 mg/kg) exhibited less toxicity than nelfinavir (LD_{50}= 600 mg/kg) and balapiravir (LD_{50} = 824 mg/kg), and the reference drugs. Further research on clinical trials is required to analyze the therapeutic efficacy of these metabolites to develop new potential drug candidates against different serotypes of DENV.

1. Introduction

Dengue, a neglected tropical disease, is a growing threat to public health, especially in developing countries, and is the most common arboviral infection in the world [1]. Dengue was listed in the top ten global health threats of 2019 by the World Health Organization (WHO), which puts nearly 40% of the worldwide population at risk of dengue infection, and about 390 million cases are reported per year [2]. Mosquito-borne dengue cases are rapidly increasing in different tropical and subtropical regions, even in the more expanded form [3–5]. WHO has planned to reduce mortality and morbidity caused by the dengue virus (DENV) by 50% and 25%, respectively, by 2020 [6]. DENV is an arthropod-borne flavivirus having antigenically distinct serotypes 1–4 (DENV-1, DENV-2, DENV-3, and DENV-4) [7, 8]. These serotypes vary from one another in amino acid sequences by 25 to 40%, and genotypes by approximately 3% [9]. However, the new serotype of DENV (DENV-5) was also isolated in 2013 and could emerge due to natural selection and genetic recombination [10].

DENV is an enveloped, single-positive-stranded RNA virus of ~11 kilobases with a single open reading frame belonging to the *Flaviviridae* family [11]. DENV is closely related to the West Nile virus, yellow fever virus, hepatitis C virus, and encephalitis viruses [11, 12]. The molecular basis

of DENV virulence remains unclear since a definitive receptor for the entry of the virus has not been recognized. The *in vivo* study suggests that DENV targets include macrophages, dendritic cells, monocytes, mast cells, and probably endothelial and hepatocytes [9]. Rapid urbanization, accelerated population growth, global warming, inefficient mosquito management, and a lack of health care facilities are the major reasons behind the increased cases of DENV infections [13].

Dengue fever, dengue hemorrhagic fever, and dengue shock syndrome are the health threats developed by DENV infection [14]. Fever, frontal headache, rash, swollen glands, nausea, vomiting, nasal stuffiness, sore throat, retro-orbital pain, abdominal, muscle, and joint pain are the most common symptoms that arise after evading DENV on human dendritic cells through vector mosquitoes *Aedes aegypti* and *Aedes albopictus* [15].

The genome of DENV encodes structural proteins; core or capsid (C), premembrane (prM or M), envelope (E), and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) required for the viral cycle [16, 17]. Nonstructural proteins are involved in viral RNA replication and evasion of host immunity, while receptor binding, fusion, maturation, and assembly of DENV are controlled by structural proteins [11, 18]. These proteins could be a target to prevent DENV infections. Due to variations in the genetic material of DENV, the development of the curative method is difficult [19, 20].

Currently available vaccines or drugs are not very satisfactory against DENV and its complications, so the development of sound, practical, and long-lasting therapeutic approaches is on the verge of controlling and preventing fatal outcomes [21, 22]. Genetically engineered mosquitoes, engineered antibodies, and the release of insect-dominant lethal and sterile insect techniques are the latest strategies to control DENV outbreaks [23]. Various *in vitro* and *in vivo* studies worldwide showed different antiviral compounds (Figure 1) from natural products inhibit protease and replication proteins of DENV (Table 1S). These compounds are further analyzed to develop them as possible antidengue drugs. Artificial intelligence (AI) has drawn a lot of attention in drug design, screening potential drug candidates, and quantitatively studying their structure-activity affinity [24, 25]. Here, we carried out molecular docking and pharmacokinetics studies of the antidengue compounds that aid drug discovery programs.

2. Methods

2.1. Selection and Preparation of Proteins and Ligands. NS2B/NS3 Protease (PDB ID: 2FOM, Resolution - 1.50 Å) [26] and NS5 Polymerase (PDB ID: 2J7U, 1.85 Å) [27] of DENV were selected as targets (Tables 2S and 3S). The open conformation of NS2B/NS3 protease is favored over the closed conformation to study ligand binding and conformation change [28]. The crystal structures of these target proteins were retrieved from the Protein Data Bank (PDB) (https://www.rscb.org/). The energy was minimized, hydrogens were added; water and ligand molecules were eliminated from protein structures using the Molecular operating environment (MOE) program [29, 30]. This refined structure was then used as a receptor for docking analyses.

2.2. Preparation of Ligands. Compounds with antidengue activities isolated from natural products were selected as ligands through a literature survey (Table 1S). Experimented molecules through *in vitro* or *in vivo* studies were examined in the computational platform to know their therapeutic potential. The chemical structures of active antidengue compounds as ligands were constructed using ChemDraw (Cambridge Soft) and processed or optimized for docking using the MOE program.

2.3. Identification of the Docking Site. The binding sites for NS2B/NS3 protease and NS5 polymerase were identified by using the Site-Finder module of MOE Software [30] (Table 4S) and cross-checked with the available literature [26, 27, 31–35]. After finding active sites, a grid box was created on receptors (target proteins). Blind docking was performed using nelfinavir and balapiravir as standard drugs for NS2B/NS3 protease and NS5 polymerase, respectively.

2.4. Physicochemical Properties and Secondary Structure of Proteins. The physicochemical properties of the dengue proteins (PDB ID: 2J7U and 2FOM) were accessed using the Expasy ProtParam web server [36, 37], which helps to determine the essential characteristics of a protein in different biological forms. The described server was used to calculate molecular weight, amino acid composition, molecular formula, theoretical isoelectric point (Pi), the total number of positive and negative residues ($+R/-R$), extinction coefficient (EC), instability index (AI), aliphatic index, and grand average of hydropathicity (GRAVY).

Secondary structure prediction is an *in silico* approach that assigns all residues from all conceivable states based solely on hydrogen bonding patterns and some geometric constraints [38]. The annotated secondary structure elements (SSEs) were predicted using the self-optimized prediction method with alignment (SOPMA) [39].

2.5. Molecular Docking Analysis. GOLD (Genetic Optimization for ligand Docking) version 4.0.1 based on a genetic algorithm with MOE version 3.12 was used to examine the binding of the ligands to the target proteins 2FOM and 2J7U of DENV. The ligand interactions, including 2D and 3D representations and distance between ligand and receptor protein interactions, were studied by the ligand interaction module in the MOE Program and visualized on the Biovia Discovery Studio Visualizer for graphical analysis and final processing.

GOLD fitness is a force field-based scoring function exploring binding modes of ligands with receptor proteins containing four terms: (a) protein-ligand hydrogen bond energy (external H-bond); (b) protein-ligand van der Waals

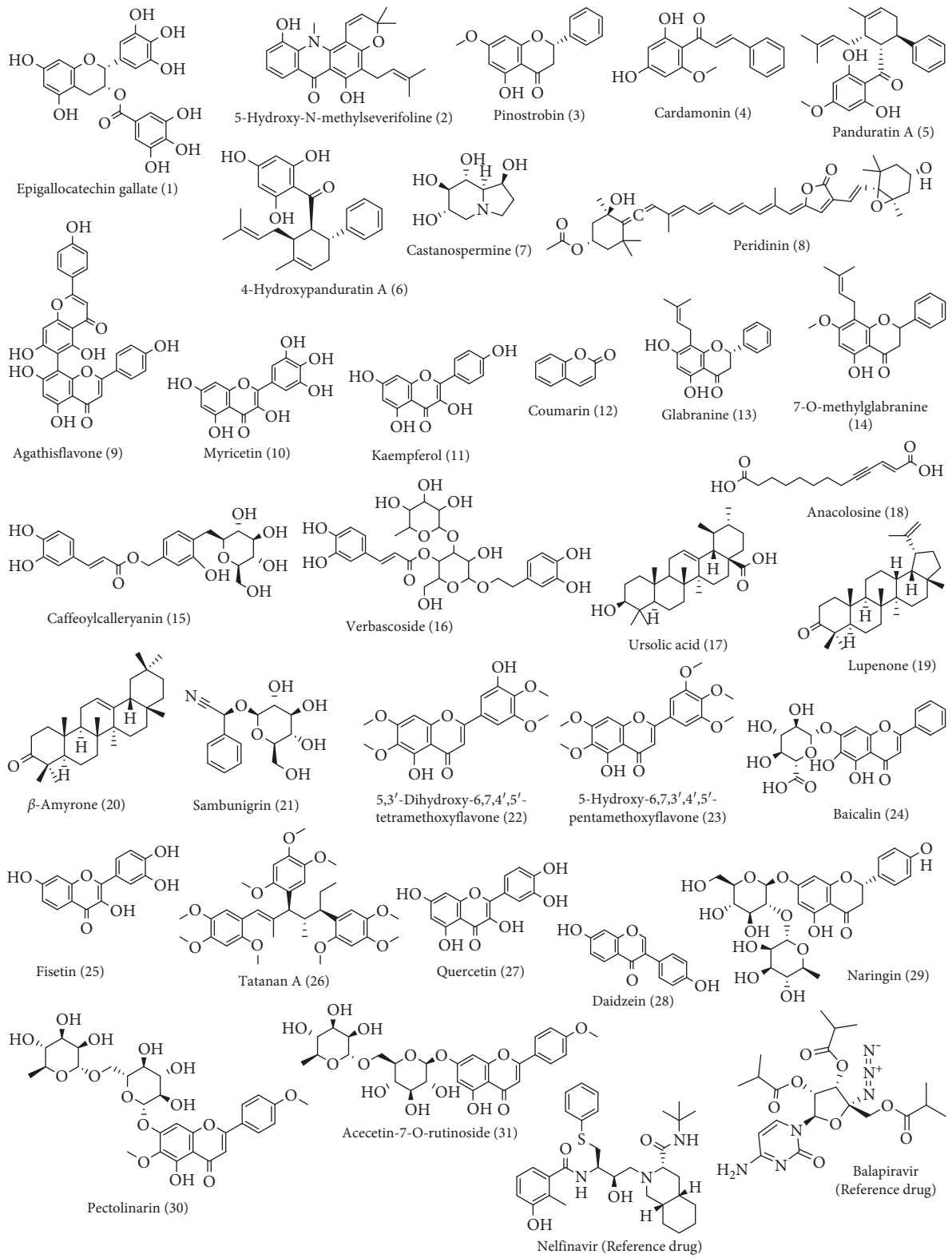

Figure 1: Antidengue compounds isolated from various medicinal plants.

energy (external vdw); (c) ligand internal van der Waals energy (internal vdw); and (d) ligand intramolecular hydrogen bond energy (internal H-bond) [40].

$$\text{GOLD fitness} = S_{\text{hb\_ext}} + S_{\text{vdw\_ext}} + S_{\text{hb\_int}} + S_{\text{vdw\_int}}, \quad (1)$$

where $S_{\text{hb\_ext}}$ = protein-ligand hydrogen-bond score; $S_{\text{vdw\_ext}}$ = protein-ligand van der Waals score; $S_{\text{hb\_int}}$ = contribution due to intramolecular hydrogen bonds in the ligand; and $S_{\text{vdw\_int}}$ = contribution due to intramolecular strain in the ligand.

The GOLD fitness score was taken as a basis to rank the binding modes of the ligand. A GOLD fitness score was obtained by subtracting intramolecular terms from GOLD fitness [40]. Higher the GOLD fitness score of ligands, higher the putative inhibitors of receptor proteins.

2.6. Estimation of Binding Energy Using a Semiempirical Method. To estimate binding energy, we applied a semiempirical method in-built to AutoDock Vina [41]. The semiempirical prediction of total binding energies is considered well suited for the relative rankings [42]. The pIC_{50} value was calculated using the formula: $pIC_{50} = -\log(IC^{*}_{50}10^{-9})$ and $\Delta G_{\text{Experimental}}$ was calculated by the equation: $\Delta G_{\text{Exp.}} = -RT \ln ({}_{p}IC_{50})$ [43].

2.7. Validation of Molecular Docking. The docking results were validated by extracting the standard nelfinavir and balapiravir from their original binding site and redocking them into the same positions. The lowest energy pose obtained on redocking and the previous docking position of compounds were superimposed, and its root mean square deviation (RMSD) was calculated. To validate the docking process, the RMSD must be within the reliable range of 2 Å [44, 45].

2.8. Prediction of Pharmacokinetic Parameters. Pharmacokinetic properties such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) of selected potent antidengue compounds were predicted by using chemoinformatic tools (pkCSM and ProTox-II) [46, 47]. *In silico* structure-based toxicity analysis by ProTox-II gives information about different levels of toxicity such as oral toxicity, organ toxicity, toxicological endpoints (carcinogenicity, cytotoxicity, hepatotoxicity, immunotoxicity, and mutagenicity), toxicological pathways, and active targets with a confidence score [46].

3. Results

3.1. Physicochemical Properties and Secondary Structure of Proteins. Expasy ProtParam allows the computation of various physical and chemical parameters for a given protein. The physicochemical properties of dengue proteins are depicted in Table 1, which displayed that the selected proteins (2J7U and chain B of 2FOM) are stable, which is given by the instability index.

Secondary structure elements of a protein like an alpha helix, beta-strands, and turns are the key constituent parts of protein structure which are displayed in Table 2 and the graphical representation is shown in Figure 1S.

3.2. Molecular Docking Analysis. GOLD fitness scores, hydrogen-bonding interactions between targets and secondary metabolites, interaction type, and bond length of the docking were shown in Tables 3, 4, 5S, and 6S. The 2D and 3D interactions of the top-scored metabolites and commercial drugs with the target proteins were shown in Figure 2 and Supplementary Figures 2S–7S. The details of the molecular properties of standard drugs and secondary metabolites are shown in Supplementary Table 7S. Supplementary Tables 8S and 9S illustrate the theoretical (calculated) and experimental binding energies of secondary metabolites.

3.2.1. Molecular Docking Analysis of NS2B/NS3 Protease. The interactions mode obtained by molecular docking for Agathisflavone (9) and the standard drug nelfinavir were illustrated in Figure 2 and Supplementary Figure 2S. The docking of ligands was evaluated based on their GOLD fitness score and lowest binding energy values (kcal/mol). Agathisflavone (9) (GOLD fitness score 64.12, $\Delta G = -8.6$ kcal/mol) showed hydrogen bonds with the amino acids Thr 149, Asn 196, and Val 175 with bond distances of 3.4 Å, 3.4 Å, and 2.1 Å, respectively. Furthermore, nelfinavir showed H-bonding residue with Trp 118 at a bond distance of 1.4 Å. Additionally, Epigallocatechin gallate (1), Pinostrobin (3), Panduratin A (5), Peridinin (8), and Pectolinarin (30) were among the top-scored compounds with NS2B/NS3 Protease (Table 3).

3.2.2. Molecular Docking Analysis for NS5-Polymerase. In silico studies showed pectolinarin (30) as a top-scored inhibitor of NS5-polymerase with a GOLD fitness score of 50.60 ($\Delta G = -7.8$ kcal/mol) and interacted with Glu 197, Ile 487, and Arg 482 amino acids with bond distances of 2.0/3.3 Å, 3.8 Å, 1.7/2.6/2.6/2.4 Å, respectively (Figure 2). It showed Π-cation interactions with Lys122 and Lys123. Acacetin-7-O-rutinoside (31) shows H-bonding interaction with Lys123, Thr126, Asn127, and Arg482 with a GOLD fitness score of 46.10 (Table 4). Similarly, balapiravir was also found to interact with Arg482 and Asn127 at bond lengths of 1.6/2.3 Å and 2.0 Å, respectively (Figure 6S).

3.3. Estimation of Binding Energy. The relationship between theoretical and experimental binding free energies, IC50, and pIC50 of secondary metabolites for NS2B/NS3 Protease and NS5-polymerase was studied (Tables 8S and 9S). The theoretical and experimental binding free energies showed a good correlation. The binding free energies of top-scored inhibitors; epigallocatechin gallate (1), pinostrobin (3), panduratin A (5), agathisflavone (9), pectolinarin (30), and acacetin-7-O-rutinoside (31) are −7.7, −7.7, −7.9, −8.6, −8.0, and −13.9 kcal/mol, respectively.

3.4. Analysis of ADMET Profiles. On predictive pkCKSM, the value of logKp greater than −2.5 indicates low skin permeability. If compounds have a logPapp value greater than 0.90, then they are considered to have high Caco-2 permeability. Another important parameter, the volume of distributions (VD), is low if logVD is less than −0.15 and high if it is more significant than 0.45 [48]. It has an impact on total clearance and half-life. Additionally, compounds with log PS > −2 would penetrate the central nervous system (CNS) and act as CNS-active drugs. However, these protease and polymerase inhibitors were found to be central nervous system inactive, so they could be good drugs against DENV infection. Different

Table 1: Physicochemical properties of dengue protein.

Physicochemical properties	NS5 (PDB ID: 2J7U)	NS2B/NS3 (PDB ID: 2FOM)	
		Chain A	Chain B
Molecular weight	73489.78	6405.85	19909.62
Theoretical pI	8.01	3.99	8.07
Molecular formula	$C_{3256}H_{5202}N_{892}O_{989}S_{38}$	$C_{265}H_{425}N_{75}O_{105}S_2$	$C_{896}H_{1409}N_{241}O_{268}S_2$
Total no. of atoms	10377	872	2816
Instability index	34.50	83.86	32.42
Aliphatic index	70.17	74.03	79.51
GRAVY	−0.640	−0.555	−0.409

Table 2: The prediction of the secondary structure of dengue protein.

Protein name	Alpha helix (Hh)	Extended strand (Ee) (%)	Beta turn (Tt) (%)	Random coil (Cc) (%)
NS5	45.04	13.39	5.20	36.38
NS2B/NS3 (chain A)	38.71	19.35	8.06	33.87
NS2B/NS3 (chain B)	11.35	31.89	15.14	41.62

Table 3: H-bonding interaction of nelfinavir and natural metabolites with DENV NS2B/NS3 protease (2FOM) with bond length, interacting residues, GOLD fitness score, and experimental IC_{50} values.

S.N.	Compound name	GOLD fitness score	Calculated binding energy (ΔG) (kcal/mol)	IC_{50} value (μM)	H-bonding residues within $4A^O$ radius	Bond length	Other interaction with resides
Reference	Nelfinavir	41.21	−8.0	—	Trp 118	1.4	—
1.	Agathisflavone (9)	64.12	−8.6	15–17.5	Thr 149 Asn 196 Val 175	3.4 3.4 2.1	—
2.	Pectolinarin (30)	59.32	−7.8	—	Asn 196 Lys 103 Gly 116 Leu 114 Leu 178	1.8 1.7 2.1 3.6/1.3 2.1	—
3.	Panduratin A (5)	54.07	−7.9	90.4 ± 2.3% at 400 ppm	Ile 194	2.2	—
4.	Peridinin (8)	53.17	−7.9	4–7	Trp 112 Leu 114	1.8 1.6	—
5.	Epigallocatechin gallate (1)	49.27	−7.7	11–18	Gly 116 Leu 178 Ala 193 Asn 181 Trp 118	2.8 1.7 2.2 2.2 1.7	—
6.	Acacetin-7-O-rutinoside (31)	48.05	−14.2	—	Gly 116 Asn 181 Ala 193	2.6 1.4 1.6	—
7.	4-Hydroxypanduratin A (6)	45.87	−7	90.4 ± 0.3% at 120 ppm	Lys 103 Asn 181	2.2 1.9	—

parameters of ADMET properties of selected potent antidengue compounds are shown in Supplementary Tables 10S–13S. The top-scored compounds were within the categorical range of having good pharmacokinetic properties.

4. Discussion

Plants-derived secondary metabolites have been screened to tackle the problems of various infectious diseases because of their effectiveness, easy accessibility, and believed to have low side effects [49]. Natural products have a wide diversity of secondary metabolites, so they are a good source of pharmacological drug candidates against DENV infections. In *in vitro* and *in vivo* assays, various compounds (Figure 1) from natural sources are shown to have antidengue properties (Table 1S). No chemical compounds have been clinically approved as DENV medicine or drugs to date.

4.1. Antidengue Properties of Some Secondary Metabolites. An antiviral assay based on cytopathic effects (CPE) of six medicinal plants (*Andrographis paniculata*, *Citrus limon*,

Table 4: H-bonding interaction of balapiravir and natural metabolites with DENV NS5 polymerase (2J7U) with bond length, interacting residues, GOLD fitness score, and experimental IC_{50} values.

S.N.	Compound name	GOLD fitness score	Calculated binding energy (ΔG) (kcal/mol)	IC_{50} value (μM)	H-bonding residues within $4A^{O}$ radius	Bond length	Other interaction with resides
Reference	Balapiravir	49.42	−12.1	—	Arg 482 Asn 127	1.6/2.3 2.0	—
1.	Pectolinarin (30)	50.60	−8.0	138.78 ± 6.10	Glu 197 Ile 487 Arg 482 Lys 122 Lys 123	2.0/3.3 3.8 1.7/2.6/2.6 /2.4	 Π-Cation Π-Cation
2.	Acacetin-7-O-rutinoside (31)	46.10	−13.9	—	Lys 123 Thr 126 Asn 127 Arg 482	3.5 2.5 2.9/2.1 2.2/2.2/2.2	 Π-Cation
3.	Caffeoylcalleryanin (15)	43.39	−7.7	6.06 ± 0.99	Asn 127 Ser 481 Arg 482	1.8 3.0 1.9/1.8	 —
4.	Naringin (29)	43.30	−9.0	289.75	Trp 485 Ser 486 His 491 Ala 489 Arg 482 Lys 123	2.6 1.5 2.2/3.7 2.1 2.1/2.4/2.1 2.4/32	 Π-Cation
5.	Glabranine (13)	40.53	−7.8	70% at 25 μM	Asn 127 Arg 482 Thr 126 Lys 122 Lys 123	2.0 2.3 2.1 – –	 Π-Cation Π-Cation
6.	7-O-Methylglabranine (14)	39.86	−7.5	70% at 25 μM	Arg 482	1.7/2.5	—
7.	Anacolosine (18)	39.84		2.5 ± 0.1	Glu 183	1.7	—

Cymbopogon citratus, *Momordica charantia*, *Ocimum sanctum*, and *Pelargonium citrosum*) on DENV-1-infected Vero E6 cells with MNTD showed that *A. paniculata*, followed by *M. charantia*, shows significant antiviral inhibitory effects in *in vitro* assays [50]. Extracts of *Boesenbergia rotunda*, *Carica papaya*, *Cissampelos pareira*, *Cladogynos orientalis*, *Dryopteris crassirhizoma*, *Eubhorbia hirta*, *Faramea hyacinthine*, *Faramea truncate*, *Flagellaria indica*, *Gastrodia elata*, *Houttuynia cordata*, *Lithospermum erythrorhizon*, *Morus alba*, *Myristica faltu*, *Ocimum sanctum*, *Piper retrofractum*, *Psidium guajava*, *Quercus lusitanica*, *Rhizophora apiculata*, *Solanum nigra*, *Smilar glabla*, *Syzgium grande*, *Syzgium companula*, *Taraxacum officinale*, *Urtica dioica*, and *Zostera marina* have shown protective activity against different serotypes of DENV in *in vitro* assays [51–58].

4.2. Protease Inhibitors. Molecules capable of inhibiting or altering the virus's protease could be potential drug candidates. Epigallocatechin gallate (1) from *Camellia sinensis* (L.) Kuntze shows antiviral activity with an EC_{50} of 14.8 ± 2.6, 18.0 ± 1.0, 11.2 ± 1.7, and 13.6 ± 0.0 μM for DENV-1, DENV-2, DENV-3, and DENV-4, respectively, in Vero cells, probably by disturbing protein configuration involved in viral infection [59]. An alkaloid, 5-hydroxy-N-methylseverifoline (2) isolated from *Atalantia buxifolia* (Poir.) Oliv. ex Benth. Twigs, demonstrated antiviral activity with an IC_{50} of 5.3 ± 0.4 μM on DENV-infected Huh-7 cells [60]. Pinostrobin (3), cardamonin (4), panduratin A (5), and 4-hydroxypanduratin A (6) isolated from *Boesenbergia rotunda* (L.) showed significant inhibition of DENV-2 NS3 protease in an *in vitro* assay [61]. Panduratin A (4) and 4-hydroxypanduratin A (5) showed competitive inhibition, while pinostrobin (3) and cardamonin (4) act as noncompetitive inhibitors toward DENV-2 NS3 protease with smaller K_i values [61]. Castanospermine (7), an alkaloid from *Castanospermum australe* A. Cunn. ex Mudie, inhibits four serotypes of DENV-infected Huh-7 and BHK-21 cells by altering the protein structure responsible for viral secretion [12]. A carotene like compound, peridinin (8), showed protective effects with an IC_{50} of 7.62 ± 0.17, 4.50 ± 0.46, 5.84 ± 0.19, and 6.51 ± 0.30 μM against DENV-1, DENV-2, DENV-3, and DENV-4, respectively [62]. Agathisflavone (9) isolated from *Cenostigma macrophyllum*,

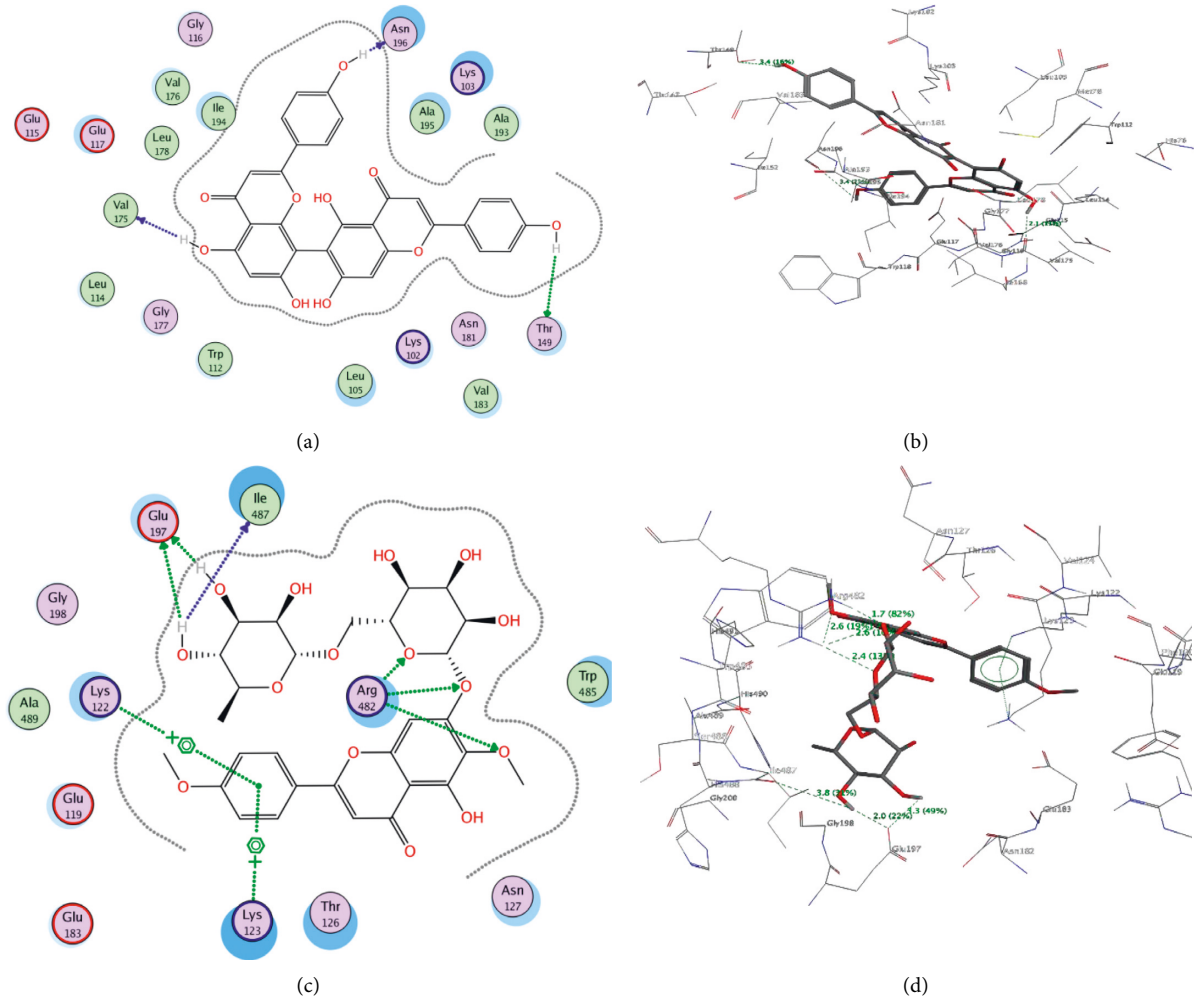

Figure 2: 2D (a) and 3D (b) interaction of agathisflavone with 2FOM (Fitness score 64.12); 2D (c) and 3D (d) interaction of pectolinarin with 2J7U (Fitness score 50.60). 2FOM is dengue virus NS2B/NS3 protease and 2J7U is dengue virus NS5 RdRP domain.

myricetin (10), and kaempferol (11) showed antiviral activity with an IC_{50} of 15.1 ± 2.2 and $17.5 \pm 1.4\,\mu$M, 22.3 ± 1.8 and $29.3 \pm 3.3\,\mu$M, and 37.8 ± 1.4 and $27.7 \pm 3.2\,\mu$M against DENV-2 and DENV-3 serine protease, respectively [63].

Phylogenetic analysis showed sequences of NS2B-NS3 and NS5 were highly conserved compared to others [64]. Thus, we have selected them as targets for molecular docking studies of potent antidengue compounds derived from natural products. The present *in silico* study aims to gather information on the probable mechanism of antidengue compounds' action through analysis of ligand-receptor binding.

DENV NS3 is a multifunctional DENV protein (618 amino acid residues) with protease domain at N-terminal, and RNA 5′-triphosphatase, RNA helicase, and nucleoside triphosphates domain in the C-terminal of the peptide, with cofactor NS2B, forms NS3-NS2B complex. It plays a pivotal role in viral polyprotein processing and replication [65–67]. Besides, NS3 also helps in the capping of nascent genomic RNA with an ATPase activity [68]. A catalytic triad (His51-Asp75-Ser135) present between two β-barrels of NS3 is highly conserved and is of prime functional importance [31]. The protein NS2B is involved in several internal cleavages and cleaving of NS2A/NS2B and NS2B/NS3 [69]. Agathisflavone (9), Pectolinarin (30), Panduratin A (5), Peridinin (8), Epigallocatechin gallate (1), and Pinostrobin (3) fit on the active site of protease (Arg482, Asn127, Glu197, Ile 487, Trp 485, Ser 486, His491, Ala489, Lys123, Thr126, Ser 481) competitively and block the enzyme activity acting, as potential inhibitors of NS3-NS2B protease of DENV. Common interacted active residues were Thr149, Asn196, Val175, Lys103, Gly116, Leu114, Leu178, Ile194, Trp112, Leu114, Gly 116, Ala193, Asn181, and Trp118. Interaction via hydrogen bonds has a noteworthy impact on drug specificity, metabolism, and adsorption [70].

4.3. Replication Inhibitors. Coumarin (12), Glabranine (13), and 7-O-methylglabranine (14) act as replication inhibitors, showing inhibition of 70% DENV-2 infection at 25 μmol/L on infected LLC-MK2 cells [71–73]. Caffeoylcalleryanin (15), verbascoside (16), and ursolic acid (17) isolated from the ethanol extract from *Arrabidaea pulchra* (Cham.) Sandwith leaves were protective against DENV-2 with EC_{50} (2.8 ± 0.4, 3.4 ± 0.4, and $3.2 \pm 0.6\,\mu$g/mL) and SI values (20.0, 3.8, and 3.1), respectively [74]. Anacolosine (18), lupenone (19),

β-amyrone (20), and (S)-sambunigrin (21) were isolated from *Anacolosa pervilleana* Baill showed protective activity against DENV [75]. 5,3′-dihydroxy-6,7,4′,5′-tetramethoxyflavone (DHTMF) (22) and 5-hydroxy-6,7,3′,4′,5′-pentamethoxyflavone (HPMF) (23) were shown to have antidengue properties with EC_{50} of 5.62 μg/mL and 4.47 μg/mL by using dengue virus-green fluorescent protein (DENV/GFP) replicon, a cell-based model [76]. Baicalin (24) isolated from *Scutellaria baicalensis* Georgi showed intracellular antiviral activity on Vero cells against DENV-2 [77]. A flavonoid, fisetin (25) inhibited DENV-2 replication with IC_{50} = 43.12 μg/mL (SI = 5.72) and decreased the RNA levels by 65% at 50 μg/mL [78]. Tatanan A (26), isolated from the ethanol extract of the *Acorus calamus,* showed antiviral activity by inhibiting the early steps of RNA replication of DENV-2 [79]. Molecular docking of artesunic acid and homoegenol derived from *M. fatua*, and andrographolide isolated from *A. paniculata* showed that the compounds are best to inhibit NS5 protein of DENV-2 [56, 80]. Similarly, the compounds suramin, naringin, quercetin, catechin, and hesperidin derived from *P. guajava* exhibited higher affinity against NS5 protein [81].

NS5 (104 KDa) is a peptide with 900 residues containing the N-terminal methyltransferase domain and an RNA-dependent RNA polymerase (RdRP) domain at the C-terminal end, responsible for pathogenesis [82, 83], and about 67% amino acid sequence identity across the four serotypes. Due to the presence of RdRP activity, it has a role in RNA replication, while the N-terminal domain is mainly associated with the capping of viral RNA. The RdRP activity was absent in the host cell while it was present in the viral cell, which indicates that a design of specific inhibitors could be a promising antiviral target with low toxicity [84]. Besides its role in RNA replication and methyltransferase activity, NS5 also has a role in the down-regulation of a host immune interferon response [13, 85, 86]. Pectolinarin (30) and Linarin (31) block the active site (Asn 196, Lys 103, Gly 116, Leu 114, Leu 178, Trp 112, Trp 118, Thr 149, Val 175, Ile 194, Asn 181, Ala 193, and Asn 181) of NS5 RNA-dependent RNA polymerase (RdRP) and alter its configuration such that a substrate-enzyme complex cannot be formed. Hence, enzyme activity is blocked, and replication of the virus is retarded preventing infection.

Besides, some secondary metabolites act as both replication and protease inhibitors against DENV. Coumarin (12) showed antiviral activity against the dengue virus by inhibiting viral proteins required for the virus cycle [73]. Flavonoids like quercetin (27), daidzein (28), and naringin (29) isolated from medicinal plants showed antidengue activity with an IC_{50} value of 35.7, 142.6, and 168.2 μg/mL, respectively, on DENV-2 infected Vero cells [87]. Quercetin (27) decreased DENV-2 RNA levels by 67% by inhibiting RNA polymerase in the viral foci reduction assay [87]. Pectolinarin (30) and acacetin-7-O-rutinoside (31) isolated from *Distictella elongata* (Vahl) Urb showed protective activity against DENV [88].

Similarly, *Pavetta tomentosa* Roxb. ex Sm., *Tarenna asiatica* (Linn.) Alston., and *Zanthoxylum limonella* (Dennst.) Alston. extracts showed larvicidal, pupicidal, and adulticidal activity against *Aedes aegypti* [89, 90]. Different herbal products (andrographolide, δ-cadinene, calarene, δ-4-carene, eugenol, pellitorine, sesamin, asarinin, and (+)-xanthoxylol-γ,γ-dimethylallylether) were shown effective against the larva of the dengue vector responsible for transmission to humans, another way to get rid of DENV risks and complications [91–94].

4.4. Pharmacokinetic Parameters and Future Direction. Evaluation of pharmacokinetic properties such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) parameters is indispensable in drug discovery. Toxicity prediction of secondary metabolites, computational methods help in gathering information that assists in clinical trials (*in vitro* and *in vivo* assays) of screening and classifying toxicity [95, 96]. The pharmacokinetic properties of natural product compounds need to be determined before recommending them as potential drug candidates. ADMET analysis revealed that high GOLD fitness scored compounds have the optimum value of parameters under study according to Lipinski's rule of five [97, 98]. Further research (*in vitro*, *ex vivo*, and *in vivo* validation experiments) is required to analyze these metabolites' therapeutic efficacy against different serotypes of DENV for the development of new potential drug candidates.

5. Conclusion

From the dawn of human civilization, we have been battling against different fatal diseases, and natural products have stood out as the best approach to medical treatment against various diseases. Even today, natural products are one of the best medicinal alternatives against viral infections. Many antidengue compounds' availabilities in herbal products are subject to further study and research. Some of these antiviral compounds could be our ultimate weapon against DENV infection. With the aid of a computational approach, we hereby perform a further study on the therapeutic efficiency of agathisflavone, pectolinarin, epigallocatechin gallate, pinostrobin, panduratin A, pectolinarin, and acacetin-7-O-rutinoside as potential DENV inhibitors.

List of Abbreviations

AI: Artificial intelligence
DENV: Dengue virus
DENV-1: Dengue virus serotype 1
DENV-2: Dengue virus serotype 2
DENV-3: Dengue virus serotype 3
DENV-4: Dengue virus serotype 4
DENV-5: Dengue virus serotype 5
EC_{50}: Half maximal effective concentration
GOLD: Genetic optimization for ligand docking
IC_{50}: Half maximal inhibitory concentration
LD_{50}: Median lethal dose
MOE: Molecular operating environment
PDB: Protein Data Bank
SI: Selectivity index
WHO: World Health Organization.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Bibek Raj Bhattarai and Bikash Adhikari contributed significantly to the literature review; Bibek Raj Bhattarai, Bikash Adhikari, Asmita Shrestha, Rishab Marahatha, Babita Aryal, and Saroj Basnet performed the in silico analysis; Binod Rayamajhee, Pramod Poudel, and Niranjan Parajuli contributed to editing and revising the manuscript. Bibek Raj Bhattarai and Bikash Adhikari have written and contributed equally to the manuscript. Niranjan Parajuli supervised the project.

Supplementary Materials

Table 1S: some antidengue compounds from natural products; Table 2S: details about molecular docking platform; Table 3S: list of targets showing the PDB ID, resolution and description of the proteins selected for docking with complexed inhibitor; Table 4S: active Site residues of NS2B/NS3 Protease and NS5 polymerase; Table 5S: H-bonding interaction of nelfinavir and natural metabolites with DENV NS2B/NS3 Protease (2FOM) with bond length, interacting residues, GOLD fitness score, and experimental IC_{50} values; Table 6S: H-bonding interaction of balapiravir and Natural metabolites with DENV NS5 Polymerase (2J7U) with bond length, interacting residues, GOLD fitness score, and experimental IC_{50} values; Table 7S: molecular Properties of standard compounds and selected secondary metabolites; Table 8S: binding free energy calculations for NS2B/NS3 Protease with inhibitors using semi-empirical method; Table 9S: binding free energy calculations for NS5 polymerase with inhibitors using semi-empirical method; Table 10S: Prediction of toxicity of selected compounds from natural products by ProTox-II; Table 11S: ADMET properties of NS3 protease inhibitors by pkCSM server; Table 12S: ADMET properties of NS5 polymerase inhibitors by pkCSM server; Table 13S: ADMET properties of NS3 protease and NS5 polymerase inhibitors by pkCSM server; Figure 1S: (A), (B), and (C) represents the graph of secondary structure prediction of proteins NS5, NS2B/NS3 (chain A), and NS2B/NS3 (chain B) respectively analyzed using SOPMA server where blue, red, green and purple colored line represents alpha helix, extended strand, beta turn, and random coil respectively: Figure 2S: 2D upper and 3D lower interaction of nelfinavir with 2FOM (Fitness score 41.21); Figure 3S: upper 2D and lower 3D interaction of pectolinarin with 2FOM (Fitness score 59.32); Figure 4S: Upper 2D and lower 3D interaction of panduratin A with FOM (Fitness score 54.07); Figure 5S: upper 2D and lower 3D interaction of peridinin with 2FOM (Fitness score 53.17); Figure 6S: 2D upper and 3D lower interaction of balapiravir with 2J7U (fitness score 49.42); Figure 7S: upper 2D and lower 3D interaction of naringin with 2J7U (fitness score 43.30). (*Supplementary Materials*).

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Review Article

Rivea hypocrateriformis (Desr.) Choisy: An Overview of Its Ethnomedicinal Uses, Phytochemistry, and Biological Activities and Prospective Research Directions

Mohammad Mukim,[1,2] Atul Kabra,[3] Christophe Hano,[4,5] Samantha Drouet,[4,5] Duangjai Tungmunnithum,[5,6] Mohit Chaturvedi,[1] Rakesh Patel,[1] Muhammad Ayaz,[7] and Daniel M. Shadrack[8]

[1]*Dr. A. P. J. Abdul Kalam University, Indore, Madhya Pradesh 452016, India*
[2]*Kota College of Pharmacy, 324005 Kota, Rajasthan, India*
[3]*University Institute of Pharma Sciences, Chandigarh University, Gharuan, Mohali, Punjab, India*
[4]*Laboratoire de Biologie des Ligneux et des Grandes Cultures, INRA USC1328, Université d'Orléans, Eure et Loir Campus, 28000 Chartres, France*
[5]*Bioactifs et Cosmétiques, GDR 3711 Cosmactifs, CNRS, Université d'Orléans, 45067 Orléans, CÉDEX 2, France*
[6]*Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayuthaya Road, Rajathevi, 10400 Bangkok, Thailand*
[7]*Department of Pharmacy, Faculty of Biological Sciences, University of Malakand, Chakdara, 18000 Dir (L), Khyber Pakhtunkhwa, Pakistan*
[8]*Department of Chemistry, Faculty of Natural and Applied Sciences, St. John's University of Tanzania, P.O. Box 47, Dodoma, Tanzania*

Correspondence should be addressed to Atul Kabra; atul.kbr@gmail.com, Christophe Hano; hano@univ-orleans.fr, Rakesh Patel; patelcip@gmail.com, and Daniel M. Shadrack; mshadrack@sjut.ac.tz

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Rivea hypocrateriformis (Desr.) Choisy is a robust woody climbing shrub of the genus *Rivea* which is widely distributed in India, Nepal, Sri Lanka, Pakistan, Bangladesh, Myanmar, and Thailand. *R. hypocrateriformis* is a promising medicinal herb with a wide range of beneficial and health-promoting properties. Since the ancient times, it has been used as a traditional medicine to treat rheumatic pain, fever, urogenital problems, snake bites, cough, piles, malaria, and skin diseases. Aside from these traditional uses, its leaves and young shoots are also cooked and eaten as a vegetable and used for the preparation of bread with millet flour. This study extensively analyzes the available information on R. hypocrateriformis botanical characterization, distribution, traditional applications, phytochemistry, pharmacology, and toxicological properties. Phytochemical investigations of the plant has revealed the presence of highly valuable secondary metabolites including alkaloids, glycosides, coumarins, flavonoids, xanthones, stilbenes, and other organic compounds. Its crude extracts and isolated compounds have revealed anovulatory, antifertility, antiarthritic, antimicrobial, anticancer, antioxidant hepatoprotective, antilithiatic, and antimitotic potentials. This review of literature clearly identifies *R. hypocrateriformis* as a potent medicinal plant with remarkable healing and health-promoting properties. Further research directions into the bioactive extracts, clinical, and toxicological evaluations to assess the beneficial health-promoting properties of this promising herb are also discussed.

1. Introduction

Medicinal plants are among the vital sources of secondary metabolites used for the management of various diseases since the establishment of the human era [1]. Even today, it is among the most useful sources for the discovery and development of novel drugs against various diseases [2, 3]. Especially, plants which are traditionally used by indigenous people to treat various diseases are more useful as their safety and efficacy are already established [4]. The discovery and clinical approval of numerous drugs using a knowledge-driven ethnomedicinal approach signify the role of medicinal plants and traditional knowledge in the discovery of novel drugs [5, 6]. Further, the development of novel and highly sensitive isolation and identification techniques and the discovery of novel molecules from these plants are easier and more cost-effective. *Convolvulaceae* is a vast and homogeneous plant family of approximately 50 genera and nearly 1,700 species [7–9]. *Rivea hypocrateriformis* (Desr.) Choisy is a woody climbing shrub belonging to the *Convolvulaceae* family and is widely distributed in India, Nepal, Sri Lanka, Pakistan, Bangladesh, Myanmar, and Thailand [9]. Its bark, stems, and leaves are traditionally used to cure a range of ailments including malaria, cancer, mental disorders, and analgesia. For instance, the indigenous people of Pakistan's Tharparkar region use this plant for the treatment of malaria and to relieve pain. The plant is reported for a wide range of biological potentials including antioxidant, anti-implantation, antimicrobial, pregnancy irruption, anticancer and antiarthritic properties [10, 11]. It is also used as a vital ingredient in the ayurvedic formulation "Rasa panchaka" used for the treatment of asthma [12]. Moreover, similar to other varieties of a related genus, such as *Rivea corymbosa* Hall and *Ipomea violacea* L. found in Mexico, this plant is also used as a hallucinogenic drug in India and as a psychoactive medicine in Pakistan [13].

Besides, young shoots and leaves of the plant are also cooked and consumed as a vegetable. The leaves are bubbled along with toppings and arranged dishes, for example, *bhaji* or *jowari* flour which was then used to prepare bread [14]. It is a rich source of micronutrients, in particular vitamin A [15, 16]. As far as we know, there is no review paper available on *R. hypocrateriformis* that was accessible in January 2022. The review paper is aimed at providing a more comprehensive analysis of the ethnomedicinal uses, phytochemistry, and biological activities. Furthermore, this review also focuses on filling some of the gaps among the currently performed studies and proposes some areas for future research on potential bioactivities of *R. hypocrateriformis*.

2. Methods

Publications were retrieved from PubMed, Google Scholar, and ScienceDirect. The strategy was using different combinations of keywords "Traditional medicines, Phytochemistry, Biological activity, Pharmacology" associated with "*Rivea hypocrateriformis*" and its synonyms. *Argyreia bona-nox* Sweet, *Argyreia uniflora* Sweet, *Convolvulus hypocrateriformis* Desr., *Lettsomia uniflora* Roxb., *Modesta coriacea* Rafin., *Rivea bona-nox* Choisy, and *Rivea fragrans* Nimmo are the synonyms of *R. hypocrateriformis*. The detailed research methodology adopted for the selection of articles for this review is stipulated as a flowchart in Figure 1.

3. Botanical Description

3.1. Taxonomical Classification and Habitat. The taxonomical classification of *R. hypocrateriformis* is Kingdom: *Plantae*; Phylum: *Tracheophyta*; Class: *Magnoliopsida*; Subclass: *Asteridae*; Order: *Solanales*; Family: *Convolvulaceae*; Genus: *Rivea* [9] It is likewise known by a variety of names, such as "*Midnapore Creeper*" in English, "*Thor-ki-bel*" or "*Phang*" in Hindi, "*Sanjvel*" in Marathi, "*Budthi Kiray*" or "*Musuttai*" in Tamil, and "*Niruboddi*" in Telugu [17]. *R. hypocrateriformis* is a woody climbing shrub found in subtropical forests of India, Nepal, Sri Lanka, Pakistan, Bangladesh, Myanmar, and Thailand. In India, it is mainly found in Assam, Bihar, Maharashtra, Rajasthan, and Tamil Nadu (Figure 2).

R. hypocrateriformis (Desr.) Choisy is a woody climbing shrub belonging to the family *Convolvulaceae*.

3.2. Morphological and Microscopical Characteristics. Morphological characterization of *R. hypocrateriformis* plant and its parts are presented in Figures 3(a)–3(d). Its flowers, usually solitary, are creamy white, typical morning glory form, flat-faced, and 6–9 cm long. Sepals unequal, ovate, blunt apically, 10–12 mm long with dense short villoses. Leaves are round-heart-shaped, blunt apically, densely appressed velvet-hairy below. Fruit are indehiscent or tardily dehiscent, dry-baccate of 2 cm long. Seeds are brown, smooth, glabrous, slightly trigonous, and surrounded by a dry white pulp. Transverse sections of the leaf showed that the upper and lower epidermis comprise of single-layered polygonal cells that cover the adhesive fingernail skin [18, 19].

4. Ethnomedicinal Uses

R. hypocrateriformis is a common ayurvedic herb, used in different ways by various local population groups due to the various beneficial uses of its bark, roots, leaves, and blossoms. Ayurvedic physicians traditionally use *R. hypocrateriformis* preparations to prevent fertility in women [20, 21]. The various ethnomedicinal usages of this herbal medicine are summarized in Table 1.

5. Phytochemistry

R. hypocrateriformis is extensively reported for the presence of important metabolites. For instance, Loganayaki et al. reported the extractive value of leaf, stem, and flower parts of the plant using polarity-directed solvent systems including chloroform, methanol, and acetone. Flowers exhibited a higher extractive value of 13.3%, followed by the flower-methanol extract 12.5%, flower-chloroform extract 11.5%, leaf-methanol extract 8.6%, stem-methanol extract 7.43%, leaf-acetone extract 5.9%, leaf-chloroform extract 2.9%,

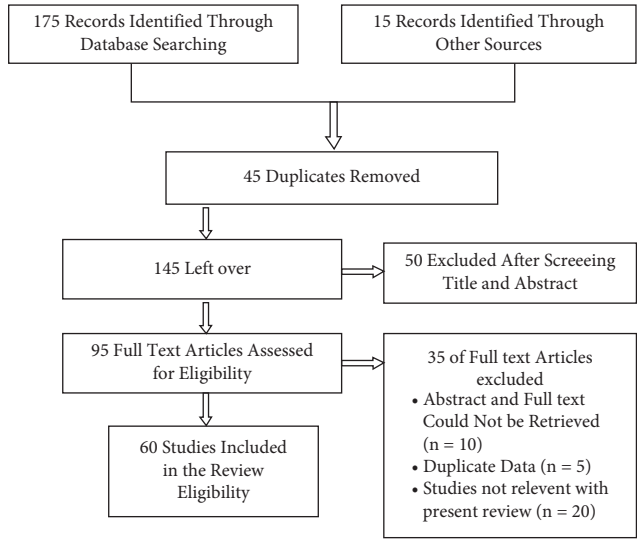


Figure 1: Flow diagram of research methodology.

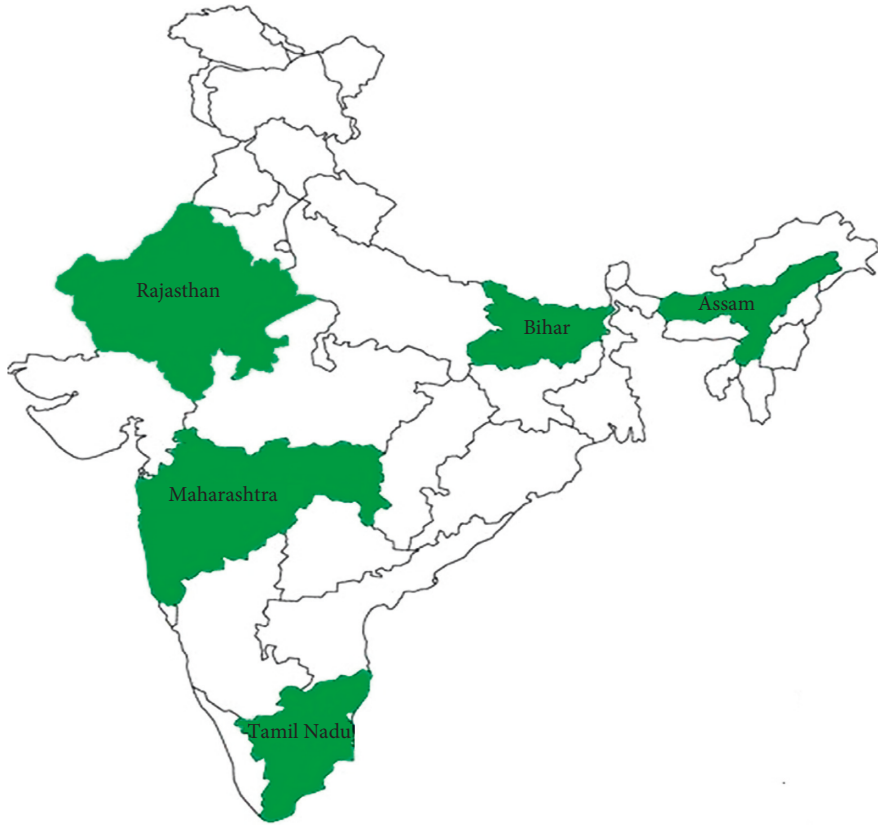
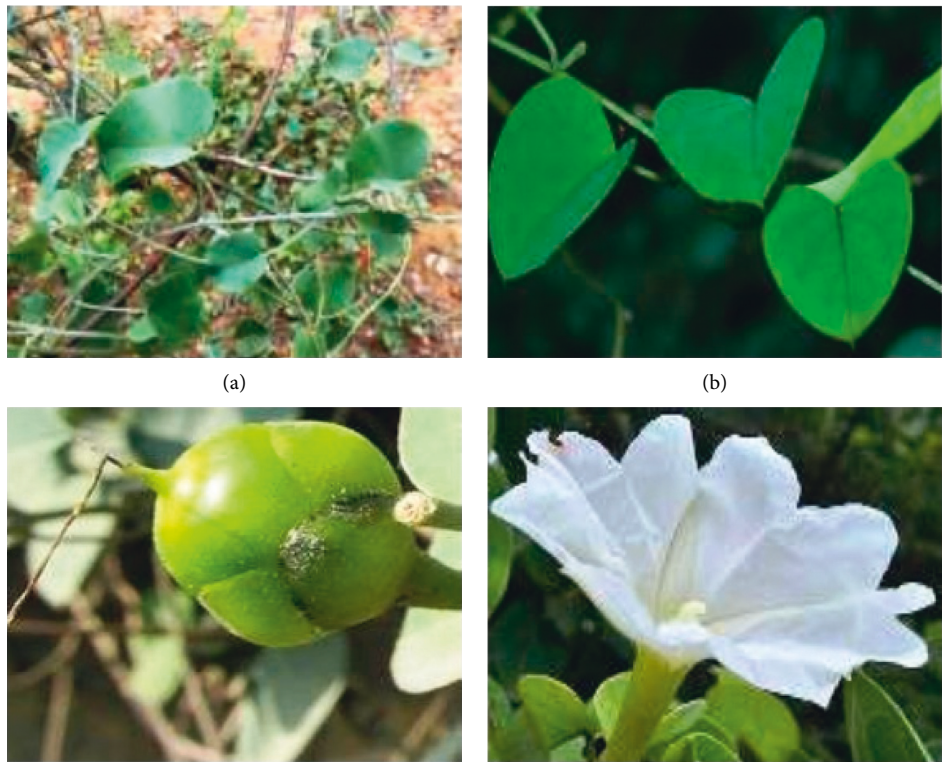


Figure 2: Natural distribution of *R. hypocrateriformis* in the India. The shaded area represents its natural habitat.

stem-acetone extract 1.87%, and stem-chloroform extract 0.7%. The same group of researchers also reported *R. hypocrateriformis* as a rich source of phenolic compounds as observed in the different extracts from leaves, stems, and flowers obtained using organic solvents. The highest total phenolic content was quantified in the flower-acetone extract and flower-methanolic extracts [39] as summarized in Table 2. These quantitative results are in agreement with recent the study [40] (Table 2). Furthermore, qualitative phytochemical screening of different parts of *R. hypocrateriformis* revealed the presence of alkaloids, flavonoids, tannins, saponins, glycosides, steroids, carbohydrates, phytosterols, and amino acid derivatives.



(a) (b) (c) (d)

Figure 3: *R. hypocrateriformis*. (a) whole plant; (b) leaf; (c) fruit; (d) flower.

Table 1: Ethnomedicinal uses of *R. hypocrateriformis*.

Plant part used	Method of administration	Usage	References
Whole plant and root	The plant juice/paste is orally taken	Treatment of snake bite	[22–24]
Whole plant	Powder	Piles and heart disease	[25]
Leaves	Cooked	Indigestion	[26]
Whole plant	Powder	Constipation	[27]
Leaves	Paste	Diarrhoea	[28,29]
Whole plant	Powder	Diuretic	[30]
Whole plant	Powder	Laxative	[30]
Stem	Powder	Cough and headache	[26]
Leaves	Juice with cow's milk	Rheumatic pain	[31]
Leaves	Juice	Skin disease of hair scalp	[31]
Whole plant and root	Plant juice/paste taken orally	Snake bite treatment	[23,24,32]
Root	Decoction	Fever	[33]
Leaves	Powder	Urogenital problem (hematuria)	[34]
Leaves	Powder	Blood purifier	[35]
Root	Paste	Cough, swelling and headache, poisonous animals bite	[22–24]
Leaves	Internal use	Stomach wounds	[36]
Leaves	Internal use (cooked)	Stomach upset and indigestion	[37,38]
Root	Powder	After parturition	[16]

5.1. Alkaloids. Three pyrrolizidine alkaloids, namely, macrophylline (1), meteloidine (2), and symlandine (3) as well as four tropane alkaloids, namely, cochlearine (4), darlingine (5), tigloidine (6), and serratanidine (7) were found in the root of *R. hypocrateriformis* [41]. Two other alkaloids were quantified in the aerial parts of the plant, namely, hypocretine 1(8i) and hypocretine 2 (8ii) [40]. The presence of aminopyrimidine pyrimethanil (9) was also reported from the roots of *R. hypocrateriformis* [41].

5.2. Glycosides. Four glycosides, namely, bergenin (10), norbergenin (11), rivebergenin A (12i), and rivebergenin B (12ii) are reported from the stem of *R. hypocrateriformis*. An

Table 2: Quantitative phytochemical content in *R. hypocrateriformis*.

Plant part	Extract/fraction	Total phenolic content	Total flavonoid content	Extractive value (%*w*/*w*)	References
Aerial	Polyphenolic	0.170 μg TAE/mg fraction	0.193 μg QAE/mg fraction	—	[40]
Leaves	Chloroform	1.1 g GAE/100 g	—	2.9	
	Acetone	2.1 g GAE/100 g	—	5.9	
	Methanolic	1.1 g GAE/100 g	—	8.6	
Stem	Chloroform	0.9 g GAE/100 g	—	0.7	
	Acetone	1.5 g GAE/100 g	—	1.87	[39]
	Methanolic	1.2 g GAE/100 g	—	7.43	
Flower	Chloroform	1.6 g GAE/100 g	—	11.5	
	Acetone	4.2 g GAE/100 g	—		
	Methanolic	3.5 g GAE/100 g	—	12.5	

TAE: tannic acid equivalent; QAE: quercetin equivalent; GAE: gallic acid equivalent.

aromatic glycoside lucuminic acid (13) and a cardiac glycoside oleandrose (14) were reported from the roots of *R. hypocrateriformis* [41].

5.3. Flavonoids. Godipurge et al. reported the presence of multifunctional compound quercetin (15) in a polyphenolic fraction of the aerial part [40]. Flavonoids including *C*-glycosides 3′-deoxymaysin (16), 6-*C*-glucopyranosylpilloin (17), *O*-glycoside peruvianoside II (18), and a prenylated flavonoid morusin (19) were identified in the roots of *R. hypocrateriformis* [41].

5.4. Xanthones. Several xanthone derivatives including dulciol B (20) and mangostenone B (21) are also reported from the roots of *R. hypocrateriformis* [41].

5.5. Stilbenes. The occurrence of blestriarene B (22) and α-viniferin (23) was reported from the roots of *R. hypocrateriformis* [41].

5.6. Coumarins. Various coumarins including tomentolide A (24) and calophyllolide (25) were reported from the root extract of the plant. Likewise, desmethylbergenin hemihydrate (26) was reported from the whole plant of *R. hypocrateriformis* [41].

5.7. Sterols and Fatty Acid Derivatives. Sterols and fatty acids including sphingosine (27) and 3S, 7S-dimethyl-tridecan-2S-ol (28) were found in the root of *R. hypocrateriformis* [41]. A long-chain fatty aldehyde pentadecanal (29), two fatty acids, namely, 2-hexyl-decanoic acid (30) and 1-palmitoyl lysophosphatidic acid (31), and 2,4-undecadienal (32) were reported from the roots of *R. hypocrateriformis* [41].

5.8. Miscellaneous Compounds. Various other compounds are also reported from the plant. Among these, *N*-acetylmuramoyl-alanine (33) belongs to the class of organic compounds known as acylamino sugars. These are organic compounds containing a sugar linked to a chain through *N*-acyl group. Two tripeptides His-His-Lys (34) and Asp-Arg-Asp (35), one bipeptide Glu-His (36) and an amino cyclitol streptidine (37), and a volatile compound methyl jasmonate (38) were reported from the roots of *R. hypocrateriformis* [41]. Structures of these bioactive phytoconstituents reported in the *R. hypocrateriformis* plant are presented in Figure 4.

6. Pharmacological Studies

Crude extracts from medicinal plants are extensively studied for various pharmacological properties [3, 42–44]. *R. hypocrateriformis* is also reported for various pharmacological potentials which might be attributed to the presence of various phytochemicals (Table 3). Many studies have shown that the presence of phenolic acids and flavonoids may be linked to cancer-prevention action [2, 48–51]. The identified phytochemicals offer protection against oxidative stress by scavenging radicals [52, 53].

Various identified phytochemicals from the plant might be implicated in the therapeutic properties of the crude extracts as they are reported for protective properties in various diseases [54, 55]. For instance, bergenin is reported to exhibit hepatoprotective, antiarrhythmic, neuroprotective, antifungal, anti-inflammatory, immunomodulatory, anti-HIV, antifungal, antihepatotoxic, wound, and ulcer healing potentials [56, 57]. Norbergenin, an *O*-methyl derivative of bergenin, is also reported to have antioxidant [58, 59] and gastroprotective [60] potentials. Likewise, calophyllolide has been reported to exhibit some biological activities including anti-inflammatory, vasodilatory, anticancer, antimicrobial, and anticoagulant properties [61]. Further, stilbene trimers including α-viniferin are reported to exhibit AChE potentials in a dose-dependent manner [62].

These findings suggest that clinical studies into the pharmacological potentials of *R. hypocrateriformis* and its derivatives could be warranted for the discovery of new potential therapeutic entities. Simultaneous *in vitro* and *in vivo* experiments on the pharmacological profile of *R. hypocrateriformis* and its derivatives might help evaluate their modes of action.

7. Toxicological Studies

The toxicological properties of the plant were reported in various studies. In a toxicological study performed on the

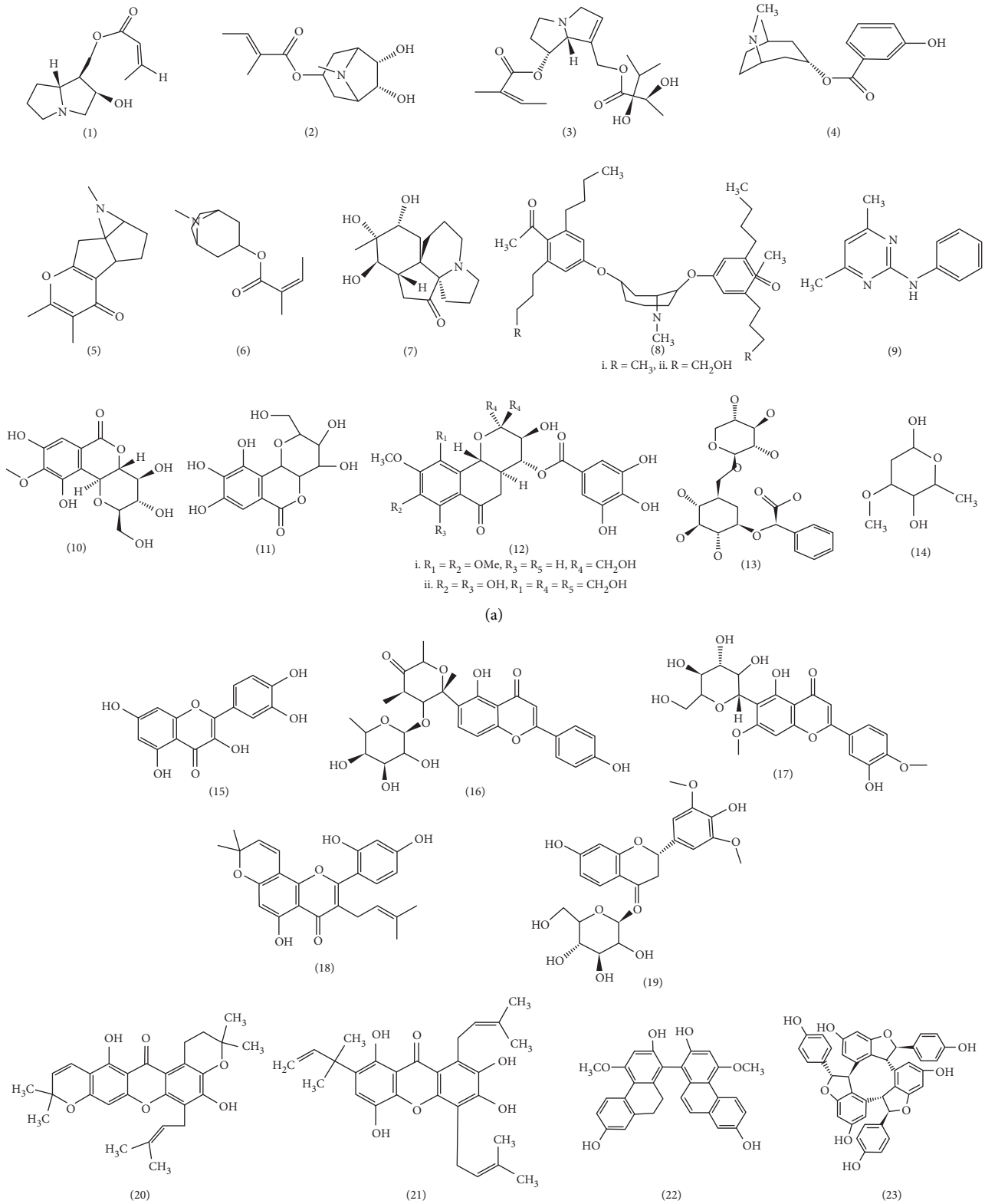


Figure 4: Continued.

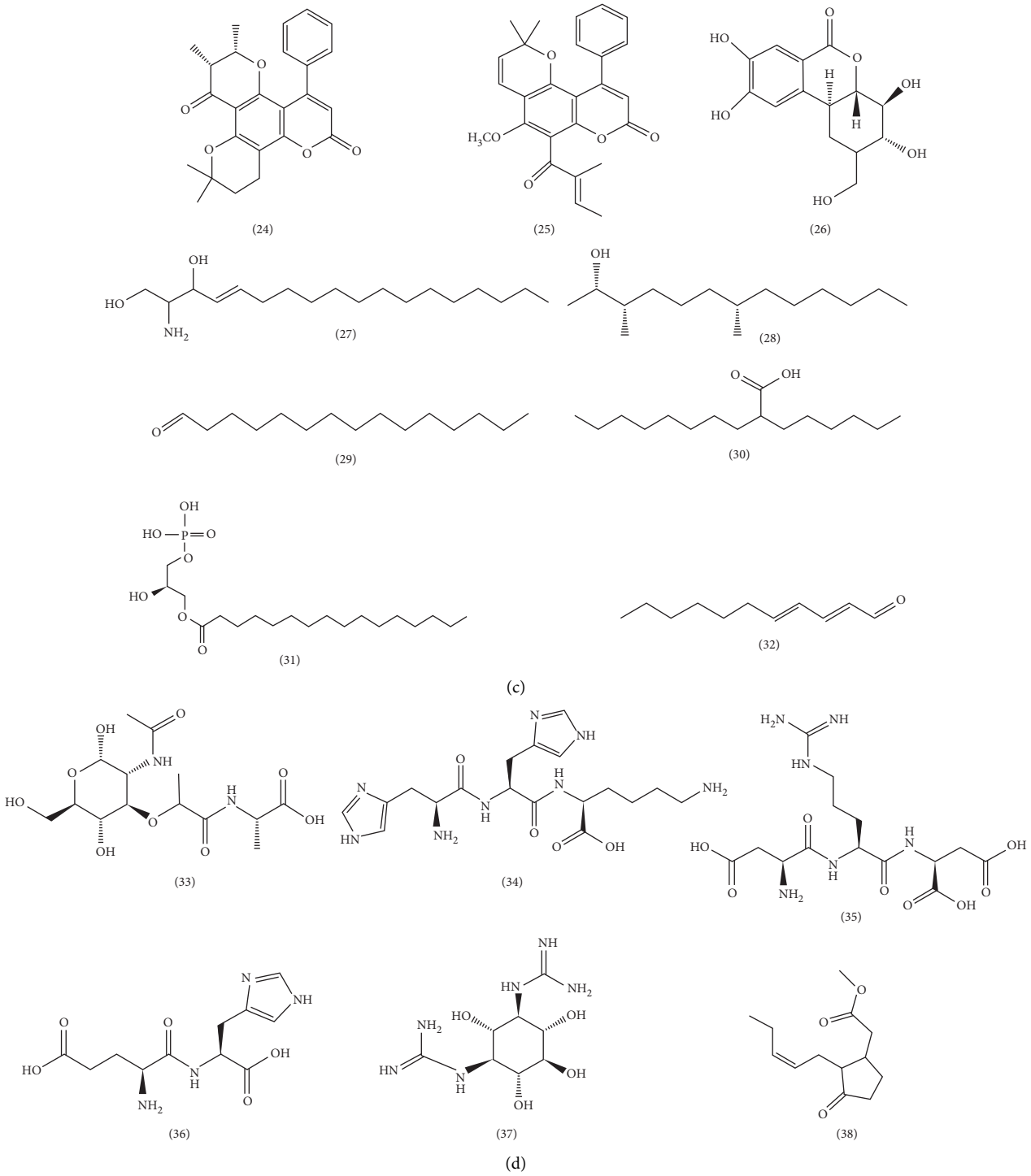


Figure 4: (a) Structure of some isolated alkaloids and glycosides from different parts of *R. hypocrateriformis*. (1) Macrophylline, (2) meteloidine, (3) symlandine, (4) cochlearine, (5) darlingine, (6) tigloidine, (7) serratanidine, (8) (i) hypocretine 1 and (ii) hypocretine 2, (9) pyrimethanil, (10) bergenin, (11) norbergenin, (12) (i) rivebergenin A and (ii) rivebergenin B, (13) lucuminic acid, (14) oleandrose. (b) Structure of some isolated flavonoids, xanthones, and stilbenes from different parts of *R. hypocrateriformis*. (15) Quercetin, (16) 3′-deoxymaysin, (17) 6-C-glucopyranosylpilloin, (18) peruvianoside II, (19) morusin, (20) dulciol B, (21) mangostenone B, (22) blestriarene B, and (23) α-viniferin. (c) Structure of some coumarins, fatty acids, and sterols from different parts of *R. hypocrateriformis*. (24) Tomentolide A, (25) calophyllolide, (26) desmethylbergenin hemihydrate, (27) sphingosine, (28) 3S, 7S-dimethyl-tridecan-2S-ol, (29) pentadecanal, (30) 2-hexyl-decanoic acid, (31) 1-palmitoyl lysophosphatidic acid, and (32) 2, 4-undecadienal. (d) Structure of some isolated other organic compounds from different parts of *R. hypocrateriformis*. (33) N-acetylmuramoyl-alanine, (34) his-his-lys, (35) asp-arg-asp, (36) glu-his, (37) streptidine, and (38) jasmonate.

Table 3: Biological activities of *R. hypocrateriformis*.

Part used	Extract/fraction	Dose tested/route of administration	Study model	Experimental models	Results	References
Antiarthritic activity						
Leaves	Methanolic	250 and 500 mg/kg, p.o.	Albino Wistar rats	Complete Freund's adjuvant (CFA)-induced arthritis	Extract showed significant anti-arthritic activity	[45]
Antimicrobial activity						
Aerial part	Au, Ag, and Au–Ag alloy NPs	25–100 μg/mL	KP, SA, BS, PA, EC, CA, TR, and CI	Agar well diffusion method	Green-synthesized AgNPs displayed very good antimicrobial potential compared to AuNPs	[40]
Aerial part	Pet. ether, chloroform, ethanol, and aqueous extract	10000, 5000, 2500, 1250 and 0.625 μg/mL	SA, BS, EC, PA, PV, AN, CA, and AF	Agar disk diffusion method	Ethanolic and aqueous extract showed higher antimicrobial potential than other extracts	[46]
Anticancer activity						
Aerial part	Au, Ag, and Au–Ag alloy NPs	1–100 μg/mL	MCF7, Sf9, Vero	MTT assay	Significant cytotoxicity on tested cancer cells in a concentration-dependent manner	[40]
Aerial part	Pet. ether, chloroform, ethanol, and aqueous	4×10^3 cells/ml	MCF-7, MCF-15, MOLT-4, HOP-62, prO	SRB assay	Chloroform and ethanolic extracts exhibited strong anticancer activity	[46]
Anovulatory effect						
Aerial part	Ethanol	200 and 400 mg/kg	Wistar albino rat	*In vivo* (effect on duration of different phases of the oestrous cycle)	Significant decrease in number of Graafian follicles and corpora lutea and significant increase in number of atretic follicles	[20]
Antioxidant activity						
Aerial part	Polyphenolic fraction	—	*In vitro*	Hydroxyl radical scavenging assay	Extracted demonstrated significant antioxidant activity	[40]
Aerial part	Au, Ag, and Au–Ag alloy NPs	10–100 μg/ml	*In vitro*	DPPH assay	NPs were capable of scavenging DPPH radicals	[40]
Leaf	Aqueous	15.51, 62.5, 250 and 1000 μg/ml	*In vitro*	DPPH assay	Aqueous extract showed highest DPPH radical scavenging activity	[15]
Leaf, stem, and fruit	Chloroform, acetone, and methanol		*In vitro*	DPPH, ABTS, and FRAP assay	Antioxidant activity was the highest in MeAA extracts, while it was intermediate in MeAM and MeA extracts.	[39]
Antifertility activity						
Aerial part	Pet. ether, chloroform, ethanol, and aqueous	200 and 400 mg/kg	Albino Wistar rats	*In vivo* (anti-implantation effect)	Ethanol extract found significant anti-implantation and interruption of early pregnancy	[20]
Whole plant	95% ethanolic extract	200 and 400 mg/kg	Albino Wistar rats	*In vivo* (anti-implantation effect)	Extract dose 400 mg/kg showed significant anti-implantation potential	[47]
Hepatoprotective activity						
Aerial part	Polyphenolic fraction	300 and 600 mg/kg	Albino Wistar rats	Paracetamol-induced hepatotoxicity	Decreased ALT, AST, ALP, and TB	[40]
Antimitotic activity						
Aerial part	Pet. ether, chloroform, ethanol, and aqueous	10 mg/ml	*In vitro*	*Allium cepa* root inhibition	Chloroform and ethanol extracts showed significant antimitotic activity	[46]

Table 3: Continued.

Part used	Extract/fraction	Dose tested/route of administration	Study model	Experimental models	Results	References
Antiproliferative activity						
Aerial part	Pet. ether, chloroform, ethanol, and aqueous	—	*In vitro*	Yeast *Saccharomyces cerevisiae* model	Chloroform and ethanol extracts showed significant antiproliferative activity	[46]
Antilithiatic activity						
Leaves	Ethanolic	2.5 ml of 0.2 g/ml solution	*In vitro*	—	Extract showed significant inhibition of calcium and phosphate accumulation	[35]
Anti-inflammatory activity						
Leaves	Ethanolic	200 and 400 mg/kg	Albino Wistar rats	Carrageenan-induced paw edema	Ethanol extracts showed significant anti-inflammatory activity	[31]
Analgesic activity						
Leaves	Ethanolic	200 and 400 mg/kg	Albino Wistar rats	Radiant heat tail flick method	Ethanol extracts showed significant analgesic activity	[31]

polyphenolic fraction of *R. hypocrateriformis*, no adverse effects or mortality were observed in the Swiss albino mice and Wistar albino rats at 4,000 mg kg^{-1} p/o dose. This was observed during 24 h period, and the extract was found to be safe at the given dose [40]. However, further detailed studies are required on different fractions and/or extracts for their toxicological effects on individual organs. Here, it should also be emphasized that the nature of *R. hypocrateriformis* can be affected by the environment and the picking time [19]. It is critical to implement authentication methods to monitor the nature and quality of the collected materials, remove the contaminated material, and ensure the biological activity associated with the resulting extracts for safety purposes, but also to ensure the reproducibility of the experiments [63, 64]. It is important to note that if the material authentication has not been done or is not sufficiently clear, its therapeutic significance is therefore meaningless. This critical prior authentication work is still conducted too infrequently and should be systematized for *R. hypocrateriformis* but also other medicinal herbs.

8. Conclusion

Based on the findings of the current study that sum up the traditional usages and pharmacological activities of its extracts and constituents, *R. hypocrateriformis* clearly appears as a promising medicinal herb with a wide range of beneficial and health-promoting properties. Nevertheless, it also appears that additional evidence from clinical studies as well as toxicological examinations are required. A particular effort should be made to put in place an adequate authentication method. Along these lines, the deliberate examinations on *R. hypocrateriformis* ought to be attempted to rationalize its ethnomedicinal uses and consider future pharmacological applications to take advantage of the properties of this herbal medicine.

Data Availability

The data used to support the findings of this study will be available on request from the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Identification of Secondary Metabolites from Mexican Plants with Antifungal Activity against Pathogenic *Candida* Species

Nancy A. Vázquez-López,[1] **Gustavo Cruz-Jiménez**,[2] **Armando Obregón-Herrera**,[1] **Estela Ruiz-Baca**,[3] **Mario Pedraza-Reyes**,[1] **Everardo López-Romero**,[1] **and Mayra Cuéllar-Cruz**[1]

[1]*Departamento de Biología, División de Ciencias Naturales y Exactas, Campus Guanajuato, Universidad de Guanajuato, Noria Alta S/N, Col. Noria Alta, C.P, Guanajuato, 36050 Guanajuato, Mexico*
[2]*Departamento de Farmacia, División de Ciencias Naturales y Exactas, Campus Guanajuato, Universidad de Guanajuato, Noria Alta S/N, Col. Noria Alta, C.P, Guanajuato, 36050 Guanajuato, Mexico*
[3]*Facultad de Ciencias Químicas, Universidad Juárez del Estado de Durango, Av. Veterinaria S/N, 34120 Durango, Mexico*

Correspondence should be addressed to Mayra Cuéllar-Cruz; mcuellar@ugto.mx

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In the last three decades, invasive fungal infections caused by *Candida* species have become an important public health problem, because they are associated with high rates of morbidity and mortality in immunocompromised and hospitalized patients. The diagnosis and treatment of candidiasis are difficult and usually inefficient. Accordingly, a diversity of available drugs, currently employed to attack candidiasis, frequently induce resistance in patients promoting toxicity due to long-term treatments. Therefore, development of accurate diagnoses and novel antifungals is of high priority to improve life's quality and expectancy of individuals infected with this pathogen. Plants are invaluable sources of new biologically active compounds. Among the plants used in Mexico in traditional herbolary medicine which have empirically been demonstrated to have antifungal activity are *Pedilanthus tithymaloides*, *Thymus vulgaris*, and *Ocimum basilicum*. In the present study, we analyzed whether these plants contain metabolites with antifungal activity against five *Candida* species. The extracts from the different plant organs were obtained by macerating them in ethyl alcohol or hexane and filtering. The obtained extracts were preserved in amber flasks at 4°C until used. The minimum inhibitory concentrations (MICs) of the active compound were determined by a microdilution assay. In addition, the following secondary metabolites were identified: linalool (3,7-dimethylocta-1,6-dien-3-ol), eugenol (4-allyl-2-methoxyphenol), limonene (1-methyl-4-(1-methylethenyl)-cyclohexene), and borneol ([(2*R*)-1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl] formate). All these compounds were found in the three plants, traditionally used in everyday life, and proved to be effective against *Candida* species and therefore a viable alternative to conventional antifungals.

1. Introduction

It has been estimated that there are over 5 million fungal species worldwide, and approximately 300 out of these are known to cause diseases in humans, while 20-25% do it with relative frequency. *Candida* species are among these pathogens. Most of the fungal infections are nontransmissible among people and routinely do not affect healthy individuals [1]. Several species from the *Candida* genus that can be classified as part of this group of pathogens are widely distributed in nature and are part of the normal microbiota in the oral cavity, the gastrointestinal tract, and the urogenital

system in human host. These organisms do not trigger infections in healthy hosts; however, some of these fungi can behave as opportunistic pathogens when the immune system of the host is compromised, causing infections called superficial candidiasis (cutaneous and mucous infections) and systemic or invasive candidiasis (infection of the bloodstream and invasive candidiasis of organs) [2, 3]. These infections amount, as a whole, to approximately 40 million per year worldwide [4]. Invasive candidiasis (IC) represents one of the most common nosocomial infections due to fungi, particularly, in cancer patients and in individuals under immunosuppression regimes [5, 6]. Candidemia can reach a mortality rate from 30 to 60% in hospitalized immunocompromised patients [7, 8]. In more than half of these mycosis cases, death occurs in the first week after the diagnosis of infection due to *Candida* [9]. *Candida albicans* has been identified as the most prevalent and pathogenic species; it is responsible for most oral and systemic candidiasis cases, as well as for community and nosocomial origin candidemia [10]. In the last decades, *Candida* non-*C. albicans* species like *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida dubliniensis*, and, recently, *Candida auris* have been increasing [11–15]. Invasive candidiasis causes significant morbidity and mortality despite intensive treatments with antifungal agents [16, 17]. The mortality attributed to this disease has not diminished significantly despite the new diagnostic methods, the new antimycotic treatment options, and the better control of the infection [18]. Starting at the second half of the last century, commercialization of amphotericin B, although toxic, helped importantly in the treatment of systemic mycoses [19]. The advent of topic azoles like miconazole and clotrimazole, and later of systemic azoles like ketoconazole, itraconazole, fluconazole, and voriconazole, has simplified the treatment of superficial and systemic mycoses, improving the healing expectancy of these infections. However, with the increase in the HIV and cancer incidences and the indiscriminate use of steroids, surgical procedures, and transplants, failed cases have been reported with the antimycotic therapy using diverse compounds like ketoconazole, fluconazole, and even amphotericin B [20]. Hence, the mortality attributed to fungi is still too high even with the current antifungal agents; thus, a greater emphasis must be placed in improving the time used for the fungicide activity of the new antifungal agents. Currently, the general treatment course with common antimycotics is too long and, hence, presents the potential of a deficient short-term fungicide effect, a diminution in compliancy and/or tolerability by patients, or even the appearance of direct resistance to the antifungal drugs [21]. A complication that arises with the treatment of mycoses is the resistance to the antifungal agents; this is defined as the capacity acquired by an organism to resist the effects of a chemotherapeutic agent to which it is habitually sensitive [22]. Plants are a viable option to obtain a wide variety of pharmaceuticals because they are easily accessible and can be applied to diverse pathologies [23]. In this way, plants constitute an excellent source of substances that can be used in the formulation of new antifungal agents [24]. However, the development of the pharmaceutical industry and the synthesis of molecules with diverse activities in the clinical field displaced the use of medicinal plants in many regions of the world, mostly in large cities [25]. Although the current available drugs are usually efficacious, the therapeutic failures and the toxicity after a long treatment are common; thus, plants are a good alternative to be explored to improve treatments of severe infections [26, 27]. It has been calculated that there are from 200 to 500 thousand species of higher plants worldwide; in Mexico, the diversity is estimated from 23 to 30 thousand species [28]. Knowledge on the chemical diversity of plants is still limited worldwide; it is calculated that the chemical structure of around 100 thousand secondary metabolites is known, and there could be at least one million of them in all the species that have not been studied yet [29]. Likewise, the biological properties of secondary metabolites are unknown; in most cases, research is centered on priority problems, like the search of anticancer agents, and they do not attempt to perform integrated studies on all the biological activities. Regarding candidiasis treatment, even though there are currently a large variety of available antifungal agents, it is increasingly more frequent that patients do not respond to treatment, causing toxicity after a long treatment. For this reason, it is necessary to identify new alternatives for the treatment of candidiasis. Plants are an invaluable source of new biological active compounds. Among the reported secondary metabolites in plants with antifungal activity are flavonoids, phenols, glycosides of phenols, and saponins. Among the plants used in traditional medicine that have empirically shown to possess antifungal activity are *Pedilanthus tithymaloides*, *Thymus vulgaris*, and *Ocimum basilicum*. In the present study, we analyzed the secondary metabolites from these plants as well as their antifungal activity against five *Candida* species.

2. Materials and Methods

2.1. Plant Material. *Pedilanthus tithymaloides* was donated and collected from a private orchard in the municipality of Leon (state of Guanajuato, Mexico). Lateral stems of the shrub, detached from the nodes, were collected. *Thymus vulgaris* and *Ocimum basilicum* plants were acquired from a nursery in Guanajuato, Mexico.

2.2. Sampling of Plants. The leaves and stems of each plant were separated, placed in individual recipients, and washed with tap water to eliminate any residues; then, they were washed again with sterile deionized water. The leaves and stems were placed separately on a grid that allowed dripping the excess water to dry the plant material. This was carried out at room temperature and away from light until use.

2.3. Extract Preparation. The plant material (leaves or stems) was placed on an analytical balance on top of a polyethylene tray until a mass of 40 g was reached and further sterilized in 2% sodium hypochlorite for 10 min. Keeping sterile conditions, this plant material was placed in a mortar, adding 100 mL of 99.5% ethyl alcohol or hexane (which was previously cooled in an ice bath), and crushed to homogeneity. The obtained macerate was filtered with a vacuum

filtration system (Kitasato flask-Büchner funnel-TYPE HVLP, 0.45 μm membrane). The obtained extracts were kept in amber flasks at 4°C until their use.

2.4. Fungal Strains and Culture Media. The strains of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis* species were obtained from the strain collection of the mycology laboratory, which is part of the Department of Biology of the Natural and Exact Sciences Division, at the *Universidad de Guanajuato*. Strains were grown in YPD (1% yeast extract, 2% Bacto-Peptone, and 2% dextrose) medium. The solid medium was supplemented with 2% agar [30].

2.5. Susceptibility Assays to the Different Extracts by Means of the Disk Diffusion Method. The minimal inhibitory concentrations (MICs) of the active compounds were determined through a microdilution assay as previously described [31]. The effect of the different extracts on the five *Candida* species was determined with yeasts in the stationary growth phase. For the latter, the five *Candida* species were grown during 48 h at 28°C under constant agitation at 120 rpm. Once the incubation period had ended, the optical density was measured at 600 nm (OD_{600nm}) and adjusted to 0.5 and 0.1 in 1 mL of sterile deionized water. The cells adjusted to the different ODs were streaked on YPD plates with the help of a sterile Digralsky loop, adding 75 μL of the suspension to each plate. Afterward, the plate was divided in sections, and with previously sterilized curved point steel tweezers, sterile filter paper disks (Whatman 40) of ca. 6 mm in diameter were placed in each section. Of each extract to be evaluated, 50 μL was placed on top of each disk (ethanol extract of stem or leaf of *P. tithymaloides*, *T. vulgaris*, or *O. basilicum*. Hexane extracts of leaf or stem of *P. tithymaloides*, *T. vulgaris*, or *O. basilicum* were also used. To assess whether the solvents used for the extracts affected or not the cell growth, ethanol and hexane were used as negative controls. Additionally, another solvent, reported as relatively innocuous, that is, dimethyl sulfoxide (DMSO), was used. As positive control, phenol at different proportions (1 : 10, 1 : 100, 1 : 1000, and 1 : 10000) and absolute phenol were used. Plates were incubated at 28°C for 36-48 h. After the incubation period, photographs were taken using the gel documentation system (GeneGenius Bio Imaging System, Artisan Technology Group, Champaign, IL, USA, from Syngene). Experiments were performed in triplicate.

2.6. Analysis of Extracts

2.6.1. Gas Chromatography Coupled to Mass Spectrometry (GC/MS). The secondary metabolites contained in the hexane extract of leaves and stems of *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* were identified by means of GC [32] coupled to a Clarus SQ8 MS (Perkin Elmer, Inc., Waltham, MA, USA) equipped with an ion deflector that allowed identifying low volatility compounds and those thermally labile. This equipment is provided with a capillary DB-5 column of phenyl methyl silicone (30 m length, 0.25 mm in diameter, and 0.25 μm of phase thickness). From each sample, 1 μL was taken and injected in splitless mode, with a total execution time of 30 min. The chromatogram was interpreted with the aid of the AMDIS software. As a previous step to the GC/MS analysis, a derivatization reaction was performed to increase the volatility and thermal stability of the polar compounds which implies to derivatize one or more polar groups of one compound to a less polar group. In 2 mL Eppendorf tubes, 2 mL of the ethanol extract was deposited and lyophilized in the Speed-vac equipment to evaporate the solvent during 3 to 5 h. The lyophilized extract was resuspended in 350 μL of pyridine, then, 50 μL of the derivatizing agent, bis-(trimethylsilyl) trifluoroacetamide (BSTFA), which contains 1% chlorotrimethylsilane, was added, and the tubes were vortexed. Afterward, the mixture was heated in a thermoblock at a constant temperature of 50°C for 1 h. Once the thermal exposure time was concluded, it was centrifuged at 1200 rpm for 4 min. From the derivatized samples, 200 μL was taken, and the metabolites were identified by means of GC/MS. The analysis was performed in triplicate.

2.6.2. High Performance Liquid Chromatography (HPLC). To obtain the pure fraction of the ethanol or hexane extracts, these were fractionated by means of HPLC (Model Altus A30, Perkin Elmer, Inc., Waltham, MA, USA) [33] with a quaternary pump and a diode detector arrangement (DDA). To inject the sample, the air sampler of the same equipment was used. Separation was performed with a *C18* column in isocratic mode with a mobile 45% acetonitrile phase and 55% water. The flow of the mobile phase was of 1.00 mL $\times$ min^{-1}, performing detection at 275 nm. A 1.5 mL volume was taken from the extracts and filtered through a 0.2 μm pore membrane; the filtered extracts were recovered in 1.5 mL Eppendorf tubes and then lyophilized. The lyophilized extract was resuspended in 1.5 mL of HPLC-grade methanol and sonicated for 30 min. The process was repeated twice. To eliminate the solvent of the collected fractions from HPLC, these were lyophilized. The obtained lyophilized samples were resuspended in 2 mL of the initial solvent of each extract, ethanol or hexane, to perform the susceptibility assays against the five *Candida* species according to the previously described protocol. Experiments were performed in triplicate.

2.6.3. Prediction of Targets for the Identified Metabolites. To evaluate whether the metabolites identified in *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* with possible antifungal activity did not have targets in human cells, the SwissTargetPrediction (http://www.swisstargetprediction.ch.) free-access server was used. This server predicts accurately the targets of the bioactive molecules based on 2D and 3D similitude measurements with known ligands [34].

2.6.4. Statistical Analysis. In order to determine which of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts showed the highest antifungal effect against the five *Candida* species, growth inhibition halos' diameters in three independent experiments were measured and reported as a mean $\pm$ standard error of the mean (SEM). Statistical differences between means were determined using a one-way ANOVA test followed by Tukey's posttest for multiple comparisons.

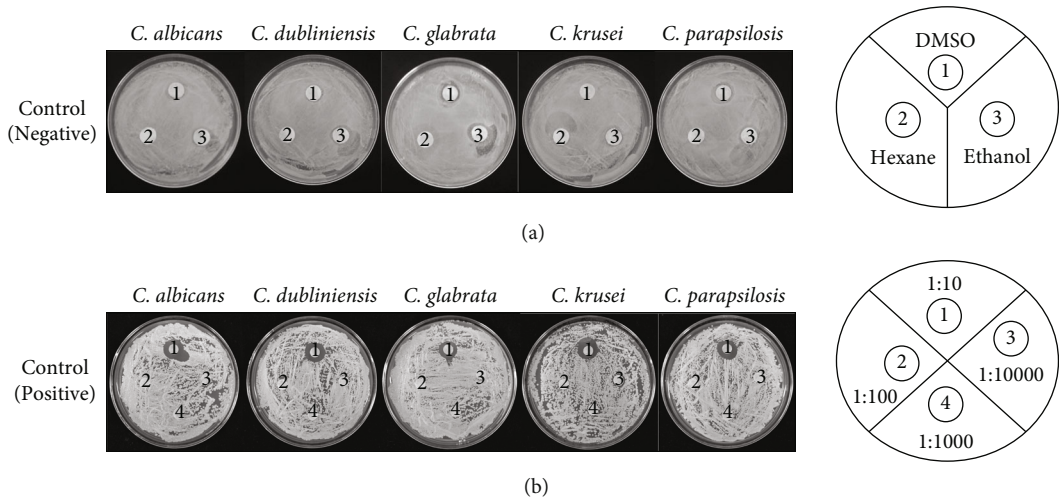


Figure 1: Susceptibility assays with the solvents used to obtain the *P. tithymaloides*, *T. vulgaris*, or *O. basilicum* extracts.

Normality and homogeneity of data were evaluated using both graphic evaluation and analytical tests such as Shapiro Wilk and Bartlett's test. Minitab 21.1 Software (State Collage PA, USA) was utilized to perform the statistical analysis. Statistical significance was considered when $p \leq 0.05$.

3. Results

3.1. Susceptibility Assays to the Different Plant Extracts with Possible Antifungal Activity. To rule out effects of the solvents employed to prepare the plant extracts, the susceptibility of the five *Candida* species to hexane and ethanol treatment was tested. Additionally, DMSO and phenol were evaluated as a negative and positive controls, respectively. Assays revealed that non-*Candida* species exhibited susceptibility to hexane, ethanol, or DMSO (Figure 1(a)). In contrast, phenol treatment, at the highest dilution (1 : 10) affected the growth of all the *Candida* strains tested (Figure 1(b)). In conclusion, ethanol and hexane were adequate to obtain the extracts from the studied plants to investigate the presence of metabolites with possible antifungal activity.

We investigated whether *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* possessed antifungal activity against five *Candida* species; to this end, ethanolic and hexane extracts prepared with stem and leaves from these plants were tested in disk diffusion assays as described in Materials and Methods. To investigate if the cell concentration impacts the antifungal efficiency of the plant extracts, the cultured *Candida* strains were tested at OD_{600nm} of 0.5 and 1.0, respectively, considering that for *Candida* species, an OD_{600nm} of 1.0 corresponds to 1×10^6 cells. It was decided to work with this amount of cells because susceptibility must be evaluated with a number of cells that will allow observing the antifungal effect of the compounds to be analyzed, which is not possible if working with a large amount of cells, where the antifungal effect is masked by the high cell density.

As shown in Figure 2(a), at both ODs, the ethanol extract of the stems presented a higher inhibition of the five *Candida* species, as compared to the leaf extracts. In contrast, except for leaf extract of *T. vulgaris*, the hexane extracts of *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* did not present a significant antifungal activity against the *Candida* species, except the leaf extract of *T. vulgaris* (Figure 2(b)). These results indicate that the metabolites with possible antifungal activity are found mainly in ethanol extracts. Furthermore, they indicate that the antifungal effect is independent from the number of cells and may correspond to the intrinsic effect of the extracts (Figures 2(a) and 2(b)).

To determine which of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts showed the highest antifungal effect against the five *Candida* species, we measured the growth inhibition halos' diameters in three independent experiments at both an OD of 0.5 (Table S1) and an OD of 0.1 (Table S2), and the means of all experiments were taken.

For the ethanol extract of the *P. tithymaloides* stem, at both ODs, the *C. dubliniensis* species presented the highest susceptibility, followed by *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. albicans* (Figures 3 and 4). For the leaf extract of *P. tithymaloides*, a nil antifungal activity was observed. Assessment of the stem extract of *T. vulgaris* revealed that *C. parapsilosis* and *C. krusei* were the most susceptible species at an OD of 0.5 and 0.1, respectively (Figures 3 and 4).

With the ethanol extract of the *O. basilicum* stem, *C. glabrata* was more susceptible at OD_{600nm} 0.5 and *C. dubliniensis* at OD_{600nm} 0.1 (Figures 3 and 4), whereas *C. albicans* and *C. parapsilosis* were the species with the highest resistance to this extract (Figures 3 and 4). With the ethanol extracts of *T. vulgaris* leaves, at an OD_{600nm} of 0.5, *C. albicans* presented the highest susceptibility (Figure 3), whereas at an $OD_{600nm}$of 0.1, it was *C. dubliniensis* (Figure 4); the resistance to this extract by *C. parapsilosis*, *C. glabrata*, and *C. krusei* at both ODs was different (Figures 3 and 4). With the stem extract of *O. basilicum*, at an OD_{600nm} of 0.5, *C. albicans* was the species with the highest resistance, followed by *C. parapsilosis*, *C. krusei*, *C. dubliniensis*, and *C. glabrata* (Figure 3). In contrast, at an OD_{600nm} of 0.1, *C. glabrata*, *C. krusei*, and *C. parapsilosis* were the species with the highest

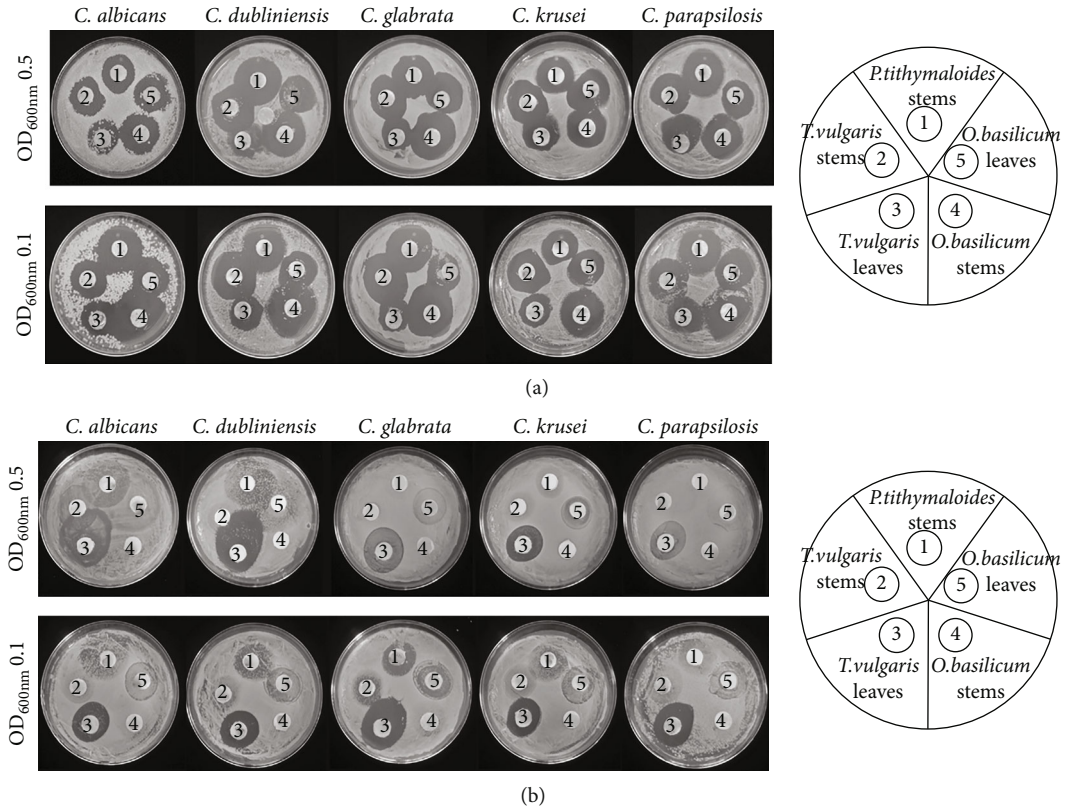


Figure 2: Susceptibility tests of stem or leaf extracts, obtained in (a) ethanol or (b) hexane, of *P. tithymaloides*, *O. Basilicum*, and *T. vulgaris* against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*.

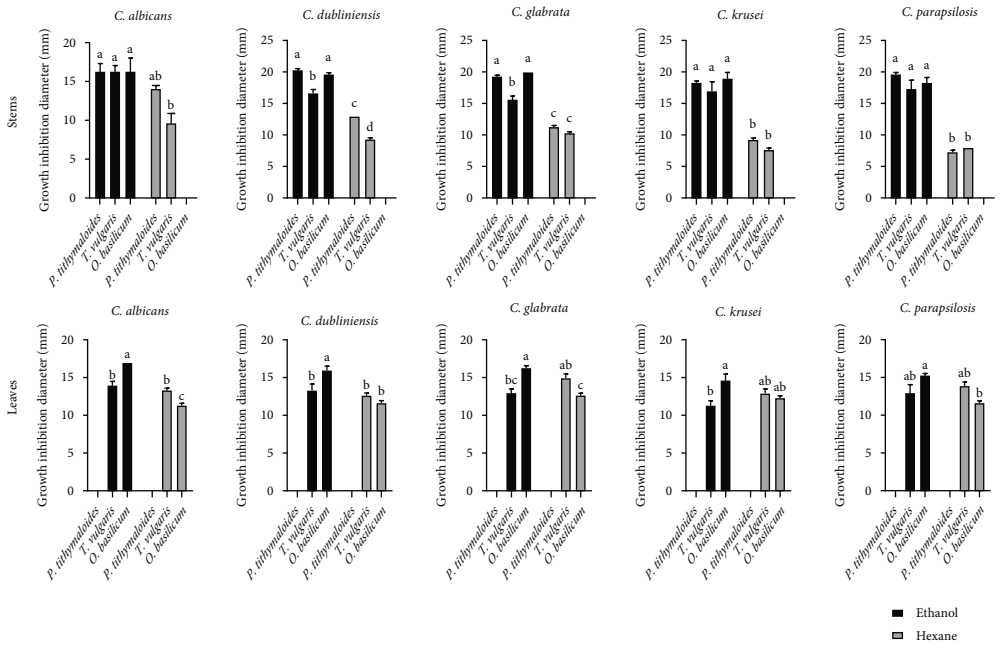


Figure 3: Diameters of the inhibition halos of the *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* extracts from stems and leaves (horizontal layout) against the five *Candida* species at an OD_{600nm} 0.5 (vertical layout). Black and dark gray bars represent ethanolic and hexanoic extracts, respectively. Different letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$). Error bars indicate the standard error of the mean (SEM, $n = 3$).

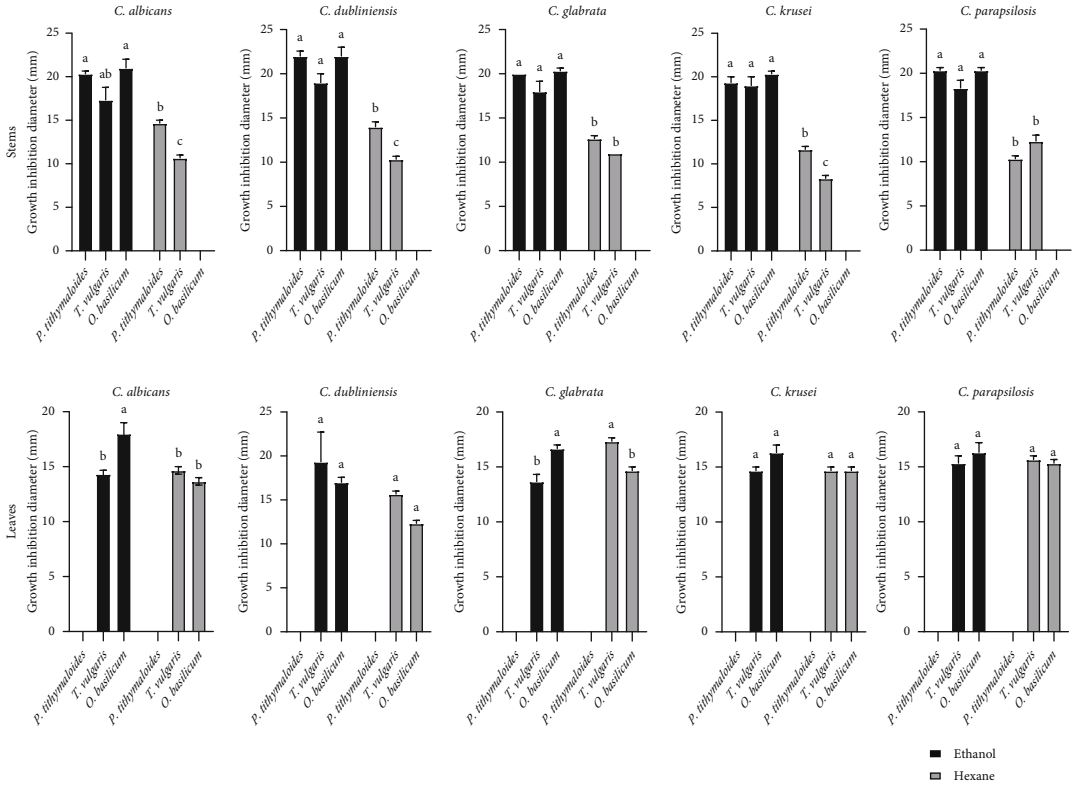


Figure 4: Diameters of the inhibition halos of the *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* extracts from stems and leaves (horizontal layout) against the five *Candida* species at an OD_{600nm} 0.1 (vertical layout). Black and dark gray bars represent ethanolic and hexanoic extracts, respectively. Different letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$). Error bars indicate the standard error of the mean (SEM, $n = 3$).

resistance to this extract (Figure 4). The highest susceptibility to the ethanol extract of *O. basilicum* leaves was shown by *C. albicans* at both ODs tested (Figures 3 and 4), whereas *C. parapsilosis* and *C. krusei* were the species with the highest resistance to this extract (Figures 3 and 4). Interestingly, the *T. vulgaris* leaf extracts prepared with hexane but not those from stems affected the growth of the five *Candida* species, when these were tested at both cell's concentrations (Figures 3 and 4).Overall, our results revealed that the solvents employed to prepare the extracts impacted the antifungal activity exhibited by the leaves and stems of the plants tested and that such effects may obey to the existence of a differential profile of metabolites with various degrees of antifungal activity.

3.2. Analysis of the Extracts Obtained with Ethanol and Hexane. The stems and leaf extracts obtained with hexane were analyzed without any GC-MS treatment, whereas the ethanol extracts were subjected to a derivatization reaction to be analyzed with this technique. The chromatograms obtained of each extract were analyzed with the AMDIS software; this approach allowed us to identify a set of secondary plant metabolites (Fig. S1). Table 1 depicts the compounds identified through GC/MS analysis of the extracts that presented antifungal activity against the five *Candida* species.

The analysis of the *T. vulgaris* extracts revealed 15 potential compounds with antifungal activity; the leaf was the plant tissue with the highest number of chemical compounds with 15 compounds, and ethanol was the extraction agent with the most extracted metabolites. Compounds like carvacrol, eugenol, carveol, and p-cymene were identified only in ethanol extracts of both leaves and stems. The thymol and thymol-methyl-ether were identified in all plant extracts. In *O. basilicum*, 11 compounds were identified in ethanol extracts (Table 1). The compounds were identified in both the leaves and stems, revealing that metabolites are present in both plant organs, but at different concentrations, this would explain why the best antifungal effect against *Candida* species is observed in stems (Figure 2(a)). Regarding *P. tithymaloides* stem extracts, eight compounds were identified. In this plant, no metabolites were identified in the leaf extracts; i.e., no antifungal activity was recorded (Figure 2). Eugenol, linalool, limonene, and borneol were found in more than one of the plants (Table 1); however, linalool was the only compound identified in the extracts of the three plants (Table 1). This finding indicates that possibly, this compound is the main one implicated in the antifungal effect against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*. Once the metabolites had been identified in the different extracts of *P. tithymaloides*, *T. vulgaris,* and *O. basilicum*, it was decided to purify the different metabolites, to assess the antifungal activity of each identified metabolite and, in this way, know which metabolite is responsible for the antifungal activity. For this, the plant

Table 1: Chemical compounds potentially responsible for the antifungal activity present in the plant extracts.

Plant extract	Plant part	Extraction agent	Identified compound	Chemical formula
T. vulgaris	Leaf Stem	Hex EtOH	Thymol methyl ether	$C_{11}H_{16}O$
	Leaf Stem	Hex EtOH	Thymol	$C_{10}H_{14}O$
	Leaf Stem	EtOH	Carvacrol	$C_{10}H_{14}O$
	Leaf Stem	EtOH	Carvacrol methyl ether	$C_{11}H_{16}O$
	Leaf	Hex	2-Chloropropionyl chloride	$C_3H_4C_{l2}O$
	Leaf	Hex	2-Bromo-2-methylbutane	$C_5H_{11}Br$
	Leaf	Hex	2,4-Dimethylhexane	C_8H_{18}
	Leaf Stem	EtOH	Eugenol	$C_{10}H_{12}O_2$
	Leaf Stem	EtOH	Methyl eugenol	$C_{11}H_{14}O_2$
	Leaf	Hex EtOH	Linalool	$C_{10}H_{18}O$
	Leaf	EtOH	Limonene	$C_{10}H_{16}$
	Leaf	EtOH	Borneol	$C_{10}H_{18}O$
	Leaf Stem	EtOH	α-Terpineol	$C_{10}H_{18}O$
	Leaf Stem	EtOH	Carveol	$C_{10}H_{16}O$
	Leaf Stem	EtOH	p-Cymene	$C_{10}H_{14}$
O. basilicum	Leaf	EtOH	Linalool	$C_{10}H_{18}O$
	Leaf	EtOH	Limonene	$C_{10}H_{16}$
	Leaf	EtOH	Borneol	$C_{10}H_{18}O$
	Leaf	EtOH	Eugenol	$C_{10}H_{12}O_2$
	Leaf Stem	EtOH	α-Bergamotene	$C_{21}H_{22}O_4$
	Leaf Stem	EtOH	Sabinene	$C_{10}H_{16}$
	Leaf Stem	EtOH	α-Pinene	$C_{10}H_{16}$
	Leaf Stem	EtOH	Germacrene D	$C_{15}H_{24}$
	Leaf Stem	EtOH	Bornyl acetate	$C_{12}H_{20}O_2$
	Leaf Stem	EtOH	α-Amorphene	$C_{15}H_{24}$
	Leaf Stem	EtOH	α-Caryophyllene	$C_{15}H_{24}$
P. tithymaloides	Stem	EtOH	Palmitic acid	$C_{16}H_{32}O_2$
	Stem	EtOH	Retinol	$C_{20}H_{28}O$
	Stem	EtOH	Myristic acid	$C_{14}H_{28}O_2$
	Stem	EtOH	Stearic acid	$C_{18}H_{36}O_2$
	Stem	EtOH	Tauric acid	$C_{12}H_{24}O_2$
	Stem	EtOH	Citronellol	$C_{10}H_{20}O$
	Stem	EtOH	Stigmasterol	$C_{29}H_{48}O$
	Stem	EtOH	Linalool	$C_{10}H_{18}O$

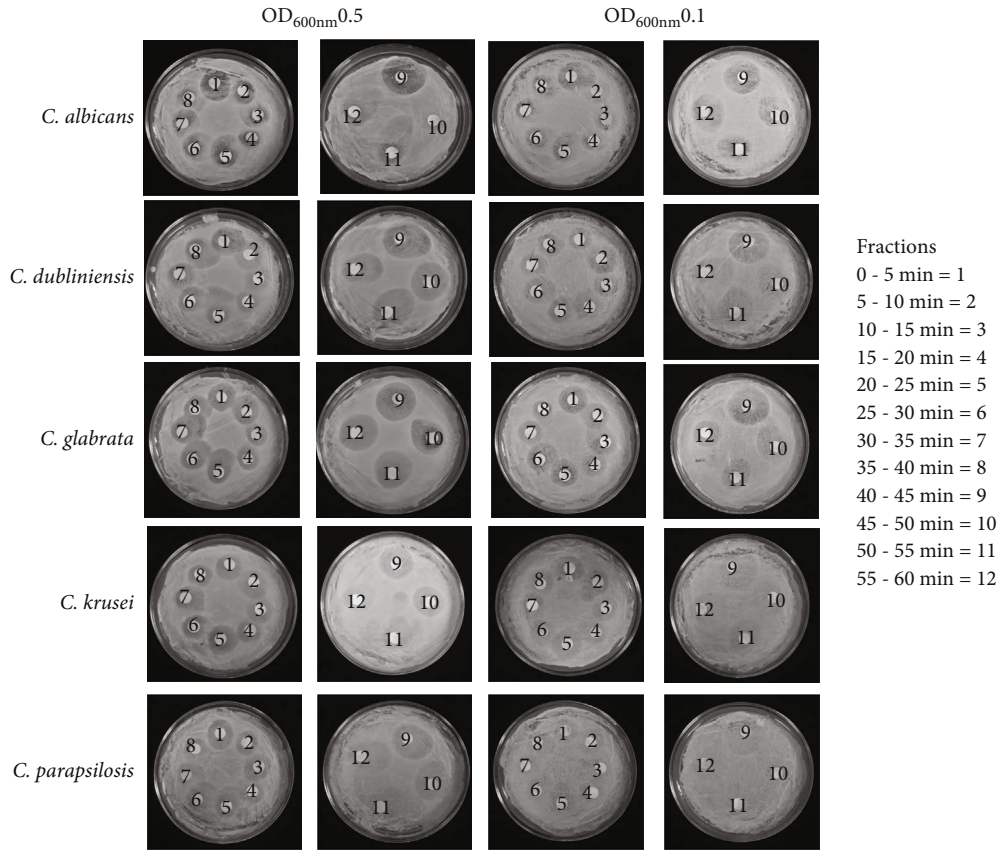


Figure 5: Susceptibility assays of the 12 pure fractions obtained from HPLC.

extracts were fractionated at 12-elution times through HPLC, collecting 12 fractions in 60 min. Each fraction was used to perform susceptibility assays against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*. Some of these fractions exhibited inhibitory activity against these fungal species, indicating that the fraction must contain some compound with antifungal activity. Figure 5 and Table 2 summarize the results of these assays.

The susceptibility assays with the different fractions obtained through HPLC revealed specific activity against some of the *Candida* species (Figure 5). To analyze these results, a table was constructed considering all experiments performed in triplicate and independently (Table 2).

For the *P. tithymaloides* stem extract, fractions 8, 9, and 12 showed antifungal activity against the five *Candida* species. The *T. vulgaris* leaf extract presented greater antifungal activity in fractions 1, 3, 5, 6, 9, 10, and 12, whereas only fraction 12 of the stem extracts presented antifungal activity. The *O. basilicum* leaf extract presented the largest number of antifungal fractions against *Candida*, these fractions were 1, 5, 6, 7, 8, 9, 10, 11, and 12 (Table 2) during the susceptibility assays. For the stem, fractions 10, 11, and 12 (Table 2) presented susceptibility to the five *Candida* species. To assess whether the four metabolites identified in the three analyzed plants could be good candidates against *Candida*, but apparently innocuous to the human cells, the targets of these metabolites were mapped using the SwissTargetPrediction software. The bioinformatics analyses revealed that the probability that any of the proteins of human cells could be an actual target was practically inexistent for the four secondary metabolites (Figure 6).

4. Discussion

In the last years, it has been reported that *Candida* species represent one of the most important causes of systemic nosocomial infections. *C. albicans* is the most important opportunistic pathogen; however, the prevalence of other species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and, recently, *C. auris* is increasing. The developments in medicine, associated with an increase in invasive procedures, increasingly aggressive immunosuppressing treatments, and the generalized use of wide-spectrum antibiotics, have favored the increase in the selective pressure and the development of antifungal resistance, contributing to the incidence of candidemia and IC. Although a variety of antifungal agents is available and because patients do not respond adequately to treatment, it is necessary to identify new antifungal agents that are safe for the patients, but highly selective against *Candida* species. The main inconvenience when designing new antifungal compounds is that the only difference between mammalian and fungal cells is the cell wall of *Candida* and, thus, the target site of excellence, leading to a lesser possibility for the effect of antifungal agents. For this reason, the search and use of new active principles, derived from natural products for alternative therapies, are utterly important. Among these natural

Table 2: Susceptibility assays with the HPLC obtained fractions.

Hexane plant extract	Number of fraction	Collecting time	Inhibitory activity on each species				
			C. albicans	*C. dubliniensis*	*C. glabrata*	*C. krusei*	*C. parapsilosis*
Stem of *P. tithymaloides*	1	0–5 min	–	–	–	–	–
	2	5–10 min	–	–	–	–	–
	3	10–15 min	–	–	–	–	–
	4	15–20 min	–	–	–	–	–
	5	20–25 min	–	–	–	–	–
	6	25–30 min	–	–	–	–	–
	7	30–35 min	–	–	–	–	–
	8	35–40 min	+	+	+	+	+
	9	40–45 min	+	+	+	+	+
	10	45–50 min	–	–	–	–	–
	11	50–55 min	–	–	–	–	–
	12	55–60 min	+	+	+	+	+
Leaf of *T. vulgaris*	1	0–5 min	–	+	+	+	+
	2	5–10 min	–	–	–	–	–
	3	10–15 min	+	–	–	–	+
	4	15–20 min	–	–	–	–	–
	5	20–25 min	+	+	+	+	–
	6	25–30 min	–	+	+	+	–
	7	30–35 min	–	–	–	–	–
	8	35–40 min	–	–	–	–	–
	9	40–45 min	–	+	+	+	+
	10	45–50 min	–	–	+	–	–
	11	50–55 min	–	–	–	–	–
	12	55–60 min	+	+	+	+	+
Stem of *T. vulgaris*	1	0–5 min	–	–	–	–	–
	2	5–10 min	–	–	–	–	–
	3	10–15 min	–	–	–	–	–
	4	15–20 min	–	–	–	–	–
	5	20–25 min	–	–	–	–	–
	6	25–30 min	–	–	–	–	–
	7	30–35 min	–	–	–	–	–
	8	35–40 min	–	–	–	–	–
	9	40–45 min	–	–	–	–	–
	10	45–50 min	–	–	–	–	–
	11	50–55 min	–	–	–	–	–
	12	55–60 min	–	–	+	+	+
Leaf of *O. basilicum*	1	0–5 min	+	+	+	–	+
	2	5–10 min	–	–	+	–	–
	3	10–15 min	–	–	–	–	–
	4	15–20 min	–	–	–	–	–
	5	20–25 min	+	–	+	+	–
	6	25–30 min	–	+	+	–	–
	7	30–35 min	+	–	+	+	–
	8	35–40 min	+	+	+	–	–
	9	40–45 min	+	+	+	+	+
	10	45–50 min	–	+	+	+	+
	11	50–55 min	+	+	+	+	+
	12	55–60 min	+	+	+	+	+

Table 2: Continued.

Hexane plant extract	Number of fraction	Collecting time	Inhibitory activity on each species				
			C. albicans	*C. dubliniensis*	*C. glabrata*	*C. krusei*	*C. parapsilosis*
Stem of *O. basilicum*	1	0–5 min	–	–	–	–	–
	2	5–10 min	–	–	–	–	–
	3	10–15 min	–	–	–	–	–
	4	15–20 min	–	–	–	–	–
	5	20–25 min	–	–	–	–	–
	6	25–30 min	–	–		–	–
	7	30–35 min	–	–	–	–	–
	8	35–40 min	–	–	+	+	+
	9	40–45 min	+	+	–	+	+
	10	45–50 min	+	+	+	+	+
	11	50–55 min	+	+	+	+	+
	12	55–60 min	+	+	+	+	+

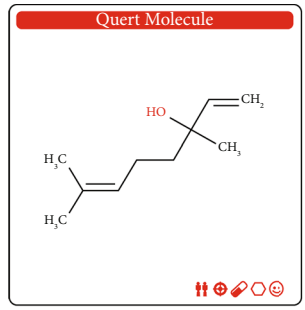
– resistant; + susceptible.

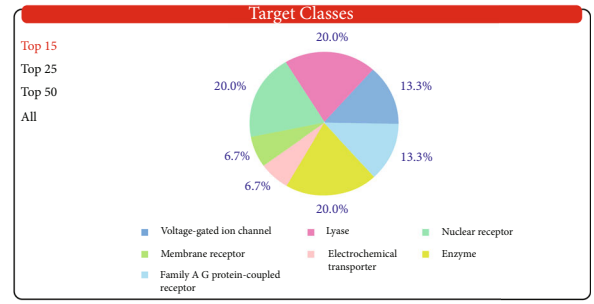
products are plants which contain a large variety of biologically active molecules, which can have different target sites and action mechanisms from those of traditional antimicrobials [35, 36]; in fact, the antifungal activity of different crude plant extracts against different microorganisms has been reported.

To identify the metabolites with antifungal activity against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*, we chose *P. tithymaloides*, *T. vulgaris*, and *O. basilicum*, which are plants used in traditional medicine to treat different diseases. Crude extracts were prepared with two different solvents: ethanol that is a solvent of polar character and hexane that is a polar. The variety of the solvents allowed obtaining different metabolites from the plants. The ethanol extracts presented higher antifungal activity (Figure 2(a)) against the five studied *Candida* species, whereas for the hexane extracts, the *T. vulgaris* leaf extract was the only one with antifungal activity against the *Candida* species (Figure 2(b)). The anatomical parts, i.e., leaves and stems, were assessed independently to analyze whether there is some difference in the composition and concentration of metabolites. This was done because some of the secondary metabolites in plant exert defense functions against predators and can also inhibit the development of insects [37], fungi [38], and bacteria [39], which determine their preferential location in one or another anatomical site. Results allowed identifying a total of 30 metabolites in *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* in leaves and/or stems. Among these metabolites, monoterpene phenols like thymol, carvacrol, and eugenol were identified in *T. vulgaris*. However, of the identified metabolites, we focused in those identified in at least two plants. In this way, linalool was identified in three plants, and eugenol, limonene, and borneol were found in *T. vulgaris* and *O. basilicum* (Table 1).

Linalool (3,7-dimethylocta-1,6-dien-3-ol) has been reported to have properties as acaricide, bactericide, and fungicide. Its antifungal activity has been tested against diverse microorganisms, such as *Fusarium moniliforme* at a dose of 1000 ppm, *Candida* species, and *Acinetobacter baumannii* [40–48]. Our results agree with other reports that have evaluated linalool as an antifungal against *Candida* species; in strains isolated from individuals with oral candidiasis, the antifungal activity was good against *C. tropicalis* and moderate against *C. albicans* [42]. In another study, the fungicide activity of linalool against 39 *C. albicans* isolates and 9 isolates of *Candida* non-*C. albicans* was evaluated and found to be effective against both *Candida* species [46, 47]. Linalool has also been found in the essential oil of *Lavandula angustifolia*; in this work, the antifungal activity against *C. albicans* was shown, as well as its participation in avoiding the morphological transition from yeast to mycelium, because linalool can inhibit the formation of the germ tube and reduce the elongation of hyphae. This finding indicates that linalool, by avoiding dimorphism, can be considered as an effective compound against *C. albicans* [41]. Besides, by preventing the morphology change in *Candida*, linalool also avoids the formation of biofilms by *C. albicans* [43] and *C. tropicalis* [44]. Biofilms are considered as virulence factors in *Candida* species, because they induce recurring candidiasis symptoms, leading to fatal outcomes. As a whole, these data show that linalool is a compound with efficient antifungal activity against the studied *Candida* species, and it is present in different plant species, as shown in this and other studies; hence, it is a good candidate to be used against these fungi. The mechanism by which terpenoids, like linalool, act as antifungal agents has not been elucidated yet; but a model has been described proposing that terpenoids induce membrane fluidization, modulating the functions of the proteins bound to the membrane and involved in signaling and transport [47]. Besides, it has been reported that terpenoids arrest the cell cycle in *Candida* [47] and other organisms, like *Staphylococcus aureus* and *Escherichia coli* [49]. Linalool exhibited an estimated low affinity for distinct families of proteins from *Homo sapiens* (Figure 6(a)).

Another identified metabolite in *T. vulgaris* and *O. basilicum* was eugenol (4-allyl-2-methoxyphenol). This compound is found in a variety of plants, like *Syzygium aromaticum* (L.) and *Myristica fragrans* (Houtt); besides, finding eugenol in *O. basilicum* agrees with other works [50, 51]. Its common

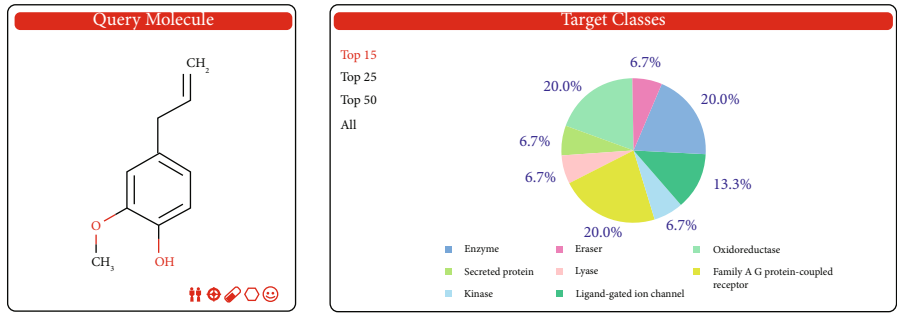




Target	Common name	Uniprot ID	chEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Transient receptor potential cation channel subfamily V member 3	TRPV3	Q8NET8	CHEMBL5522	Voltage-gated ion channel	0.0630257148888	1 / 0
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.0630257148888	33 / 0
Carbonic anhydrase I	CA1	P00915	CHEMBL261	Lyase	0.0630257148888	27/ 0
Carbonic anhydrase IV	CA4	P22748	CHEMBL3729	Lyase	0.0630257148888	11 / 0
Transient receptor potential cation channel subfamily M member 8	TRPM8	Q7Z2W7	CHEMBL1075319	Voltage-gated ion channel	0.0630257148888	1 / 0
Mineralocorticoid receptor	NR3C2	P08235	CHEMBL1994	Nuclear receptor	0.053517944289	34 / 0
Glucocorticoid receptor	NR3C1	P04150	CHEMBL2034	Nuclear receptor	0.053517944289	52 / 0
Progesterone receptor	PGR	P06401	CHEMBL208	Nuclear receptor	0.053517944289	74 / 0
Sigma opioid receptor	SIGMAR1	Q99720	CHEMBL287	Membrane receptor	0.053517944289	27 / 0
Dopamine transporter (by homology)	SLC6A3	Q01959	CHEMBL238	Electrochemical transporter	0.053517944289	101 / 0
Squalene monooxygenase	SQLE	Q14534	CHEMBL3592	Enzyme	0.053517944289	0 / 4
Indoleamine 2,3-dioxygenase	IDO1	P14902	CHEMBL4685	Enzyme	0.053517944289	9 / 0
Estradiol 17-beta-dehydrogenase 2	HSD17B2	P37059	CHEMBL2789	Enzyme	0.053517944289	57 / 0
Dopamine D2 receptor (by homology	DRD2	P14416	CHEMBL217	Family A G protien coupled receptor	0.053517944289	13 / 0
Muscarinic acetylcholine receptor M4	CHRM4	P08173	CHEMBL1821	Family A G protien coupled receptor	0.053517944289	11 / 0
Mu opioid receptor	OPRM1	P35372	CHEMBL233	Family A G protien coupled receptor	0.053517944289	8 / 0
Delta opioid receptor	OPRD1	P41143	CHEMBL236	Family A G protien coupled receptor	0.053517944289	5 / 0
Kappa opioid receptor	OPRK1	P41145	CHEMBL237	Family A G protien coupled receptor	0.053517944289	29 / 0
Adrenergic receptor alpha-2	ADRA2C	P18825	CHEMBL1916	Family A G protien coupled receptor	0.0439186325197	13 / 0
Heme oxygenase 1 (by homology)	HMOX1	P09601	CHEMBL2823	Enzyme	0.0439186325197	36 /0
Tyrosine-protien kinase JAK 1	JAK1	P23458	CHEMBL2835	Kinase	0.0439186325197	60 /0
Tyrosine-protien kinase JAK 2	JAK2	O60674	CHEMBL2971	Kinase	0.0439186325197	86 /0

(a)

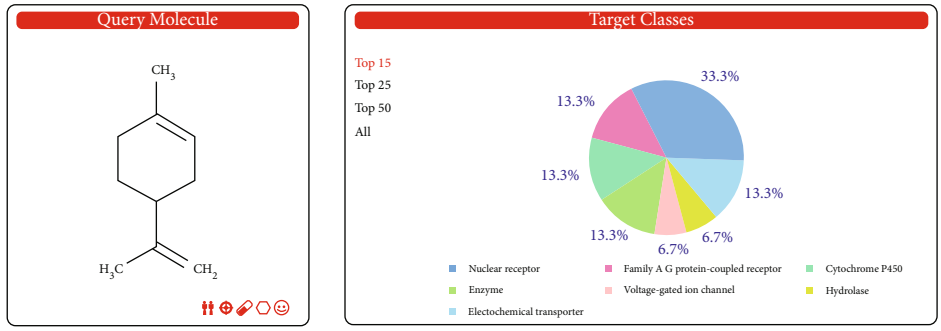
Figure 6: Continued.



Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Fatty acid desaturase 1	FADS1	O60427	CHEMBL5840	Enzyme	0.133391037839	8 / 0
Histone deacetylase 6	HDAC6	Q9UBN7	CHEMBL1865	Eraser	0.133391037839	10 / 0
Egl nine homolog 1	EGLN1	Q9GZT9	CHEMBL5697	Oxidoreductase	0.125075959828	4 / 0
Vascular endothelial growth factor A	VEGFA	P15692	CHEMBL1783	Secreted protein	0.125075959828	1 / 0
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.125075959828	199 / 15
G-protein coupled receptor 84	GPR84	Q9NQS5	CHEMBL3714079	Family A G protein-coupled receptor	0.125075959828	18 / 0
Cyclooxygenase-1	PTGS1	P23219	CHEMBL221	Oxidoreductase	0.125075959828	35 / 13
D-amino-acid oxidase	DAO	P14920	CHEMBL5485	Enzyme	0.125075959828	27 / 0
Poly [ADP-ribose] polymerase-1	PARP1	P09874	CHEMBL3105	Enzyme	0.125075959828	32 / 0
Tyrosine-protein kinase SRC	SRC	P12931	CHEMBL267	Kinase	0.125075959828	10 / 0
Adenosine A1 receptor	ADORA1	P30542	CHEMBL226	Family A G protein-coupled receptor	0.125075959828	28 / 0
Adenosine A2a receptor	ADORA2A	P29274	CHEMBL251	Family A G protein-coupled receptor	0.125075959828	17 / 0
Steroid 5-alpha-reductase 1	SRD5A1	P18405	CHEMBL1787	Oxidoreductase	0.125075959828	3 / 0
Neuronal acetylcholine receptor subunit alpha-3	CHRNA3	P32297	CHEMBL3068	Ligand-gated ion channel	0.125075959828	1 / 0
Neuronal acetylcholine receptor protein alpha-4 subunit (by homology)	CHRNA4	P43681	CHEMBL1882	Ligand-gated ion channel	0.125075959828	3 / 0
Interleukin-8 receptor B	CXCR2	P25025	CHEMBL2434	Family A G protein-coupled receptor	0.125075959828	20 / 0
dCTP pyrophosphatase 1	DCTPP1	Q9H773	CHEMBL3769292	Enzyme	0.125075959828	5 / 0
Alkaline phosphatase, tissue-nonspecific isozyme	ALPL	P05186	CHEMBL5979	Enzyme	0.125075959828	15 / 0
Methionine aminopeptidase 2	METAP2	P50579	CHEMBL3922	Protease	0.116739032206	5 / 0
Carbonyl reductase [NADPH] 1	CBR1	P16152	CHEMBL5586	Enzyme	0.116739032206	1 / 0
Calcium-activated potassium channel subunit alpha-1	KCNMA1	Q12791	CHEMBL4304	Voltage-gated ion channel	0.116739032206	11 / 0
Arachidonate 15-lipoxygenase	ALOX15	P16050	CHEMBL2903	Enzyme	0.116739032206	33 / 11
Neuronal acetylcholine receptor; alpha3/beta4	CHRNA3 CHRNB4	P32297 P30926	CHEMBL1907594	Ligand-gated ion channel	0.116739032206	1 / 0
Vascular endothelial	KDR	P35968	CHEMBL279	Kinase	0.116739032206	32 / 0

(b)

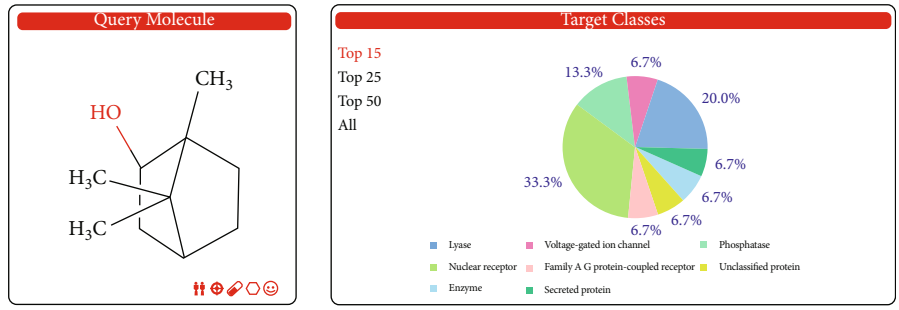
Figure 6: Continued.



Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Peroxisome proliferator-activated receptor alpha	PPARA	Q07869	CHEMBL239	Nuclear receptor	0.146856850103	1 / 7
Cannabinoid receptor 2	CNR2	P34972	CHEMBL253	Family A G protein-coupled receptor	0.146856850103	1 / 1
LXR-alpha	NR1H3	Q13133	CHEMBL2808	Nuclear receptor	0.0043083186041	0 / 14
Cytochrome P450 19A1	CYP19A1	P11511	CHEMBL1978	Cytochrome P450	0.023827432783	0 / 127
Anandamide amidohydrolase	FAAH	000519	CHEMBL2243	Enzyme	0.0	0 / 10
Vanilloid receptor	TRPV1	Q8NER1	CHEMBL4794	Voltage-gated ion channel	0.0	0 / 2
Androgen Receptor (by homology)	AR	P10275	CHEMBL1871	Nuclear receptor	0.0	0 / 47
Estrogen receptor alpha	ESR1	P03372	CHEMBL206	Nuclear receptor	0.0	0 / 27
Muscarinic acetylcholine receptor M2	CHRM2	P08172	CHEMBL211	Family A G protein-coupled receptor	0.0	0 / 1
Acetylcholinesterase	ACHE	P22303	CHEMBL220	Hydrolase	0.0	4 / 1
Norepinephrine transporter	SLC6A2	P23975	CHEMBL222	Electochemical transporter	0.0	0 / 1
Serotonin transporter	SLC6A4	P31645	CHEMBL228	Electochemical transporter	0.0	0 / 1
Cytochrome P450 2C19	CYP2C19	P33261	CHEMBL3622	Cytochrome P450	0.0	0 / 1
Squalene monooxygenase	SQLE	Q14534	CHEMBL3592	Enzyme	0.0	0 / 5
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0.0	0 / 32
Butyrylcholinesterase	BCHE	P06276	CHEMBL1914	Hydrolase	0.0	0 / 1
Protein-tyrosine phosphatase 1B	PTPN1	P18031	CHEMBL335	Phosphatase	0.0	0 / 25
Nuclear receptor subfamily 1 gropu 1 member 3 (by homology)	NR1I3	Q14994	CHEMBL5503	Nuclear receptor	0.0	0 / 2
Cyclooxygenase-1	PTGS1	P23219	CHEMBL221	Oxidoreductase	0.0	0 / 2
DNA topoisomerase I	TOP1	P11387	CHEMBL1781	Isomerase	0.0	0 / 2
Adenosine A1 receptor	ADORA1	P30542	CHEMBL226	Family A G protein-coupled receptor	0.0	0 / 1
Adenosine A2a receptor	ADORA2A	P29274	CHEMBL251	Family A G protein-coupled receptor	0.0	0 / 1
Adenosine A3 receptor	ADORA3	P0DMS8	CHEMBL256	Family A G protein-coupled receptor	0.0	0 / 2
Arachidonate 5-lipoxygenase	ALOX5	P09917	CHEMBL215	Oxidoreductase	0.0	0 / 4
Nuclear receptor ROR-gamma	RORC	P51449	CHEMBL1741186	Nuclear receptor	0.0	0 / 9
Sterol regulatory element-binding protein 2	SREBF2	Q12772	CHEMBL1795166	Unclassified protein	0.0	0 / 1
Niemann-Pick C1-like	NPC1L1	Q9UHC9	CHEMBL2027	Other membrane	0.0	0 / 5

(c)

Figure 6: Continued.



Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.340608982509	3 / 2
Carbonic anhydrase I	CA1	P00915	CHEMBL261	Lyase	0.340608982509	3 / 2
Carbonic anhydrase IV	CA4	P22748	CHEMBL3729	Lyase	0.340608982509	2 / 2
Transient receptor potential cation channel subfamily M member 8	TRPM8	Q7Z2W7	CHEMBL1075319	Voltage-gated ion channel	0.302383432167	1 / 1
Dual specificity phosphatase Cdc25A	CDC25A	P30304	CHEMBL3775	Phosphatase	0.197404892104	0 / 7
Dual specificity phosphatase Cdc25B	CDC25B	P30305	CHEMBL4804	Phosphatase	0.197404892104	0 / 5
Nuclear receptor subfamily 1 group I member 3	NR1I3	Q14994	CHEMBL5503	Nuclear receptor	0.197404892104	0 / 2
Androgen Receptor	AR	P10275	CHEMBL1871	Nuclear receptor	0.177829917574	34 / 17
Bile acid receptor FXR	NR1H4	Q96RI1	CHEMBL2047	Nuclear receptor	0.139879779447	0 / 12
G-protein coupled bile acid receptor 1	GPBAR1	Q8TDU6	CHEMBL5409	Family A G protein-coupled receptor	0.139879779447	0 / 16
Sonic hedgehog protein (by homology)	SHH	Q15465	CHEMBL5602	Unclassified protein	0.139879779447	0 / 10
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0.101228839251	5 / 5
Estrogen receptor alpha	ESR1	P03372	CHEMBL206	Nuclear receptor	0.0918391658524	1 / 6
UDP-glucuronosyltransferase 2B7	UGT2B7	P16662	CHEMBL4370	Enzyme	0.0822880706974	8 / 24
Testis-specific androgen-binding protein	SHBG	P04278	CHEMBL3305	Secreted protein	0.0822880706974	2 / 22
DNA polymerase alpha subunit	POLA1	P09884	CHEMBL1828	Transferase	0.0727163610114	0 / 1
Niemann-Pick C1-like protein 1	NPC1L1	Q9UHC9	CHEMBL2027	Other membrane protein	0.0727163610114	0 / 7
11-beta-hydroxysteroid dehydrogenase 1	HSD11B1	P28845	CHEMBL4235	Enzyme	0.053517944289	9 / 11
LXR-alpha	NR1H3	Q13133	CHEMBL2808	Nuclear receptor	0.053517944289	0 / 10
Glucose-6-phosphate 1-dehydrogenase	G6PD	P11413	CHEMBL5347	Enzyme	0.0439186325197	0 / 7
GABA-B receptor (by homology)	GABBR1	Q9UBS5	CHEMBL2064	Family A G protein-coupled receptor	0.0439186325197	0 / 4
Glucocorticoid receptor	NR3C1	P04150	CHEMBL2034	Nuclear receptor	0.0439186325197	20 / 0
Dopamine D2 receptor (by homology)	DRD2	P14416	CHEMBL217	Family A G protein-coupled	0.0439186325197	5 / 0

(d)

Figure 6: Results of the target sites prediction in human cells: (a) Linalool (3,7-dimethylocta-1,6-dien-3-ol; SMILE: CC(O)(C=C)CCC=C(C)C. B) Eugenol (4-Allyl-2-methoxyphenol; SMILE: COC1=CC(CC=C)=CC=C1O. (c) Limonene (1-methyl-4-(1-methyletenyl)-cyclohexene; SMILE: CC1=CCC(CC1)C(=C)C. (d) Borneol ([(2*R*)-1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl] formate; SMILE: CC1(C2CCC1(C(C2)O)C)C. Green bars indicate the estimated probability that a protein could be an actual target of the secondary metabolite, at less probability the lesser it is that an actual target is implicated.

name comes from the plant *Eugenia caryophyllata* (syn. *Syzygium aromaticum*) [50] where it was found for the first time. This compound, like linalool, has been shown to have antifungal activity against *Candida* species [47]. The antifungal activity of this compound has been investigated for almost four decades [52]. These authors analyzed the antifungal activity of eugenol in 31 strains of *C. albicans* and found that it had indeed antifungal activity against this fungus [52]. Several years later, the activity of eugenol against several opportunistic fungi, including *C. albicans*, was investigated, and authors found that the analyzed strains were inhibited by this metabolite [53]. Aiming at administering eugenol as a treatment against fungi, toxicity studies were performed in mice, but the maximal tolerated dose was of 62.5 mg/kg; therefore, treatment of mycoses with eugenol is not allowed [52]. To be able to use eugenol as an antifungal drug, derivatives of this metabolite have been synthetized, finding that a derivative known as peracetyl glucoside, which is more potent and less cytotoxic than eugenol, inhibited the growth of *C. albicans*, *C. glabrata*, and *C. tropicalis* [54]. In a later work, it was demonstrated that the use of a new derivative of eugenol against these fungi inhibited 90% the growth of *C. glabrata* [44, 45]. These data together with our findings indicated that the eugenol in the fractions of two of the analyzed plants is possibly responsible for the observed antifungal activity (Table 1). The action mechanism of eugenol in fungi has already been studied, and it is suggested that because eugenol is lipophilic, it can enter the fatty acid chains of the lipid bilayer of the membrane, upsetting its fluidity and permeability [55, 56]. It has also been reported that eugenol inhibits the ATPase activity and that it is capable of producing oxidative stress [57]. The analysis by SwissTargetPrediction revealed a low affinity of eugenol for enzymes from *H. sapiens* (Figure 6(b)) which would be beneficial for the treatment of candidiasis.

Limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) is another of the compounds identified in *T. vulgaris* and *O. basilicum*; this cyclic monoterpene has been identified in a large variety of citrus plants and other plants, i.e., *Thapsia villosa*, *Dyssodia decipiens*, *Helichrysum italicum* [58, 59]. Other reports agree with our results by reporting limonene as a compound of *Thymus vulgaris* [60–62]. Limonene is widely used in the pharmaceutical industry as insecticide and antimicrobial [63–67]. Few studies have analyzed the antimicrobial effects of limonene; however, there are some works that evaluated the biological activity of limonene against species of genera like *Aspergillus*, *Trichophyton*, and *Candida*, reporting the antifungal efficiency of this compound [68, 69]. Our results agree with studies that have evaluated limonene as an antifungal against species of *Candida*, in which it was efficient against clinical isolates of *C. albicans* strains, with a MIC of 12.5–188.4 $\mu g\,mL^{-1}$ [59]. Another study demonstrated the antifungal activity of limonene on the planktonic growth of 35 clinical isolates and two standard strains of *C. albicans*, in which the fungicide activity was efficient in all cases at a 20 mM concentration [68]. No specific mechanisms on how limonene induces cellular disturbances have been proposed; however, it is known that limonene exerts an inhibitory effect on the formation of the germ tube in *C. albicans*, affecting the morphological change from yeast to mycelium [46, 47, 70, 71], which is an important virulence factor for the pathogenicity of this species [72]. The hydrophobicity of limonene apparently facilitates the dissolution of lipids located in the microbial plasmatic membrane, inducing a loss of the membrane's integrity and, thereby, affecting functions like permeability, signaling, and transport [73, 74]. Besides, it affects respiration and the energetic metabolism by interfering with the ATP synthesis through the inhibition of the respiratory complex and ATPase activities [75, 76]. These data support the capacity of limonene to act as an efficient antifungal against *Candida*, because it is a component of a large variety of plants, and as shown in this and other studies, it is capable of acting on multiple targets. The aforementioned makes this compound a good candidate to be used against fungi. This compound was identified in this work as one of the metabolites responsible for the antifungal activity of *T. vulgaris* and *O. basilicum*. In addition, limonene exhibits a low activity on family A of G protein-coupled receptor, nuclear receptor, and cytochrome P450 (Figure 6(c)).

The fourth metabolite identified in *T. vulgaris* and *O. basilicum* was borneol ([(2*R*)-1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl] formate). It is an organic compound belonging to the bicyclic monoterpenoid class. Its presence has been reported in more than 260 plants, mainly in those belonging to the *Lamiaceae* family, including *T. vulgaris* and *O. basilicum* species [60–62]. These reports agree with our present results (Table 1). Borneol is widely used in the pharmaceutical industry and as an antimicrobial agent [77–82]. The fungicide activity reported in other studies on borneol against *C. albicans* is effective at a MIC of 0.320 mg/mL [83], which places this compound as a potential candidate for the treatment of candidiasis. Like other terpenoid compounds, as linalool and limonene, borneol interrupts the integrity of the microbial plasmatic membrane [46, 47, 73, 74] and affects the yeast to mycelium morphological change in *C. albicans* [71, 84]. In addition, borneol is found in a large variety of plants, and the analysis *in silico* indicated a low affinity for proteins such as lyase, phosphatase, nuclear receptor, and family A of G protein-coupled receptor (Figure 6(d)). These observations and results described in this work allowed us to propose thus terpenoid as one of the compounds mainly responsible for the antifungal activity of *T. vulgaris* and *O. basilicum.* However, besides borneol, linalool, eugenol, and limonene were identified as additional candidate drugs with anti-*Candida* activity. These results together with our *in silico* studies suggest a low percentage of binding of these drugs to human targets, but future studies will be required to fully elucidate their mechanism of action.

5. Conclusions

Linalool, eugenol, limonene, and borneol found in *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* plants, traditionally of everyday use, are effective compounds against *Candida* species, thus allowing them to be considered as viable alternatives to traditional antifungal agents once their toxicity has been discarded in animal models.

Data Availability

The data used to support the findings of this study are included within this article.

Conflicts of Interest

The authors declare that there were no conflicts of interest with any organization or entity with a financial interest or financial conflict with the material discussed in this work.

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Supplementary Materials

Figure S1: representative chromatograms obtained from each extract that were analyzed with the AMDIS software, in which some of the secondary metabolites present in the studied plants could be identified. Table S1: diameters of the inhibition halos of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts against the five *Candida* species at an OD_{600nm} 0.5. Superscript letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$), $n = 3$. Lowercase letters indicate significant differences on inhibition halos between stem extracts for each *Candida* species. Uppercase letters indicate significant differences on inhibition halos between leave extracts for each *Candida* species. EtOH: ethanol; Hex: hexane; NGI: no growth inhibition; SD: standard deviation. Table S2: diameters of the inhibition halos of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts against the five *Candida* species at an OD_{600nm} 0.1. Superscript letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$), $n = 3$. Lowercase letters indicate significant differences on inhibition halos between stem extracts for each *Candida* species. Uppercase letters indicate significant differences on inhibition halos between leave extracts for each *Candida* species. EtOH: ethanol; Hex: hexane; NGI: no growth inhibition; SD: standard deviation. *(Supplementary Materials)*

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Research Article

Spray-Dried Microencapsulation of Oregano (*Lippia graveolens*) Polyphenols with Maltodextrin Enhances Their Stability during *In Vitro* Digestion

Manuel de Jesús Bernal-Millán,[1] **Erick Paul Gutiérrez-Grijalva**,[2] **Laura Contreras-Angulo**,[1] **M. Dolores Muy-Rangel**,[1] **L. Xochitl López-Martínez**,[1] **and J. Basilio Heredia**[1]

[1]*Nutraceuticals and Functional Foods Laboratory, Centro de Investigación en Alimentación y Desarrollo, A.C., Carretera a Eldorado Km. 5.5, Col. Campo El Diez, CP, 80110 Culiacán, Sinaloa, Mexico*
[2]*Cátedras CONACYT-Centro de Investigación en Alimentación y Desarrollo, A.C., Carretera a Eldorado Km. 5.5, Col. Campo El Diez, CP 80110, Mexico*

Correspondence should be addressed to J. Basilio Heredia; jbheredia@ciad.mx

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The effect of *in vitro* gastrointestinal digestion on the release of microencapsulated phenolic compounds was evaluated through an optimized spray drying process. A stock extract of oregano phenolic compounds was developed and microencapsulated in a spray dryer following a central composite rotatable design, controlling the variables, inlet temperature (111.7-168.2°C), and percentage of wall material (5.8-34.1%). Optimum drying conditions for spray drying were decided based on different yield percentages (Y %) and encapsulated phenolic compounds (EPC). The analyzed physical properties were morphology measured by electron microscopy and humidity; other properties evaluated were the content of total phenolic compounds, antioxidant capacity determined by DPPH and ABTS assays, and phenolic profile by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS). During the gastrointestinal simulation, a stability of 85% was determined in the intestinal stage. Microencapsulation technology by spray drying is an excellent selection to stabilize and protect bioactive compounds of oregano and promote its use as a functional ingredient.

1. Introduction

Oregano is the name given to a variety of plants, generally belonging to the Lamiaceae and Verbenaceae botanical families that share similar flavor and odor [1]. Oregano is a shrub plant distributed in semiarid climates in Mexico, Central America, and Europe [2]. Oregano leaves have been used since ancient times in cuisine and traditional medicine to stimulate menstruation and as antivenom, and diluted infusions of oregano are used to treat respiratory diseases and stomach infections [3, 4]. Currently, the number of studies to test its properties as a food preservative, pesticide, and antimicrobial has grown [5]. However, research is oriented to the study of its functional and nutraceutical properties, which are attributed to its phytochemical compounds [2, 6]; these include essential oils and phenolic compounds, which are secondary metabolites generated by plants as a defense against different biotic and abiotic factors [7].

Oregano provides numerous health benefits thanks to its abundant amount of antioxidants, namely, rosmarinic acid, carvacrol, thymol, limonene, quercetin, pinene, ocimene, caryophyllene, and other phenolic compounds (polyphenols, flavones, and flavonols) [8]. The functionality attributed to phenolic compounds is related to their hydroxyl groups. Phenolics may act as scavengers and stabilizers of free radicals and reactive oxygen species, preventing and delaying the onset of noncommunicable diseases like cancer [9–11]. However, generally, bioactive phenolic compounds

must be bioaccessible and bioavailable, so they can be absorbed and reach systemic circulation and distributed to organs and tissues [12]. Bioaccessibility is defined as the number of polyphenols present in the intestine due to its release from the food matrix, which is available to be absorbed through the intestinal barrier [12].

However, it has been recently reported that oregano phenolic compounds can be easily degraded and metabolized when exposed to a simulated gastrointestinal system. Gutiérrez-Grijalva et al. [13], who evaluated the *in vitro* bioaccessibility of phenolic extracts of oregano (7.74% bioaccessibility), indicate that phenolic compounds are released before reaching the intestinal phase, causing the degradation and transformation of these compounds and with it the loss of bioaccessibility. Microencapsulation has become important as an emerging technology for protecting phenolic compounds both from environmental factors and from conditions in the digestive phases. Therefore, selecting an appropriate encapsulation method and wall material is crucial to protect this type of compound. Martínez-Ramírez et al. [14], evaluated the bioaccessibility of xoconostle (*Opuntia joconostle*) microencapsulated phenolic compounds by spray drying, using 30% maltodextrin-gum Arabic mixture as wall material. They achieved an increase in bioaccessibility from 42 to 64%, concluding that the encapsulation process was favorable. This study was aimed at evaluating the effect of *in vitro* gastrointestinal digestion on the release of phenolic compounds from Mexican oregano (*Lippia graveolens*) microencapsulated by spray drying.

2. Materials and Methods

2.1. Plant Material. Oregano (*Lippia graveolens)* was obtained in the Temohaya indigenous area, Municipality of Mezquital, Durango (coordinates N: 23.299722; W: 104.509167). The oregano leaves were dried in an Excalibur Food Dehydrator Parallax Hyperware (Sacramento, CA) at 40°C for 24 h, and ground in an Ika Werke M20 grinder (Wilmington, NC, USA) until a fine powder was obtained with a sieve #40. The oregano powder was stored at −20°C until use.

2.2. Extraction of Polyphenols. Polyphenol-rich extracts were prepared as follows: 1 g of dried oregano and 10 mL of distilled water were stirred and homogenized in a stir plate (Thermo Scientific Cimarec) for two hours in the absence of light. Then, the slurry was collected and vacuum filtered with Whatman # 4 paper. Subsequently, the extract obtained was centrifuged at 6,000 rpm for 15 min; the supernatant was collected and stored at 4°C for later use. This technique was carried out repeatedly until approximately 5 l of extract stock was obtained.

2.3. Experimental Design and Statistical Analysis. A central composite design was used to optimize the microencapsulation using the drying chamber temperature (X1: 111.7–168.2°C) and the percentage of encapsulating material (X2: 5.8-34.1%) as variables. The coded and uncoded levels of the independent variables are shown in Table 1. The range of each variable was selected based on preliminary tests (data not shown). Table 2 shows the experimental design used and 13 experimental runs performed. Two response variables were evaluated: % Y (yield percentage) and EPC (encapsulated phenolic compounds). Statistical significance was assessed using an analysis of variance, and the results were considered significant when $p < 0.05$. The coefficient of determination (R^2) was also evaluated to determine the model's suitability. Graphical and numerical optimizations were used to find the optimal levels of the independent variables (% Y and EPC). Additional confirmation experiments were conducted to verify optimal conditions.

Table 1: Central composite design used to optimize the microencapsulation process of oregano (*Lippia graveolens*) phenolics.

	Factor levels				
Independent factors	-1.41421	-1	0	1	1.41421
X1: temperature (°C)	111.7	120.0	140.0	160.0	168.2
X2: wall material (%)	5.8	10.0	20.0	30.0	34.1

Table 2: Central composite design used to optimize the microencapsulation process of oregano (*Lippia graveolens*) extracts.

Run	Coded levels	Factor 1: temperature (°C)	Factor 2: wall material (%)
1	00	140	20
2	—	120	10
3	a0	111.7	20
4	0a	140	5.8
5	00	140	20
6	0A	140	34.1
7	00	140	20
8	-+	120	30
9	++	160	30
10	+-	160	10
11	00	140	20
12	00	140	20
13	A0	168.2	20

Factorial (+, -), axial (A, a), and central (0) runs.

2.4. Preparation of the Microcapsules. An aliquot of 150 mL of stock extract was mixed with maltodextrin 10 DE as wall material. The mixture was homogenized on a stir plate at 600 rpm until completely dissolved. Subsequently, the mixture was fed to a Spray Dryer Yamato ADL311S. Wall material (%) and inlet temperature were applied according to the design (Table 2). The constant conditions were atomization pressure at 0.1 MPa, feed flow at 5 mL/min, and airflow at 0.32 m^3/min. The recovered powders were weighed to obtain the yield of the process and stored in an amber glass bottle at room temperature for analysis.

2.5. Process Yield. The encapsulation yield of the process was calculated using a gravimetric technique as the relationship

between the numbers of solids fed into the dryer concerning the solids recovered at the exit of the dryer and was reported as a percentage [15].

2.6. Total Phenolic Content. Total phenolic compounds were extracted following the method of Cilek et al. [16], with some modifications. A 200 mg of encapsulated phenolic sample was dissolved in 2 mL of ethanol/acetic acid/water mixture (50 : 8 : 42) to disrupt spray-dried particles and allow phenolic compounds to release. This mixture was stirred using Vortex-Genie 2 for 1 min and filtered with a nylon microfilter (0.45 μm). The total phenolic content was determined using the Folin-Ciocalteu reagent, following the methodology described by Swain and Hillis [17], with some modifications. The reaction mixture was prepared by combining 10 μL of the sample, 230 μL of distilled water, 10 μL of Folin-Ciocalteu reagent, and 25 sodium carbonate solution (4 N). The reaction mixture was incubated for 2 h before reading the absorbance at 725 nm using a 96-well using a Synergy HT microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). The results were expressed as milligrams of gallic acid equivalents (mg GAE)/gram sample.

2.7. Polyphenol Content on the Surface of the Microparticles. The phenolic concentration on the surface of microparticles was determined following the report by Cilek et al. [16]. A sample of 200 mg of microcapsules was dispersed in 2 mL of ethanol/methanol mixture (50 : 50) for 1 min. The content of phenolic compounds on the surface of the microparticles was measured and quantified with the method described in the total phenolic content section.

2.8. Microencapsulation Yield of Polyphenols. These were calculated by subtracting the amount of total phenolic compounds and the content of surface phenolic compounds [18].

$$\text{Encapsulated PC} = \text{total phenolics} - \text{phenolic compounds on the surface.} \quad (1)$$

2.9. Antioxidant Capacity. Two different methods were used to determine the antioxidant capacity of microencapsulates. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was performed as described by Thaipong et al. [19], and the assay of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) according to Karadag et al. [20]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard, and the results are expressed in micromole equivalent Trolox (TE)/gram sample.

2.10. Identification and Quantification of Polyphenols by UPLC-qTOF-MS/MS. The identification and quantification of oregano polyphenols entrapped in the microparticles were performed following the report of Gutiérrez-Grijalva et al. [21] using a UPLC class H equipment (Waters Corporation, USA) coupled to a G2-XS QT of the mass analyzer (Quadrupole and Time of Flight) using a UPLC BEH C18 column (1.7 μm × 2.1 mm × 100 mm) at 40°C. Phenolic compounds were separated with a gradient elution solution A (water-formic acid 0.1%) and solution B (acetonitrile) at a 0.3 mL/min flow rate. The gradient elution procedure was as follows: 0 min, 95% (A); 5 min, 70% (A); 9 min, 30% (A); 14 min, 0% (A); 14.5 min, 0% (A); 15 min, 95% (A); and 16 min, 95% (A). The ionization of the compounds was carried out by electrospray (ESI), and the parameters used consisted of a capillary voltage of 1.5 kV, sampling cone: 30 V, desolvation gas of 800 (L/h), and a temperature of 500°C. A 0-30 V collision ramp was used. The identification of compounds was done using the North American Mass-Bank Database (MoNA). The content of phenolic compounds was expressed in milligrams per 100 g sample.

The quantification of phenolic compounds by UPLC was performed as a function of the peak area of the maximum absorption wavelength. The standards used were caffeic acid, luteolin, naringenin, phloretin, and quercetin.

2.11. Morphology and Moisture. Microparticle morphology was analyzed by an environmental scanning electron microscope (model EVO-50, Carl, Zeiss, Germany). The sample without any previous treatment was placed on a sample holder with the help of an adhesive double-sided carbon tape. The observation was made under high vacuum conditions, with a secondary electron detector (SE1) and an acceleration voltage of 10-15 kV (×2000 and ×4000 magnification). The amount of water in the microcapsules was determined using a gravimetric analysis using the AOAC method 925.09 (1997) [22].

2.12. In Vitro Gastrointestinal Digestion. An *in vitro* digestion model was conducted using the method reported by Flores et al. [23], with some modifications. Briefly, *in vitro* digestion consisted of a 3-step process that simulates digestion in the mouth, stomach, and small intestine, mimicking the pH, chemical composition of the digestive fluids, temperature (37°C), and transit times. The composition of the artificial digestive juices is listed in Table 3.

Powder samples (1 g) were placed in 50 mL corning tubes and incubated for 5 min at 37°C in a Model 290400S incubator (Boekel Scientific, Feasterville, PA) at 55 rpm. The samples were digested as follows: the oral phase began by adding 1.71 mL of salivary and an incubation time of 5 min; after that, the stomach phase began when 3.42 mL of gastric juice was added and gently mixed for 2 h. In the end, the intestinal phase consisted of adding 3.42 mL of intestinal juice to the mix and incubating for 2 h. At the end of the *in vitro* digestion process, the samples were centrifuged at 6000 rpm for 15 min, and the resulting supernatant called digesta was stored at -20°C before further analysis.

Microencapsulated samples without oregano extracts were used as blank. At the end of each digestive phase, the whole digested sample in the tube was centrifuged under the conditions mentioned above, and the supernatants were kept frozen until further use (–20°C).

Table 3: Composition of simulated digestive juices.

Salivary juice	Gastric juice	Intestinal juice
500 mL distilled water	500 mL distilled water	500 mL distilled water
58.5 mg NaCl	2.752 g NaCl	7.012 g NaCl
74.5 mg KCl	0.824 g KCl	0.564 g KCl
1.05 g $NaHCO_3$	0.266 g NaH_2PO_4	3.388 g $NaHCO_3$
	0.399 g $CaCl_2.2H_2O$	80.0 mg KH_2PO_4
0.2 g urea	0.306 g NH_4Cl	50.0 mg $MgCl_2$
	0.085 g urea	0.1 g urea
Adjuncts		
0.5 g mucin	2.5 g pepsin	9.0 g pancreatin
1.0 g α-amylase	3.0 g mucin	1.5 g lipase
pH		
6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2

3. Results and Discussion

Table 4 shows the % Y and EPC of the 13 experiments performed to optimize the microencapsulation of oregano extract. The % Y values ranged from 34.73 to 71.85%, and the highest values were found under the experimental conditions X1 = 140°C and X2 = 20%.

The ANOVA analysis showed that a quadratic model is significant for the response variables yield (%) and encapsulated phenolic compounds as a function of the inlet temperature and percentage of wall material (Tables 5 and 6). Also, both the linear term and the quadratic term of the wall material were significant for the yield response variable (Figure 1). This model showed a determination coefficient $R^2 = 79.36\%$, which might suggest there is high variability in yields. Regarding the encapsulated phenolic compounds (response variable), the linear and quadratic terms of the two factors, temperature and wall material, were significant, showing a determination coefficient $R^2 = 87.29\%$ for this model. Furthermore, it has been reported that yields above 50% are considered optimal, which agrees with our results.

3.1. Antioxidant Characterization of the Optimal Microencapsulate. The phenolic content by the Folin-Ciocalteu assay of the microencapsulates (Table 7) was 14.05 mg GAE/g of sample (this result was calculated using the difference between total phenolic and superficial phenolics). In contrast to the microencapsulates, nonencapsulated oregano extracts had a total phenolic content of 33.66 mg GAE/g of the sample. This difference might occur as a result of the spray-drying process, in which atomization of the feed material results in very fine mist-like droplets with an increased surface area, which involves higher exposure of the extracts to the heat that might degrade phenolics.

Moreover, due to the atomization, some parts of the wall material can be removed from the core material even after homogenization [24]; these partially covered microcapsules are easily affected by heat. Another factor that could be related to this result is that when wall material is added to an extract, the spatial distribution of phenolics is dispersed, and its quantification per unit of mass decreases [25]. Our results were similar to those reported by Ruiz-Canizales et al. [26], for maltodextrin microencapsulated extracts of blue corn phenolics (13.9 mg GAE/g sample), but higher than those found by Tolun et al. [27], where they microencapsulated grape phenolic compounds (5.4-8.5 mg GAE/g sample).

Regarding the antioxidant capacity of the microencapsulate, we obtained values of 50.83 μmol ET/g sample and 85.17 μmol ET/g sample for the inhibition of the DPPH and ABTS radicals, respectively. In contrast, Cilek et al. [16] showed a lower antioxidant capacity for extracts of cherry pomace microencapsulated with a mixture of maltodextrin/gum Arabic using the DPPH method (17.98 μmol ET/g). Also, Saénz et al. [18] indicated an antioxidant capacity of microencapsulated cactus peel extracts (*Opuntia ficus-indica*) with maltodextrin 10 DE using the ABTS method (19.2 μmol ET/g). The differences between each report of microencapsulated phenolic-rich extracts might be attributed to the concentration of microencapsulated phenolics and the type of compound that is encapsulated.

Moreover, our results showed that our microencapsulated oregano extracts have the potential to be used as antioxidant ingredients in formulations of functional beverages and foodstuff.

3.2. Identification and Quantification of Polyphenols by UPLC-qTOF-MS/MS. Microencapsulated phenolic compounds were identified by comparing the fragments obtained in each sample spectrum with the spectra provided by the MassBank of North America (MoNA) database. Twelve compounds were identified, mostly flavonoids and one phenolic acid. The flavonoids luteolin-7-glucoside, scutellarin, apigenin-7-glucoside, luteolin, and apigenin belong to the subgroup of flavones; 4 of the flavonoids are flavanones: taxifolin, eriodictyol, naringenin, and pinocembrin; one flavonol identified as quercetin, and a dihydrochalcone identified as phloretin (Table 8). It has been previously reported that most of the phenolic compounds obtained by methanolic extracts of *Lippia graveolens* belong to the flavone subgroup of flavonoids [4, 13, 21]. Regarding the microencapsulated phenolic acids, only caffeic acid was identified, and it should be noted that in an extract without microencapsulation, two other phenolic acids were identified, namely, gallic and vanillic acids indicating that during the drying process, these two phenolic acids were degraded.

Quantification was performed based on available commercial standards (Table 8). The phenolic compounds that predominate in higher concentrations belong to the group of flavanones. This profile was similar to previous studies [4, 6, 13] even though the extraction method was 100% water. It is worth mentioning that in this study, one of the flavonoids found in the highest concentration was naringenin, which has already been reported as predominant in methanolic extracts of the *L. graveolens* species [28].

3.3. Particle Morphology. One of the essential characteristics to consider in a microencapsulate is size and shape. Electron microscopy micrographs showed that oregano microcapsules

Table 4: Experimental values of the optimization.

Experimental run	Coded variables		Experimental values	
	X1	X2	% *Y*	EPC (mg GAE/430 mg IGAS)
1	-1	-1	58.93	251.15
4	1	-1	53.85	212.59
5	-1	1	40.22	150.59
7	1	1	48.15	207.10
2	-1.41421	0	34.76	138.00
12	1.41421	0	69.39	260.63
9	0	-1.41421	66.92	259.57
6	0	1.41421	55.30	220.29
11	0	0	69.05	277.39
10	0	0	66.42	284.38
8	0	0	69.27	276.55
3	0	0	71.85	282.36
13	0	0	68.61	281.40

% *Y*: yield percentage; EPC: encapsulated phenolic compounds; mg GAE: milligrams of gallic acid equivalents; IGAS: milligrams of initial gallic acid solution.

Table 5: Analysis of variance of the effect of temperature (°C) and wall material (%), adjusted to a quadratic model on the yield (powder recovery rate) of the encapsulation process.

Source	DF	Sum of squares	Mean squares	*F*	*p*
Model	5	1416.50	283.30	5.38	0.0239
A-inlet temperature	1	335.53	335.53	6.38	0.0395
B-% WM	1	208.49	208.49	3.96	0.0868
AB	1	42.33	42.33	0.80	0.3996
A^2	1	703.63	703.63	13.37	0.0081
B^2	1	213.51	213.51	4.06	0.0838
Residual	7	368.40	52.63		
Lack of fit	3	353.44	117.81	31.50	0.0031
Pure error	4	14.96	3.74		
Total					
$R^2 = 79.35\%$					

Table 6: Analysis of variance of the effect of temperature (°C) and wall material (%), adjusted to a quadratic model, on the content of encapsulated phenolic compounds recovered after the encapsulation process.

Source	DF	Sum of squares	Mean squares	*F*	*p* value
Model	5	25942.68	5188.54	9.62	0.0049
A-inlet temperature	1	4597.07	4597.07	8.52	0.0224
B-% WM	1	3279.71	3279.71	6.08	0.0431
AB	1	2240.55	2240.55	4.15	0.0809
A^2	1	13524.35	13524.35	25.07	0.0016
B^2	1	3934.76	3934.76	7.29	0.0306
Residual	7	3776.05	539.44		
Lack of fit	3	3731.52	1243.84	111.74	0.0003
Pure error	4	44.53	11.13		
Total	12	29718.72			
$R^2 = 87.25\%$					

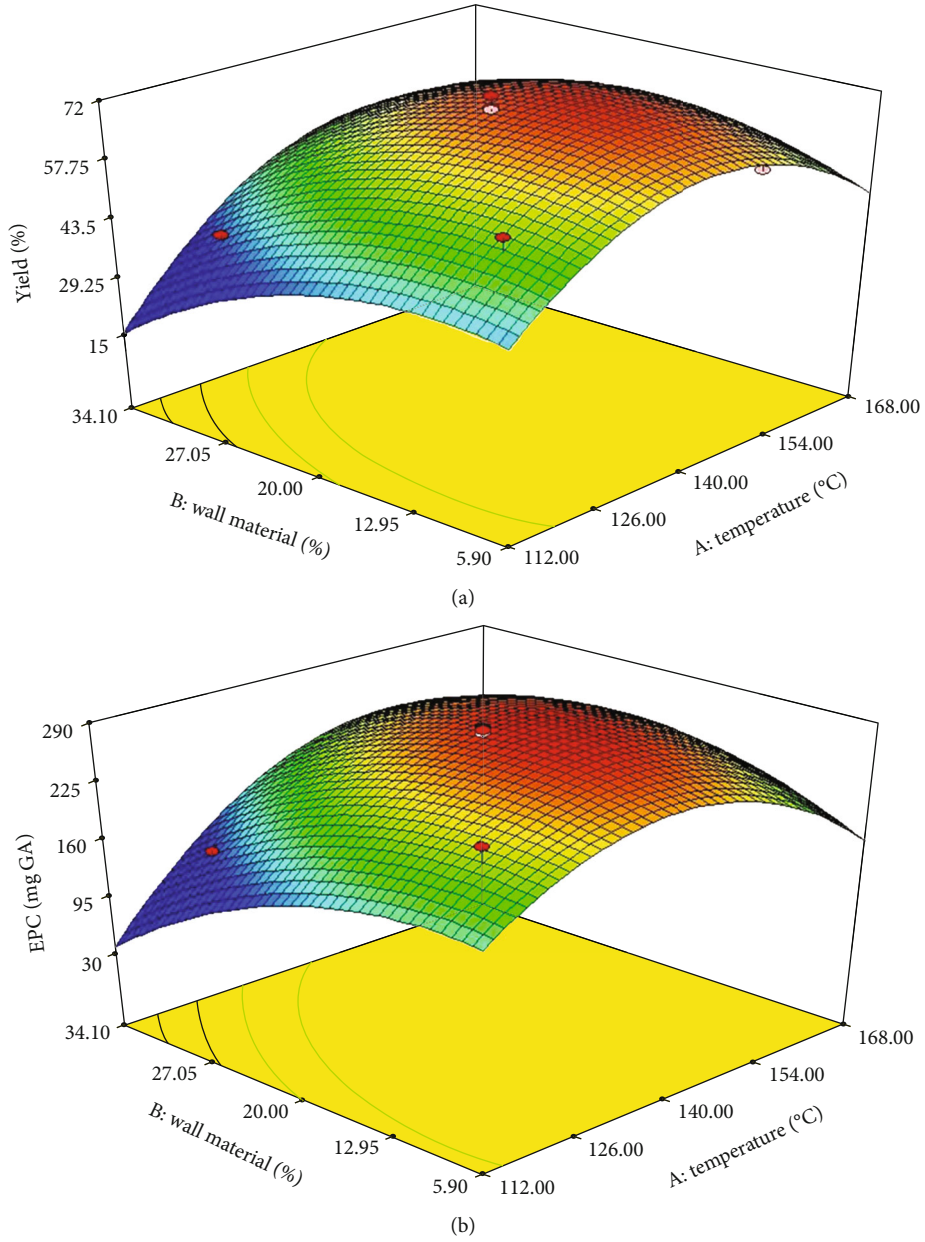

Figure 1: Response surface for (a) yield (%) and (b) encapsulated phenolic compound in microencapsulated oregano (*Lippia graveolens*) extracts.

Table 7: Content of encapsulated phenolic compounds and antioxidant capacity of microencapsulated oregano (*Lippia graveolens*) extracts.

Sample	TPC (mg GAE/g sample)	SPC (mg GAE/g sample)	EPC (mg GAE/g sample)	AC by DPPH (μmol TE/g sample)	AC by ABTS (μmol TE/g sample)
Optimized microcapsules	14.45 ± 0.34	0.40 ± 0.02	14.05 ± 0.31	50.83 ± 0.25	85.17 ± 2.07

TPC: total phenolic compounds; SPC: surface phenolic compounds; EPC: encapsulated phenolic compounds; AC: antioxidant capacity. Data shown as means ± standard deviation of three replicates ($n = 3$).

have a particle size between 2 and 12 μm and a spherical shape with depressions (Figure 2). The spherical form of the atomized powders is based on the principle of generation of droplets by spraying and conversion of these droplets into particles by evaporation of the solvent [29]. A similar study by Rezende et al. [30], show that most samples presented spherical conformation, without agglomeration, irregular shapes, and few fissures. However, some showed a smooth surface characteristic of microparticles produced by a spray-drying process. They mention that roughness in the microparticles is usually attributed to particle shrinkage due to the drastic loss of moisture followed by cooling [30].

Table 8: Identification and quantification of phenolic compounds in microencapsulated powder of oregano (*Lippia graveolens*) extracts.

TR (min)	MS^2	$[M-H]^-$	MS^2 (m/z)	Identification	Compound type	Quantification
4.15	180.04	179.03	134.03, 135.04, 179.03	Caffeic acid	Phenolic acid	147.55 ± 6.92^a
5.41	448.10	447.09	284.03, 285.03, 447.09	Luteolin-7-glucoside	Flavone	130.11 ± 14.40^e
5.49	462.08	461.07	164.98, 285.04, 461.07	Scutellarin	Flavone	3.51 ± 0.15^e
5.65	304.05	303.05	125.02, 285.03, 303.04	Taxifolin	Flavanone	249.06 ± 31.01^e
6.08	432.10	431.09	268.03, 269.04, 431.09	Apigenin-7-glucoside	Flavone	20.09 ± 2.09^e
7.30	288.06	287.05	135.04, 151.00, 287.05	Eriodictyol	Flavanone	112.44 ± 9.54^e
7.36	286.04	285.03	133.02, 151.00, 285.03	Luteolin	Flavone	96.93 ± 4.61^b
7.38	302.04	301.03	151.00, 178.99, 301.03	Quercetin	Flavonol	18.87 ± 1.99^c
7.95	270.05	269.04	117.03, 151.00, 269.04	Apigenin	Flavone	28.75 ± 2.51^e
7.97	272.06	271.06	119.04, 151.00, 271.06	Naringenin	Flavanone	204.65 ± 17.39^e
8.01	274.08	273.07	123.04, 167.03, 273.07	Phloretin	Dihydrochalcone	21.35 ± 1.43^d
9.45	256.07	255.06	151.00, 213.05, 255.06	Pinocembrin	Flavanone	28.20 ± 1.70^e

[a]μg caffeic acid/g microencapsulated, [b]μg luteolin /g microencapsulated, [c]μg quercetin /g microencapsulated, [d]:μg floretin /g microencapsulated, [e]μg quercetin equivalent/g microencapsulated. Results are shown as mean ± standard deviation of three replicates ($n = 3$).

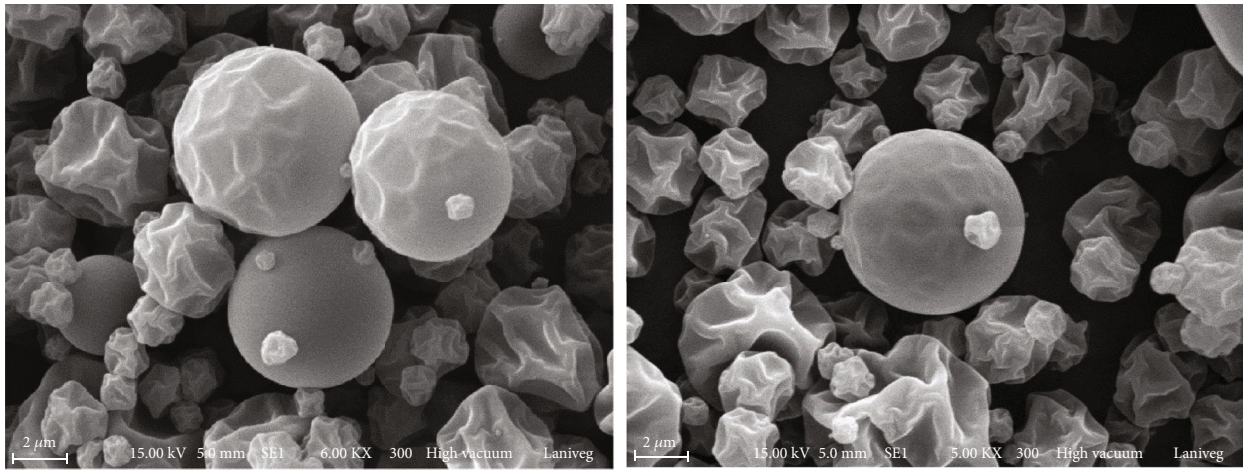

Figure 2: Micrographs of the microparticle structure of phenolic compounds of oregano (L*ippia graveolens*) produced with maltodextrin 10 DE as wall material, using spray drying.

Regarding particle size, González et al. [31] found a unimodal particle size distribution in OLE-SA microparticles, with sizes ranging from 0.25 μm to 20 μm. Regarding this, minimal variability in size is desirable not to affect the sensory properties of the final product where it is applied. Furthermore, the pressure of the atomization air and the hydrolysis degree of maltodextrin may also influence the microcapsules characteristics. In this sense, it has been reported that higher dextrose equivalents in maltodextrin might yield a smoother surface in the microcapsules. In this study, we used low hydrolysis degree maltodextrin for its characteristics, such as longer shelf life and high glass transition temperature [32]. Furthermore, Ruiz-Canizales et al. [26], obtained maltodextrin-coated microparticles from blue corn phenolics with a spherical particle size of 1 to 10 μm, with marked depressions. Also, Çam et al. [33] analyzed the structure of microencapsulates of maltodextrin and gum Arabic for phenolics from pomegranate peel, reporting an average particle size of 10 μm and spherical shape. The particles obtained showed low moisture (3.55%) and higher yield (77.42%), while low humidity prevents phenolic compound oxidation, and yields of >50% are considered optimal for microencapsulation with maltodextrin [25, 34].

3.4. Stability of Oregano Microencapsulates. To evaluate the release of the microencapsulated oregano phenolic compounds during gastrointestinal digestion, we evaluated the total phenolic content at the end of each digestive phase. The results (Figure 3) showed a release of phenolics in the salivary stage of 83.34%. In comparison, in the gastric phase, there was an increase presenting 91.38%, and finally, in the intestinal stage, there was a slight decrease, finding a release of 85.05%. There were no significant differences between the three phases among the release of encapsulated phenolic compounds and the control. This may indicate that there was no significant degradation of phenolic compounds,

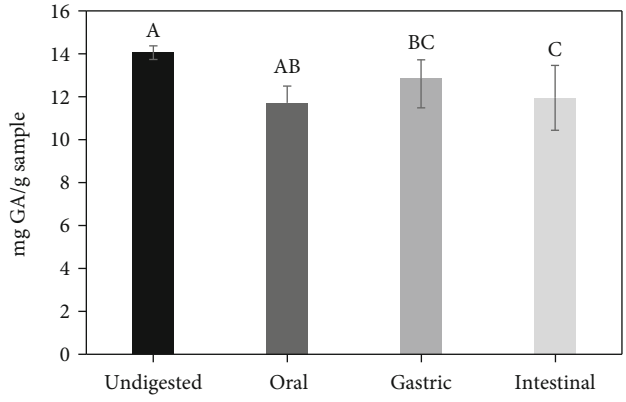

Figure 3: Total phenolic content of microencapsulated extracts during the in vitro digestion process.

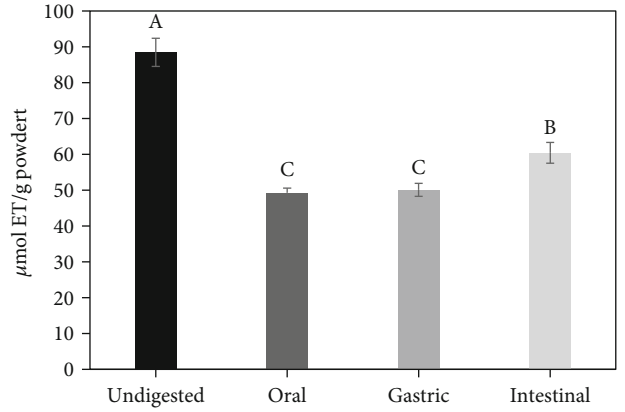

Figure 4: Antioxidant capacity of microencapsulated extracts during the in vitro digestion process.

and then, it could be assumed that the use of the microencapsulation process was an effective protector. In a previous study Gutiérrez-Grijalva et al. [13], the bioaccessibility of phenolics in nonencapsulated oregano extract was evaluated, finding a bioaccessibility in the intestinal stage of 7.46%. The authors mentioned that pH changes during *in vitro* digestion are among the main factors affecting the stability of phenolic compounds, causing their degradation or metabolism [35, 36].

Similar results were obtained by Ruiz-Canizales et al. [26], who evaluated the release of microencapsulated blue corn phenolics with maltodextrin 30 DE and showed a release of phenolics from the matrix of 98% and 88% during the gastric and intestinal phases, respectively. The authors stated that acid solubilization and hydrolysis of the polymer matrix (maltodextrin) could occur in gastric conditions. In intestinal conditions, enzymatic hydrolysis can occur, as well as interferences can happen in the Folin-Ciocalteu test due to the presence of reducing sugars. One of them is glucose, a powerful reducing sugar that composes maltodextrin molecules. Additionally, the study by Ruiz-Canizales et al. [26] obtained a percentage of antioxidant capacity in the intestinal stage of 60% compared to their control, similar to what we found in our study (Figure 4). The antioxidant capacity results of our microencapsulated extracts were found at 88.46, 49.34, 50.08, and 60.43 μmol ET/g powder for the undigested, oral, gastric, and intestinal phases, respectively.

Martínez Cifuentes [37] submitted to an *in vitro* digestion process free and microencapsulated extracts of taxo (*Passiflora mollisima*), blackberry (*Rubus glaucus* Benth), and mortiño (*Vaccinium floribundum* Kunth). Microencapsulation was performed using maltodextrin and maltodextrin-gum Arabic. The author found that the phenolic content of free lyophilized extracts decreased 30, 57, and 50% during the gastric phase for taxo, blackberry, and mortiño, respectively. Furthermore, microencapsulated extracts of these fruits showed a bioaccessibility from 72 to 83%, suggesting that microencapsulation protected phenolics from degradation and metabolism due to pH changes, as previously mentioned. It was also shown that microencapsulated protected phenolics even at the end of the intestinal phase, where an increase in antioxidant capacity was observed in taxo and blackberry of 133% and 166%, respectively. This was similar to the study by Ydjedd et al. [38], who evaluated the effect of gastrointestinal digestion *in vitro* on encapsulated and nonencapsulated phenolic compounds of ripe carob pulp, showing an antioxidant capacity of the encapsulates up to 10 times greater in the intestinal phase compared to the nonencapsulated ones.

This might be attributed to the fact that the antioxidant capacity depends on the type and concentration of phenolic compounds present in the microencapsulated extracts since there are a variety of chemical structures that, by interacting with the wall material, may or may not withstand the gastric conditions.

4. Conclusion

We found that the optimal conditions to microencapsulate oregano phenolics by spray drying are an inlet temperature of 145°C and a percentage of wall material of 16%. Moreover, the aim of this work was to optimize the microencapsulation conditions of oregano phenolics with low DE maltodextrin, to enhance the bioaccessibility of oregano phenolics. This was achieved as we reported that the stability was around 85%, which suggests that maltodextrin microencapsulation is a suitable and optimal alternative for protecting these phytochemicals. However, further studies should be performed for this technology to be used in the development of functional foods or dietary supplements.

Data Availability

All data are included within the article.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Research Article

Antioxidant, Antimicrobial, and Protein Kinase Inhibition Profiling of C. ambrosioides Seed Extracts along with RP-HPLC

Saira Bano,[1] Muhammad Waleed Baig,[1] Mohammad K. Okla,[2] Syeda Saniya Zahra,[1] Nosheen Akhtar,[3] Wahidah H. Al-Qahtani,[4] Hamada AbdElgawad,[5] and Ihsan-Ul Haq[1]

[1]*Department of Pharmacy, Quaid-i-Azam University, Islamabad 45320, Pakistan*
[2]*Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*
[3]*Department of Biological Sciences, National University of Medical Sciences, Rawalpindi 46000, Pakistan*
[4]*Department of Food Sciences & Nutrition, College of Food & Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia*
[5]*Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Ant-werp 2020, Groenenborgerlaan 171, Antwerp, Belgium*

Correspondence should be addressed to Ihsan-Ul Haq; ihsn99@yahoo.com

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The validation of underexplored traditional plant remedies represents a reservoir of novel leads for drug discovery. In line with this, in vitro total phenolics and flavonoids content, multimode antioxidants, antimicrobial, cytotoxicity, and protein kinase inhibition assays were conducted on C. ambrosioides seed extracts in addition to RP-HPLC. Methanol extract exhibited highest total phenolics ($64.6 \pm 0.6\,\mu$g gallic acid equivalent/mg) and flavonoids ($50.9 \pm 0.5\,\mu$g quercetin equivalent/mg) content. RP-HPLC quantified rutin ($1.98\,\mu$g/mg) in methanol extract whereas quercetin ($0.322\,\mu$g/mg) and kaempferol ($2.86\,\mu$g/mg) in methanol-distilled water extract. Methanol extract exhibited highest ascorbic acid equivalent (AAE) free radical (DPPH) scavenging (IC_{50} of $110.7 \pm 5\,\mu$g/ml), total antioxidant capacity ($110.6 \pm 2.2\,\mu$g AAE/mg), and total reducing power ($94.30 \pm 0.46\,\mu$g AAE/mg). Highest antibacterial activity against K. pneumonia (14 ± 1.61 mm ZOI) and antifungal activity against F. solani (17 ± 1.38 mm ZOI) were shown by n-hexane and chloroform extracts, respectively. Ethyl acetate extract exhibited highest brine shrimps cytotoxicity (LC_{50} of $125\,\mu$g/ml). A noteworthy protein kinase inhibitory potential was shown by ethanol extract with a 20 ± 1.27 mm bald zone. Therapeutic potential of medicinal plants can be completely explored by using multiple solvent system. This study makes C. ambrosioides, a resourceful prospect for the bioactivity-guided isolation of lead compounds.

1. Introduction

Plants have formed the basis for a sophisticated traditional system of medicines that paved the way to provide potential remedies for various ailments [1]. Wide spectrum biological properties possessed by plant secondary metabolites have made them an important source of bioactive leads. The chemical diversity and versatility of plant-based remedies emphasize the need of critical screening via modern approaches based on ethnopharmacological fingerprints. Advancements in the science of drug discovery have led to effective scrutiny of plants having potential medicinal benefits. It reinvigorated pharmaceutical firms and practitioners towards plant-oriented research and instilled ardor into quest of new drug moieties [2].

Evaluation of numerous plant species and herbs showed that if antioxidant potential is present in plants then they can be used for the treatment of cardiovascular diseases, hyperglycemia, Alzheimer, and even cancer. Teas, vegetables, and fruits prevent serious health complaints like cardiovascular diseases and cancer owing to the presence of antioxidants and numerous other chemical entities. Vitamins, carotenoids, anthocyanins, flavonoids, and various other polyphenolic components are the keystones of antioxidant, antimicrobial, and anticancer potential of plant products [3].

Genus Chenopodium belongs to the family chenopodiaceae. It has 102 genera and 1700 species [4]. Chenopodium ambrosioides (wormseed) is an annual or perennial erect branched aromatic herb indigenous to subtropical and temperate regions including Central America, South America, and Brazil [5]. It is naturally growing in Margalla hills of Pakistan [6] and is commonly known as Gandi Buti [7]. For centuries, different preparations of C. ambrosioides have been utilized by native people due to its dietary and medicinal importance. It is rich in terpenoids and flavonoids components that exhibit profound pharmacological activities such as anthelmintic, antioxidant, antitumoral, antileshmanial, antiinflammatory, wound healing, and cancer chemoprevention [8]. In Brazil, this folk medicine is used to treat respiratory problems, tuberculosis, and rheumatism [5] and has also proved effective in the treatment of uterine hemorrhage [9]. The essential oil (chenopodium oil) obtained through hydrodistillation of the plant is a mixture of ascaridole, isoascaridole, p-cymene, limonene, and alpha terpinene with known medicinal values [10–12]. Despite already documented phenomenol biological attributes of C. ambrosioides, its restorative potential is further explored in the present study. Fourteen solvent systems having a wide polarity distribution were used on the seeds to make extracts. To the best of our knowledge, this is the first report on RP HPLC, antibacterial, antifungal, cytotoxic, and protein kinase inhibitory potential of seed extracts.

2. Methodology

2.1. Protocols Adopted Are Well Developed and Cited Properly

2.1.1. Collection and Identification. Plant material was collected from the vicinity of the Quaid-i-Azam University Islamabad and identified as C. ambroisides by Professor Dr. Rizwana Aleem Qureshi. A voucher specimen (PHM-494) was deposited at the herbarium of medicinal plants, Quaid-i-Azam University Islamabad, Pakistan.

2.1.2. Preparation of Crude Extracts. Deteriorated seeds were removed, rinsed with tap water, shade dried for four weeks, and then pulverized to coarse powder. Extraction was carried out in 14 different solvents of varying polarity either alone or in 1 : 1 proportion which includes n-hexane (NH), chloroform (CHL), ethyl acetate (EA), acetone (Act), methanol (Me), ethanol (Eth), distilled water (Dw), ethyl acetate-n-hexane(EA-NH), ethanol-ethyl acetate (Eth-EA), chloroform-methanol (CHL-Me), ethyl acetate-methanol (EA-Me), acetone-methanol (Act-Me), acetone-distilled water (Act-Dw), and methanol-distilled water (Me-Dw). The weighted amount of plant powder (60 g) was macerated in different solvent systems separately, followed by intermittent shaking and ultrasonication. On the third day, it was filtered by muslin cloth and fine filtered using Whatman no. 1 filter paper. The filtrates were evaporated to dryness in a rotary evaporator at 45°C under reduced pressure. The process was repeated twice by using the same individual marcs. The dried crude extracts thus obtained were combined and stored in preweighed vials at -80°C till further analysis.

Extract recovery was calculated as

%Extract recovery = $(A/B) \times 100$.

A = weight of crude extract.

B = weight of the respective powdered plant material.

2.2. Phytochemical Analysis

2.2.1. Total Phenolics Content Determination (TPC). The phenolic content in the test sample was determined by the use of a 10 percent phenol-Ciocalteu (FC) agent, as described [13]. Gallic acid and DMSO have been used as positive and negative standards. The extract solution (20 μl; 4 mg/ml DMSO) and FC solution (9 : l) were mixed in 96-well plates. After 5 minutes, add 90 μl sodium carbonate (6% w/v), incubate for 30 minutes at 37°C (Germany), and record the absorption at 630 nm with a microplate reader. The calibration curve is calculated using gallic acid (2.5, 5, 10, and 20 μg/ml). The process was repeated twice. The resulting phenolic content is expressed as equivalent to μg gallic acid per mg extract (μg GAE/mg).

2.2.2. Total Flavonoids Content Determination (TFC). In 96 well plates, mix 20 μl sample solution (4 mg/ml DMSO), 10 μl of 1 M potassium acetate (98.15 g/L), 10 μl of aluminium chloride (10% w/v), and 160 μl of distilled water was incubated at room temperature for 30 min. Finally, the microplate reader is set, and the absorption is measured at 415 nm. The calibration curve is drawn with quercetin at a final concentration of 2.5, 5, 10, 20, and 40 μg/ml. And the resulting flavonoids were expressed after triple analysis in the form of an equivalent of μg of quercetin per mg extract (μg QE/mg) [14].

2.3. RP-HPLC. RP-HPLC with analytical columns has been used to quantify polyphenols using the methodology described [13, 15]. Reference compounds, i.e., catechins, quercetin, gallic acid, caffeic acid, myricetin, rutin, apigenin, and kaempferol; the final concentration was prepared with samples diluted by methanol to 50 μg/ml. Two mobile phases were used, the mobile phase A contains 5 : 10 : 85 : 1 acetonitrile-methanol-water- acetic acid, while the mobile phase B contains 40 : 60 : 1 acetonitrile-methanol-acetic acid. The flow rate was kept at 1 ml/min. Each sample contains 20 μl aliquots (10 mg/ml methanol) in the column, which is allowed to be reconditioned for 10 minutes before the next analysis. Mobile phase A is isocratic, and B gradient volume is 0–50% in 0-20 minutes, 50–100% in 20-25 minutes, and 100% in 25-30 minutes. The samples were shown absorbance at different wavelengths, e.g., rutin was determined at 257 nm, gallic acid and catechin at 279 nm, apigenin and caffeic acid at 325 nm, and kaempferol, quercetin, and myricetin at 368 nm.

3. Biological Evaluation

3.1. Free Radical Scavenging (DPPH) Assay. The sample solution (20 μl, 4 mg/ml DMSO) and 180 μl of the DPPH agent (9.2 mg/100 ml ethanol) were added to the 96 wells,

and then the incubation at 37°C for 1 hour in the dark cabin. The absorption was measured using a microplate reader at 517 nm. Ascorbic acid is the reference standard. The experiments were repeated three times. The percentage of sample radical bleaching potential is calculated by this formula:

$\%\text{scavenging activity} = (1 - \text{Ab}_s/\text{Ab}_c) * 100.$

Ab_s = absorbance of DPPH solution with sample.

Ab_c = absorbance of negative control (containing the reagent solution without sample).

A sample with a scavenging of >50% at 400 μg/ml was tested in lower concentrations using three-fold serial dilution methodology to find the IC_{50} value. The corresponding IC_{50} values are calculated using the table curve software [16, 17].

3.2. Total Antioxidant Capacity Determination. A 100 μl sample solution (DMSO 4 mg/ml) was mixed with a 1 ml mixture (0.6 m sulfuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate), incubated at 90° C for 95 min, and cooled at room temperature, and absorbance was taken at 645 nm using a spectrophotometer. Ascorbic acid is used as a positive control agent, and DMSO is used as a negative control agent. The antioxidant potential of each solvent extract is calculated after three-step analysis, and the result is expressed as microgram (μg AAE/mg) equivalent to the mg equivalent [13].

3.3. Total Reducing Power. A mixture containing 200 μl of each sample (4 mg/ml DMSO), 500 μl of 0.2 M phosphate buffer (pH 6.6), and 1% potassium ferricyanide [$K_3Fe(CN)_6$] was incubated for 20 min at 50°C. Afterward, 500 μl of 10% TCA was added, centrifuged at 3000 rpm for 10 min, supernatant fluid (500 μl) was transferred to Eppendorf tube, mixed with ferric chloride (500 μl), and distilled water (100 μl). Absorbance was recorded at 700 nm. DMSO and ascorbic acid (4 mg/ml) were used as negative and positive controls. The results were represented as μg ascorbic acid equivalent per mg of extract (μg AAE/mg) after triplicate analysis [16].

3.4. Antimicrobial Assays. The first activity of the test sample against bacterial and fungal strains has been investigated using the diffusion method of agar discs [14]. About 100 μl of each test strain is distributed on a presteriled agar plates. The filter paper disc (6 mm diameter) was infused with a test extract of 5 μl (100 μg) each. Afterward, discs were placed on previously seeded agar plates. The discs impregnated with (4 mg/ml of DMSO) and clotrimazole (4 mg/ml of DMSO) served as positive controls, while the DMSO-injected discs served as negative controls. After 24 hours at 37°C (antimicrobial) and 24 hours at 25°C (antimicrobial) incubation, the diameter and control of the growth inhibition zone around the sample were measured to the closest mm via vernier caliper and recorded after triple analysis of measuring.

3.5. Brine Shrimp Toxicity Assay. Hatched nauplii (Artemia salina) in sea water (34 g/L sea salt + 6 mg/L yeast) were collected. Different subdilutions of the test sample were tested to determine the lethal concentration at 1000, 500, 250, and 125 μg/ml. A precise count of 20 nauplii were transferred to each well-containing seawater. The corresponding microliters of each concentration were added to each well, and the final volume of each well was made up to 300 μl with seawater. DMSO concentration did not exceed 1%. Doxorubicin and DMSO are positive and negative controls. After 24 hours of incubation at 30°C, the plates were examined with a reverse microscope, and the dead nauplii that were settled at the bottom were counted in each well. LC_{50} was calculated accordingly for the extracts with ≥50% mortality at highest concentration using table curve software 2D version 4.

3.6. Protein Kinase Inhibition Assay. Protein kinase inhibition potential of sample extracts was evaluated according to the procedure reported previously [16, 18]. The 24-hour regenerated Streptomyces culture (100 μl) in Trypton soy broth spread to small-scale ISP4 plates on the lawn. A 6 mm diameter sterile filter paper disc was loaded with a test sample of 5 μl (100 μg) and placed on a newly planted plate. The surfactant-wetted discs function as positive controls, while the DMSO-infused discs function as negative controls. These plates were incubated for 72 hours and allowed hyphae to develop. After incubation, the growth inhibition zone is measured and recorded around each disc with a vernier caliper. The development of a bald area around the disc suggests that phosphorylation inhibition is possible by the samples. The bald area shows that hyphae formation is inhibited, while the clear area shows that Streptomyces killing thus exhibiting the cytotoxicity of the sample.

3.7. Statistical Analysis. The results of cytotoxic, antimicrobial, enzyme, and phytochemical studies were expressed as a mean ± SD in three-fold analysis. Further statistical analysis is carried out using a one-way variance analysis (ANOVA) using Statistix 8.1.

4. Results and Discussion

4.1. Percentage Yield. In total, 14 different extracts of the seeds of C. ambrosioides have been prepared with different solvents. Maximum extract yield (10.01% *w*/*w*) was obtained when Me-Dw was used as the extraction solvent (Table 1). Minimum yield was given by NH extracts, i.e., 2.17%. The solvent system, the plant material, the extraction technique, and the extraction time strongly influence the recovery of bioactive constituents [19]. Biological activities do not correspond with the extract yields, and low-yielding solvents might show more noticeable affects. It depends on distinct solubilities of diverse plant metabolites in different solvents [20]. This information is critical to large-scale extraction optimization after sound activity observation [2].

4.2. Total Phenolics Content Determination. The results of the phenolic content showed that Me was the most capable solvent to extract polyphenols (Figure 1; Table 2). Maximum phenolics content was quantified in the Me extract (64.6 ± 0.6 μg GAE/mg). On the contrary, the NH extraction quantified the lowest concentrations of phenolic acids, i.e., 3.2 ± 0.3 μg GAE/mg. The consumption of plant phenolics is associated with a decrease in the incidence of cancer, degeneration, and heart disease [21, 22] because they act as a singlet oxygen suppressor, metal chelator, and reduction

Table 1: The percentage of extract recovery from C. ambrosioides seeds extracted with various solvents.

Sr. no	Solvent extract code	% extract recovery
1	NH	2.17
2	CHL	5.00
3	EA	5.83
4	Act	5.17
5	Me	5.00
6	Eth	5.33
7	Dw	7.67
8	EA-NH	2.21
9	CHL-Me	8.33
10	EA-Me	6.17
11	Eth-EA	5.67
12	Act-Me	5.83
13	Act-Dw	8.50
14	Me-Dw	10.01

agent. The antioxidant potential may be caused by the presence of hydroxyls, methyls, double bonds, or ketones in the phenolic molecules [23].

Values are presented as mean ± standard error after triplicate analysis. (a)–(e) represent the significance of results. Samples that display different alphabets were significantly different at $p < 0.05$.

4.3. Total Flavonoids Content Determination. The determination of the total flavonoids content indicated that the highest quantity of quercetin-equivalent (QE) flavonoids in the Me extract of the seeds was quantified, i.e., $50.9 \pm 0.5\,\mu$ g QE/mg (Figure 1; Table 2). NH extract showed minimum flavonoids detection, i.e., $1.5 \pm 0.3\,\mu$g QE/mg. Flavonoids have antioxidant effects because they have a high antioxidant potential by removing and stabilizing free radicals involved in the oxidation process [24].

4.4. RP-HPLC. The raw extracts are quantitatively analyzed using reverse-phase HPLC technology to identify and quantify polyphenols. The sample peak is compared to the retention time of the reference compounds (caffeic acid, rutin, gallic acid, quercetin, apigenin, catechin, myricetin, and kaempferol) and the UV absorption spectrum. Polyphenols were detected only in Me and Me-Dw extracts, respectively.

The HPLC quantitative profiling shows that rutin is quantified in the extracts of Me seeds, i.e., 1.98 μg/mg extract (Figure 2(b); Table 3). Significant amounts of quercetin and kaempferol were quantified in Me-Dw (0.322 and 2.86 μg/mg, respectively) extract of seed (Figure 2(c)). All quantified polyphenols have clinical uses, i.e., rutin has a significant anticancer, antioxidant, and anti-inflammatory effect [25] and antimicrobial properties [26]. Likewise, kaempferol and quercetin have antimicrobial properties [27], antioxidant, and cytotoxic potential [28]. It can be concluded that C. ambrosioides seeds have a remarkable antimicrobial, antioxidant, and cytotoxic potential. The current evaluation may be due to the detection and quantification of polyphenols such as kaempferol, quercetin, and rutin.

Free radicals produced by the body are important biological substances. These reactive oxygen species (hydroxyl radical, hydrogen peroxide, superoxide radical, peroxyl, and peroxynitrite) are expressed more, cause oxidative stress, and are associated with the etiology of some diseases [29]. The determination of antioxidant potential using different methods has been used. Each test shows different antioxidant mechanisms, such as the elimination of free radicals, the decomposition of peroxides, and the prevention of chain initiation.

4.5. Free Radical Scavenging (DPPH) Assay. A total of 12 extracts exhibited ≥50% scavenging at 400 μg/ml concentration (Figure 3; Table 2). Me extracts showed the highest potential for DPPH bleaching, i.e., $80.12 \pm 1.53\%$ (IC_{50} 110.7 μg/ml). The free radical testing of DPPH is a simple, fast, reliable, cost-effective, and convenient way of studying the antioxidant properties of plant extracts [30]. 2, 2-Diphenyl-1-picrylhydrazyl, known as DPPH radical (molecular formula $C_{18}H_{12}N_5O_6$) is a cell permeable stable free radical. The delocalization of spare electron over the entire molecule does not allow the molecule to dimerise. The mechanism is based on the antioxidant ability to decolorize DPPH by forming yellow-colored diphenyl picrylhydrazine. ($DPPH_2$) results in quantitative measurement of the change in the absorbance value [31]. The results indicate that seeds may be a good source of antioxidants.

4.6. Total Antioxidant Capacity Determination. The total antioxidant capacity of crude extracts is estimated using phosphate molybdenum-based methods. The Me extract has the highest antioxidant capacity, that is, $110.6 \pm 2.2\,\mu$g AAE/mg (Figure 4; Table 2). NH extract revealed lowest antioxidant potential (11.2 ± 0.7 AAE/mg) among all the seed extracts. The phosphomolybdenum based total determination of antioxidant capacity relied on the conversion of MO (VI) to MO (V), which results in green-colored phosphomolybdate complex at acidic pH. This change of colour is noted by spectrophotometer and intensity of colour signifies antioxidant capability [32]. A human cell has been attacked by approximately 10,000 oxidative hits per day from reactive oxygen species that induce alteration of genetic damage which is an initial step towards development of mutagenesis [33]. The emergence of reactive oxygen species is an important factor in pathological problems such as protein oxidation, initiation of mutation of DNA, lipid peroxidation, and cellular degeneration leading to diabetes, cancer, Parkinson's, inflammatory, Alzheimer's, and cardiovascular diseases [29]. Natural antioxidants have been in continuous use for many years. Phytochemicals owing to antioxidant properties prevent development of degenerative diseases [34]. The presence of antioxidant capacity in the test extracts is attributed to the detection and quantification of phytochemicals in the analysis of TPC, TFC, and RP-HPLC in the present study.

4.7. Total Reducing Power Assay. Me ± extract exhibited maximum reducing power, i.e., $94.3 \pm 0.6\,\mu$g AAE/mg extract (Figure 4; Table 2). On the other hand, lowest

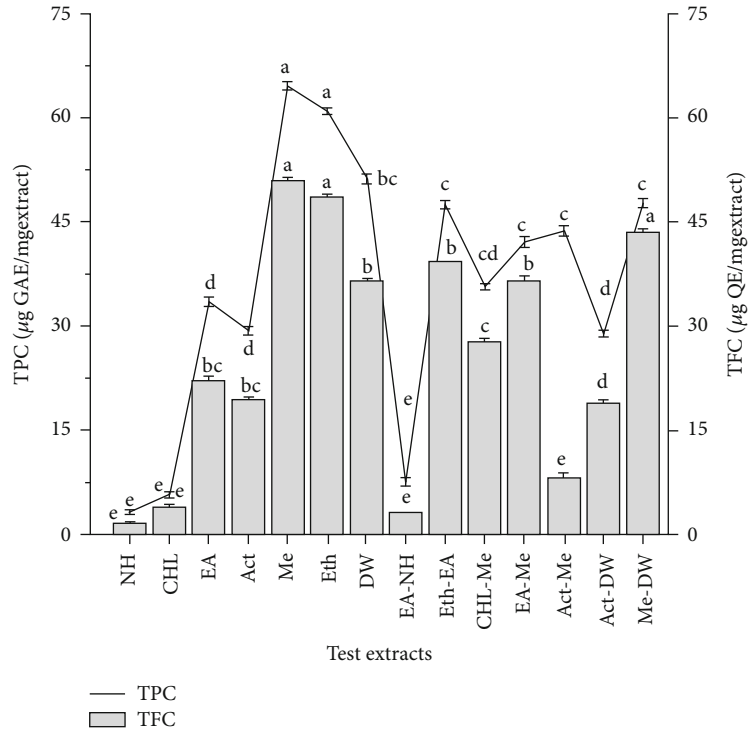

Figure 1: Total phenolics content (TPC) and total flavonoids content (TFC) determination in seed extracts of C. ambrosioides. Values are presented as mean ± standard error after triplicate analysis. (a)–(e) represent the significance of results. Samples that display different alphabets were significantly different at $p < 0.05$.

Table 2: Phytochemical analysis and antioxidant potential of different solvent extracts of C. ambrosioides.

No.	Extracts	Total phenolics content	Total flavonoids content	Free radical scavenging		Total antioxidant capacity	Total reducing power
		μg/mg	μg/mg	% inhibition	IC_{50} (μg/ml)	μg/mg	μg/mg
1	NH	3.2 ± 0.3^{e}	1.5 ± 0.3^{e}	$10.38 \pm 0.78_{e}$	—	11.2 ± 0.7^{e}	13.7 ± 0.8^{e}
2	CHL	5.7 ± 0.4^{e}	3.9 ± 0.4^{e}	$18.53 \pm 1.21_{e}$	—	32.5 ± 0.8^{e}	15.4 ± 0.7^{e}
3	EA	33.5 ± 0.7^{d}	22.3 ± 0.5^{bc}	60 ± 1.27^{b}	329 ± 5.0^{e}	50.6 ± 2.5^{d}	45.2 ± 0.9^{c}
4	Act	29.3 ± 0.6^{d}	19.5 ± 0.3^{bc}	59 ± 1.53^{b}	332 ± 3.0^{e}	35.6 ± 2.1^{d}	33.9 ± 0.9^{c}
5	Me	64.6 ± 0.6^{a}	50.9 ± 0.5^{a}	$80.12 \pm 1.53_{a}$	110.7 ± 5.0^{a}	110.6 ± 2.2^{a}	94.3 ± 0.6^{a}
6	Eth	60.9 ± 0.5^{a}	48.6 ± 0.4^{a}	78.6 ± 1.21^{a}	198 ± 4.0^{b}	99.7 ± 2.2^{a}	89.9 ± 0.9^{a}
7	DW	51.2 ± 0.7^{bc}	36.5 ± 0.3^{b}	56 ± 1.21^{b}	278 ± 3.0^{c}	71.8 ± 2.4^{b}	68 ± 0.2^{b}
8	EA-NH	7.6 ± 0.6^{e}	3.2 ± 0.3^{e}	52 ± 0.68^{b}	380 ± 3.0^{e}	37.3 ± 2.3^{e}	26.5 ± 0.2^{d}
9	Eth-EA	47.5 ± 0.6^{c}	39.4 ± 0.2^{b}	$68.31 \pm 1.21_{b}$	218 ± 2.0^{b}	87.8 ± 2.2^{b}	80.2 ± 0.9^{a}
10	CHL-Me	35.7 ± 0.4^{cd}	27.7 ± 0.4^{c}	$65.38 \pm 1.21_{b}$	294 ± 4.0^{c}	65.9 ± 2.4^{b}	61.9 ± 0.2^{b}
11	EA-Me	42.1 ± 0.7^{c}	36.6 ± 0.7^{b}	67.2 ± 0.78^{b}	226 ± 7.0^{b}	68 ± 2.9^{b}	62.7 ± 0.7^{b}
12	Act-Me	43.7 ± 0.7^{c}	8.2 ± 0.6^{e}	$57.6 \pm 1.08_{bc}$	360 ± 6.0^{e}	38.2 ± 2.7^{e}	33.3 ± 0.8^{c}
13	Act-DW	28.9 ± 0.5^{d}	19 ± 0.4^{d}	61 ± 0.51^{b}	308 ± 4.0^{e}	51.5 ± 2.1^{d}	45.6 ± 0.7^{c}
14	Me-DW	47.7 ± 0.6^{c}	43.4 ± 0.7^{a}	71 ± 0.49^{a}	200 ± 7.0^{b}	87.7 ± 1.7^{b}	78.2 ± 0.5^{b}

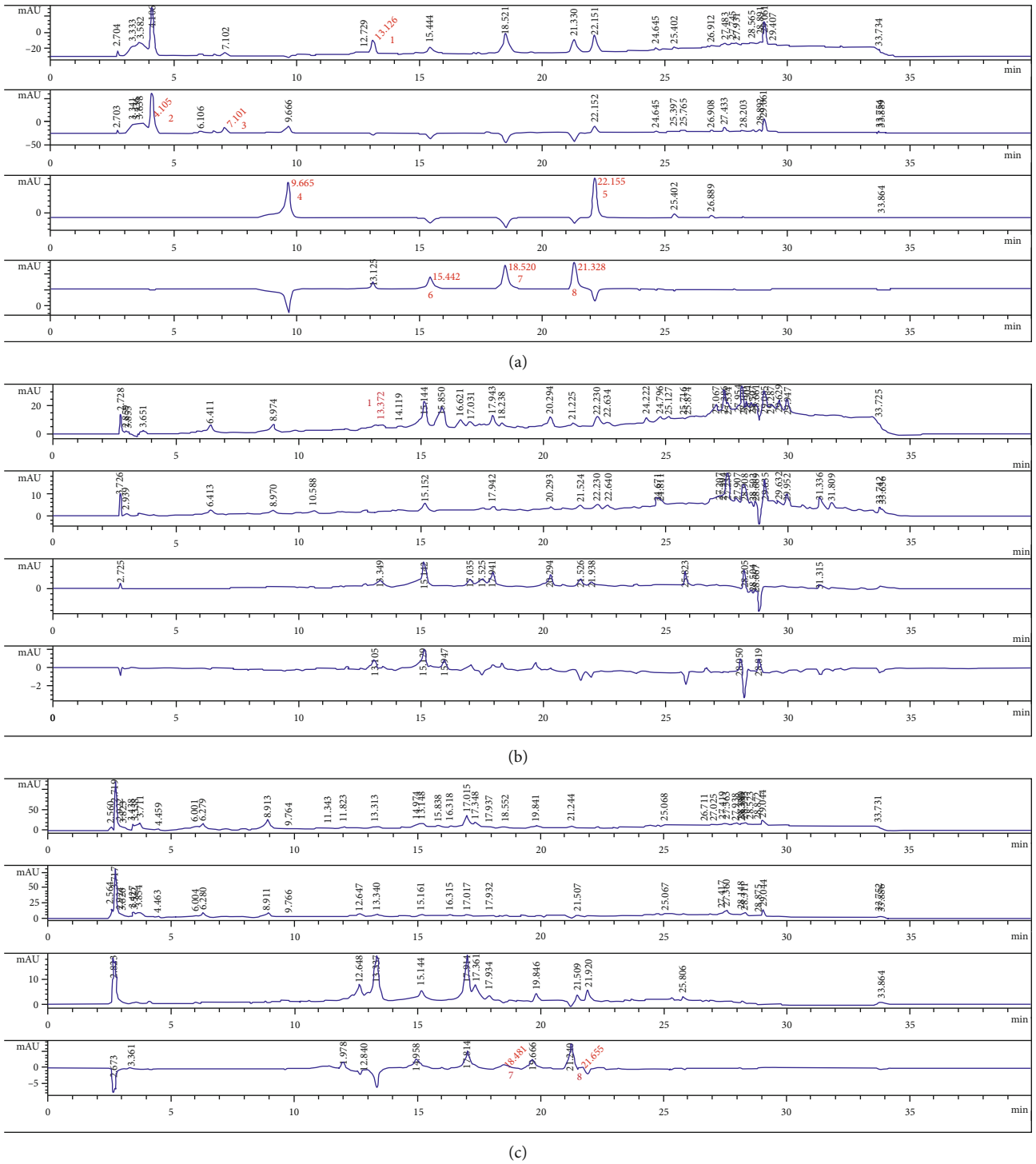

Figure 2: HPLC chromatograms of (a) standards, (b) Me extract of seed, and (c) Me-Dw extract of seed. 1; rutin. 2; gallic acid. 3; catechin. 4; caffeic acid. 5; apigenin. 6; myricetin. 7; quercetin. 8; kaempferol.

reduction potential was observed as $13.7 \pm 0.9\,\mu$g AAE/mg in NH extract. Reductones are thought to have caused the ability to exercise antioxidant effects by giving hydrogen atoms that cause chains of free radicals to break down [16]. Plants produce specific bioactive molecules that make them very effective antioxidants due to their strong H-producing capacity [35]. Assay findings further strengthen the antioxidant potential of C. ambrosioides seeds by indicating the presence of reductones.

4.8. Antimicrobial Assays. Medical research on infectious disease control is developing rapidly. However, drug abuse and resistance to antimicrobials still develop, and worldwide dispersion necessitates the development of advanced

Table 3: HPLC-DAD analysis of different solvent extracts of C. ambrosioides using standard polyphenols.

Extract name	Polyphenols (μg/mg extract)							
	GA	Rutin	CA	Catechin	AP Seed	Myrecetin	Quercetin	Kaemp
Me	—	1.98	—	—	—	—	—	—
Me-Dw	—	—	—	—	—	—	0.322	2.86

–: not detected; GA: gallic acid; CA: caffeic acid; AP: apigenin; Kaemp: kaempferol.

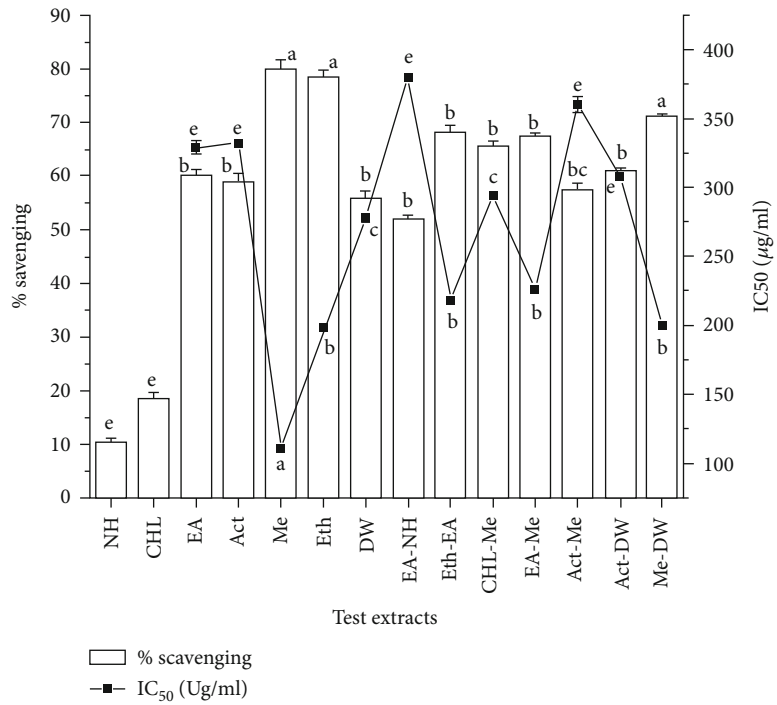

Figure 3: The determination of the percentage of free radical scavenging activity and the IC_{50} values of various solvent extracts in the seeds of C. ambrosioides. The values are presented as the mean average after triplicate experiment. (a)–(e) represent the significance of results. Samples that display different alphabets were significantly different at $p < 0.05$.

scientific approaches to establish novel therapeutic remedies [36]. Almost, all extracts have shown lower to moderate antibacterial potential. Maximum antibacterial activity was depicted when NH was employed to extract phytoconstituents (Table 4). Standard drug cefixime exhibited significantly higher activity against S. aureus, M. luteus, and K. pneumonia with 16 ± 0.7, 25 ± 1.04, 18 ± 0.81, and 17 ± 1.12 mm ZOI, respectively. The lack of growth inhibition areas around the impregnated discs confirmed the nontoxic effects of DMSO. The antimicrobial effect of active samples is difficult to attribute to more than one active principle because the different chemical compositions of each extract make it difficult to recognize. In addition to the main ingredients, minor components can also play an important role in extract biological activity [37]. It is possible that multiple botanical chemicals and essential oils present in plants produce synergistic or antagonistic effects [11]. Phenolic compounds might be responsible for obvious antimicrobial activity. Their antibacterial actions include affecting the function of the cytoplasmic membrane, disturbing the metabolism of energy, and affecting the synthesis of nucleic acids [38]. HPLC-based detection of rutin, kaempferol, and quercetin may be considered to be responsible for the current antibacterial activity being investigated.

All crude extracts were also tested for antimicrobial potential against five filamentous fungi strains. The extract showed that the test strain had a significant growth inhibition zone (Table 5).

The extracts of CHL and Act-Me seeds showed the greatest activity against F. solani and A. fumigatus with 17 ± 1.38 and 12 ± 1.42 mm ZOI, respectively (Table 5). Mucor specie and A. flavus strain were sensitive to EA-Me and Dw extracts with 14 ± 1.21 and 10 ± 0.78 mm ZOI, respectively. Maximum activity against A. niger was shown by Act-Me (15 ± 1.24 mm ZOI) extract. Standard drug clotrimazole (10 μg/disc) exhibited maximum activity. The lack of a growth inhibition zone around a DMSO-impregnated disc confirms the nontoxic effect of DMSO. The results are in accordance with the antifungal activity of essential oil obtained through hydrodistillation from aerial parts of C.

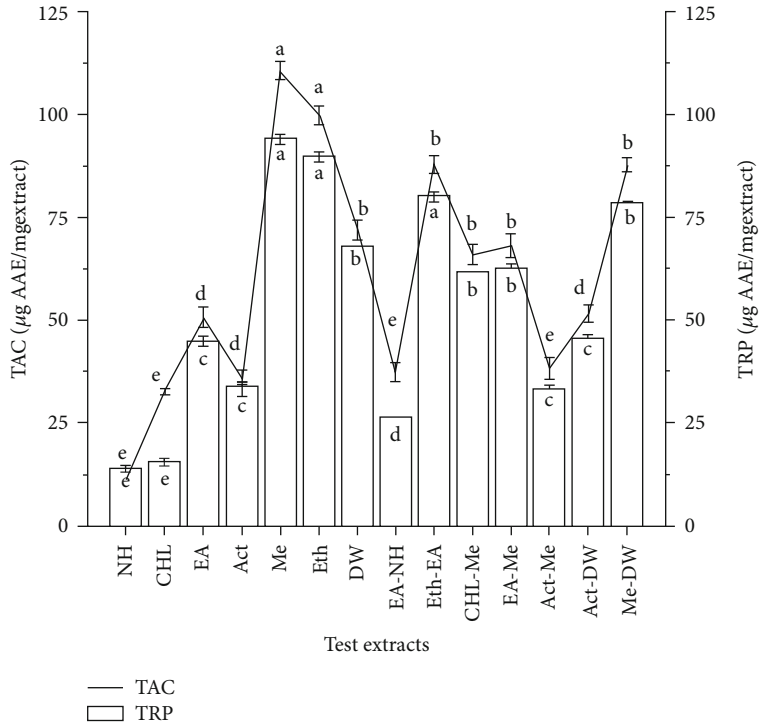

Figure 4: The total determination of antioxidant capability and reduction power in C. ambrosioides seed extracts. After a triplicate investigation, the values are displayed as mean ± average. (a)–(e) represent the significance of results. Samples that display different alphabets were significantly different at $p < 0.05$.

Table 4: Antibacterial activity of C. ambrosioides seed extracts against pathogenic bacteria.

Extract name	Zone of inhibition (mm) at 100 μg/disc		
	K. pneumoniae	M. luteus	S. aureus
NH	14 ± 1.61^{ab}	13 ± 1.30^{b}	9 ± 0.79^{b}
CHL	13 ± 1.39^{ab}	10 ± 0.97^{bc}	8 ± 0.69^{c}
EA	12 ± 1.27^{b}	10 ± 0.83^{bc}	8 ± 0.63^{c}
Act	13 ± 1.23^{ab}	8 ± 0.55^{d}	7 ± 0.55^{cd}
Me	7 ± 0.95^{e}	10 ± 0.71^{c}	8 ± 0.49^{c}
Eth	6 ± 0.79^{e}	9 ± 0.59^{cd}	8 ± 0.43^{c}
Dw	6 ± 0.83^{e}	8 ± 0.45^{d}	8 ± 0.39^{c}
EA-NH	9 ± 0.98^{d}	9 ± 0.81^{cd}	9 ± 0.67^{b}
CHL-Me	11 ± 0.81^{c}	10 ± 0.78^{c}	8 ± 0.61^{b}
EA-Me	8 ± 0.68^{d}	11 ± 0.98^{c}	9 ± 0.71^{b}
Eth-EA	10 ± 1.07^{cd}	11 ± 1.08^{c}	9 ± 0.81^{b}
Act-Me	12 ± 1.12^{c}	—	9 ± 0.73^{b}
Act-Dw	11 ± 1.11^{c}	7 ± 0.47^{d}	8 ± 0.53^{c}
Me-Dw	9 ± 0.61^{d}	8 ± 0.63^{d}	9 ± 0.83^{b}
Cefixime	17 ± 1.12^{a}	25 ± 1.04^{a}	16 ± 0.7^{a}
DMSO	—	—	—

Values are represented as mean ± standard error of triplicate experiments. –: no activity. Concentration of sample: 100 μg per disc. (a)–(e) represent the significance of results. Samples that display different alphabets were significantly different at $p < 0.05$.

ambrosioides [11, 39]. Different mechanisms are considered responsible for the antifungal effects of biomolecules, i.e., inhibition of cell wall formation, cell membrane disruption, mitochondrial dysfunction, and inhibition of cell division [40].

4.9. Brine Shrimp Cytotoxicity Assay. The cytotoxicity analysis of shrimp provides an effective preproposal method for predicting antimalarial, antimicrobial, insecticidal, and antitumor activities. Different solvent extracts of C. ambrosioides seeds showed cytotoxicity to brine shrimps on 24-hour exposure period in a concentration-dependent manner. The analysis revealed that brine shrimp cytotoxic activity with the lowest LC_{50} was shown by EA and CHL-Me extracts, i.e., 125 and 130 μg/ml, respectively (Table 6). The remaining extracts revealed their cytotoxicity in the following order: CHL > Act – Me > NH > EA – NH > Act > Eth – EA > Eth > Me > EA – Me > Act – Dw. Plant extracts with an LC_{50} of less than 1000 μg/ml were considered toxic to cells [16]. Most samples have LC_{50} values below 1000 μg/ml which represents a prominent cytotoxicity profile of crude extracts of C. ambrosioides seeds. The cytotoxicity observed in raw extracts may be due to the presence of secondary metabolites like phenols, flavonoids, terpenes [41], and other constituents which are not detected by HPLC analysis. This preliminary screening warrants detection of further polyphenols followed by isolation of potent compounds.

4.10. Protein Kinase Inhibition Potential. Protein kinase inhibitors are unique compounds that are specially characterized for inhibiting oncogenic kinase [42]. Protein kinase

Table 5: The antifungal activity of different solvents extracts of seeds of C. ambrosioides.

Extract name	Zone of inhibition (mm) at 100 μg/disc				
	F. solani	A. fumigatus	Mucor spp.	A. flavus	A. niger
NH	—	—	7 ± 0.43^{d}	7 ± 0.44^{c}	7 ± 0.39^{e}
CHL	17 ± 1.38^{b}	8 ± 0.51^{c}	10 ± 0.87^{bc}	—	9 ± 0.64^{cd}
EA	—	7 ± 0.31^{c}	12 ± 0.97^{b}	—	7 ± 0.25
Act	—	—	13 ± 1.13^{b}	7 ± 0.29^{c}	13 ± 1.21^{bc}
Me	8 ± 0.46^{c}	9 ± 0.67^{c}	—	—	13 ± 1.05^{bc}
Eth	8 ± 0.52^{c}	—	7 ± 0.39^{d}	—	9 ± 0.58^{cd}
Dw	—	8 ± 0.59^{c}	—	10 ± 0.78^{b}	7 ± 0.37^{e}
EA-NH	9 ± 0.63^{c}	—	8 ± 0.46^{d}	8 ± 0.61^{bc}	8 ± 0.49^{d}
CHL-Me	—	—	12 ± 1.13^{b}	8 ± 0.53^{bc}	8 ± 0.53^{d}
EA-Me	9 ± 0.69^{c}	12 ± 1.42^{bc}	14 ± 1.21^{b}	7 ± 0.39^{c}	11 ± 0.93^{c}
Eth-EA	8 ± 0.49^{c}	—	10 ± 0.87^{bc}	—	10 ± 0.97^{c}
Act-Me	9 ± 0.63^{c}	7 ± 0.47^{c}	13 ± 1.23^{b}	7 ± 0.37^{c}	15 ± 1.24^{b}
Act-Dw	9 ± 0.55^{c}	—	9 ± 0.59^{c}	—	7 ± 0.31^{e}
Me-Dw	—	8 ± 0.81^{c}	12 ± 0.93^{b}	9 ± 0.71^{b}	7 ± 0.42^{e}
Clotrimazole	30 ± 1.54^{a}	31 ± 0.9^{a}	30 ± 1.2^{a}	29 ± 0.5^{a}	31 ± 0.8^{a}
DMSO	—	—	—	—	—

Values are represented as mean ± standard error of three separate experiments. –: no activity. Clotrimazole (10 μg/disc) was positive control. (a)–(e) represent the significance of results. Samples that display different alphabets were significantly different at $p < 0.05$.

Table 6: Cytotoxic potential of different solvent extracts of C. ambrosioides seed.

Extract name	Brine shrimp lethality		Protein kinase inhibition	
	% mortality 1000 μg/ml	LC_{50} μg/ml	Diameter (mm) at 100 μg/disc Clear zone	Bald zone
NH	100	152^{bc}	—	11 ± 0.83^{b}
CHL	100	133^{b}	—	10 ± 0.49^{b}
EA	97.5	125^{b}	—	10 ± 0.61^{b}
Act	93	179^{bc}	—	12 ± 0.91^{b}
Me	100	250^{c}	—	20 ± 1.34^{a}
Eth	97.5	228^{c}	—	20 ± 1.27^{a}
Dw	35	$>1000^{e}$	—	—
EA-NH	97	168	8 ± 0.68	—
CHL-Me	90	130^{b}	—	—
EA-Me	80	255^{c}	—	—
Eth-EA	97	185^{bc}	10 ± 0.78	—
Act-me	95	137^{b}	15 ± 1.08	—
Act-Dw	52	971^{d}	9 ± 0.51	—
Me-Dw	50	$>1000^{e}$	11 ± 0.49	—
Doxorubicin	—	5.93^{a}	—	—
Surfactin	—	—	—	21^{a}
DMSO	—	—	—	—
1% DMSO in water	—	—	—	—

The values are presented as an average mean with standard error after triple analysis. –: no activity or not applicable. (a)–(e) represent the significance of results. Samples that display different alphabets were significantly different at $p < 0.05$.

inhibitor (PKg2) in the sample blocks Streptomyces aero hyphal formation, thus suggesting that it inhibits the proliferation of cancerous cells. The results are summarized in Table 6. Among all seed extracts, only unisolvent extracts showed remarkably significant inhibition of the formation of hyphae at 100 μg/disc. The maximum activity of the Me and Eth extracts is shown, that is, 20 ± 1.34 and 20 ± 1.27 mm bald zone of inhibition, respectively (Table 5). The

nontoxic effect of DMSO was confirmed by the absence of ZOI. Positive control surfactin (20 μg/disc) showed 21 mm ZOI. The abnormal activity of protein kinases is overexpression, mutation, or deregulation, avoiding physiological processes and causing life-threatening cancers. The ability of samples and extracts to bind to the active or inactive sites of a kinase as a whole is crucial for the development of new drugs to prevent chemotherapy. The results are consistent with previous reports in which extracts of Me from various sources show an anti-inflammatory reaction by directly inhibiting several interleukins-1-receptor and mTOR kinases [43, 44]. The activity exhibited by Me-Dw extract of C. ambrosioides is believed to inhibit cancer linked several kinases (ABL1, CLK1, MET, and NEK4), and the existence of quercetin (detectable by HPLC fingerprints) is attributed to this activity [45]. In this study, various extracts show significant inhibition potentials of kinases, which may be a powerful source of chemoprevention drugs.

5. Conclusion

Extraction in wide polarity solvents is crucial to determine therapeutic potential. Plant biochemicals are dependent on the polarity of extraction solvents. The quantification of RP-HPLC (rutin, quercetin, and kaempferol), as well as the good antioxidant potential, makes the seeds of C. ambrosioides a good source of antioxidants. Significant results were obtained by extracts in antibacterial, antifungal, cytotoxic salt shrimp, and protein kinase inhibition studies. The optimal solvent system should be used for extraction on a preparative scale to improve productivity. The current study also demonstrates promising potential of C. ambrosioides seeds for the discovery of novel bioactive moieties through bioactivity guided isolation.

Data Availability

All the data is original based on extensive research and can be provided as supplementary data if required.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

S.B. performed experiments, analyzed and interpreted the data, did software statistics, and wrote and revised the manuscript. M.W.B. and S.S.Z. assisted in in vitro experiments, acquisition of the data, and critical review of the manuscript. M.K.O. and N.A. assisted in data analysis and interpretation and made critical revisions. W.H.A.-Q. and H.A. contributed to the acquisition of the data and critical review of the manuscript. I.-u.H. conceptualized and designed the study, supervised execution of experiments, critically revised the manuscript, and approved the final version of this manuscript. All authors have read and agreed to the published version of the manuscript.

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Research Article

Identification of Volatile Compounds and Insecticidal Activity of Essential Oils from *Origanum compactum* Benth. and *Rosmarinus officinalis* L. against *Callosobruchus maculatus* (Fab.)

Asmae Baghouz,[1] Yamna Bouchelta,[1] Imane Es-safi,[1] Mohammed Bourhia,[2] El Moussaoui Abdelfattah,[3] Abdullah A. Alarfaj,[4] Abdurahman Hajinur Hirad,[4] Hiba-Allah Nafidi,[5] and Raja Guemmouh[1]

[1]*Laboratory of Biotechnology, Conservation and Valorization of Natural Resources, Department of Biology, University of Sidi Mohamed Ben Abdellah, Faculty of Science Dhar El MahrazB.P, 1796 Atlas, Fez 30003, Morocco*
[2]*Laboratory of Chemistry, Biochemistry Nutrition and Environment, Faculty of Medicine and Pharmacy, University Hassan II, Casablanca 20000, Morocco*
[3]*Laboratory of Biotechnology, Environment Agrifood and Health, Faculty of Science, University of Sidi Mohamed Ben Abdellah, Fez 30050, Morocco*
[4]*Department of Botany and Microbiology College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*
[5]*Department of Food Science, Faculty of Agricultural and Food Sciences, Laval University, Quebec, University Street QC G1V 0A6 2325, Canada*

Correspondence should be addressed to Mohammed Bourhia; bourhiamohammed@gmail.com

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This work was undertaken to investigate the volatile compounds and insecticidal activity of essential oils (EOs) from *Origanum compactum* Benth. and *Rosmarinus officinalis* L. against the crop pest *Callosobruchus maculatus* (Fab.). Essential oils of *Origanum compactum* (EOC) and *Rosmarinus officinalis L.* (EOR) were extracted by use of hydrodistillation, and their volatile compounds were profiled by gas chromatography-mass spectrometry (GC-MS). The insecticidal activity of extracted EOC and EOR was evaluated against *C. maculatus*. GC-MS analysis revealed that carvacrol (70.88%) and 1,8-cineole (62.35%) were the major constituents of EOC and EOR, respectively. EOC exhibited a potent insecticidal activity with calculated LC_{50} values of 6.77 and 3.57 μL/L air, 24 and 48 h posttreatment, respectively. Comparable LC_{50} values were obtained for EOR recording 6.25 and 3.82 μL/L, 48 h posttreatment. The effects of fumigation by the tested EOs on fertility (egg hatching) and the emergence of adult *C. maculatus* were also investigated. Notably, EOC completely abolished egg fertility judged by the abrogation of emergence of adults, regardless of the tested dose. By contrast, EOR completely inhibited the fertility and the emergence of *C. maculatus* adults at the dose of 16 μL/10 g. The outcome of the present study highlights the utility of the EOs from *O. compactum* Benth. and *R. officinalis* L. as natural sources of effective and ecofriendly pest-control agents.

1. Introduction

Nowadays, many environmental challenges influence agriculture worldwide. In addition to poor soil quality and cultivation techniques, there are problems related to insect pests. Pests attacking stored food legumes result in significant damage and loss of both quality and quantity. While losses attributed to pests are estimated to be around 40% in Africa, they do not exceed 3% in developed countries [1].

Despite the magnitude of losses caused by insect pests, a limited number of studies have shed light on pests in Africa. In Morocco, *C. maculatus* causes serious damage to stored legume foods, where *C. maculatus* larvae develop and feed on the cotyledons of legumes, particularly when no measures are taken. Pests are capable of destroying a crop within 4-5 months according to the Food and Agriculture Organization (FAO). Losses due to insect pests reached 35% of global agricultural production [2].

C. maculatus can be considered as one of the most growing challenges throughout the tropical and subtropical regions. It is a worrying pest of several pulses including *Vigna unguiculata*, *Cicer arietinum*, *Glycine max*, and *Phaseolus vulgaris*. These pulses are an important food source for millions of people based in tropical and subtropical areas. Cowpea seeds are most attacked pulses by *C. maculatus* and cause maximum damage, which could reach 2–5 kg seeds within 45–90 days when stored under optimal temperature (30 ± 10 C) and moisture conditions ($75 \pm 3\%$) [3].

Insecticides represent one of the most used control methods to manage insect pests. However, the resistance of insects to modern insecticides is still a great challenge facing chemical insecticides. In addition, these chemicals can possess risks to consumers and cause even harmful effects in the long term [4]. The plant kingdom represents a potentially effective alternative as a source of natural pest-control agents. Aromatic plants contain essential oils (EOs) that possess natural insecticidal activities. Hence, the insect-controlling potential of plant-derived EOs has been widely tested against pests attacking stored grains through their insecticidal potencies [5–8]. In this context, several researchers have reported lethal effects of EOs against plant and human pests [9, 10].

This work aimed to investigate the profile of volatile compounds and fumigant activity of EOs from *Origanum compactum* Benth. and *Rosmarinus officinalis* L. against *Callosobruchus maculatus* (Fab).

2. Materials and Methods

2.1. Insect Breeding. *C. maculatus* was obtained from a local warehouse and subsequently bred in glass jars with 500 g of *Vigna unguiculata* seeds. Jars were maintained at a temperature of 27 ± 1 °C, relative humidity of $70 \pm 5\%$, and a photoperiod of 14 h (light)/10 h (dark).

2.2. Plant Material. *O. compactum* was harvested from the region of Taounate from Morocco, whereas *R. officinalis* was harvested from the region of Taza, Morocco. Thereafter, the studied plants were identified by a botanist before being deposited at the Herbarium of Sidi Mohamed Ben Abdellah University. Next, the leaves were cleaned and dried in the shade at room temperature for 15 days.

2.3. Extraction of Essential Oils. One hundred grams of *O. compactum* and *R. officinalis* leaves were soaked in 750 mL of distilled water before being extracted at 100°C by use of a Clevenger apparatus for 4 h. The obtained EOs were dehydrated with anhydrous sodium sulfate before being stored in a refrigerator at 4°C until further use [11].

2.4. Gas Chromatography-Mass Spectrometry Analysis. The volatile compounds of the studied plants were determined by using GC-MS. Briefly, 0.1 μL of the sample was injected for analysis using a gas chromatograph coupled to a mass spectrophotometer (Agilent Technologies 5973 with an Agilent 19091S-433 HP-5MS column, 30 m long, 0.25 mm inner diameter, and 0.25 μm film thickness of the stationary phase) in positive mode. Helium was used as the carrier gas, with a typical pressure range (psi) of 0.9 mL/sec. The oven temperature program was set between 60 and 300°C for 10 min and then held at 300°C for 20 min. The detector temperature was set at 250°C, whilst the injector temperature was set at 260°C. Identification of compounds was performed by comparing retention times with standards of the database [12].

2.5. Insecticidal Activity Test. The insecticidal activity test was carried out to evaluate the activity of the essential oils in a vapor phase as reported in earlier work [11]. To achieve this goal, Whatman paper discs (3×3 cm) impregnated with different concentrations of the tested EOs (4, 8, 12, 16, and 20 μL/L of air) were attached to the inner surface of the stoppers of each jar to avoid their direct contact with the insects. Next, 10 g of cowpea seed and five pairs of *C. maculatus* aged from 0 to 48 h were separately introduced into each jar. Total mortality of insect individuals by each dose was recorded daily for 5 days. Egg-laying capacity of the females of *C. maculatus* was calculated by use of a magnifying binocular. Jars were subsequently maintained at a temperature of 27 ± 1°C, relative humidity of $70 \pm 5\%$, and a photoperiod of 14 h (light)/10 h (dark) until the emergence phase of adults.

2.6. Statistical Analysis. The results were expressed as means (±SD). A two-way analysis of variance (ANOVA) was used to analyze the effect of varying doses and exposure periods on mortality and fecundity of females and the emergence of adult *C. maculatus*. Significant differences between treatments were calculated by using Tukey's multiple range tests ($p < 0.05$). The lethal concentration LC_{50}, LC_{90}, chi-square, and 95% confidence intervals for each regression coefficient were calculated by use of probit analysis [13]. A significant difference was considered when $p < 0.05$.

3. Results and Discussion

3.1. Analysis of Essential Oil Components. The results of the volatile compounds profile of EOC are given in Figure 1 and Table 1. In this sense, the analysis showed the presence of 12 major compounds representing 99.89% of the total oil composition. EOC was majorly composed of carvacrol (70.88%) followed by caryophyllene oxide (7.97%), o-cymene (5.68%), and thymol (5.16%). Concerning the

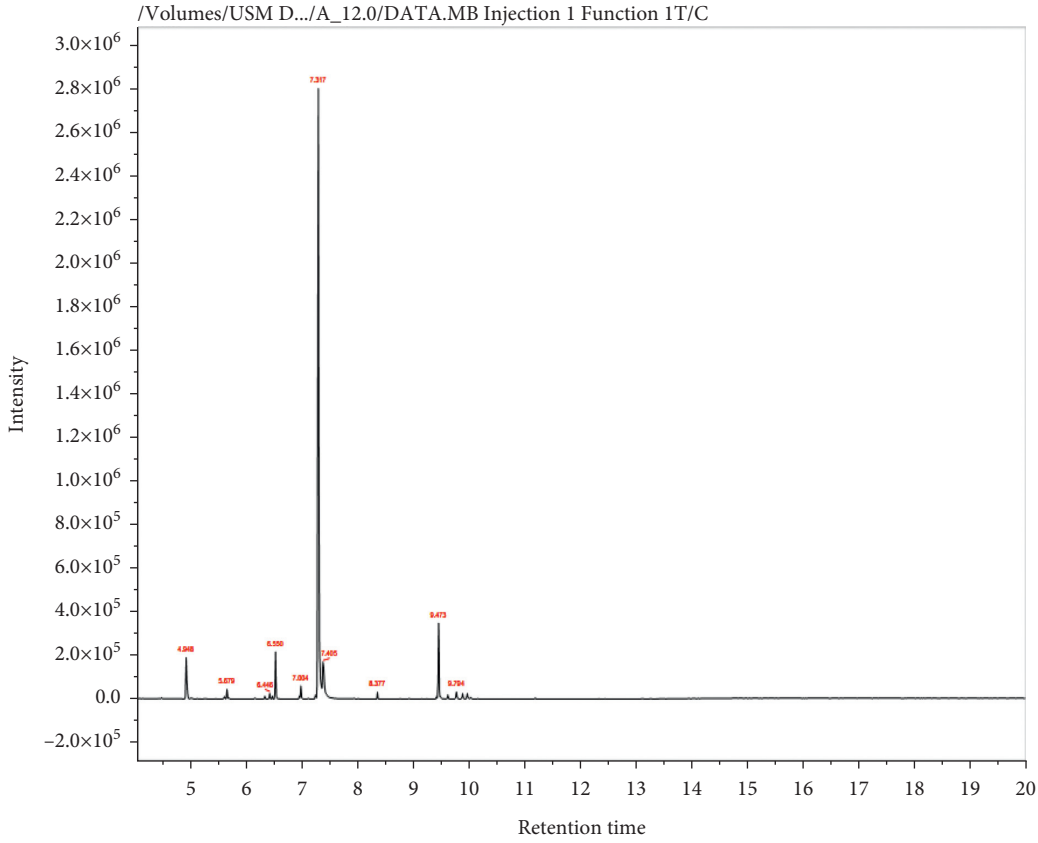

Figure 1: Chromatographic profile of EOC identified by GC-MS.

Table 1: Volatile compounds of EOC identified by GC-MS.

Peak	RT	Compound	Chemical formula	Chemical class	RI		Area (%)
					Cal	Lit	
1	4.94	o-Cymene	C10H14	MO.H	1024	1024	5.68
2	5.67	Terpinolene	C10H16	MO.H	1280	1282	0.93
3	6.44	β-Terpineol	C10H18O	MO.O	1143	1144	0.53
4	6.55	α-Terpineol	C10H20O	MO.O	1163	1164	4.58
5	7.00	Piperitenone	C10H14O	MO.O	1341	1343	1.34
6	7.31	Carvacrol	C10H14O	MO.O	1297	1299	70.88
7	7.40	Thymol	C10H14O	MO.O	1290	1290	5.16
8	8.37	Trans-Caryophyllene	C15H24	ST.H	1594	1598	0.60
9	9.47	Caryophyllene oxide	C15H24O	ST.O	1986	1986	7.97
10	9.79	Adamantanone	C10H14O	MO.O	1309	1311	0.86
11	9.90	Naphthalene	C11H10O	O	1445	1447	0.65
12	9.98	Camphene	C10H16	MO.H	1065	1028	0.81
			Monoterpene hydrate (MO.H)				7.42
			Monoterpene oxygenated (MO.O)				83.25
			Sesquiterpenes hydrate (ST.H)				0.60
			Sesquiterpenes oxygenated (ST.O)				7.97
			Others (O)				0.65
			Total identified (%)				99.89

RI, retention indices; Lit, literature; Cal, calculate; RT, retention time in minutes.

volatile compounds profile of EOR, GC-MS analysis revealed the presence of nine major compounds representing 99.89% of the total oil composition. EOR was mainly composed of 1,8-cineole (62.35%), camphor (23.14%), borneol (5.51%), and camphene (4.10%) (Figure 2 and Table 2).

3.2. Insecticidal Activity Test

3.2.1. Effect on Adult Mortality. Insecticidal activity of EOC and EOR against the adults of *C. maculatus* is given in Table 3. Statistical analysis revealed that the observed insecticidal effect is both time and dose-dependent. EOC exhibited significantly

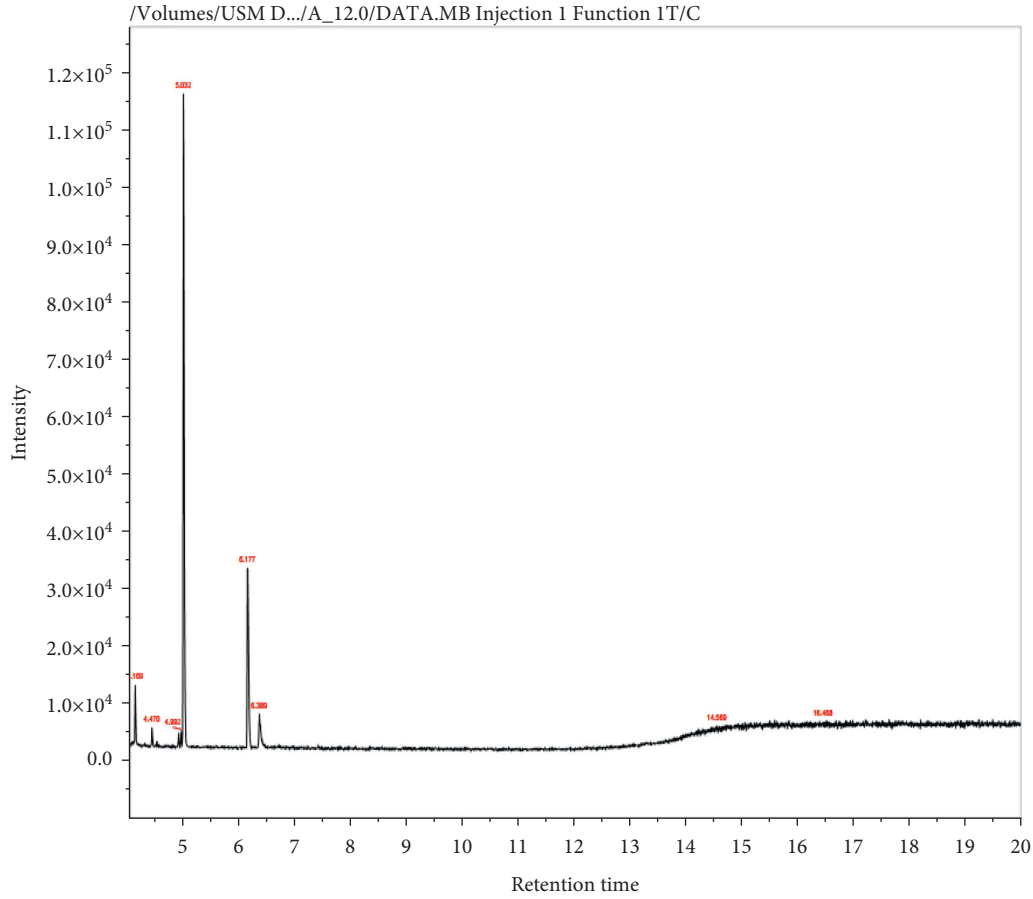

Figure 2: Chromatographic profile of EOR volatile compounds identified by GC-MS.

Table 2: Volatile compounds of EOR identified by GC-MS.

Peak	RT	Compound	Chemical formula	Chemical class	RI		Area (%)
					Cal	Lit	
1	4.16	Camphene	C10H16	MO.H	1085	1068	4.10
2	4.47	Cis-Ocimene	C10H16	MO.H	1037	1037	1.30
3	4.94	o-Cymene	C10H14	MO.H	1024	1024	1.07
4	4.99	Limonene	C10H16	MO.H	1028	1029	0.91
5	5.03	1,8-Cineol	C10H18O	MO.O	1186	1186	62.35
6	6.17	Camphor	C10H16O	MO.O	1146	1146	23.14
7	6.38	Borneol	C10H18O	MO.O	1169	1169	5.51
8	14.56	Santolinyl acetate	C12H20O2	O	1172	1174	0.80
9	16.46	Butanoic acid	C11H22O2	O	1196	1197	0.72
			Monoterpene hydrate (MO.H)				7.38
			Monoterpene oxygenated (MO.O)				91
			Sesquiterpenes hydrate (ST.H)				0
			Sesquiterpenes oxygenated (ST.O)				0
			Others (O)				1.52
			Total identified (%)				99.90

RI, retention indices; RT, retention time in minutes.

high mortality rate as a function of increasing concentrations ($F = 156.60$; df = 5,48; $p < 0.0001$) and exposure time ($F = 102.25$; df = 2,48; $p < 0.0001$), whereas EOR showed significant variation in *C. maculatus* mortalities at different concentrations ($F = 348.49$; df = 5, 36; $p < 0.0001$) and was highly significant with increasing exposure time ($F = 229.8$; df = 2, 36; $p < 0.0001$). The LC_{50} value for EOC was 6.77 and 3.57 μL/L air 24 h and 48 h postexposure, respectively; whereas, the LC_{90} ranged from 35.90 to 15.17 μL/L, respectively. The LC_{50} value for EOR ranged from 6.25 to 3.82 μL/L 48-hour post-exposure, whereas the LC_{90} ranged from 20.70 to 12.40 μL/L (Table 3).

Table 3: Lethal concentrations (μL/L) and chi-square (χ^2) values for EOC and EOR against adult *C. maculatus*.

Essential oils	Days	LC_{50} (μL/L)	95% CI	LC_{90} (μL/L)	95% CI	d*f*	χ^2
EOC	1	6.77	0.58–10.99	35.90	17.98–50242.031	3	1.23
	2	3.57	0.039–6.20	15.17	9.39–302.76	3	1.32
EOR	1	6.25	2.39–8.99	20.70	13.41–103.89	3	2.28
	2	3.82	0.42–6.05	12.40	8.23–51.18	3	2.66

Table 4: Effect of essential oils on mortality of *C. maculatus* as a function of concentrations and exposure times.

Essential oils	Doses (μL/L of air/10 g)	Exposure time (h)			
		24 h	48 h	72 h	96 h
EOC	4	36.66±5.77	66.66±5.27	90±1.0	100±0
	8	53.33±5.77	73.33±6.54	96.66±5.70	100±0
	12	63.33±5.77	86.66±6.54	100±0	100±0
	16	73.33±5.27	96.66±5.70	100±0	100±0
	20	93.33±3.74	100±0	100±0	100±0
	Control	0±0	0±0	0±0	0±0
EOR	4	36.66±3.35	63.33±3.11	100±0	100±0
	8	46.66±5.77	70±0.0	100±0	100±0
	12	63.33±3.07	86.66±6.54	100±0	100±0
	16	76.66±2.01	100±0	100±0	100±0
	20	100±0	100±0	100±0	100±0
	Control	0±0	0±0	0±0	0±0

As given in Table 4, both EOC and EOR showed dose and exposure time-dependent insecticidal activities, leading to100% of adult mortality 72 h postexposure. At the highest dose, EOC induced 90.0% of adult mortality (20 μL/L air/10 g) 24 and 48-hour posttreatment, whereas at the highest dose, EOR exhibited 100% of adult mortality 24-hour posttreatment. No mortalities were recorded in control groups.

3.2.2. Effect on Fecundity. The fecundity of *C. maculatus* females was strongly affected by the insecticidal effects of EOs tested. The obtained results showed a significant decrease in the number of eggs laid by females after being exposed to the vapor of EOC and EOR relative to the control (Figure 3 and Table 5). At the highest dose used for testing (20 μL/10 g seeds), the two EOs completely inhibited the fecundity of females relative to the control value of 196.66 ± 11.54. ANOVA analysis indicated that the EO-mediated toxicity against *C. maculatus* fecundity was highly significant as a function of increasing concentrations ($F = 1123.48$; df = 5, 24; $p < 0.0001$). Moreover, there is no significant difference between EOC and EOR towards the fecundity of females ($F = 6.31$; df = 1, 24; $P = 0.0191$). This can be explained by the fact that *C. maculatus* has sensitivity towards EOs of the tested aromatic plants.

3.2.3. Effect on Fertility. The obtained results showed that both EOC and EOR significantly reduced the egg hatchability when compared to the control in a dose and time-dependent manner (Figure 4 and Table 5). For all tested EOC doses, egg hatching was not recorded compared to the control fecundity rate of 94.02 ± 4.08.

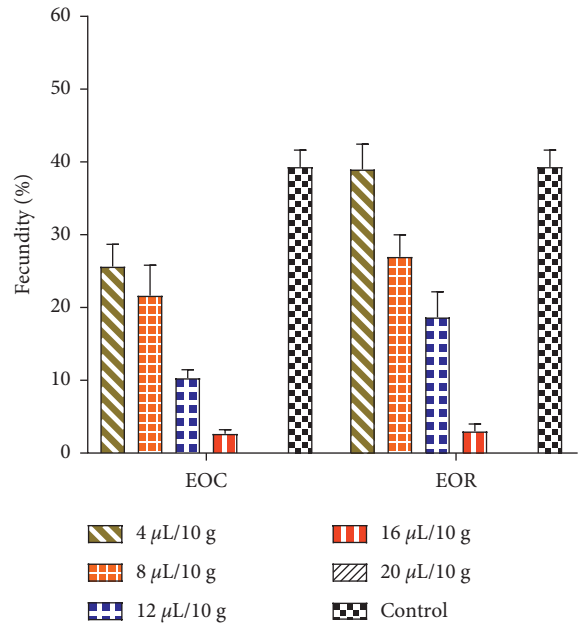

Figure 3: Effects of essential oil of EOC and EOC on the fecundity of *C. maculatus* (mean ± SD).

Similarly, EOR exhibited a potent egg hatching inhibitory effect, wherein the dose of 16 μL/10 g, resulted in complete abrogation of egg hatchability (Figure 4; Table 5). EOC possessed a toxic effect on the fertility of *C. maculatus* eggs irrespective of the tested dose, whereas EOR inhibited the fertility at the highest tested dose. Therefore, EOC exhibited a far more potent inhibitory effect on egg hatchability than EOR.

Table 5: Effects of EOC and EOR on fecundity, fertility, and emergence of *C. maculatus*.

Dose (μL/L)	Fecundity		Fertility (%)		Adult emergence (%)	
	EOC	EOR	EOC	EOR	EOC	EOR
4	23.66 ± 5.03	37.33 ± 5.50	0 ± 0	53.03 ± 7.27	0 ± 0	39.03 ± 4.69
8	21.66 ± 4.16	27 ± 3	0 ± 0	41.63 ± 4.64	0 ± 0	22.12 ± 1.25
12	10.33 ± 1.15	18.66 ± 3.51	0 ± 0	22.77 ± 3.92	0 ± 0	14.24 ± 1.33
16	2.66 ± 0.57	3 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Control	196.66 ± 11.54	196.66 ± 11.54	94.02 ± 4.08	94.02 ± 4.08	89.97 ± 0.89	89.97 ± 0.89

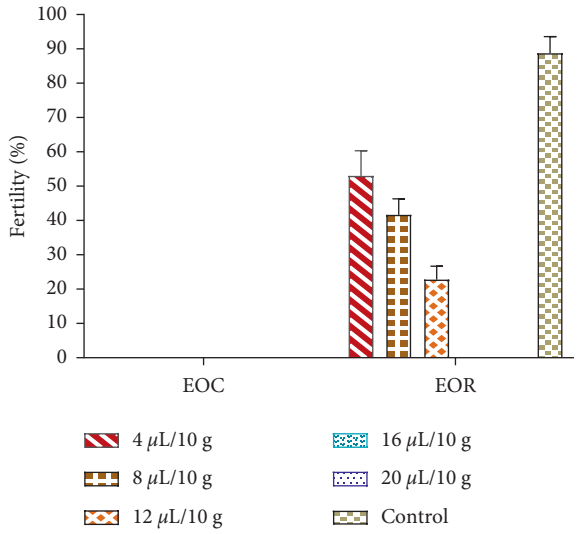

Figure 4: Effects of essential oil of EOR on the fertility of *C. maculatus* (mean ± SD).

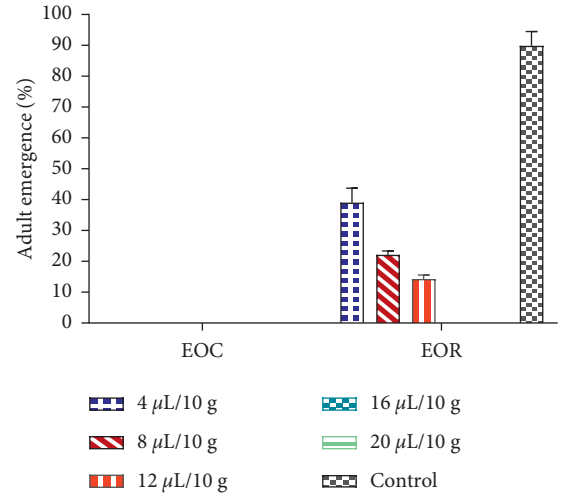

Figure 5: Effects of EOC and EOR on the emergence rate of *C. maculatus* adults.

3.2.4. Effect on Adult Emergence. The obtained results showed no *C. maculatus* adults' emergence in *Vigna unguiculata*. Seeds provisory treated with EOC regardless of the used dose; meanwhile, the total inhibition of *C. maculatus* adults' emergence by EOR was recorded for the hastiest dose used for testing 16 μL/10 g (Figure 5 and Table 5). Therefore, EOC exhibited higher inhibitory activity on the emergence rate of *C. maculatus* adults as compared to that of EOR.

4. Discussion

The obtained results showed that EOC was higher in carvacrol (70.88%), which is in agreement with a previously published study [14], reporting that carvacrol was the major constituent of EO extracted from *Origanum compactum* Benth. (43.97%). Additionally, other studies reported variable concentrations of carvacrol present in EO of *O. compactum* of 47.85% and 31.22% [15]. The current study, the result of which are reported here, found that 1,8-cineole (62.35%) was the major compound of EOR. Similarly, these findings were conforming to those reported by Ait-Ouazzou and co-workers [16], who showed that 1,8-cineole was the main constituent of the EO extracted from *R. officinalis* (43.99%).

Considering the insecticidal activity of the tested EOs against *C. maculatus*, the obtained results clearly indicated that EOC efficiently controlled *C. maculatus*, which is in agreement with previous reports [17], which revealed that EO of *O. compactum* possessed high insecticidal activity against *Spodoptera littoralis* larvae, with an LD_{50} of 0.05 mL/larva. Similarly, *O. compactum* possessed the insecticidal effect against adults of *Musca domestica* and *Mayetiola destructor* [17–19]. In this study, EOR was shown to be effective against *C. maculatus*, which is corroborated by findings reported by another previous work [20]. In that study, authors reported that EO of *R. officinalis* was bioactive against *C. maculatus*. Accordingly, Douiri and co-workers reported the insecticidal effect for EO of *Rosmarinus species* on *C. maculatus* males and females with LC_{50} varying from 5.51 to 2.43 μL/L air and 6.80 to 3.04 μL/L air, respectively [21].

In the present work, the insecticidal effect of the studied oils resulted in a significant reduction in the number of eggs laid per female. It is thus fitting to conclude that our results were comparable with those reported by Douiri and co-workers [22], who showed that EOs from *Asteraceae* species efficiently controlled *C. maculatus* potently impacting their fecundity, longevity, fertility (89.03–93.40%), and success rate (80–90%). In addition, LC_{50} was determined to be 2.5 and 23.3 μL/L of air for females and 2.56 and 46.07 μL/L for males. In this context, Bounechada et al. stated that the leaf powder of *Ocimum basilicum* completely abrogated the emergence of *Trogoderma granarium*, which

indicated that *Ocimum basilicum* might serve as an ecofriendly control agent specifically for this pest species [2].

Furthermore, it was reported that at a dose of 33.3 μL/L, the essential oils of *Melaleuca quinquenervia* and *Ocimum gratissimum* significantly reduced the oviposition of the *C. maculatus* females by 98.78% ± 0.87 and 99.94 ± 0.35%, respectively [23, 24]. In this study, the obtained results showed that EOs efficiently controlled the fertility of *C. maculatus* (hatching eggs). In this case, EOC completely inhibited the hatching of eggs laid by the female *C. maculatus*, regardless of the used dose. Meanwhile, the total inhibition of hatching eggs by EOR was obtained by the highest dose used. Specifically, at the dose of 400 μL, EOs extracted from *O. basilicum* and *O. gratissimum* inhibited the hatching of eggs of *C. maculatus* [25]. Similarly, Ketoh et al. stated that *C. schoenanthus* EO inhibited hatching egg and development of neonate *C. maculatus* larvae at the dose of 33.3 μL/mL [26]. In addition, EO of *Z. multiflora* has been previously reported to exhibit a strong insecticidal effect against eggs, larvae, and adults of *C. maculatus* [27].

Our results also showed that the tested EOs efficiently controlled the emergence of *C. maculatus* adults. EOC has completely prevented the total emergence of adults irrespective of the concentration used, whereas EOR prevented the emergence of *C. maculatus* when applied at the highest dose. Moussa Kéïta et al. [25] with a drop in the emergence of *C. maculatus* adults to 0 and 4% follow exposure to EOs of *Ocimum basilicum* and *Ocimum gratissimum*. It was also reported that the emergence of *C. maculatus* F_1 adults was significantly inhibited by EO of *Alpinia calcarata* at concentrations of 0.80 g/L using fumigant toxicity [28]. Similarly, the emergence of *C. maculatus* has been previously reported to be also controlled by *Allium sativum* [21].

EOs from aromatic plants exhibit a potent insecticidal effect by fumigation, contact, and repulsion assays [5, 8, 29]. EOs are known for their ovicidal, repellent, and insecticidal activities against various insects attacking stored products [29]. The mechanism of action (MOA) of EOs against insects was investigated by Renoz and co-workers who reported that EOs resulted in *Sitophilus granarius* death by altering a variety of key biological processes and activities, namely, muscular and neurological systems, cellular respiration, protein synthesis, development, reproduction, and insects' behavior [30]. Rajendran et al. reported that terpenoids have gained particular attention among other constituents of EOs because of their potent fumigant effect against stored grain insects [31]. In this context, it has been postulated that *C. maculatus* could absorb EOs along with their components, for example, terpenoids. Consequently, the toxicity of the tested EOs in the current study is hypothesized to be attributed to the presence of carvacrol [32, 33].

In the present work, the insecticidal activity of both EOC and EOR could be due to bioactive compounds identified in the oils, particularly carvacrol, which is known for its bioactivity including the insecticidal effect [34]. In the current study, carvacrol was found to be the major component in EOC with 70.88%, whereas 1,8-cineole was reported to be the dominant constituent in EOR with 62.35%. Taken together, these monoterpene compounds could be responsible for the biological activity of these EOs. Additionally, 1,8-cineole, borneol, and thymol have been previously reported to exert adverse toxicities against *S. oryzae* adults at the lowest dose (0.1 μL/720 mL volume), 24 h posttreatment by fumigation [35]. Camphor and linalool caused 100% mortality for *R. dominica* adults, and this has been attributed to monoterpenoids contained in EOs accounting for the observed insecticidal activity [4]. It was also reported that carvacrol, linalool, thymol, terpineol, and eugenol inhibited the emergence of *A. obtectus* adults [36, 37]. Citral and 1,8-cineole contained in EO were found to be ovicides and strong inhibitors of the emergence of adult houseflies. Eugenol and (−)-menthone powerfully inhibited adult emergence *C. maculatus* adults [38]. The MOA by which terpenes can exert this insecticidal effect have been reported in an earlier work [28]. EO constituents can operate synergistically or individually depending on which insect pest is being targeted. For example, the two components D-limonene and α-terpineol showed a synergistic toxicity against *Trichoplusia ni*, whereas no correlation was found with toxicity against *Spodoptera frugiperda*. The MOA underlying the synergistic interaction of 1,8-cineole and camphor, the major constituent of the EO of *R. officinalis* against *Trichoplusia ni*, has already been reported by Tak et al. [39], who showed that 1,8-cineole enhances the penetration of camphor into the blood circulation through the insect's body wall referred to as integument. The MOA of the reported insecticidal activity of EOs has been thoroughly investigated by Rattan and co-workers [40], who reported that EOs and their components, particularly thymol, result in insect death through the inhibition of acetylcholinesterase, thereby leading to its accumulation eventually causing hyperstimulation of nicotinic and muscarinic receptors and disrupted neurotransmission. Moreover, it might act by blocking the octopamine receptors through tyramine receptors cascade or by disruption of the octopaminergic system [40].

5. Conclusion

The obtained results revealed that the studied EOs efficiently controlled the insect life cycle, which could be attributed to its richness in specific bioactive monoterpenoid alcohols such as carvacrol. Taken together, the outcome of the present study highlights the benefits of the EOs extracted from *Origanum compactum* Benth. and *Rosmarinus officinalis* L. as effective ecofriendly pest-control agents. Further investigation is therefore warranted to evaluate the safety of these EOs and their nontarget toxicities against mammals and humans.

Data Availability

The data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Tinctoride A, a New Hopan-Type Triterpenoic Peracid from the Thallus of Lichen *Parmotrema Tinctorum* (Despr. ex Nyl.) Hale

Le-Thuy-Thuy-Trang Hoang[1], **Thanh-Nha Tran**[1], **Thi-Phi-Giao Vo**[2,3], **Hoang-Vinh-Truong Phan**[4,5], **Phan-Si-Nguyen Dong**[6], **Dinh-Tri Mai**[6,7], **Ngoc-An Nguyen**[6], **Thi-Minh-Suong Huynh**[8], **Hong Truong Luu**[9], **Tien-Dung Le**[10], **Minh-Trung Dao**[1], **and Van-Kieu Nguyen**[4,5]

[1]*Department of Environmental Engineering, Thu Dau Mot University, Binh Duong, Vietnam*
[2]*Department of Ecology-Evolutionary Biology, University of Science, 227 Nguyen Van Cu Str., Dist. 5, Ho Chi Minh City, Vietnam*
[3]*Vietnam National University Ho Chi Minh City, Linh Trung Ward, Thu Duc City, Vietnam*
[4]*Institute of Fundamental and Applied Sciences, Duy Tan University, Ho Chi Minh City 700000, Vietnam*
[5]*Faculty of Natural Sciences, Duy Tan University, Da Nang 550000, Vietnam*
[6]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam*
[7]*Institute of Chemical Technology, Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam*
[8]*University of Natural Resources and Environment Ho Chi Minh City, Ho Chi Minh City, Vietnam*
[9]*Southern Institute of Ecology, Institute of Applied Materials Science, Vietnam Academy of Science and Technology, 1D, TL29 Street, District 12, Ho Chi Minh City, Vietnam*
[10]*Institute of Applied Materials Science, Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam*

Correspondence should be addressed to Van-Kieu Nguyen; nguyenvankieu2@duytan.edu.vn

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A new hopan-type triterpenoic peracid, tinctoride A (1), together with three known compounds, zeorin (2), 6β,22-dihydroxyhopane (3), and ergosterol peroxide (4), was isolated from *Parmotrema tinctorum* (Despr. ex Nyl.) Hale. Their chemical structures were identified by extensive 1D and 2D NMR analysis and high-resolution mass spectroscopy and compared with those reported in the literature. The enzyme inhibitory potential of compounds 1–3 against α-glucosidase was investigated, exhibiting nil to weak inhibitory activity.

1. Introduction

Lichens, which are symbiotic organisms having the characteristics of mycobionts and fungi, comprise 17,000 species distributed from tropical to polar regions [1–3]. This unique ability produces various chemical constituents that are found in most of these organisms [4]. Previous chemical investigations of lichens have confirmed the presence of many kinds of aromatic secondary metabolites, such as depsides, depsidones, dibenzofuranes, xanthones, and anthraquinones [5]. Most of these compounds are unique to lichens, but some can also be obtained from fungi and plants [2]. Biological markers have indicated that lichens have potent antioxidant, anticancer, antiarthritic, anti-inflammatory, antihuman immunodeficiency virus (anti-HIV), and antiherpes simplex virus 1 (anti-HSV-1) activities [4, 5].

Parmotrema tinctorum (Despr. ex Nyl.) Hale (Parmeliaceae) (lichenised Ascomycota) is a foliose lichen growing abundantly in various countries, commonly on rocks and trees in moist tropical and temperate areas [6, 7]. The extract of *P. tinctorum* exhibits significant α-glucosidase, α-amylase, and aldose reductase inhibition [7], and some isolated

compounds manifest moderate in advanced glycation end product formation inhibition activities [8]. However, only a few publications have reported the chemical investigation of *P. tinctorum*, resulting in the isolation of some monoaromatic and aromatic compounds [8–12].

The present paper reports the results of the isolation and elucidation of a new triterpenoid (1) and three known compounds (2-4) from *P. tinctorum*. Moreover, the enzyme inhibitory activity of compounds 1-3 against α-glucosidase is described.

2. Materials and Methods

2.1. General Experimental Procedure. Column chromatography (CC) was performed on 60–120-mesh silica gel. The IR spectra were measured on Frontier FTIR/NIR spectrometers (Perkin Elmer, USA). NMR spectra (1D and 2D) were recorded on a Bruker Avance spectrometer at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. HRESIMS were recorded on a Bruker MicrOTOF-Q II mass spectrometer. Thin-layer chromatography was carried out on precoated Kieselgel 60 F_{254} or silica gel 60 RP-18 $F_{254}S$ (Merck). Spots were visualized by spraying with 5% vanillin in acidic aqueous solution, followed by heating.

2.2. Plant Material. The thalli of lichen *P. tinctorum* were collected in the Lam Dong province of Vietnam in May 2020. The scientific name of the plant was identified by Dr. Vo Thi Phi Giao, Faculty of Biology-Biotechnology, University of Science, National University, Ho Chi Minh City, Vietnam. A voucher specimen PHH0011338 was deposited in the Herbarium of the University of Science, Ho Chi Minh City Vietnam National University (PHH).

2.3. Isolation and Purification. The air-dried roots (2.5 kg) were extracted exhaustively with ethyl acetate (10 L × 3) and methanol (10 L × 3) at room temperature. The filtered solution was evaporated under reduced pressure to afford two crudes: E (105.2 g) and M (135.5 g). Crude E was washed with ethyl acetate to yield the liquid EL (23.5 g) and precipitate EP (75.8 g). EL was applied to Sephadex LH-20 and eluted with methanol to obtain EL1–3, and EL2 was then fractionated using C-18 silica gel CC with a gradient system of water-methanol (1 : 8), affording five fractions (EL2A–E). Fraction EL2D (16.6 g) was selected for further fractionation by silica gel CC, using a solvent system consisting of n-hexane/$CHCl_3$/ethyl acetate/acetone (4 : 0.2 : 0.2 : 0.2) to afford fractions EL2D1–D5. Subfraction EL2D3 (1.43 g) was rechromatographed by silica gel CC using an n-hexane/$CHCl_3$/ethyl acetate/acetone (5 : 0.2 : 0.2 : 0.2) solvent system, affording compounds 1 (1.6 mg) and 4 (25.0 mg). Fraction EL2D1 (0.63 g) was rechromatographed to afford compounds 2 (3.2 mg) and 3 (5.2 mg), by the same procedure used for EL2D3.

2.3.1. Tinctoride. A (1). Colorless gum; IR (KBr, cm^{-1}): 3564, 2925, 2849, 1739, 1709, 1459, 1374, 1247, 1032; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 4.66 (1H, dd, J = 4.8, 11.6 Hz, H-1), 4.58 (1H, dd, J = 4.4, 12.4 Hz, H-3), 2.36 (1H, m, H-21), 2.33 (1H, m, H-22), 2.02 (3H, s, H-34), 1.97 (3H, s, H-32), 1.89 (1H, m, H-2a), 1.86 (1H, m, H-20a), 1.66 (1H, m, H-2b), 1.62 (1H, m, H-20a), 1.54 (2H, m, H-6), 1.52 (1H, m, H-19a), 1.48 (2H, m, H-16), 1.47 (2H, m, H-9, H-12a), 1.45 (1H, m, H-11a), 1.43 (1H, m, H-20a), 1.40 (1H, m, H-7a), 1.32 (2H, m, H-12b, H-13), 1.31 (2H, m, H-15), 1.30 (1H, m, H-11b), 1.23 (1H, m, H-17), 1.20 (1H, m, H-7b), 1.13 (3H, d, J = 6.4 Hz, H-29), 1.00 (3H, s, H-25), 0.94 (3H, s, H-26), 0.90 (3H, s, H-27), 0.89 (1H, m, H-19b), 0.83 (6H, s, H-23, H-24), 0.75 (1H, d, J = 2.8, 11.2 Hz, H-5), 0.70 (3H, s, H-28); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 182.0 (C-30), 170.5 (C-33), 170.3 (C-31), 80.5 (C-1), 76.5 (C-3), 53.8 (C-17), 52.9 (C-5), 50.8 (C-9), 48.7 (C-13), 44.4 (C-18), 42.7 (C-21), 42.3 (C-22), 42.3 (C-8), 42.1 (C-14), 42.1 (C-10), 41.0 (C-19), 37.9 (C-4), 33.5 (C-15), 33.1 (C-7), 30.1 (C-2), 28.0 (C-23), 26.7 (C-20), 23.9 (C-12), 23.0 (C-11), 21.9 (C-34), 21.2 (C-32), 19.9 (C-16), 17.9 (C-6), 17.8 (C-29), 17.0 (C-26), 16.7 (C-27), 16.2 (C-24), 15.8 (C-28), 13.0 (C-25); HRESIMS m/z: $[M + Na]^{+597.3794}$ for $C_{34}H_{54}O_7Na$ (calcd. 597.3767).

2.4. α-Glucosidase Activity Test. The α-glucosidase inhibitory activity of the compounds was investigated using a method from Wan et al. [13]. All samples were examined in triplicate at different concentrations to obtain the IC_{50} value of each compound. The mean values and standard deviations were retained. Briefly, 120 μL of extract were preincubated with 20 μL of α-glucosidase (1 unit/mL) in 0.1 M potassium phosphate buffer (pH 6.8) at 37°C for 15 min. The reaction was then initiated by adding 20 μL of 5 mM para-nitrophenyl-α-D-glucopyranoside (PNPG) in 0.1 M potassium phosphate buffer, and the mixture was further incubated for 15 min. The reaction was terminated by the addition of 80 μL of 0.2 M Na_2CO_3 in 0.1 M potassium phosphate buffer, and the absorbance of the mixture was recorded at 405 nm. The results were expressed as % inhibition of enzyme activity and calculated according to the following equation:

$$\text{Inhibition}\,(\%) = \left[1 - \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right)\right] \times 100. \quad (1)$$

3. Results and Discussion

3.1. Structural Elucidation. The ethyl acetate extract of *P. tinctorum* was separated by CC over silica gel normal phase and reversed-phase RP C-18, and sephadex LH-20 to afford a new triterpene, tinctoride A (1), and three known compounds, zeorin (2) [14], 6β,22-dihydroxyhopane (3) [15], and ergosterol peroxide (4) [16] (Figure 1).

Compound 1 was purified as a colorless gum with molecular formula $C_{34}H_{54}O_7$, as assumed by the sodiated molecular ion peak at m/z 597.3794 (calcd. for $C_{34}H_{54}O_7$ + Na, 597.3767) on the HRESI mass spectrum. The ^{13}C NMR and HSQC spectra of 1 revealed 34 carbon signals, including 9 methyl, 9 methylene, 8 methine, and 8 quaternary carbons. In addition, the ^{1}H NMR spectrum showed six singlets (δ_H 1.00, 0.94, 0.90, 0.83 × 2, 0.70) and one doublet (δ_H 1.13, d, 6.4), and the key HMBC correlations were observed from six methyl groups, H_3-23 (δ_H 0.83), H_3-24 (δ_H 0.83), H_3-25 (δ_H 1.00), H_3-26 (δ_H 0.94),

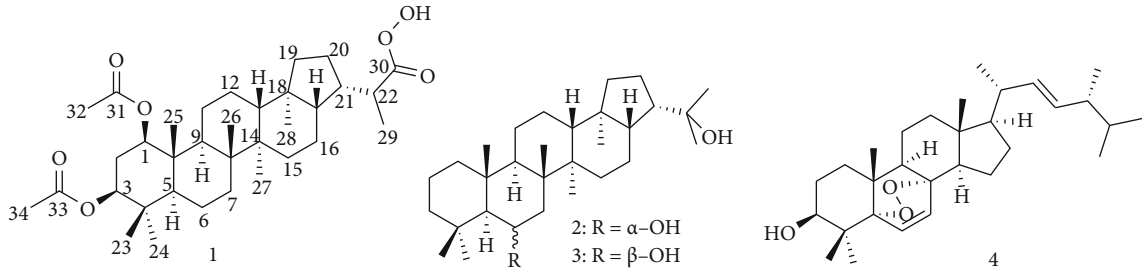

Figure 1: Chemical structure of compounds 1-4.

H_3-27 (δ_H 0.90), and H_3-28 (δ_H 0.70), to their attached quaternary carbons C-4 (δ_C 37.9), C-4, C-10, C-8 (δ_C 42.3), C-14 (δ_C 42.1), and C-18 (δ_C 44.4), respectively, suggesting that 1 was a hopan-type triterpenoid [17], related to the known cometabolites 2, 3. In addition, the HMBC correlations from the methyl protons at δ_H 2.02 (H_3-34) to C-33 (δ_C 170.5) and δ_H 1.97 (H_3-32) to C-31 (δ_C 170.3) suggested the occurrence of two acetoxycarbonyl groups located at C-1 and C-3, respectively, which was further backed up by the HMBC crosspeak of the oxymethine proton H-1 (δ_H 4.66) to C-31 and H-3 (δ_H 4.58) to C-33. Furthermore, the key HMBC of the doublet methyl H_3-29 (δ_H 1.13, d, $J = 6.4$ Hz) to C-21 (δ_C 42.7), C-22 (δ_C 42.3), and C-30 (δ_C 182.0) indicated that a methyl group belonging to the isopropyl moiety located at C-21 (ring E) was oxidated to a carboxyl function (Supplementary material Figure S1). The FTIR spectrum of compound 1 also revealed the presence of free OH, OH stretching, C=O stretching, and OH bending vibrations of the peracid functional group at 3564, 3293, 1739, and 1459 cm^{-1}, respectively [18], indicating a peracid moiety located at C-30, which was further clearly evidenced by the value of its chemical shift at δ_C 182.0, instead of approximately 184 ppm in the case of an acid function [15], and also by the molecular formula $C_{34}H_{54}O_7$ from the HRESI mass spectrum. In addition, the chromatography experiment between 1 and the crude fraction revealed that 1 was a minor substance in *P. tinctorum* (Supplementary material Figure S2).

As to the relative stereochemistry of C-1 and C-3, the elevated coupling constant of H-1 ($J_{H-1,H-2} = 11.6$, 4.8 Hz) and H-3 ($J_{H-2,H-3} = 12.4$, 4.4 Hz) determined the axial position of both of these oxymethine protons. Furthermore, the syn-orientation (β-orientation) of H-1 (δ_H 4.66), H-3 (δ_H 4.58), and H-5 (δ_H 0.75) was definitely indicated by pair-to-pair NOESY interactions (Supplementary material Figure S1). From all the above data, compound 1, namely, tinctoride A, was readily elucidated as 1β,3β-diacetoxy-21α-hopan-29-oic peracid.

3.2. α-Glucosidase Inhibition Assay. The enzyme inhibition of compounds 1-3 against α-glucosidase was evaluated (Supplementary material Table S1). Triterpenoids 1–3 exhibited weak (compound 2, IC_{50} 258.87 μM) or no (compounds 1 and 3) α-glucosidase inhibitory activity compared with acarbose (positive control, IC_{50} 108.08 μM). Among these compounds, 2 showed the highest α-glucosidase inhibitory activity, similar to that reported in the previous study [19], while the 6β-OH orientation of compound 3 led to a decrease in its α-glucosidase inhibitory activity, in spite of its molecular formula being closely related to compound 2. It is worth noting that the stereochemistry of C-6 hydroxyl substitutions in hopan-type triterpenoids could play an important role in their α-glucosidase inhibitory activities. The extract of *P. tinctorum*, however, displayed significant α-glucosidase inhibition [7], which indicates that the hopan-type triterpenoids are not the most potent α-glucosidase inhibitory component in the ethyl acetate fraction of *P. tinctorum*. Due to the reports of various phenolic compounds [6, 8, 9, 11, 12], further isolation of other compounds from the ethyl acetate fraction, such as phenolic compounds or other types of triterpenoids, will be important research for the discovery of active compounds against α-glucosidase.

4. Conclusions

Three hopan-type terpenoids, including one new peracid, tinctoride A, and two known compounds, zeorin (2) and 6β,22-dihydroxyhopane (3), together with ergosterol peroxide (4), were isolated from the lichen *P. tinctorum*. Their chemical structures were identified by extensive 1D and 2D NMR analysis and high-resolution mass spectroscopy and compared with those reported in the literature. To the best of our knowledge, compound 1 is a new peracid, while compounds 2–4 have not previously been isolated from *P. tinctorum*. Compounds 1-3 displayed nil or weak inhibitory activity against α-glucosidase.

Data Availability

The data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

Acknowledgments

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Supplementary Materials

Supplementary material relating to this article is available online, alongside Table S1 and Figures S1–S10. *(Supplementary Materials)*

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Review Article

Ethnobotanical Uses, Chemical Constituents, and Application of *Plantago lanceolata* L.

Limenew Abate[1,2] **Rakesh Kumar Bachheti**[1,2] **Mesfin Getachew Tadesse**[2] **and Archana Bachheti**[3]

[1]*Centre of Excellence in Nanotechnology, Ethiopia*
[2]*Department of Industrial Chemistry, Addis Ababa Sciences and Technology University, P.O. Box 16417, Addis Ababa, Ethiopia*
[3]*Department of Environment Science, Graphic Era (Deemed to be University), Dehradun-248002, Uttarakhand, India*

Correspondence should be addressed to Rakesh Kumar Bachheti; rkbachheti@gmail.com

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The medicinal benefits of *P. lanceolata* L. have been acknowledged worldwide for hundreds of years. The plant is now distributed worldwide, especially in temperate zones. This review gives an overview of ethnomedicinal use, phytochemistry, pharmacological activities, and other potential application of *P. lanceolate* L. Several effective chemical constituents such as polyphenols, tannins, flavonoids, alkaloids, terpenoids, iridoid glycosides, fatty acids, and polysaccharides are found in *P. lanceolata* L., which contribute to its exerting specific therapeutic effects. Correspondingly, studies have found that *P. lanceolata* L. has different biological activities, including antioxidant, antibacterial, wound-healing, anti-inflammatory, cytotoxic, and antiulcerogenic activity. The plant also treats various diseases related to the skin, respiratory organs, digestive organs, reproduction, circulation, cancer, pain relief, and infections. The plant has many applications in cosmetics such as lotion and creams; it is also used as an excellent indicator to know the presence and absence of heavy metals and the accumulation in industrial and urban areas. The plant suppresses soil nitrogen mineralization in agriculture due to allelochemicals such as aucubin. The biological activities, medicinal properties, and industrial application of *P. lanceolata* mainly depend on the activities of the responsible, active chemical constituents. However, this field still needs more study to determine the exact mechanisms and the main bioactive compound activity accountable for these activities. Also, most of the studies have been performed in vitro, so further in vivo studies are recommended for the future.

1. Introduction

Under the plant kingdom, medicinal plants have been mainly used by local peoples found in developing countries, especially in resource-limited areas. Many peoples in this region directly or indirectly use medicinal plants to satisfy their primary health care needs [1]. Consumers' interest in using herbal products for personal and health care has grown worldwide [2]. From the World Health Organization's (WHO) perspective, medicinal plants (MP) have become popular. Approximately 4000 million people utilize herbal remedies regularly [3]. Phytomedicines, derived from seeds, roots, leaves, fruits, bark, seeds, and flowers of medicinal plants, can treat diseases [4]. Many researchers have given more attention to medicinal plants because they can generate many uses and applications in medicine and pharmacy [5]. It is estimated that half of the pharmaceutical drugs are derived from medicinal plants due to their capacity of the chemical constituents that bring therapeutic effects [6].

Plantago is a genus of medicinal plants belonging to the Plantaginaceae family [7]. It has around 275 species that grow annually and permanently [8]. Its name comes from the Latin "planta," meaning "sole," to represent the broad leaves lying touching the ground [9]. It is known for its pharmaceutical activities [10]. *Plantago* has a wide range of uses, including raw materials for salads, soups, baking, and animal feed to improve health and reduce antibiotic use

[8]. Phytochemicals derived from root, leaf, and stem of genus Plantago have shown medicinal potential [11]. *P. major* L., *P. halepensis* Miller, *P. lentiscus* D., *P. trimula* L., and *P. lanceolata* L. are the most common species [12].

P. lanceolata L. is a well-known species of the genus *Plantago*; it is widely distributed in meadows, roadside strips, pastures, and green areas in the temperate world 800 m above sea level [13, 14]. It has been used for medicinal purposes to treat diseases such as wound healing, inflammation, cancer, respiratory system disorder, blood circulation, reproductive system, and digestive organs [13]. It has various applications as cosmetics [15], as metal removal from polluted areas [16], as an additive in foods [17], and as an insecticide [18]. The extracts of the plant also showed different properties as antioxidant [19], antibacterial [20], anti-inflammatory [21], rheological [22], and viscoelastic [22] (Figure 1). Phytochemicals in the root, leaf, and seed of *P. lanceolata* L. include iridoid glycosides, polyphenols, polysaccharides, and flavonoids, which have therapeutic potential [11].

The available information about *P. lanceolata* L. is scattered and not all in one site. There is much literature on ethnomedicine, phytochemistry, and pharmacological activities of *P. lanceolata* L. The current review brings together all of the disparate information on the various possible applications of extracts and bioactive compounds obtained from *P. lanceolate* L. in one location.

2. Materials and Methods

Published research papers, review papers, proceedings, short communications, and book chapters describing *P. lanceolate* L. or *Ribwort plantain* are the primary information for writing this article. More than 100 publications were obtained from 1993 to 2021. In the search process, keyword phytochemistry of *P. lanceolata* L, traditional medicinal use of *P. lanceolata* L., ethnomedicinal use of *P. lanceolata* L., and bioactive compounds isolated from the different parts of the plant, history, and distribution about the plant were used. We classified the data according to ethnomedicinal, pharmacological activities, phytochemistry, and application of *P. lanceolata* L. ChemDraw was used to draw the structure of bioactive compounds, while EndNote performed reference writing. We use the Natural products database for Africa (NDA) to write the botanical name and the local name of the medicinal plant.

3. History and Distribution

P. lanceolata L.is an international species distributed in European countries from Iceland found south and east of Spain and Asia's Northern and Central parts. Historically, the plants originated from the Eurasia continent; however, they slowly expanded worldwide, including the colonizers from Europe. Historically, *P. lanceolata* L. for medicinal purposes started from ancient Greeks and Roman peoples [21]. Also, in the country China, the plants were used 3000 years ago [17]. Most of the time, this plant is considered a weed and wild plant, but it is the most cultivated plant, and in small amounts, it is also cultivated in Romania. However, it is a new crop in the UK [15]. Even though the species are common and native to Europe, the North part of Africa, the south and west part of Asia, and Europe [23], currently, they occur in every aspect of the world, such as the USA, Australia, New Zealand, Japan, and in many countries of Africa [23]. It became cultivated in temperate zones and naturalized in many continents except Antarctica [24].

4. Ethnomedicinal Use of *Plantago lanceolate* L.

Different people use *P. lanceolata* L. leaf as emollient, demulcent, and expectorant. It is effective for dysmenorrhea, abdominal pain, laxatives, and astringents [10]. The inflamed wounds can be treated by applying the leaf powder of *P. lanceolata* L. [15]. It effectively stops bleeding and encourages the treatment of damaged tissue [9]. In church ceremonies, the leaves of *P. lanceolata* L. were utilized as incense smoke. This plant's inflorescences were combined with Helichrysum stalks and burned to perfume clothes and rooms [17]. The seeds of *P. lanceolata* L. are essential in treating parasitic worms; the mucilage from the plant is used as a laxative and alleviates irritated membranes. Eye lotion is highly treated with distilled water obtained from the whole parts of the plant [9]. Eye illness wound repairing, antibacterial, anti-inflammatory, antiasthmatic, and diuretic properties are also treated by the aerial parts of *P. lanceolata* L. [25]. Mixing juice from the plant with honey or wine relieves gout, and arthritis can be treated by consuming crushed leaves with salt. It is also used as a topical application for skin diseases [8]. Ethnomedicinal uses of various parts of *P. lanceolata* L. in different countries have been summarized in Table 1.

5. Pharmacological Activities

Many studies have investigated the cytotoxic, antispasmodic, antibacterial, antioxidant, anti-inflammatory, and wound healing effects of different portions of the *P. lanceolata* L. (Table 2) [31–34]. Methanolic, 30% acetonitrile, 80% methanol, 80% ethanol, and hot water extract of leaves, roots, flowers, fruits, and seeds of *P. lanceolata* L. had been studied for their bioactivities. These extracts showed strong, cytotoxic, antiobesity, anti-inflammatory, wound healing, antioxidant, and antimicrobial effects [35].

5.1. Antioxidant Activities. Different studies were conducted to test the antioxidant activities of the *P. lanceolata* L. extracts using different antioxidant methods such as cupric reducing antioxidant capacity (CUPRAC), oxygen radical absorbance capacity (ORAC), dimethyl-4-phenylenediamine (DMPD), ferric reducing antioxidant power (FRAP), 2, 2′ -azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), oxygen radical absorbance capacity (ORAC), and DPPH (2,2-diphenyl-1-picrylhydrazyl) β-carotene bleaching method [27]. Various extraction procedures were employed to get extracts from the aerial portion of *P. lanceolata* L. including supercritical fluid extraction (SFE), ultrasound-assisted

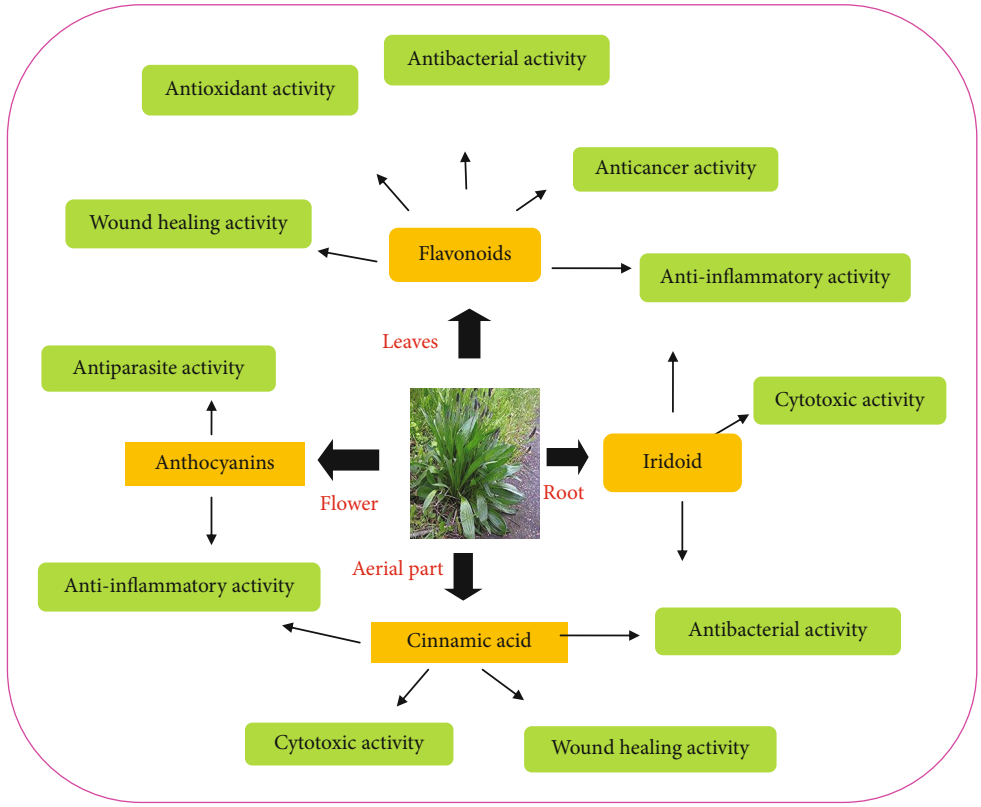


Figure 1: Biological activity of *P. lanceolate* L.

extraction (UE), and Soxhlet (SOX). The antioxidant activities of these extracts were examined for antioxidant activities according to linoleic acid/β-carotene and DPPH assays. The results showed that the plant has a strong antioxidant potential [36]. In another study, the aerial part of the plant extract was also performed for antioxidant activities using some antioxidant assays such as reducing power (FRAP assay), lipid peroxidation, superoxide anion and NO scavenger capacity, hydroxyl radical, and DPPH radical. The findings suggest that *P. lanceolata* L. has antioxidant properties comparable to the synthetic antioxidant BHT [21]. Furthermore, using different solvent extractions such as aqueous, methanol, and ethanol leaf extracts of the plant also showed antioxidant activity potential based on the result obtained from antioxidant assays [36, 37]. Acidified methanol ((80%) and HCl (1%)) extracts of root, stem, flower, and fruit parts of *P. lanceolata* L. exhibited significantly higher antioxidant capacities compared to the value of the plant *M. neglecta*. Methanol (60%) extracts from the seeds of the plants also showed an antioxidant behavior using assays like DPPH, OH radical scavenging, and cellular antioxidant activity [4].

P. lanceolata L. antioxidant activity is influenced by several factors. According to research, the extracted solvent affects antioxidant activity. For example, in one study, ethanol extracts were found to have a stronger antioxidant capacity than water and methanol extracts [28]. Parts of the plant such as aerial, leaves, root, and flower also affect the antioxidant behavior. The type and concentration of phytochemicals responsible for antioxidant activities vary from one part of a plant to another [8]. Different solvents have different free radical scavenging activities on the same part of the plant. For instance, methanol, acetone, ethyl acetate, chloroform and n-hexane leaf extracts had the value of IC50 1.81, 2.02, 0.56, 0.41, and 0.41 μg/m, respectively, based on DPPH assay [1]. The antioxidant properties of herbal products are mainly attributed to phenolic compounds such as flavonoids and polyphenolic derivates (cinnamic acid, p-coumaric acid, syringic acid, vanillic acid, and salicylic acid), compounds that are found in the leaves of *P. lanceolata* L. [21]. Antioxidant behaviors of different parts of the *P. lanceolate* L. with their assay are listed in Table 3.

5.2. Antimicrobial Activities. Medicinal herbs, shrubs, and trees and their products have shown the potential with antimicrobial agents [42]. A study was conducted to show the effects of the extracts of *P. lanceolata* L. on antibacterial activities against *monocytogenes*, *Streptococcus*, *S. aureus*, *Salmonella*, and *E. coli* species. The agar disc diffusion method showed that the leaf extracts of the plant have better antibacterial activity against selected bacterial pathogens [20]. *P. lanceolata L.* leaf extracts also showed antibacterial activity against *S. pneumoniae*, MRSA, *S. aureus*, *S. boydii*, *E. coli*, and *K. pneumoniae* using various solvents such as water, methanol, and acetone [43]. The antibacterial tests on leaf extracts of *P. lanceolata* L. were also done against some bacterial species like *K. pneumoniae*, S. boydii, *E. coli*, *S. pneumoniae*, *MRSA*, and *S. aureus*; the result showed that a higher degree of antimicrobial activity was observed with

Table 1: Ethnomedicinal use of *P. lanceolata* L.

Parts of the plant	Ethnomedicinal use	Country	Reference
Aerial parts	(i) Treatment of decoction internally, embolism, diarrhea for children, infusion cough, expectorant	Turkey	[4]
Leaves	(i) Bath with the infusion of the plant with *R. canina* to treat infertility (ii) Fresh leaves spread on a cloth and heated and put on the skin can reduce poultice wound healing. The mixture of water plants, flour, and black pepper used for decoction internally (iii) When the flour of the plant is added in boiled water, treat colds (iv) Treat hemorrhoids, cancer, disorders like gynecological decoction if it is eaten cooked and decoction as tea (v) If the leaf is mixed in boiled milk, decrease muscle pain, stop diabetes, kyphos, coughs, and menstrual aches	Algeria	[25]
	(vi) Small leaves are inserted in the nostrils to heal a headache	South Africa	[26]
	(vii) Used for discontinuing too much bleeding and wound repairing, anti-inflammatory, cough medication, antibacterial cause, sore throat treatment, and antidiarrheal	South Africa	[1]; [27]
	(viii) Used to treat insect and snake bites, cervicitis, rectal fissures, hemorrhoids, cuts, and abscess (ix) Used for wound repairing, intestinal and internal disorder, stomach pain, maturation of an abscess, diabetes, burn treatment, shortness of breath	Duzce	[28]
	(x) Infusion is essential for emollient, expectorant, and demulcent (xi) Burned wounds can be treated when powdered leaves are applied (xii) The heated leaves are essential for wet dressing for wounds and swellings	Pakistan	[10]; [9]
	(xiii) To alleviate the problems on external animal skin parasites, use ground leaves or juice from fresh leaves	Poland	[17]
	(xiv) Prepare an infusion of the leaves; then, use it to wash the eyes	Island of Mauritius	[3]
	(xv)The juice of the freshly squeezed leaf is pasted with butter and made into ointment	Ethiopia	[29]
Root	(i) Root juices are used to cure earache	South Africa	[30]
	(ii) The mixture of its root with the root of *M. vulgare* with equal amounts is a medicine for the bite of rattlesnakes	Not specified	[9]
Seed	(i) Extracts used as purgative and laxative	Pakistan	[10]
	(ii) Seeds were also commonly used as a natural laxative due to their high content of fibers, also having external uses for skin inflammations and wound healing, and also used as a rubefacient	Baghdad-Iraq	[24]

MIC and MBC values in the range of 6.25 to 25%, respectively [37]. In another research, methanol extract of leaves of *P. lanceolata* L. was found to inhibit *S. aureus* and *P. mirabilis* more than ethanol extract. However, the ethanol extract displayed better activity than the methanol extract against *E. coli* and *K. pneumoniae* [31]. The value of antibacterial activities (inhibition zone) can be affected by the type of solvent used to extract bioactive compounds, type of bacteria species, and parts of the plant (Table 4) [33].

5.3. Wound Healing Activities. Different practices have been used for centuries to treat injuries due to burning. Among those, 1/3 of medicinal plants have been used for wound healing caused by burning. Studies performed on extracts obtained from the leaves of *P. lanceolata* L. have shown a wound healing effect [45]. Aqueous and methanol extract of *P. lanceolata* L. showed wound healing potential by reducing the levels of TBARs in mice and rats. Furthermore, *P. lanceolata* L. was revealed to have the ability to enhance tissue Zn^{+2} and Cu^{+2} levels, both of which are essential indicators in the wound healing formation process [46]. The aqueous aerial parts of *P. lanceolata* L. also showed wound healing activities on 48 injured rats. The burned surface area of rats decreases by 10% when the extracts are placed on the surface [47]. In another research, wound healing activities were also observed when P. *lanceolata* L. extracts were applied to the skin of donkeys and Sprague-Dawley rats [48, 49].

5.4. Anti-Inflammatory Activities. The biological answer for the immune system caused by different factors such as pathogens, cell damage, cut, and compounds that cause toxicity is termed inflammation [17]. The disorders like gastritis, tumors, arthritis, atherosclerosis, and others involve inflammation in their progress [34]. Different studies have been carried out to assess the anti-inflammatory properties of different parts of *P. lanceolat*e L. The anti-inflammatory efficacy of methanol extract aerial parts of the plant was investigated using COX-1 and 12-LOX inhibition. The result confirmed that COX-1 inhibitory activity (IC50) was 2.00, and for that of 12-LOX, the inhibitory activity (IC50) was 0.75 [21]. In vivo anti-inflammatory activities of *P. lanceolata*L. dichloromethane extract were examined using an in vitro enzymatic assay. The result indicated anti-inflammatory characteristics in mice using 160 mg/kg, 80 mg/kg, and 40 mg/kg [34].

Table 2: Antioxidant behaviors of different parts of the plant.

Plant's part (s)	Solvent used	Concentration of solvent	Response to antioxidant assay	Reference
Whole plant	Ethanol	80%	1100 μmol Fe^{2+}/g DW in FRAP, and for ORAC, it is 3500 μmol TE/g DW	[38]
	Mixture of methanol and hydrochloric acid	80%:1%	For FRAP =201.4 μmol Fe^{2+}/g DW and for ORAC = 930.5 μmol TE/g DW,	[4]
Aerial	Methanol	80%	IC_{50} = 4.20 μg/ml in DPPH, IC_{50} = 236.12 μg/ml in hydroxyl radical scavenging, IC_{50} = 23.85 superoxide anion scavenging IC_{50} = 24.83 for lipid peroxidation.	[21]
	Ethanol	80%	1100 μmol Fe^{2+}/g DW in FRAP and 3500 μmol TE/g DW for ORAC	[38]
	Mixture of methanol and water	40%:1%	IC_{50} = 24.83 mg/mL in lipid Peroxidation & FRAP= 109.80 mg of AAE/g of DW	[21]
Leaves	Acetonitrile	30%	DPPH radical inhibition at 25 μg/ml is 12.77% to 15.78%	[39]
	Mixture of methanol with hydrochloric acid	80%:1%	130.4 μmol Fe^{2+}/g DW	[4]
	Water and dilute hydrochloric acid	…..	29.39 = DPPH in %, 137.83 in FRAP μM TE/5 mg	[40]
Root	Methanol and hydrochloric acid	80%:1%	190.1 μmol Fe^{2+}/g DW	[4]
Fruit		80%:1%	255.2 μmol Fe^{2+}/g DW	
Seeds	Methanol (60%)	60%	118.58 in μmol TE/g in DPPH 499.53 μmol TE/g in hydroxyl radical scavenging assays 27.00 μmol QE/g in cellular antioxidant activity assay	[41]
Flower	Methanol and hydrochloric acid	80%:1%	369.1 μmol Fe^{2+}/g DW	[4]

5.5. Cytotoxic Activity. A study was conducted to test the cytotoxic activities of *P. lanceolata* L. extract using an MTT assay. The result confirmed that chloroform leaf extract of the plants showed a good cell feasibility report in the range of 100% to 75.35% on the mouse leukemic macrophage cell line (RAW 264.7). The secondary phytocompounds like terpenoids and phenols could be responsible for this cytotoxicity effect [1]. The cytotoxicity activities of aerial part of *P. lanceolata* L. extracts were also conducted on human cell line such as MRC-5, HT-29, MCF7, and HeLa. The result showed a stronger cytotoxic activity due to some bioactive compounds such as gallic acid, luteolin-7-O-glucoside chlorogenic, apigenin, and vanillic [21]. The cytotoxic effects of aqueous leaf extracts of *P. lanceolata* L. on MCF-7 cells were investigated, and the results revealed that the plant's leaf extracts decreased MCF-7 cell proliferation [50]. In another study, bioactive compounds from methanol extract of the plant showed the cytotoxic activities on MCF-7 with the value of GI_{50} = 114.45, TGI > 240, and LC_{50} > 250 [32, 51]. This showed that methanolic *P. lanceolata* L. leaf extracts exhibit cytotoxicity against breast cancer cell lines. The result also showed that the leaf extract of *P. lanceolata* L. decreased the proliferation of CAL51 triple-negative breast cancer cells but had only a minor effect on MCF7, AMJ13, and MDAMB breast cancer cells.

5.6. Antispasmodic Activity. Plantago species have been found to have a wide range of biological activities, including cytotoxic, anti-inflammatory, antioxidant, and antispasmodic properties [52]. The aerial parts of *P. lanceolata* L. was examined for antispasmodic activity on isolated ileum and trachea of the guinea-pig [21]. The result indicated that the *P. lanceolata* L. extract suppressed the contractions of the guinea-pig ileum generated by diverse compounds such as acetylcholine, histamine, potassium, and barium ions. The compounds aucubin, lavandulifolioside, isoacteoside, catalpol peracetate, plantamajoside, acteoside, and luteolin (Figure 2 and Table 2) inhibited the ACh-induced contractions of the guinea-pig ileum [31]. Flavonoids also possess antispasmodic activities for *P. lanceolata* L. [21].

6. Phytochemistry of *P. lanceolata* L.

A study showed that different concentrations of bioactive compounds such as flavonoids [58], coumarins [59], lipids and cinnamic acids [60], and tannins [61] are found in the whole or separated parts of *P. lanceolata* L. such as flowers, leaves, and roots. For instance, in the whole part of the plant, the average amount of the main classes of compounds: flavonoids, coumarins, lipids, cinnamic acid content, and phenolic content were 358, 9, 1120, 200, and 1368 μg/g of DW, respectively [54]. The levels of total phenolics and various groups of phenolic compounds in *P. lanceolata* L. extracts ranged from 12.2 ± 1.7 (stem) to 35.3 ± 2.8 (leaf) mg GAE/g DW [4]. The following subsections explain some bioactive compounds found in *P. lanceolata* L.

6.1. Phenolic Compounds. Different types of phenolic compounds are recently reported in *P. lanceolata* L. Some of these compounds are 3,4-dihydroxyphenylacetic acid, (+)-catechin, pyrocatechol, vanillin, verbascoside, epicatechin, taxifolin, hesperidin, rosmarinic acid, pinoresinol,

Table 3: Antibacterial properties of different solvent extracts of *P. lanceolata* L. on gram-positive and negative bacteria species based on inhibition zone response.

Microorganism	Solvent used	Concentration of solvent	Inhibition zone(mm)	Reference
S. aureus	Chloroform	……	8	[20]
	Acetone	95%	16.3	[43]
	Methanol	95%	17.3	
	Ethanol	……	14	[20]
	Methanol	…..	15.8	
	Water	……	16.3	[43]
	Methanol	70%	8	
	Petroleum ether	20 μl	23	[5]
	Chloroform /methanol	20 μl	23	
E.coli	Methanol	70%	6	[20]
	Chloroform /methanol	20 μl	24	[5]
	Ethyl acetate extract	…..	8	[33]
	Chloroform	…..	7.6	[44]
	Petroleum ether	20 μl	24	[5]
	Acetone	95%	12.7	
	Methanol	95%	15	[43]
	Water	…..	13.3	
	Ethanol	…..	15	[44]
S. pneumoniae	Acetone	95%	10.7	
	Methanol	95%	10.3	[20]
	Water	…..	13.6	
S.boydii	Acetone	95%	16	
	Methanol	95%	16.6	[43]
	Water	…..	15.3	
K. pneumoniae	Acetone	95%	17	[43]
	Ethyl acetate	…..	8	[33]
	Methanol	95%	26	[43]
	Water	…..	15.7	
C. albicans	Acetone	…..	8.7	
	Methanol	…..	15.3	[43]
	Water	…..	22	
S. aureus	Methanol	70%	9	[20]
L. monocytogenes	Methanol	70%	16	
S. agalactiae	Petroleum ether	20 μl	17	
	Chloroform /methanol	20 μl	19	[5]
	Methanol	20 μl	16	
P. mirabilis	Methanol	…..	8	[33]
P. aeruginosa	Chloroform	50mg	9	[5]
	Ethanol	50mg	16	

eriodictyol, and kaempferol (Figure 3) [62]. Different concentrations of phenolic bioactive compounds such as gallic acid ($18 \pm 1\,\mu$g/g), protocatechuic acid ($92 \pm 0.2\,\mu$g/g), 3,4-dihydroxyphenylacetic acid ($9 \pm 0.2\,\mu$g/g), caffeic acid ($156 \pm 4\,\mu$g/g), vanillic acid ($90 \pm 1\,\mu$g/g), syringic acid ($31 \pm 1\,\mu$g/g), and vanillin 26 ± 2 (μg/g) were found in the methanolic extract of *P. lanceolata* L. [35]. In other studies, the concentration of bioactive compounds like gallic acid (2.73 mg/g), protocatechuic acid (24.11 mg/g), vanillin (9.18 mg/g), p-coumaric acid (61.16 mg/g), kaempferol (43.64 mg/g), luteolin (5.35 mg/g), apigenin (8.27 mg/g) [63], p-hydroxybenzoic acid (149.46 mg/g), 2,5-dihydroxybenzoic acid (16.20 mg/g), protocatechuic acid (103.48 mg/g), vanillic acid (411.52 mg/g), gallic acid (212.01 mg/g), apigenin

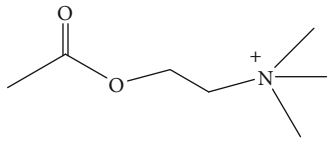
Table 4: Pharmacological activities of different parts of *P. lanceolata* L.

Part used	Pharmacological activities	Bioactive compounds	Reference
Aerial part	Antioxidant, anti-inflammatory and cytotoxic activities	P-hydroxybenzoic, vanillic, gallic acid, chlorogenic acid, apigenin, luteolin and luteolin-7-O-glucoside	[21]
Aerial parts	Wound healing, eye infections	Not identified	[25]
Aerial parts and leaves	Wound healing activity	Iridoidglucosides	[49]
Aerial parts and leaves	Cytotoxic activity	Flavonoids	[21]
Aerial parts, leaves, seeds and whole plan	Anti-inflammatory activity	–	[21, 53]
Flowers	Antimicrobial and antioxidant activities	Flavonoids and anthocyanin	[54]
Flowers, leaves, and roots	Antimicrobial activity	Phenolics and flavonoid	[21]
Flowers, leaves, and roots	Antimicrobial activities	Flavonoids, coumarins, lipids, cinnamic acids, lignans, and phenolic compound	[54]
Leave	Cytotoxic activity	Luteolin-7-*O*–glucoside	[32]
Leave	Anti-inflammatory	Polyphenols	[53]
Leave	UV-protecting and fibroblast growth-stimulating activity	Phenolic and flavonoid compounds	[55]
Leave	Antimicrobial activity	Polyphenolic compounds and flavonoids	[8]
Leave	Antioxidant activity	Flavonoids, tannins and saponins	[1]
Leave	Anti-inflammatory activity	Aucubin and flavonoids	[53]
Leave	Anti-ulcer activity	Polyphenol and flavonoids	[21]
Leave	Free radical scavenging activities	Polyphenolic compounds, iridoid glucosides, phenylethanoid glycoside, aucubin and flavonoids	[17]
Leave, flower, root	Antioxidant activity	Phenolic compounds	[4]
Leaves	Anti-ulcer activity	–	[56]
Seed	Wound healing activity	Catalpol, aucubin, and acteoside	[57]
Seed	Anti-inflammatory activity	Iridoid glucosides	[53]
Seed	Parasite worms	Iridoid glucosides	[9]
Whole part	Wound healing activity	Iridoid glucosides	[49]
Whole plant, leaves	Anti-obesity	–	[4]

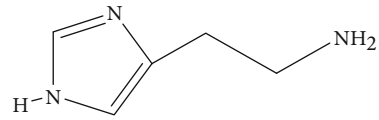
(184.38 mg/g), luteolin-7-O-glucoside (119.15 mg/g), and quercetin-3-O-glucoside (34.67 mg/g) [21] was found.

6.2. Flavonoids. Several flavonoid bioactive substances, including luteolin-7-O-glucuronide, luteolin, apigenin, luteolin-7-O-glucoside, and quercetin-3-O-D-galactopyranoside, were found in *P. lanceolata* L. Other flavonoids such as 3, 5, 7, 4-tetrahydroxyflavonol, apigenin-6,8-di-C-glucoside, luteolin-7-Oglucoside, and 7-O-glucuronide-3′-glucoside, as well as quercetin-3-rutinoside, 7-O-glucuronide, and apigenin-7-O-glucoside, were also identified in *P. lanceolata* L. (Figure 4) [11, 64]. Some flavonoids like cinnamic acids (Figure 5) are present in of *P. lanceolata* L. [30]. Aqueous extraction of the plant contains some flavonoids such as catechin with its derivatives, epicatechin with its derivative, and luteolin derivatives. The ethanol extracts of the plant also contain epicatechin, luteolin, epicatechin, and luteolin derivatives [21]. In the latest study, a new flavonoid compound called isorhamnetin 3-*O*-α-L-$^4C_1$-arabinopyranosyl-(1⟶2)-β-D-$^4C_1$-glucopyranoside) was isolated from the leaves of *P. lanceolata* L. [65].

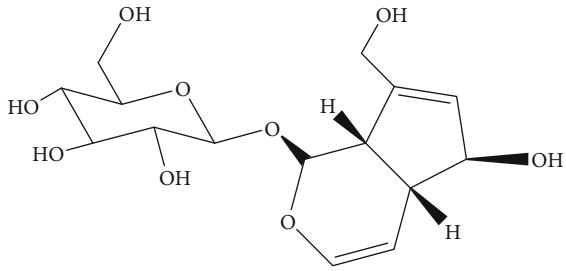
6.3. Iridoid Glycosides. Several iridoid glycosides are isolated from the leaves of *P. lanceolata* L. Aucubin and catalpol are the main iridoid glycosides present in it; asperuloside, globularin, gardoside, geniposidic acid, mayoroside, melittoside, and desacetylasperuloside acid methyl ester are also present in the leaf of *P. lanceolata* L. [66]. The study that was performed to know acteoside content in Plantago species using the HPTLC method indicated that *P. lanceolata* L. has a significantly higher acetonide than *P. reniformis* Beck, *P. atrata* Hoppe, *P. holosteum* Scop, *P. schwarzenbergiana* Schur, and *P. coronopus* L. [11]. The maturity of *P. lanceolata* L. leaves affects the contents of iridoid glycosides; for instance, catalpol is found in the highest quantity in intermediate and immature age leaves, while aucubin is found in them the less amount [15]. Catalpol, aucubin, acteoside, and verbascoside are the most important bioactive compounds obtained from *P. lanceolata* L. These compounds gave the plant a potential of anti-inflammatory, antioxidant, antineoplastic, and hepatoprotective [67]. In another research, a new phenolic compound, named phenylethanoid glycoside 2-(3, 4-dihydroxyphenyl) ethyl *O*-α-L-arabinofuranosyl-(1⟶2)-[α-L1C4rhamnopyranosyl (1⟶3)] [*E*-caffeoyl-1⟶4]-β-D-



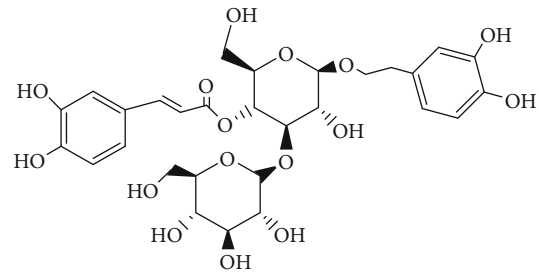
Acetylcholine



Histamine

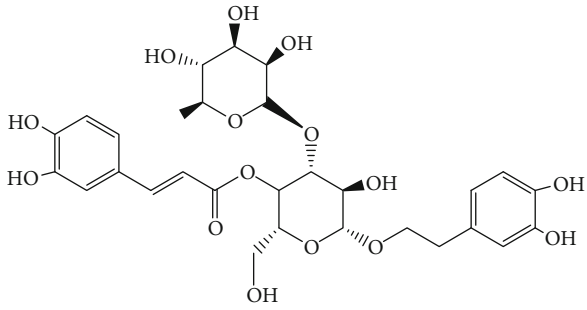


Aucubin

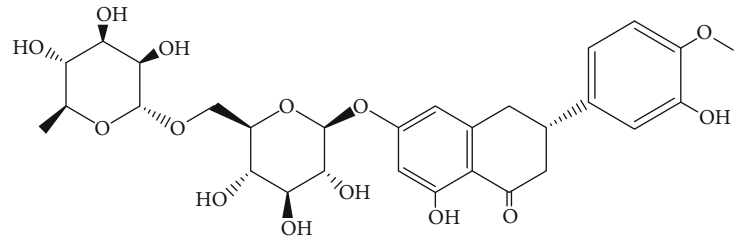


Plantamajoside

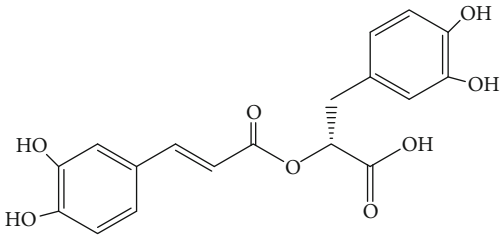
Figure 2: Structures of bioactive compounds used as antispasmodic activity.



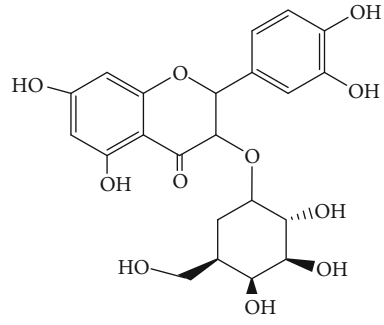
Verbascoside



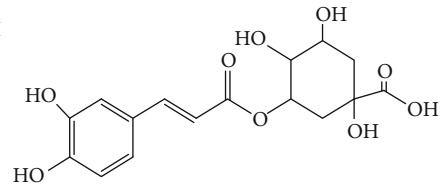
Hesperidin



Rosmarinic acid



Hyperoside



Chlorogenic acid

Figure 3: Major phenolic compounds of *P. lanceolata* L.

4C1 glucopyranoside (Figure 6) was isolated from *P. lanceolata* L. leaves [65].

6.4. Volatile Oil and Essential Oils. The isolation of volatile components from aqueous *P. lanceolata* L. extracts was studied using hydrodistillation [68]. The findings confirmed the presence of monoterpenes, sesquiterpenes, oxidized monoterpenes, oxidized diterpenes, apocarotenoids, and aldehydes. The other compounds present were ketones, phenols, phenolic ethers, esters, aliphatic hydrocarbons, aromatic hydrocarbons, oxidized sesquiterpenes, alcohols, and fatty acids [15]. The volatile oils in the fruits and leaves of *P. lanceolata* L. were identified using GC-MS analytical techniques. The result confirmed the presence of 6-(3-hydroxy-

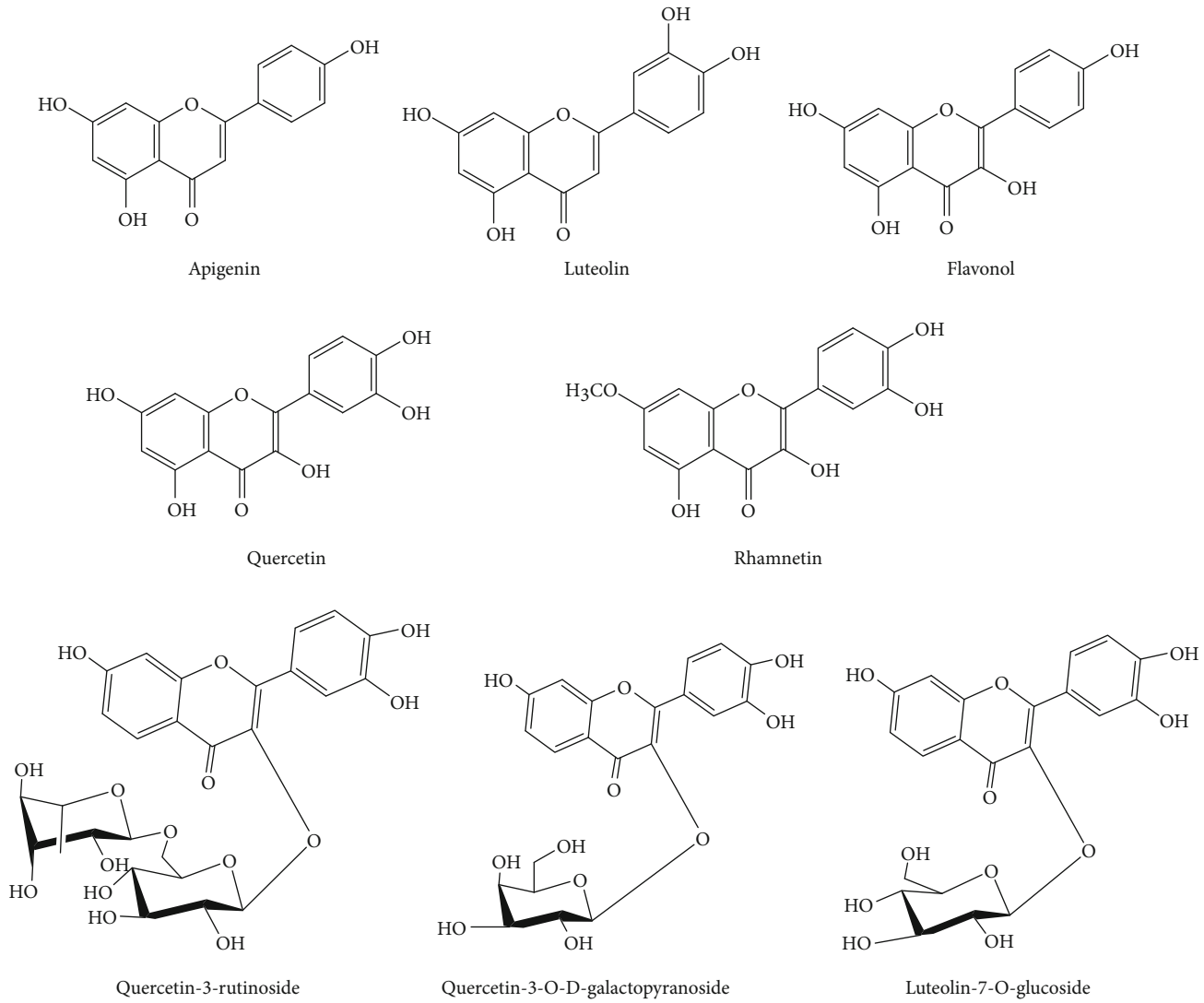


Figure 4: Structures of important flavonoids isolated from *P. lanceolata* L.

1-butenyl)-1,5,5-trimethyl-7-oxabicyclo [4,1,0] heptane-3-ol and (E),4(3-oxo-2,6,6-trimethylcyclo-hex-2-en-1-yl)-3-buten-2-ol, benzoic acid, oct-1-en-3-ol, oct-1-en-3-ol, and vanillic acid (Figure 7) [15].

Varicose fatty acid compounds present in the n-hexane extract of *P. lanceolate* L. leaves were identified using GC-MS. Some of the fatty acids observed in the GC-MS data were palmitic acid, myristic acid, and stearic acid (Figure 8) [24]. An investigation of the plant's proximate composition analysis also confirmed the presence of polyunsaturated fatty acids in *P. lanceolata* L. leaf extract [69]. In aqueous extracts on *P. lanceolata* L., some fatty acid components, including capric acid, palmitic acid, and margaric acid, were detected. Additional fatty acids such as linolenic acid, myristic acid, pentadecanoic acid, and linoleic acid were detected using GC-FID and GC-MS methods [68].

6.5. Phenolic Carboxylic Acid. Phenolic compounds are important bioactive compounds in *P. lanceolata* L. [28]. From the leaves of *P. lanceolata* L., phenolic compounds such as p-hydroxybenzoic acid, protocatechuic, gentisic, chlorogenic, and neochlorogenic acid were isolated [66]. Aqueous extracts of dried leaves of *P. lanceolata* L. contain benzoic acid derivatives, gallic acid, and benzoic acid, and ethanol extracts of the plant also contain some phenolic compounds like caffeic acid derivatives, ferulic acid, benzoic acid derivatives, ferulic acid, and benzoic acid (Figure 8) [65].

6.6. Terpenoids. Terpenoids are essential compounds in the genus *Plantago* [70]. Different classes of terpenoids were reported in *P. lanceolata* L. These include the (E)-β-farnesene, (E)-α-bergamotene, and sesquiterpenes (E)-β-caryophyllene. Also, other terpenoids like C11 homoterpene (E)-4,8-dimethyl-1,3,7 nonatriene (DMNT) and monoterpene (E)-β-ocimene are also present in the plant [71]. Many terpenoids such as loliolide, ursolic acid, and oleanolic acid (Figure 9) are detected in petroleum ether and chloroform/methanol extract *P. lanceolata* L. leaves [16].

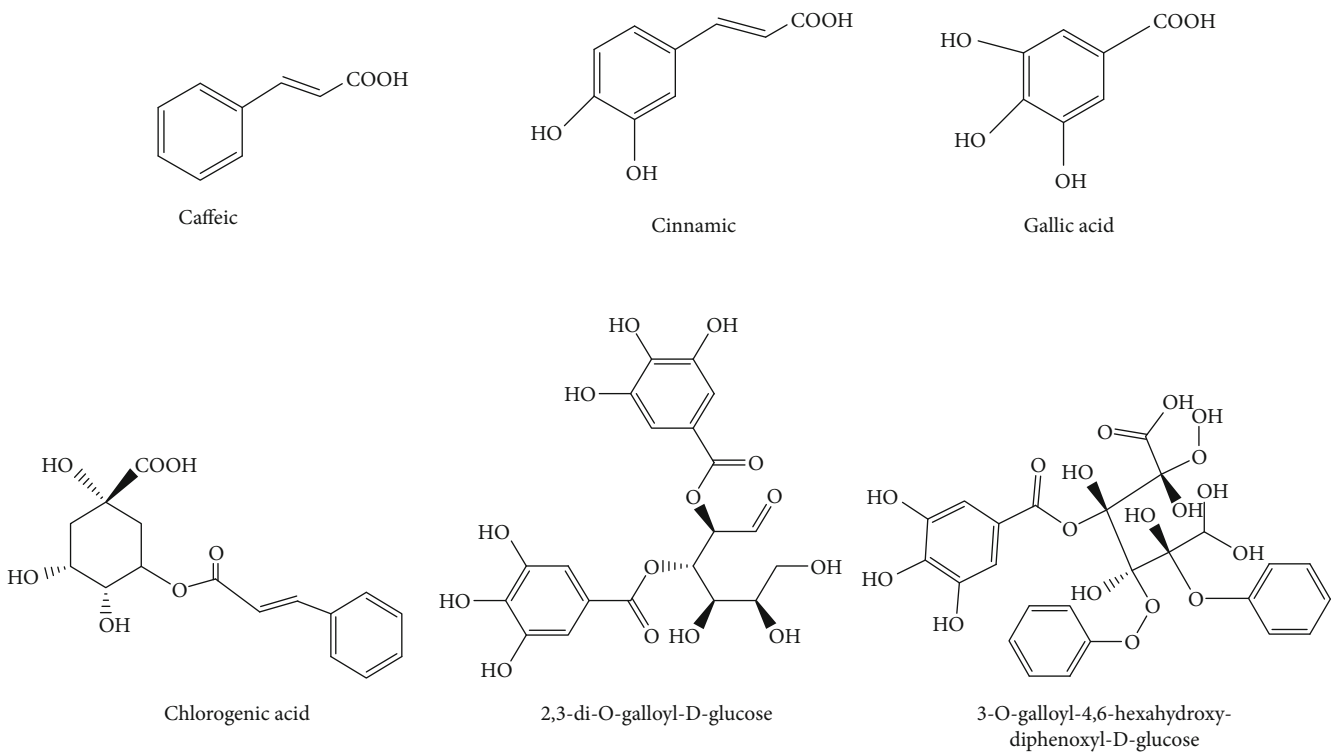


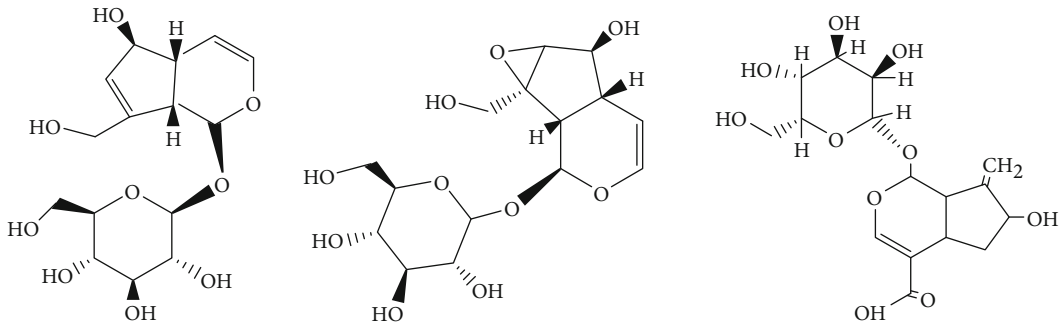
Figure 5: Cinnamic derivatives isolated from *P. lanceolata* L.

6.7. Acteoside. Acteoside (Act), a phenylethanoid glycoside, is an active compound in several plants and traditional herbal medicines [72]. Acteoside (Figure 10) is one of the main bioactive compounds in *P. lanceolate* L. [67, 73]. A study indicated that the aerial parts of *P. lanceolata* L. had much more acetonide than other Plantago species such as *P. atrata* Hoppe, *P. coronopus* L., *P. reniformis* Beck, *P. holosteum* Scop, and *P. schwarzenbergiana* Schur, according to HPTL technique quantification data [11]. Acetonide is also present in ethanolic extracts of *P. lanceolata* L. Antispasmodic action was conferred by the presence of these chemicals in the plant [31].

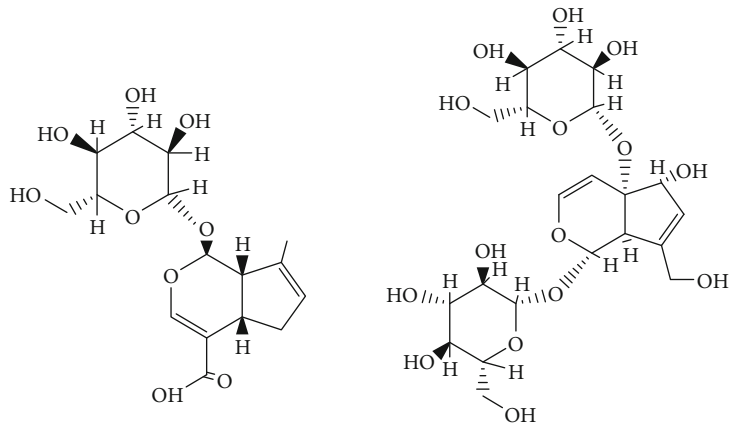
6.8. Polysaccharides. Some polysaccharides such as L-mannose, D-glucuronic acid, D-glucose, D-galactose, D-galacturonic acid, L-arabinose, D-mannose, and minor proportions of L-fructose and D-xylose are present in different parts of *P. lanceolata* L. [15]. Pectic, rhamnogalacturonan, arabinogalactan, and α-D-glucan polysaccharides were also isolated from the leaves [40]. The leaves of *P. lanceolata* L. contain galacturonic acid from 62.64% to 70.58%, arabinose content from 37.36% to 29.42%, galacturonic acid 35.8%, and glucuronic 21.9%. At the same time, rhamnose was found only in traces [74].

6.9. Other Bioactive Compounds. A study was conducted for isolating bioactive compounds from methanol extract of leaf of *P. lanceolata* L. using silica gel column chromatography techniques. The ^{1}H NMR and ^{13}C-NMR spectrum afforded one important compound, 6″-O-ethyl-4″-acetyl verbascoside (Figure 10). This compound possesses the plant to have antioxidant and antibacterial activities [5], and using DEPT-135, FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectra, a second bioactive compound named as(E)-butyl 2-(4-(2-(2–hydroxyl-2-methyl cyclohexyl)ethyl)-7-methyloctahydro-1H-inden-1-yl)-5-methylhept-4-enoate was isolated using methanol as a solvent [28]. Important phytochemicals such as hexahydro-pseudo-ionone, diheptyl phthalate, and phytol were extracted from leaves of *P. lanceolata* L. The plant leaf extract also contains bioactive chemicals such as ditridecyl phthalate, hexahydro farnesyl acetone, stigmasterol methyl ether, stearyl aldehyde, alpha-bisabolene epoxide, and allantoin (Figure 11) [24]. Bioactive anthocyanins such cyanidin glycoside, delphinidin glycoside, peonidin glycoside, and petunidin glycoside were identified in the flower of *P. lanceolata* L. [75].

6.10. Minerals. The Plantago leaf extracts possess different metallic elements such as arsenic, cadmium, copper, and cobalt. Metals such as iron, nickel, lead, zinc, magnesium, sodium, calcium, and phosphorus are also found in the leaves of the plant [44]. On other investigations, some metallic elements like nickel and cobalt also found in the leaves of *P. lanceolata* L.; however, the plant's roots, on the other hand, have the largest quantities of Ni and Co compared to the leaves [76]. Cadmium concentrations in the leaves of *P. lanceolata* L. ranged from 0.89 to 0.44 mg/kg [76, 77]. A study also indicated that from the washed leaves of *P. lanceolata* L., some metallic elements like lead, iron, Manganese, cadmium, zinc, and lead were also analyzed [21, 69]. In a study conducted on analyzing the nutritional requirements of grazing livestock in *P. lanceolata* L. and other species, the highest

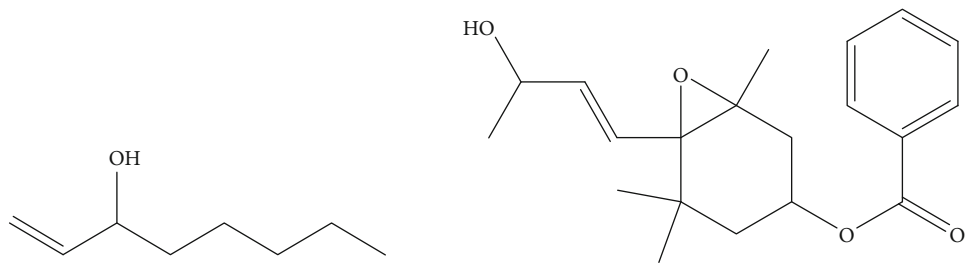


Aucubin Catapol Gardoside



Geniposidic acid Melittoside

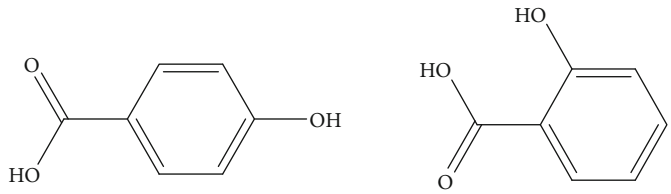
Figure 6: Iridoid glucosides and phenylethanoid glycosides isolated from *P. lanceolata* L.



Oct-1en-3-ol, n(E)

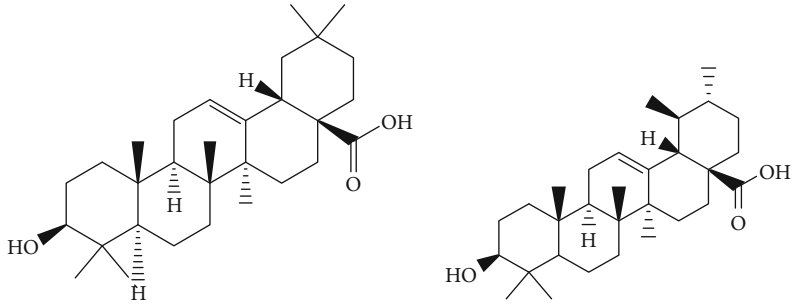
6-(3-hydroxy-1-butenyl)-1,5,5,-trimethyl-7-oxaicyclo[4,1,0]heptan-3-ol benzoic acid

Figure 7: Major volatile oils from *P. lanceolata* L.



P-hydroxy benzoic acid Salicylic acid

Figure 8: Some phenolic compounds from *P. lanceolata* L.



Oleanolic acid Ursolic acid

Figure 9: Some terpenoids in the leaves of *P. lanceolata* L.

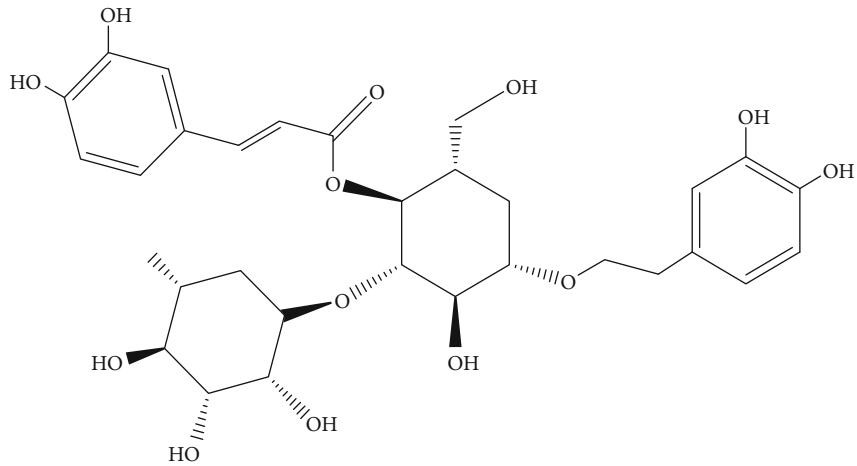


Figure 10: Structure of acteoside.

phosphorus and potassium were found in *P. lanceolata* L. before the flowering period [78].

7. Application of *P. lanceolata* L.

7.1. Cosmetics. P. lanceolata L.is included in the list of cosmetic plants. It is used in the cosmetic industry in many European industries [15]. Aqueous infusions and stabilized fresh juice from leaves of *Plantago* species are used in cosmetics [17]. The leaves of *P. lanceolata* L. can be used to manufacture lotion, creams, and face masks in the European industry. The presence of salicylic acid in the plant's leaves effectively reduces existent skin impurities and optimizes the skin appearance due to its antibacterial, keratolytic, and anti-inflammatory action [15].

7.2. Biological Activities. P. lanceolata L. leaves are used externally to treat sores and wounds and internally treat bronchitis, antibacterial, astringent, anti-inflammatory, emollient, antitussive, furuncles, bug, and snake bites [79]. Ethanolic extract of the aerial portions of *P.* lanceolata L. was examined for the antispasmodic activities against guinea-pig ileum and trachea. The result showed that various agonists such as acetylcholine, histamine, barium, and potassium ions inhibited the guinea-pig ileum's contractions [31]. European manufacturers use *P.* lanceolata L. alone or in combination with other plants for different medicinal purposes: in Finland and Romania used for digestion expectorant; in Slovenia, Italy and Romania used as astringent, soothing irritations, and antimicrobial; and in Poland and Belgium used for various forms such as herbal tea, tablets, and syrup [79]. Traditionally, coughs, dysentery, and diarrhea can be treated using tea from the plant's leaves. Blisters, rashes, swelling, and insect stings are also treated with leaves of *P. lanceolata* L. Mucilage from *P. lanceolata* L. seed has been shown to lower cholesterol levels in the blood [80].

7.3. Metal Removal. P. lanceolata L. can also be used as a metal indicator and metal removal from the atmosphere during air pollution [76]. *P. lanceolata* L. can be used as a good bioindicator for heavy metal accumulation in urban and industrial areas. Data on accumulative capacity allow us to recommend this species to indicate the presence of metals like lead, zinc, and cadmium [81]. Studies assessing Cu resistance by a microorganism called rhizosphere of *P. lanceolata* L. have shown potential agents for bioremoval of Cu and bacterial stimulation of Cu bio adsorption by this plant species [82]. *P. lanceolata* L. is the best indicator of environmental pollution. A study on environmental pollution in an urban area of Poland using *P. lanceolata* L. as

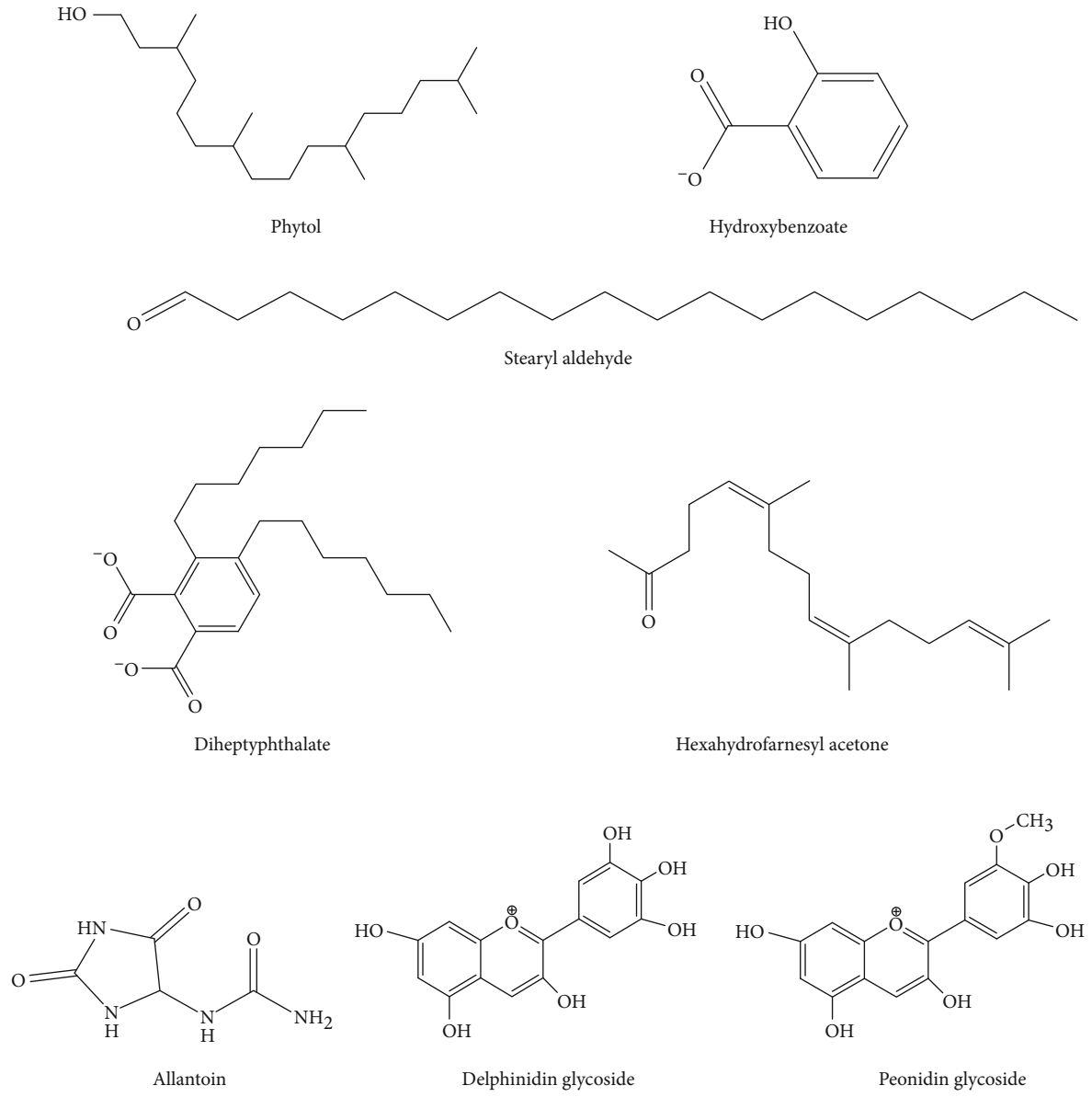


Figure 11: Some other bioactive compounds from *P. lanceolata* L.

indicators gives information about the concentration of metal in the area [16, 83]. The studies were carried out to determine the metal concentration in samples taken from the metallurgical site. The result showed that some metals such as Cd, Zn, and Pb had been detected in *P.* lanceolata L. which exceeded the permitted limits ($Zn > 300\,mg\,kg^{-1}$, $Pb > 100\,mg\,kg^{-1}$, and $Cd > 4\,mg\,kg^{-1}$). In the plant material, unwashed samples had significantly more significant Zn, Cd, Pb, Mn, and Fe than washed ones. This revealed the plant's ability to remove metals from highly contaminated environments [16]. Regardless of high concentrations of heavy metals in soil, especially Ni, Zn, and Cr, *P. lanceolata* L. showed remarkable tolerance to ecophysiological conditions of serpentine soils. This indicates the potential application of this species in the remediation of heavy metal–polluted soils [84].

7.4. Additive in Foods. P. lanceolata L. is an edible plant in Italy, and its leaves are used as an additive to some foods like wine, salads, tea, tincture, and macerate, or it can be eaten like lettuce. It is also used for animal nutrition like rabbits; when given to porkers, it can enhance the taste of meat and increase the number of unsaturated acids [17]. Leaves of various species of *Plantago* are taken as cooked or raw. Only young leaves are consumed in the form of salads [85]. The leaves of *P. lanceolata* L. are significant as cooked vegetables and soups. People used to eat the plants during the spring when vegetables were in short supply. The leaves are significant in preparing macerate, an infusion, juice, wine, tincture, and tea [17]. Dried leaves of *P. lanceolate* L. are used as a tea and appetizer and are good for digestion. The fresh leaves are topically applied with cream from cows' milk and bread or clay as a suppurative [86].

7.5. Insecticide. P. lanceolata L. extracts can be used as an insecticide to control insects. Secondary metabolites such as glycosylated iridoids produced from the plant contribute to these insecticide activities [18]. Specifically, plants attribute the polar molecules aucubin and catalpol (Figure 3) to this effect [87]. The concentration of catalpol in *P. lanceolata* L. showed an increase under herbivore attack. Consequently, the reduction in the oviposition of *L. coffeella* on leaves treated with the methanol polar fraction of the *P. lanceolata* L. extract may be induced by catalpol or aucubin [88].

7.6. Agriculture. P. lanceolata L. has a significant role in agricultural application. The advantages of this plant in the agriculture sector lie in its high content of valuable substances for human and grazing animals [14]. *P. lanceolata* L. has emerged as forage with the ability to reduce reactive nitrogen (N) losses, in particular N leaching, from grazing dairy systems [89]. It is most commonly used on farms as part of mixed pasture swards. It has a more prominent contribution when grass production decreases and gaps in the sward, especially in low-fertility dryland pastures. Where the grass or legume growth is poor, *P. lanceolata* L. contributes less than 20% of the sward [90]. In *P. lanceolata* L., chemical aucubin is responsible for nitrogen mineralization and nitrification [91]. Fertilization may affect not only plant species diversity but also insect dynamics by altering plant nitrogen supplies. *P. lanceolata* L. is grown on the farm to improve trophic levels and species interactions in managed grassland ecosystems, which occurred due to fertilizer [91]. *P. lanceolata* L. influences the distribution of soil mineral N in dairy grassland on peat soil. It has been recognized as a potential relief approach for reducing nitrogen (N) losses [92]. The iridoid glycosides and catalpol, as well as the phenylpropanoid glycoside and verbascoside, may be responsible for these effects [93].

8. Conclusion

P. lanceolata L. is one of the well-known species of the genus *Plantago*. It is distributed in European countries and the northern and central parts of Asia. *P. lanceolata* L. plays a vital role in managing certain ailments and diseases such as antimicrobial, wound-healing, anti-inflammatory, cytotoxic, and antispasmodic. It has many applications like cosmetics, pharmaceutical, antibacterial, synergetic, insecticide, metal indicators, heavy metal removal from the polluted area, and food additives. Phytochemicals such as iridoid glycoside, fatty acids, phenol, flavonoids, tannins, alkaloids, terpenoids, steroids, coumarins, saponins, glycosides, and quinines are present in different parts of *P. lanceolata* L. The biological activities and medicinal properties of *P. lanceolata* L. mainly depend on the activities of the chemical constituents. This field still needs more study to determine the exact mechanisms and the main bioactive compounds responsible for treating specific diseases. It is of great importance to investigate their chemical profile and biopotential. Most of the research has been done in vitro; further in vivo investigations of *P. lanceolata* L. are required.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

LA and AB have drafted the review. MGT prepared different tables and figures required for the manuscript, provided guidance during the development of idea, and wrote and revised the manuscript. The authors read and approved the final manuscript.

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Research Article

Essential Oils from *Artemisia herba alba* Asso., *Maticaria Recutita* L., and *Dittrichia Viscosa* L. (*Asteraceae*): A Promising Source of Eco-Friendly Agents to Control *Callosobruchus maculatus* Fab. Warehouse Pest

Allali Aimad,[1] **Mohammed Bourhia**,[2] **Hadin Hana**,[1] **Rezouki Sanae**,[1] **Ahmad Mohammad Salamatullah**,[3] **Walid Soufan**,[4] **Hail Z. Rihan**,[5,6] **Lahcen Ouahmane**,[2] **El Abdali Youness**,[7] **Eloutassi Noureddine**,[8] **and Fadli Mohamed**[1]

[1]*Laboratory of Plant Animal and Agro-industry Productions, Faculty of Sciences, University of Ibn Tofail, Kenitra, Morocco*
[2]*Laboratory of Microbial Biotechnology, Agro-sciences and Environment (BioMAgE), Cadi Ayyad University, Marrakesh, Morocco*
[3]*Department of Food Science & Nutrition, College of Food and Agricultural Sciences, King Saud University, 11 P. O. Box 2460, Riyadh 11451, Saudi Arabia*
[4]*Plant Production Department, College of Food and Agriculture Sciences, King Saud University, P. O. Box 2460, Riyadh 11451, Saudi Arabia*
[5]*School of Biological Sciences, Faculty of Science and Environment, University of Plymouth, Drake Circus, PL4 8AA, Plymouth, UK*
[6]*Phytome Life Sciences, Launceston, PL15 7AB, UK*
[7]*Laboratory of Biotechnology Environment Agri-food and Health, Faculty of Sciences Dhar El Mahraz, Sidi Mohammed Ben Abdellah University, Fez, Morocco*
[8]*Regional Center for the Trades of Education and Training, Fez, Morocco*

Correspondence should be addressed to Allali Aimad; aimad.allali@uit.ac.ma and Mohammed Bourhia; bourhiamohammed@gmail.com

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Callosobruchus maculatus (Fab.) (*C. maculatus*) is one of the major pests of legume seeds in storage causing significant damage, leading to food insecurity and low income for farmers. This work was planned to develop eco-friendly agents from essential oils of *Artemisia herba alba* Asso. (AEO), *Maticaria Recutita* L. (MEO), and *Dittrichia Viscosa* L. (DEO) to control *C. maculatus*. To achieve this goal, essential oils (EOs) were extracted by hydro-distillation using Clevenger apparatus before being characterized by GC-MS. EOs were used for testing purposes using three different tests, namely, inhalation toxicity, contact toxicity, and repellency tests. GC-MS analysis of EOs showed the presence of 16 potentially active compounds in AEO and 38 in MEO, whilst 15 compounds were identified in DEO. AEO was higher in thujone (57.6%) and chrysanthenone (11.8%). Santolina alcohol (40.7%) and germacrene D (8.9%) were the major compounds identified in MEO, whereas isocostic acid (72.3%) was the chief compound of DEO. The obtained findings showed that the studied EOs showed considerable insecticidal activity against *C. maculatus* with a lethal dose (LC_{50}) of 3.78, 8.86, and 14.34 μL/1 liter of air by AEO, MEO, and DEO, respectively. At 1 μL/1 liter of air, the oviposition reduction rate was 90.02%, 70.65%, and 48.23% by AEO, MEO, and DEO, respectively, whereas the emergence reduction rate was 87.32%, 60.08%, and 32.24% by AEO, MEO, and DEO, respectively. With increasing doses up to 20 μL/L, the reduction of individual emergence reached 98.8% by AEO of 24 h after treatment. AEO, MEO, and DEO showed significant repellent effects against adults of *C. maculatus* with repulsion percentages of 60.83%, 50.83%, and 72.5%, respectively. The outcome of this work suggests that the essential oils of the studied plants, particularly *Artemisia herba alba* Asso. oils, can constitute a natural and environmentally friendly alternative to develop new bioinsecticides for the control of *C. maculatus*.

1. Introduction

Aromatic and medicinal plants and their extracts have been traditionally used as plant-protection agents in various ancient cultures history recorded. However, chemically synthesized insecticides have gradually replaced natural insecticides during the 20th century. With the massive and sometimes irrational use of chemically synthetic insecticides generated in recent years, people are becoming increasingly aware of their harmful effects on human health and the environment besides their effectiveness in crop protection. Most of these synthetic insecticides are nondegradable and accumulate in the environment or the human body through food chains, often causing chronic diseases and other severe physiological disorders [1–5]. Although these chemicals have been more strictly regulated, it is necessary to explore new, safe, and environmentally friendly alternatives with considerable insecticidal efficacy.

Plant EOs are mixtures of bioactive, fragrant, volatile, and lipophilic secondary metabolites that are also synthesized to defend against various plant pathogens. Essential oils have been scientifically studied worldwide to control postharvest pests of cereals, legumes, citrus, tomatoes, and food-borne microorganisms [6–9].

Several studies on natural bioinsecticides have revealed that essential oils along with other secondary plant metabolites (terpenoids, polyphenols, steroids, and alkaloids) synthesized by aromatic and medicinal plants are responsible for several biological activities against plant pests, especially essential oils which have shown important insecticidal, nematicidal, acaricidal, and larvicidal properties [10–20]. Essential oils not only act as poisons or neurotropic on the nervous system of insects [21–23] but also can affect cytochrome P450, which plays a key role in the insect detoxification system, alter insect metabolism (e.g., ATP synthesis, Krebs cycle, oxidative phosphorylation, and others) [24]. Overall, the final action of EOs may be the result of more than one specific interaction with the targets, so that the possibility of insects developing resistance is greatly reduced [25].

Callosobruchus maculatus (Fab.) (Chrysomelidae), known as cowpea weevil, is one of the most well-known storage pests causing damage to chickpea (*Cicer arietinum*) and other leguminous crops, particularly in Africa and tropical and South American regions [26–28]. The negative economic impacts caused by this insect are related to the penetration of the larvae to feed inside the grain causing a significant loss of weight and a reduction in nutritional values and germination potential [29–32].

The *Asteraceae* family contains a large number of plant species, with more than 1600 genera and over 23,000 species. Some of these species, such as *Matricaria recutita* L., *Artemisia herba alba* Asso., and *Dittrichia viscosa* L. have already been reported to have important medicinal and nutraceutical applications. Plants belonging to the family *Asteraceae* can be used as insecticides thanks to the variety of bioactive molecules present in the EOs of several species. Therefore, this study aimed to evaluate the efficacy of EOs from *Artemisia herba alba* Asso. and *Maticaria Recutita* L. and *Dittrichia Viscosa* L. in the control of *C. maculatus* because no similar has been done up to date.

2. Materials and Methods

2.1. Animal Material. For testing purposes, *C. maculatus* was isolated from a sample of chickpeas stored in the Fez. city of Fez-Morocco. Mass rearing of *C. maculatus* was performed in glass jars containing Cicer arietinum (chickpea) seeds. The jars were maintained at a temperature of $25 \pm 1^{\circ}C$, relative humidity, and a photoperiod of 14:00 (light)/10:00 (dark) to obtain several successive generations of the insect. The nonwinged form of the adults with greater reproductive capacity was selected for testing. The active/inactive form was determined both by the presence of flight activity, the size of the elytra, and the intensity of pigmentation on the elytra [33].

2.2. Plant Material. In this work, three medicinal and aromatic plants growing in different regions in Morocco were collected for testing purposes (Table 1).

2.3. Extraction of Essential Oils. The aerial parts of the plants were dried in the shade and ventilated area with a temperature of about 25°C for 5 days before distillation. The extraction of EOs from different aromatic and medicinal plants was conducted using a Clevenger apparatus [34]. Briefly, 200 g of each plant aerials part previously crushed was introduced into a flask with 1200 mL of distilled water. Next, the whole was boiled for 3 hours until complete extraction. The obtained essential oils were dehydrated on anhydrous sodium sulfate and stored at 4°C until further use.

The essential oil yield in percentage was calculated by the following formula:

$$Y_{HE} = \frac{M_{HE}}{M_D} \times 100. \quad (1)$$

Here, Y_{HE} is the yield of essential oil (%), M_{HE} is the mass of the EO (g), and M_D is the mass of dry plant matter (g).

2.4. Analysis of the Chemical Composition of Essential Oils. The analysis of the essential oils was performed by gas chromatography-mass spectrometry (GC-MS). The coupling was performed using an Agilent-Technologies 6890 N Network GC system equipped with a flame ionization detector and an HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Temperature programming from 35°C to 250°C, with a gradient of 5°C/min. Retention indices were determined by gas chromatography on two fused silica capillary columns (30 m × 0.25 mm) from Agilent Technologies, LittleFalls, CA, USA, with temperature programming from 35°C to 250°C at a rate of 5°C/min, with the lower and upper temperatures maintained for 3 and 10 minutes,

Table 1: Plant species used for the extraction of essential oils.

Species studied	Voucher specimen ID	Family	Sample site	Harvest period	Parts used
A. herba alba	BA22/13009	Asteraceae	Boulemane	June 2019	Leaves
M. recutita	BM19/09549	Asteraceae	Taounate	April 2019	Leaves
D. viscosa	BD01/00282	Asteraceae	Fez	August 2019	Leaves

respectively. The carrier gas (helium) flow rate was 1.0 mL/min. A 1.0 μL sample was injected in splitless mode. The essential oil constituents were identified by comparing their mass spectra with those of the NIST02GC/MS library data and the Adams.

2.5. Toxicity of Essential Oils

2.5.1. Contact Toxicity. To evaluate the effect of each EO, 100 g of seed infested by five pairs of insects (male and female) aged 0 to 48 h were used. The tests were packaged in glass containers (1 L) duly closed with a perforated lid and covered with a thin transparent cloth. The essential oils were added directly to the seeds with a pipette, followed by manual shaking for 2 minutes. After 48 h of confinement, adult mortality was assessed [35]. A control for each test was performed under the same conditions without the EOs. Based on the results obtained in the preliminary tests, treatments with increasing concentrations (1, 5, 10, 20 μL/1 liter of air) were performed. After 24 hours, mortality was assessed, and dead insects were removed. Eggs deposited in the grains were also counted after 12 days from the start of the tests, followed by a regular count of emerged insects from Day 28 after confinement.

The observed mortality rate was corrected by the Abbott formula as follows:

$$\mathbf{Pc} = 100 \times \frac{\mathrm{P0} - \mathbf{Pt}}{100 - \mathbf{Pt}}. \quad (2)$$

Here, **Pc** is the corrected mortality percentage (%), **Po** is the mortality observed in the test, and **Pt** is the mortality observed in the control.

The reduction percentage in the number of eggs and adults emerging in each concentration of essential oil was calculated using the following formula:

$$\mathbf{PR} = \frac{\mathbf{NC} - \mathbf{NT}}{\mathbf{NC}} \times 100. \quad (3)$$

Here, PR is the percentage of oviposition (%), NC is the number of eggs laid in the control, and NT is the number of eggs in the experiment.

2.5.2. Inhalation Toxicity. In 1 L glass jars with tightly closed lids, each containing 10 *C. maculatus* bruchids (male and female) ranging in age from 0 to 48 h, small cotton balls were suspended with a thread attached to the inside of each lid. Using a micropipette, doses of 1 μL, 5 μL, 10 μL, and 20 μL/L of each essential oil were each deposited into the above cotton balls. For each dose, three replicates were performed. The comparison was made to a control sample (cotton without essential oil) [36].

The observed mortality rate is corrected by the Abbott formula and calculated as follows:

$$\mathbf{Pc} = 100 \times \frac{\mathbf{P0} - \mathbf{Pt}}{100 - \mathbf{Pt}}. \quad (4)$$

Here, Pc is the corrected mortality percentage (%), *P*o is the mortality observed in the test, and *P*t is the mortality observed in the control.

2.6. Repulsion Test. The repellent effect of the studied essential oils on C. *maculatus* adults was evaluated using the preferential area method on filter paper described by McDonald et al. [37]. Briefly, 9 cm diameter filter paper discs used for this purpose were divided into two halves with 31.80 cm^2 area in each. Next, 0.5 mL of each EOs solutions previously prepared in acetone (1, 5, 10, and 20 µL/mL of acetone) was uniformly spread on one of the two disc halves in order to obtain doses such as 0.016, 0.079, 0.157, and 0.315 µL/cm^2 per disc. While the other half received only 0.5 mL of acetone. The Petri dishes were then sealed with Parafilm. After 30 minutes, the number of bruchids presents on the half of the disc treated with the essential oil was counted against the number on the untreated part. Three replicates for each experiment were done under the same environmental conditions as the insect breeding.

The percentage of repulsion (PR) was calculated according to the following formula [38]:

$$\mathbf{PR} = \frac{\mathbf{NC} - \mathbf{NT}}{\mathbf{NC} + \mathbf{NT}} \times 100. \quad (5)$$

Here, **PR** is the percentage of repulsion (%), **NC** is the number of insects in the control area, and **NT** is the number of insects in the treated area.

The average percentage of repulsion was calculated for each EO assigned to one of the different repulsive classes ranging from 0% to 100% [39].

2.7. Data Analysis. SPSS for Windows® (version 21.0) statistical software was used to perform analysis. Data were treated with a one-way analysis of variance (ANOVA) to determine the difference between the extreme values of the group. Fisher's minimum significant difference (LSD) test was used to separate significant from nonsignificant means at $\alpha = 0.05$. Lethal concentrations LC_{50} and LC_{95} with their confidence intervals were determined using the probit method [40].

3. Results

3.1. Essential Oil Yield. The extracted EOs were found to be different in terms of specific characteristics, especially in odor and color. The values of the essential oil yields varied

from one species to another with highly significant differences ($F = 1179.71$; $P < 0.001$). From Figure 1, it can be seen that the highest yield of EOs was recorded for *A. herba alba* with a value of $1.18 \pm 0.15\%$, followed by *M. recutita* ($0.45 \pm 012\%$) and *D. viscose* ($0.23 \pm 0.06\%$).

3.2. Chemical Composition of Essential Oils. The compounds identified in the EOs of the three studied plants along with their retention index and percentages are figured in Figures 2–4 and Table 2, whereas the chemical formulas of the chief compounds are presented in Table 3. The obtained results revealed the presence of 16 potentially bioactive compounds in AEO and 38 in MEO, whilst 15 compounds were identified in DEO. The main compounds identified in AEO were thujone (57.6%) and chrysanthenone (11.8%). Santolina alcohol (40.7%) and germacrene D (8.9%) were the major compounds identified in MEO, whereas, isocostic acid (72.3%) was the chief compound of DEO.

3.3. Inhalation Toxicity of Essential Oils. In this test, EOs at different doses (0, 1, 5, 10, and 20 µL/1 litre air) were used to evaluate their toxicity against *C. maculatus* through inhalation. Adult mortality was recorded every 24 hours for 4 days. The obtained results are illustrated in Figures 5–7.

According to the results obtained, most of the essential oils studied showed a considerable insecticidal effect with a significant difference ($P < 0.001$) in the longevity of the treated adults depending on the dose and duration of exposure. The mortality of *C. maculatus* adults increased with increasing doses and duration of exposure to essential oils. A reduction in mortality was observed in chickpea bruchid adults treated with the lowest dose (1 µL) of *A. herba alba* essential oils causing total mortality after 96 h of exposure, thus reflecting their powerful insecticidal effect. At higher doses, and after 96 h of exposure, all three essential oils show a total insecticidal effect at the 20 µL dose.

The probit method (Table 4) allows calculating the lethal concentrations (LC50) which cause the mortality of 50% of the adults of *C. maculatus* treated with essential oils. The results obtained (Table 3) allow us to classify the tested EO from the most toxic to the least toxic according to the following order: *A. herba alba*, *M. recutita*, and *D. viscosa* whose LC50 are, respectively, 2.18, 4.09, and 19.96 µL/1 litre of air volume.

3.4. Contact Toxicity of Essential Oils. The action of essential oils of different aromatic plants by direct contact on the biology of the *C. maculatus* insect was also studied. In this regard, the tests performed concerned the longevity of the adults, the fecundity of the females, and the viability of the eggs. In view of the results illustrated in Figures 8 to 10, all the essential oils tested showed a more or less significant insecticidal effect depending on the dose and duration of exposure.

In general, mortality of *C. maculatus* adults increased significantly ($P < 0.001$) with increasing EO doses, or when contact time with the oil was extended to nearly 96 h. EOA

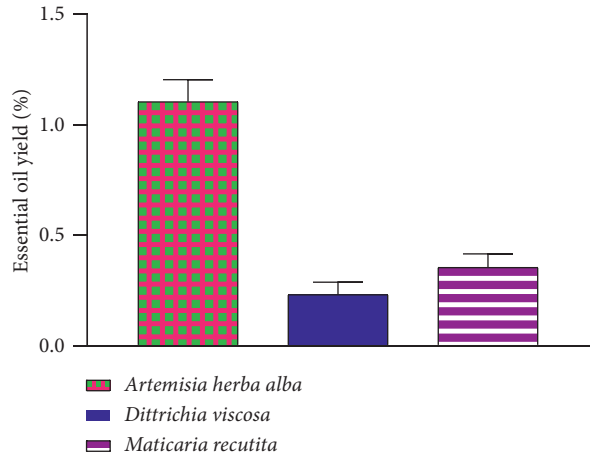


Figure 1: Essential oil yield of the studied plants.

was found to be the most toxic against *C. maculatus* with total mortality (100%) recorded for a dose of (5 µL) 4 days postcontact. In the case of EOM and EOD, total mortality (100%) was achieved by increasing the doses up to 10 µL.

Based on the results of the contact test with EOs at different doses, the lethal concentrations LC_{50} and LC_{95} causing mortality of 50% and 95% of *C. maculatus* adults respectively were calculated according to the Probit method (Table 5), and the obtained values are shown in Table 4. This allowed us to classify the EOs tested by contact according to their degree of toxicity from the most toxic to the least toxic using the following classification: EMA, EOM, and EOD whose LC50 are respectively 3.78, 8.86, and 14.17 µL/1 litre of air.

3.5. Effect of Direct Contact with Essential Oils on Fecundity and Emergence of Callosobruchus maculatus Fab. Individuals. Fecundity in *C. maculatus* occurred within 24 h after mating. In this contact test, the fecundity process was evaluated in the presence of EOs of different aromatic plants. The obtained results are displayed in Figure 11. From this figure, it can be seen that the number of eggs laid varies according to the EOs and the doses used. Thus, the average fecundity in the control batches was 184.67 ± 17.78 eggs/five females. This process decreased significantly ($P < 0.001$) with increasing doses of EOs. Indeed, at the highest dose (20 µL), EOA, EOD, and EOM significantly affect the fertility of *C. maculatus,* which was reduced to 6 ± 2, 19.33 ± 7.50, and 57.66 ± 12.58 eggs/female, so that the percentage of reduction was of 96.79%, 89.69%, and 68.95%, respectively (Figure 12).

After the emerged individuals of *C. maculatus* completed their developmental cycle, they were subjected to evaluation of emergence under different doses of EOs and the obtained results of the test are presented in Figures 13 and 14. In this regard, EOs significantly reduced number of *C. maculatus* emergences when compared to the control batches (111.67 ± 4.44) ($P < 0.001$). Moreover, at 20 µL/1 litre of air, only 1.33 ± 0.57, 9.67 ± 2.31, and 21.67 ± 2.03 individuals emerged in the presence of EOA, EOM, and EAD respectively.

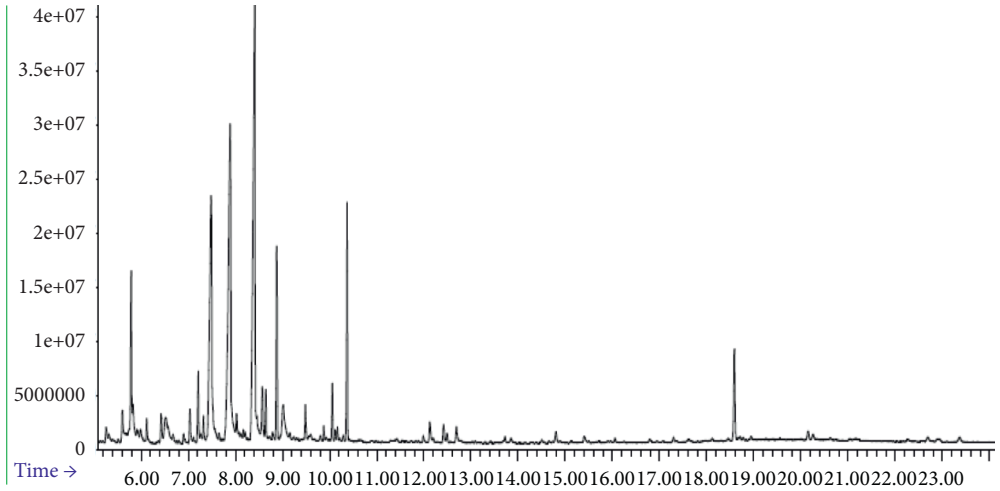


Figure 2: Chromatogram of *Artemisia herba alba* Asso.essential oil.

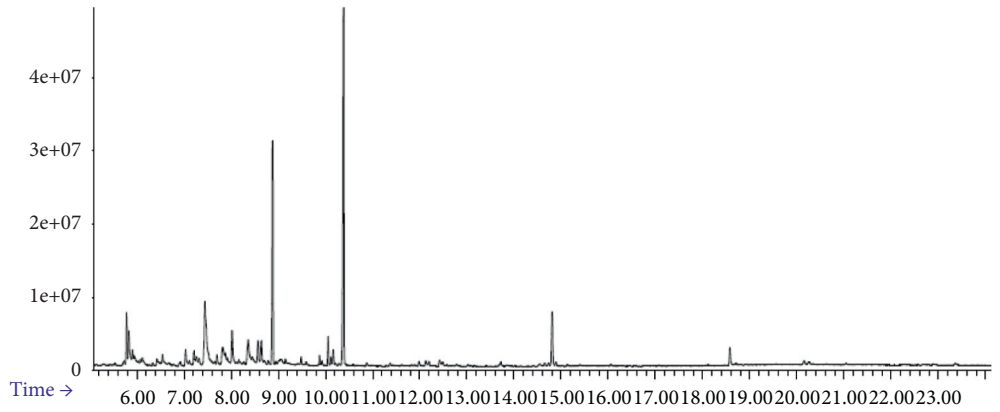


Figure 3: Chromatogram of *Matricaria recutita* L. essential oil.

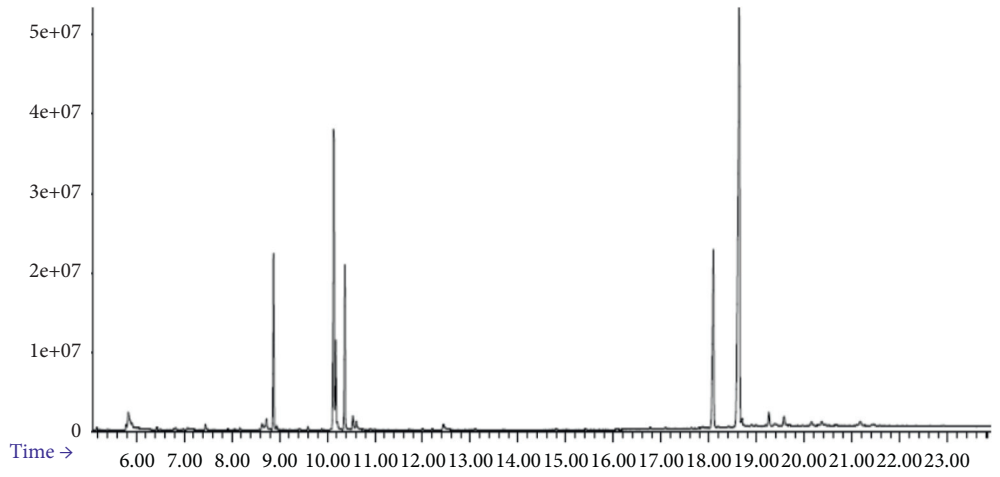


Figure 4: Chromatogram of *Dittrichia viscosa* L. essential oil.

3.6. Repulsive Activity of Essential Oils. The results of the repulsion test are given in Figure 15. Overall, the EOs were found to be repulsive to adults of *C. maculatus*. The obtained results showed that the repulsive activity of the studied EOs was in a dose-dependent manner. From Table 5, it can be seen that with increasing concentration up to 20 µL, the mean of repulsive activity by EOD reached 72.5%. Regarding the repulsive activity, the analysis of variance showed significant differences ($P < 0.001$) in EOM and EOD towards *C. maculates* (Figure 15).

Table 2: Chemical composition of essential oils from *Artemisia herba alba* Asso., *Matricaria recutita* L., and *Dittrichia viscosa* L.

Compounds	RI	Relative percentage		
		A. herba alba	*M. recutita*	*D. viscosa*
α-Pinene	937	0.6	5.7	–
Camphene	954	2.3	0.2	–
Sabinene	972	0.7	0.1	–
b-Pinene	974	–	0.3	–
Myrcene	992	–	0.5	–
Yomogi alcohol	986	–	3.0	–
p-Cymene	1025	0.2	0.1	–
Limonene	1028	–	3.1	–
1,8-Cineole	1030	11.7	0.3	–
Santolina alcohol	1037	–	**40.7**	–
g-Terpinene	1072	0.3	–	–
Artemesia alcohol	1080	–	4.3	–
a-Thujone	1109	**29**	–	–
b-Thujone	1112	**28.6**	–	–
Campholenal	1121	–	0.1	–
(E)-Pinocarveol	1139	–	1.3	–
Camphor	1146	0.2	–	–
Chrysanthenone	1158	14.8	–	–
Borneol	1164	0.1	0.3	–
Pinocarvone	1166	–	1.9	–
Santolinaacetate	–	–	2.2	–
Terpinen-4-ol	1173	–	0.3	–
trans-Pinocarveol	1178	1.9	–	–
a-Terpineol	1199	–	0.2	0.1
Hexylisovalerate	–	–	0.1	–
Acetate de bornyl	1289	0.1	1.0	–
Phenethylacetate	–	–	0.1	–
trans-Sabinylacetate	1299	0.3	–	–
α-Copaene	1377	–	0.1	–
b-Bourbonene	1380	0.2	–	–
β-Maaliene	*	–	0.4	–
β-Elemene	1392	–	0.6	–
B-Caryophyllène	1419	–	1.7	1.8
(E)-β-Farnesene	1456	–	4.0	–
γ-Curcumene	1479	–	–	0.3
α-Humulene	1480	–	0.4	–
Germacrene D	1492	4.3	8.9	–
δ-Selinene	1493	–	–	0.2
α-Muurolene	1503	–	1.0	0.7
δ-Cadinene	1520	–	1.1	–
trans-γ-Cadinene	1513	–	–	0.1
(Z)-Nerolidol	1536	–	0.3	–
Germacrene D-4-ol	1570	–	1.6	–
Caryophylleneoxide	1579	–	0.9	–
Caryophylladienol	–	–	2.8	–
10-Epi-γ-eudesmol	1623	–	–	3.4
Caryophylla-4(14),8(15)-dien-5-ol	1636	–	–	0.6
Epi-a-cadinol	1639		1.2	–
Epi-α-muurolol	1640	–	1.6	–
α-muurolol	1641	–	0.6	–
a-Cadinol	1643	–	1.4	–
Cedr-8(15)-en-9-α-ol	1650	–	–	0.4
Selin-11-en-4-α-ol (=Kongol)	1653	–	–	2.5
Ylangenal	1675	–	–	2.4
Junipercamphor	1692	–	–	5.1
Isocosticacid	1890	–	–	**72.3**
Phytolacetate	2223	–	–	0.9
Monoterpenes		**90.4**	**62.5**	**0.1**
Sesquiterpenes		**4.5**	**30.8**	**89.8**

Table 2: Continued.

Compounds	RI	Relative percentage		
		A. herba alba	*M. recutita*	*D. viscosa*
Others		**0.4**	**3.3**	**0.9**
Total identified		**95.3%**	**96.6%**	**90.8%**

Table 3: Major compounds of essential oils of *Artemisia herba alba* Asso., *Matricaria recutita* L., and *Dittrichia viscosa* L.

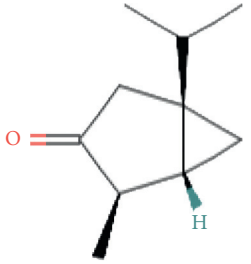
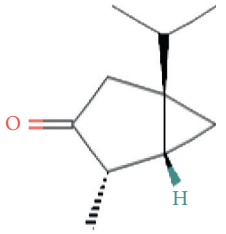
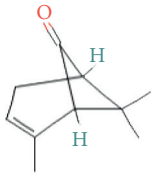
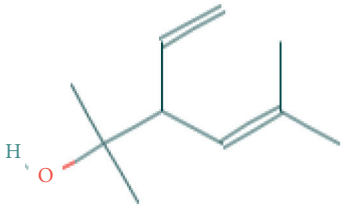
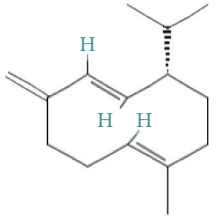
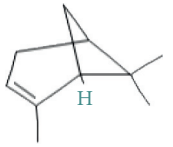
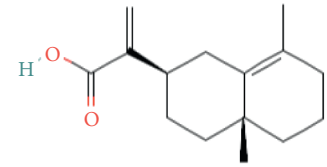
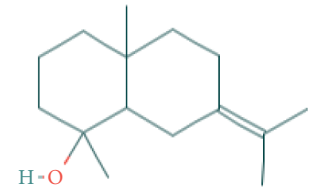
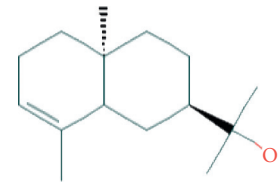
Plant species	Major compounds	Relative percentage	Chemical formula	Chemical structure
A. herba alba	a-Thujone	29	C10H16O	
	b-Thujone	28.6	$C_{10}H_{16}O$	
	Chrysanthenone	14.8	$C_{10}H_{14}O$	
	Santolina alcohol	40.7	$C_{10}H_{14}O$	
M. recutita	Germacrene D	8.9	$C_{15}H_{24}$	
	Alpha-pinene	5.7	**$C_{10}H_{16}$**	

Table 3: Continued.

Plant species	Major compounds	Relative percentage	Chemical formula	Chemical structure
	Isocostic acid	72.3	$C_{15}H_{22}O2$	
D. Viscosa	Juniper camphor	5.1	$C_{15}H_{26}O$	
	10-Epi-γ-eudesmol	3.4	$C_{15}H_{26}O$	

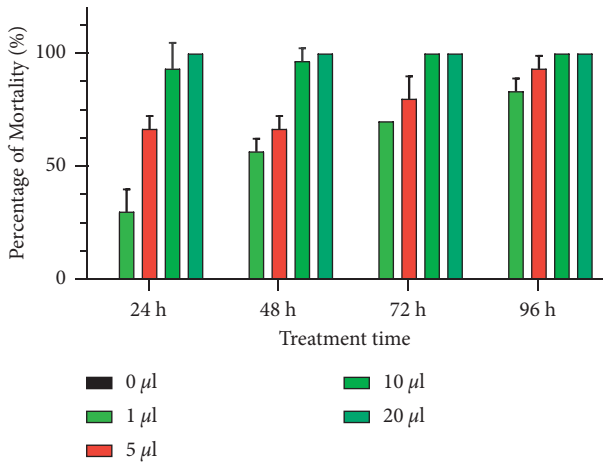


Figure 5: Mortality rate of *Callosobruchus maculatus* Fab. adults exposed to the inhalation test of *Artemisia herba alba* L. essential oils.

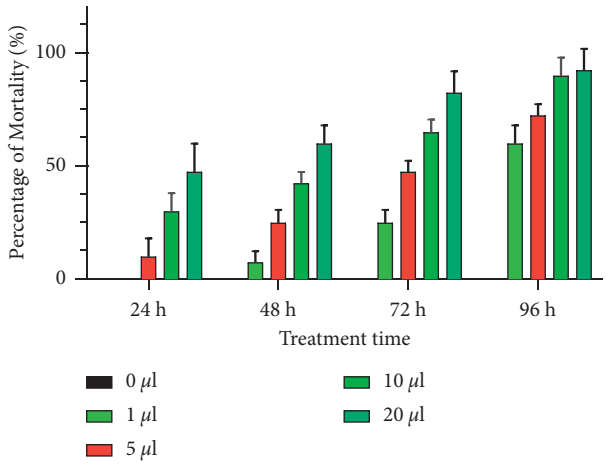


Figure 6: Mortality rate of *Callosobruchus maculatus* Fab. adults exposed to the inhalation test of *Dittrichia viscosa* L. essential oils.

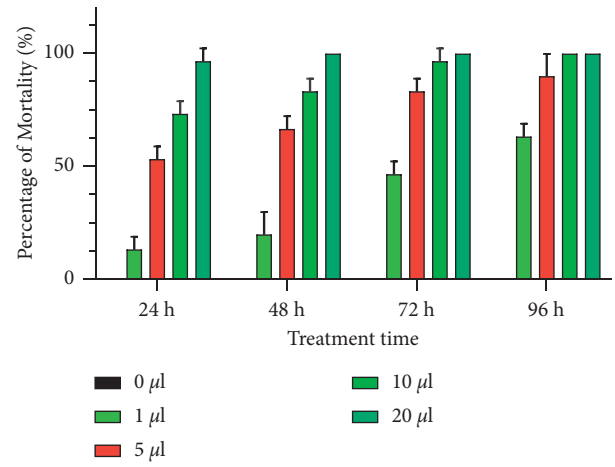


Figure 7: Mortality rate of *Callosobruchus maculatus* Fab. adults exposed to the contact test of *Matricaria recutita* essential oils.

The percentage of repellency calculated by the method of McDonald et al. [37] showed that the EOA and EOD were repulsive with respective rates of 60.83% and 72.5%, whereas, EAM was moderately repulsive (50.83%).

4. Discussion

This study aimed to investigate the chemical composition and insecticidal activity of EOs from *A. herba alba*, *M. Recutita*., and *D. Viscosa* on *C. maculatus*. The obtained results in this context showed that EOs yields in these plants ranged from $0.23 \pm 0.06\%$ to $1.18 \pm 0.15\%$. It is thus fitting that these yields were relatively low when compared to some industrially exploited plants as sources of essential oils [41]. Many factors influence the yield, content, physicochemical characteristics, and chemical composition of essential oils such as plant species, environmental conditions, extraction technique, drying, harvesting time and environment, cultivation practices, and age of plant material [39].

Table 4: LC50 and LC95 values (μL/1 litre of air) calculated based on the mortality of *C. maculatus* adults after 24 h exposure to various concentrations of essential oils in the inhalation test.

Treatment	DF	Slope + ES	LC_{50} (IC 95%)	LC_{95} (IC 95%)	χ^2
A. herba alba	2	1.91 ± 0.17	2.18 (0.12; 5.26)	15.82 (6.22; 7720.86)	8.46
M. recutita	2	2.02 ± 0.18	4.09 (1.42; 7.99)	26.73 (11.99; 519.77)	5.81
D. viscosa	2	2.09 ± 0.3	19.96 (16.27; 27.09)	14.66 (12.95; 17.43)	1.24

C50 and LC90 were the concentration of causing 50% and 90% mortality against larvae after 24 h; slope: measures of inclination of the line probit (p) = constant + Bx (covariates x transformed using log base 10; SE = standard error; 95% CI: 95% confidence interval; χ^2 = chi-square.

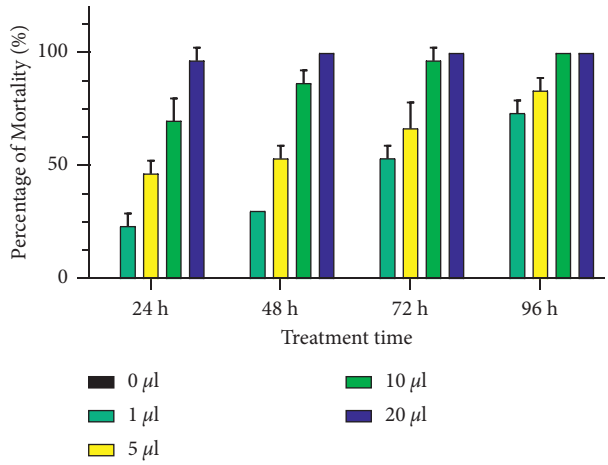


Figure 8: Mortality rate of *Callosobruchus maculatus* Fab. adults exposed to the contact test of *Artemisia herba alba* L. essential oils.

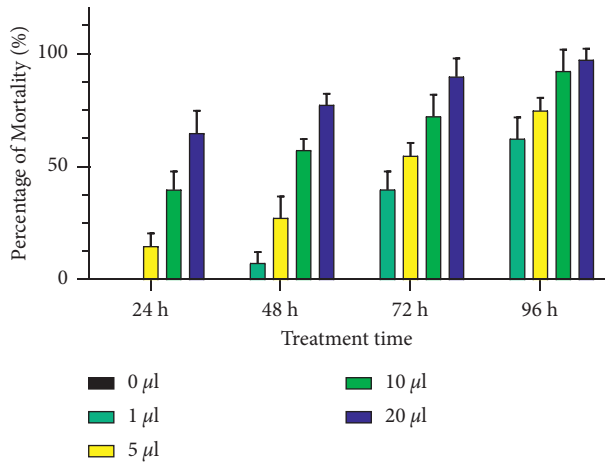


Figure 9: Mortality rate of *Callosobruchus maculatus* Fab. adults exposed to a contact test of *Dittrichia viscosa* L. essential oils.

The chemical composition of EOs investigated here was similar to that described in previous investigations. Our findings revealed that AEO was higher in thujone (57.6%) and chrysanthenone (11.8%), whilst MEO was higher in santolina alcohol (40.7%) and germacrene D (8.9%). Isocostic acid (72.3%) was the chief compound of DEO so that these results were consistent with prior works [41–43].

This study evaluated the insecticidal activity of EOs by inhalation on adult individuals of *C. maculatus* using multiple doses and exposure times. The obtained results indicated that inhalation treatments with EOs from the studied aromatic plants considerably affect the longevity of

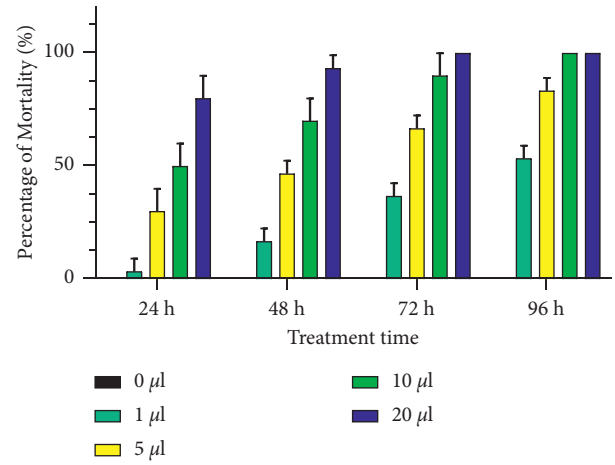


Figure 10: Mortality rate of *Callosobruchus maculatus* Fab. adults exposed to a contact test of *Matricaria recutita* L. essential oils.

C. maculatus adults. Overall, the mortality averages of *C. maculatus* adults became increasingly important with increasing dose and duration of exposure to EOs. Our results were in harmony with those obtained by many authors [36, 44–46], who reported the insecticidal effect of EOs of many aromatic plants. The studied EOs here provide new perspectives in the control of *C. maculatus*. For comparison purposes, Mahmoudvand et al. [47]observed that EOs of *Lippia citrodora* L., *Rosmarinus officinalis* L., *Mentha piperita* L., and *Juniperus Sabina* L. caused mortality of genus *Callosobruchus*. Aboua et al. [48] showed that EOs of *Melaleuca quinquenervia* L., *Citrusaurantifolia* (Christm.), and *Ageratum conyzoides* L. possessed fumigation toxicity effects against this pest. *Carum copticum*and *Vitex pseudo-negundo* oils also posed effects on the genus *Callosobruchus* at different stages of insect development [49].

In this work, three EOs extracted from Moroccan aromatic plants were tested by direct contact at different doses in order to evaluate their insecticidal effects on *C. maculatus* adults along with their reproduction (fecundity and emergence). In this instance, the results showed that the EOs of the studied aromatic plants had well-controlled the longevity of the insect and clearly limited its fecundity and emergence in a dose- and time-dependent manner. In this sense, the EOA was the most effective, whereas, EOM was the least effective in terms of toxicity against *C. maculatus*.

Numerous studies have demonstrated the action of EOs on the longevity of adults of different species of stored grain pests. Due to their high volatility, EOs and their constituents, particularly monoterpenes, exert insecticidal effects, and reduce or disturb the insect growth at different stages of their

Table 5: LC50 and LC95 values (μL/1 litre of air) calculated for mortality of *C. maculatus* adults after 24 h of postcontact with various concentrations of essential oils.

Treatment	DF	Slope + ES	LC_{50} (IC 95%)	LC_{95} (IC 95%)	χ^2
A. herba alba	2	1.62 + 0.15	3.78*	39.24*	16.15
M. recutita	2	2.06 + 0.21	8.86 (7.55, 10.48)	55.62 (39.22, 92.19)	1.63
D. viscosa	2	2.47 + 0.30	14.17 (12.24, 16.99)	65.31 (44.98, 193.93)	0.35

C50 and LC90 were the concentration causing 50% and 90% mortality against larvae after 24 h of posttreatment; slope: measures of inclination of the line probit (p) = constant + Bx (covariates x transformed using using log based 10; SE = standard error; 95% CI: 95% confidence interval; χ^2 = chi-square. *Wide confidence intervals were excluded from the calculation.

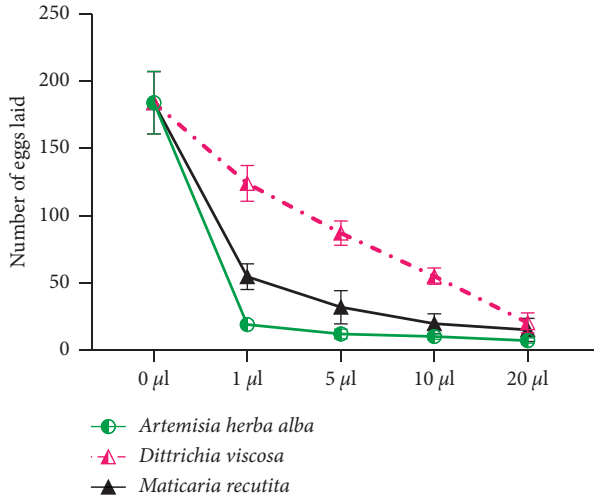


Figure 11: Effects of essential oils on the number of eggs laid by females of *Callosobruchus maculatus* Fab.

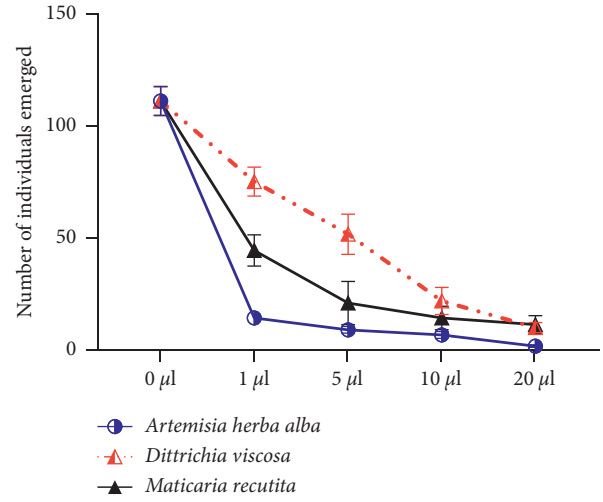


Figure 13: Effects of essential oils on the number of individuals emerging after the completion of life cycle.

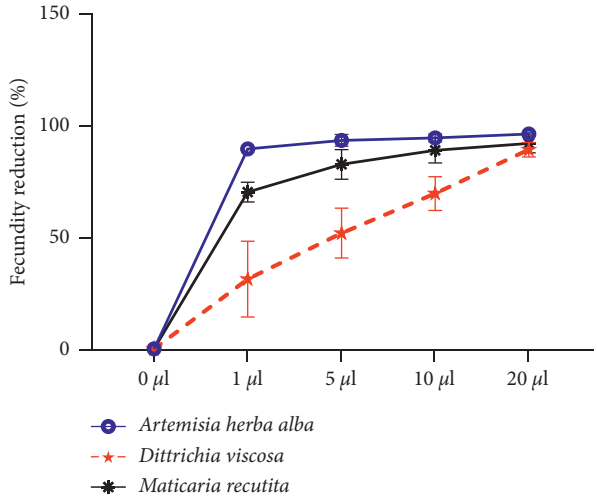


Figure 12: Rate of the fertility reduction in females under the effect of EOs.

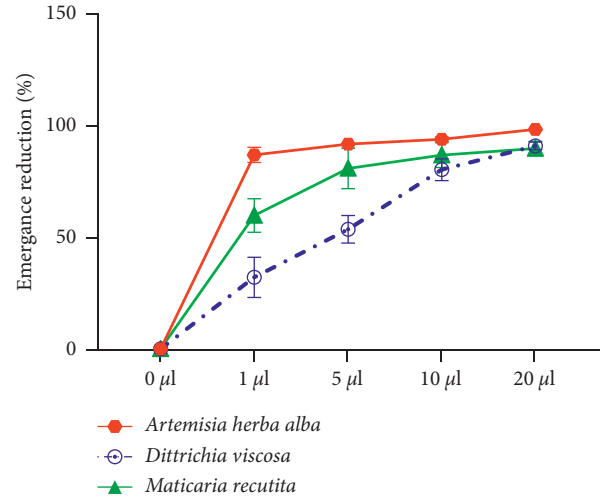


Figure 14: Rate of the reduction of *Callosobruchus maculatus* Fab. emergence under the effect of essential oils.

life [45, 50–53]. It was reported that EOs' efficiencies are closely related to the phytochemical profile and the entomological target [54].

A. herba alba Asso. is among the most important species of the family Asteraceae, which has been the subject of various chemical and biological studies, including insecticidal properties [55, 56]. It is thus fitting that our results are in agreement with those reported by Hussain [57], who investigated the insecticidal properties of *A. herba alba* Asso on other insects. In addition, Abdelgaleil et al. [58] and Aggarwal et al. [59] reported that the insecticidal properties of many *Artemisia* species are due to the presence of 1,8-cineole. Furthermore, Bachrouch et al. [60] reported that the high percentages of α Thujone, 1,8-cineole, and norboran-2-one in the essential oil of *A. herba alba* conferred it the best insecticidal potential against two stored commodity insects

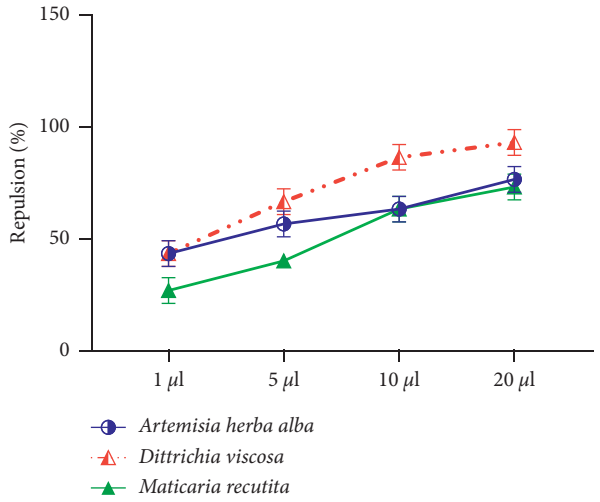


Figure 15: Repulsive effects of essential oils against *Callosobruchus maculatus* Fab.

including *Oryzaephilus surinamensis* (L.) and *Tribolium castaneum* (Herbst). Therefore, we can confirm that the insecticidal effects of the studied EOs in this study could be due to the thujone identified by GC-MS in EOA.

For better understating this insecticidal effect of EOs, the study conducted by Senthil-Nathan [61] revealed that botanical chemicals affect the metabolism and growth of insects through physiological processes and enzymatic activities. In this way, understanding the biochemical effects of pesticides on insects can certainly provide safe pest control strategies [62]. In this sense, and according to França et al. [63], botanical insecticides can act on insects as acetylcholinesterase (AChE) inhibitors. So that the inhibition of AChE by phytochemicals, especially monoterpenoids, causes blockage of the neurotransmitter acetylcholine at the synaptic cleft [64], which is the case for the EOs tested in our study, whose essences are rich in compounds with a strong insecticidal effect against *C. maculatus*. Indeed, the pharmacological effect occurred at the level of chloride channels, octopamine receptors, tyramine receptors, acetylcholine esterase, nicotinic acetylcholine receptors (nAChR), and sodium channels in *C. maculatus* treated with EOs may be due to the individual activity of single compound or due to synergistic relationship of several chemical compounds because EOs contain a complex mixture of constituents that can interact with many target molecules [22]. EO compounds can also act with a variety of other targets including g-aminobutyric acid (GABA) gated.

A study was carried out by François Renoz et al. [65] on the mode of action of the EO of *M. arvensis* tested by contact on adult Sitophilus granarius, leading to rapid paralysis and alteration of walking behavior. This study revealed that the EOs of Mentha arvensis induced dramatic physiological changes in the exposed insects. In this case, the majority of differentially expressed proteins (DEPs) related to the muscle and nervous system were dysregulated along with serious problems affecting cellular respiration, protein synthesis, and detoxification process. These results suggest that EOs are capable of affecting a variety of biological processes.

The obtained findings showed that a significant reduction in fecundity and emergence rate was recorded. This is could be explained by ovicidal and larvicidal activities of the tested EOs that potentially creep into eggs, which in turn result in the blockage of embyogenesis [65, 66]. The ovicidal activity of the EOs would be due to the direct effects of their compounds leading to the inhibition of the metabolic activity of the eggs. This is the case of piperitone isolated from the EOs of *Cymbopogon schoenanthus* L. on the eggs of *Callosobruchus maculatus*. Moreover, ß-arasone identified in the EOs of *Acorus calamus* L. showed toxicity on the eggs of *Callosobruchus chinensis*, *Sitophilus oryzae* L., and *Sitophilus granarius* L. [67]. In addition, according to Schmidt et al. [67], EOs have a sterilizing effect on eggs. Previous works carried out on *Acanthoscelides obtectus* belonging to the family Bruchidae, showed high sensitivity of eggs to the vapors of Eos from *Lavandula hybrid* L., *Rosmarinus officinalis* L., and *Eucalyptus globules* L. [68].

The percentage of repulsion effect evaluated by the method of McDonald et al. [37] showed that the EOA, EOD, and EOM were repulsive towards *C. maculatus* with a respective rate of 60%. In this context, Papachristos and Stamopoulos [69] showed that EOs of 13 aromatic plants, five of them *Monardella viridis* L., *Eucalyptus globulus* L., *Melaleuca microphylla* L., *Rosmarinus officinalis* L., and *Lysimachia hybrid* L. showed significant repulsive effects against *Theileria orientalis* L. and *Clonorchis sinensis* L. Furthermore, these authors reported that the most toxic EO exhibited simultaneously repulsive and reproductive inhibitory effects on bean bruchid, so that our results were in agreement with this literature.

Further works aim to evaluate potential toxicities of the studied EOs on nontarget organisms, and humans will be more appreciated prior to developing insecticide-based essential oils.

5. Conclusion

This study concluded that the essential oils from *Artemisia herba alba* Asso. and *Maticaria Recutita* L. and *Dittrichia Viscosa* L. well controlled the development of *Callosobruchus maculatus* with more attention can be paid to *Artemisia herba alba* Asso. essential oil rich in alpha thujone, beta-thujone, and chrysanthenone. The obtained results in this study showed that the use of essential oils as a biological pesticide could be incorporated in the management program for the control of chickpea pests as a safe alternative form.

Data Availability

Data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

A. A., M. B., H. H., R. S., and A. M. S. contributed to original draft preparation and review and editing. W. S., H. Z. R., L. O., and E. A. Y. carried out formal analysis and review and editing. E. N. and F. M. performed conceptualization and supervision.

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Review Article

Research Advances in Antitumor Mechanism of Evodiamine

Luning Li,[1] **Cunxin Zhang**,[2] **Chen Huang**,[1] **Xinchen Tian**,[3] **Wenxue Sun**,[1,4] **and Shulong Jiang**[1]

[1]*Tumor Laboratory, Jining No. 1 People's Hospital, Jining Medical University, Jining, Shandong 272000, China*
[2]*Department of Spine Surgery, Jining No. 1 People's Hospital, Jining Medical University, Jining, Shandong 272000, China*
[3]*Cheeloo College of Medicine, Shandong University, Ji'nan, Shandong 250100, China*
[4]*Department of Pharmacy, Jining No. 1 People's Hospital, Jining Medical University, Jining, Shandong 272000, China*

Correspondence should be addressed to Wenxue Sun; sunwenxue121@126.com and Shulong Jiang; jnsljiang@163.com

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Evodiamine is a natural alkaloid extracted from Fructus Evodia. This bioactive alkaloid has been reported to have a wide range of biological activities, including anti-injury, antiobesity, vasodilator, and anti-inflammatory effects. In recent years, it has been found that evodiamine has tumor-suppressive effects on a variety of tumors. There is growing evidence that evodiamine can inhibit the rapid proliferation of tumor cells, induce cell cycle arrest at a certain phase, increase the incidence of apoptosis, promote autophagy, inhibit microangiogenesis and migration, and regulate immunotherapy. Evodiamine can inhibit Wnt/β-catenin, mTOR, NF-κB, PI3K/AKT, JAK-STAT, and other signaling pathways in various cancer cells, and it can significantly downregulate the expression of many tumor markers, such as VEGF and COX-2. These facts partially explain the antitumor mechanism of evodiamine. In this article, the antitumor mechanism of evodiamine was reviewed to provide the basis for its clinical application and therapeutic development in the future.

1. Introduction

The traditional Chinese medicine *Fructus Evodia* is the dry fruit of the plant *Evodia rutaecarpa*, and it was first mentioned in "Shennong Materia Medica Classic." It has been found that *E. rutaecarpa* has analgesic, antidiarrheal, antiemetic, and anti-inflammatory effects in clinical practice [1–5]. The dried, powdered fruit (2.5 kg) of *E. rutaecarpa* was defatted with n-hexane (2 × 5 L) and extracted with acetone (3 × 5 L) at room temperature to yield an extract (137 g). The crude acetone extract was then eluted with n-hexane: acetone (9 : 1–19 : 1) on a silica gel column to produce evodiamine (6.17 g) [6]. Evodiamine was also purified from Fructus Evodia by a high-performance liquid chromatography method with a gradient elution of acetonitrile-tetrahydrofuran-0.02% phosphoric acid at the detection wavelength of 220 nm [7, 8].

Previous studies have shown that evodiamine has many biological activities, including anti-inflammation [9–14], antiobesity [12], analgesia [14], and promotion of vasodilation [15–18]. These activities are related to a wide range of targets, such as caspase 3 and transient receptor potential vanilloid 1 (TRPV1) [19–22]. In recent years, evodiamine has been found to have antitumor activity against a variety of tumor cells, involving various mechanisms that cause cancer cell death, such as induction of cell cycle arrest, promotion of cell apoptosis, promotion of autophagy, and inhibition of tumor invasion and metastasis [23–30] (Figure 1). The signaling pathways involved in the antitumor effect of evodiamine include the classical apoptosis pathway, endoplasmic reticulum stress, MAPK, PI3K-Akt, and JAK-STAT [31–34]. In this article, the antitumor mechanism of evodiamine was reviewed based on research trends in recent years, and the ways through which evodiamine could exert its antitumor effect were summarized to provide a basis for its clinical application and in-depth research.

2. Molecular Mechanisms of Antitumor Effect of Evodiamine

2.1. Inhibition of Tumor Cell Proliferation and Cell Cycle. There is evidence that evodiamine reduces the occurrence of tumors by inhibiting the proliferation of cancer cells and changing the cell cycle. Previous studies have shown that

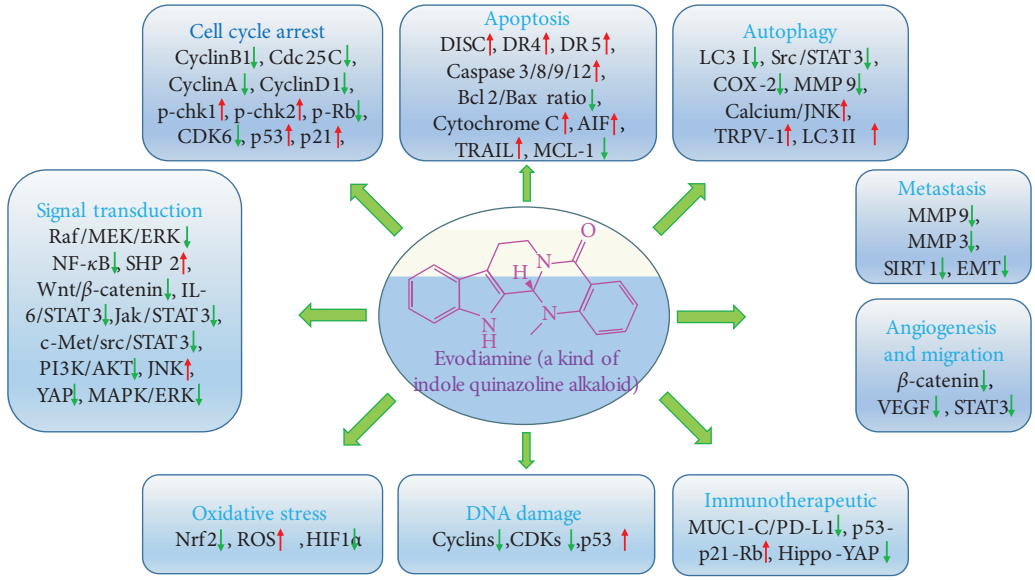

Figure 1: Synopsis of the multifactorial mode of action of evodiamine-type drugs.

evodiamine can inhibit the proliferation of human cancer cell lines, such as breast cancer [35], colon cancer [36], liver cancer [37, 38], cholangiocarcinoma [27], osteosarcoma [39], and melanoma [40] in a dose- and time-dependent manner (Table 1). One of the distinguishing features of tumor cells is their dysregulation of the cell cycle caused by the abnormal expression of cyclins and/or the abnormal replication of DNA. The activity and expression states of CDC25 C, cyclin, and cyclin-dependent kinases (CDKs) regulate the stability of the cell cycle. Many studies have shown that arresting tumor cell cycle progression is an effective strategy to inhibit tumor proliferation [41–45].

Previous studies have reported that evodiamine may block the cell cycle progression and significantly increase the percentage of G2/M phase cells in tumors (Table 2). Zhou et al. found that evodiamine affected the cell cycle progression in human osteosarcoma cells U2OS. The same authors reported that the expression levels of cyclin B1, CDC25 C, and CDC2 changed significantly, which may be related to evodiamine-dependent downregulation of the phosphorylation of MEK and ERK and inhibition of the Raf/MEK/ERK signaling pathway [46]. Moreover, in human lung cancer cells with G2/M phase arrest after evodiamine treatment, cyclin A and cyclin B1 expression levels decreased, whereas those of p-Chk1 and p-Chk2 increased [47]. In addition, evodiamine interferes with the colorectal cancer cell lines COLO205 and HT-29, causing the cell cycle to stagnate in the G2/M phase, which has been shown to be related to changes in cyclin B1/CDC25 C expression and is controlled by JNK [48]. According to the above studies, evodiamine induces the G2/M cycle arrest by affecting signaling factors involved in multiple signaling pathways regulating cyclins, CDKs, and CHK.

Evodiamine may also cause some tumor cells to stagnate at the G0/G1 phase (Table 2). Interestingly, Du et al. found evidence of evodiamine-induced downregulation of cyclin D1 and CDK6 in human breast cancer MDA-MB-231 cells; these cells stagnated at the G0/G1 phase in the presence of evodiamine treatment [49]. In another study, after using evodiamine, the cells were seeded in the upper well of a Transwell and incubated with 10 μg/mL PGI/AMF and 10 μg/mL PGI/AMF +6 μmol/L evodiamine for 28 h. Normal cells served as the control group. We then used these cells to treat human breast cancer MCF-7 and MDA-MB-231 cells, and tumor cells were arrested at the G0/G1 phase; p53 and p21 were upregulated; and p-Rb, cyclin B, and cyclin A were downregulated [50]. Based on these results, evodiamine keeps the cell cycle in the G0/G1 phase by targeting the p53-p21-Rb signaling pathway and affecting the expression of G0/G1 phase cyclin-related proteins.

2.2. Promotion of Apoptosis in Tumor Cells. Apoptosis is a very important and highly conservative cell death model that can effectively inhibit tumorigenesis. The classical process of cell apoptosis involves caspases as the core proteins that activate other proenzymes in a sequence, which involves multiple signaling pathways leading to cellular apoptosis [51–55]. The promotion effects of evodiamine on apoptosis signal transduction pathways can be divided into extrinsic and intrinsic categories. The extrinsic pathway involves the activation of the death receptor pathway, whereas the intrinsic pathway includes the activation of the mitochondrial and endoplasmic reticulum pathways.

Evodiamine induces the expression of death receptors on the surface of tumor cells and promotes the binding of death receptors to their ligands, thereby accelerating the formation of DISC and leading to apoptosis (Table 3). According to Khan et al., evodiamine can upregulate the death receptors DR4 and DR5 in U87 glioblastoma cells under the synergistic effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which leads to the activation of caspases 3 and 8 and the induction of apoptosis in tumor cells [56].

Table 1: Inhibition of tumor cell activity and proliferation by evodiamine in cancer cell lines.

Cell line	Cell type	Specific results	Mode of action	References
MCF-7/ADR	Breast cancer	The IC_{50} values of MCF-7 cells treated with evodiamine for 24, 48, and 72 h were 7.68 μM, 0.64 μM, and 0.30 μM, respectively. The IC_{50} values of the MCF-7/ADR cells were higher than those of MCF-7, with values of 24.47 μM, 1.26 μM, and 0.47 μM for 24, 48, and 72 h of treatment with evodiamine, respectively.	Inhibiting cell activity via the regulation of Ras/MEK/ERK pathway	[35]
HCT-116/L-OHP	Colorectal cancer	The cells were treated with evodiamine at 0.4, 0.8, and 1.6 μM for 24, 48, and 72 h, respectively. Evodiamine significantly enhanced rhodamine 123 accumulation and caused a significant decrease in the IC_{50} level in HCT-116/L-OHP cells.	Inhibiting the p50/p65 NF-kappaB pathway	[36]
HepG2, Huh-7, and Hep3B	Hepatocellular carcinoma	The cells were treated with evodiamine at 0.25, 0.5, 1, 2, 4, 6, and 8 μM for 72 h. Evodiamine inhibited the proliferation of HCC cells in a dose-dependent manner under hypoxia (survival fraction: 1.0–0.3).	Downregulating HIF-1α under hypoxia	[37]
HepG2 and Bel-7402	Hepatocellular carcinoma	Evodiamine treatment at a range of concentrations (0, 0.25, 1, 4, 8, 16, and 32 μM) inhibited HepG2 cells and Bel-7402 growth. The IC_{50} values of HepG2 and Bel-7402 cells at 48 h were 14.7 μM and 16 μM, respectively.	Affecting the Hippo-Yes-associated protein signaling pathway	[38]
HuCCT-1 and TFK-1	Cholangiocarcinoma	Two CCA cell lines, HuCCT-1 and TFK-1, were treated with evodiamine at different concentrations (0, 5, 10, 20, or 40 μM) for different time periods (24, 48, or 72 h). Cell viability: 80%–20% (HuCCT-1); 75%–25% (TFK-1).	Inducing IL-6/STAT3 signaling inhibition.	[27]
143B and MG63	Osteosarcoma	143B cells were treated with evodiamine at 0.5, 1, 2, 4, 6, 8, 16, and 32 μM. MG63 cells were treated with evodiamine at 0.25, 0.5, 1, 2, 4, 6, 8, and 16 μM. Cell viability was measured at 24, 48, and 72 h. Relative growth rate: 95%–15% (143B); 98%–30% (MG63).	Inhibiting Wnt/beta-catenin signaling pathway	[39]
A-375	Melanoma	The cells were treated with evodiamine at 5, 7.5, 10, 12.5, and 15 μM for 24, 48, and 72 h. Relative cell viability: 1.0–0.2.	Inducing reactive oxygen species-dependent apoptosis and necrosis	[40]

Table 2: Induction of cell cycle arrest by evodiamine in cancer cell lines in vitro.

Cell line	Cell type	Specific results	Mode of action	References
U2OS	Osteosarcoma	Evodiamine treatment at a range of concentrations (0, 3, 6, and 12 μM) triggered G2/M cell cycle arrest (9%–77%) for 24 h.	Inhibiting the Raf/MEK/ERK signaling pathway	[46]
A549	Lung cancer	Evodiamine treatment at a range of concentrations (0, 1, 2, and 4 μM) triggered G2/M cell cycle arrest (15.8%–86.2%) for 24 h.	Downregulation of ERK	[47]
COLO205 and HT-29	Colorectal carcinoma	Evodiamine treatment at a range of concentrations (0, 1, 2, and 4 μM) triggered G2/M cell cycle arrest for 24 h; evodiamine treatment at 2 μM triggered G2/M cell cycle arrest for 6, 12, and 24 h.	JNK activation	[48]
MDA-MB-231	Breast cancer	Exposure to different concentrations of evodiamine (0, 15, 30, 60, 90, and 120 μM) caused G0/G1 arrest of MDA-MB-231 (53.29%–60.12%) for 24 h.	Decreasing Bcl-2, cyclin D1, and cyclin-dependent kinase 6 expression	[49]
MCF-7 and MDA-MB-231	Breast cancer	Cell cycle analyses in monolayer culture (condition with FBS, without FBS), sphere culture condition. Evodiamine (0, 100, and 200 nM) mildly but significantly increased the proportion of cells at the G1 stage.	Activating the p53-p21-Rb pathway	[50]

Evodiamine can transform the tumor cells' mitochondrial membrane into a state of increased permeability, thereby causing the irreversible release of various apoptosis promoters, including cytochrome C and AIF, into the cytoplasm or the nucleus and promoting the occurrence of apoptosis (Table 3). Mohan et al. found that evodiamine

Table 3: Induction of apoptosis and autophagy by evodiamine in cancer cell lines in vitro.

Cell line	Cell type	Specific results	Mode of action	References
U87	Glioblastoma	The cells were treated with evodiamine (10 μM) and TRAIL (50 ng/ml) either separately or in combination for 24 h. TRAIL alone did not induce apoptosis, whereas evodiamine significantly induced apoptosis in U87 glioblastoma cells after 24 h (apoptosis rate: 35%–70%).	Sensitizing cells to TRAIL via the death receptor pathway	[56]
A549 and H1299	Lung epithelial cancer	Evodiamine (20–40 μM) treatment of A549 cells for 24 h significantly increased the number of apoptotic cells by sixfold.	Activating both the intrinsic and the extrinsic apoptosis pathways	[57]
U937	Leukemia	The cells were treated with evodiamine (0.2–0.8 μmol/L) for 18 h. Evodiamine induced apoptosis in a dose-dependent manner (sub-G1 population: 5%–45%).	Activating caspase-dependent and caspase-independent pathways	[58]
MC3 and HSC4	Oral cancer	The cells were treated with various concentrations of evodiamine (0.25, 0.5, and 1 μM for MC3 cells; and 0.2, 2, and 20 μM for HSC4 cells) for 24 h. The apoptotic effect of evodiamine was confirmed by 4′-6-diamidino-2-phenylindole (DAPI) staining. Evodiamine-treated cells displayed nuclear condensation and fragmentation—hallmarks of apoptosis.	Inhibiting the AKT pathway	[59]
H446 and H1688	Small cell lung cancer	The induction of apoptosis was detected in H446 or H1688 cells after 24 h of treatment with 10 μM evodiamine. The apoptosis rate of evodiamine-treated H446 (~15%) or H1688 (~11%) cells was much higher than that of the untreated cells (blank control, ~5% or ~4%).	Activating mitochondria-dependent and endoplasmic reticulum stress-induced pathways	[60]
U87-MG	Glioblastomas	The percentage of autophagy induced by evodiamine (0, 0.1, 6, 10 μM) increased to a maximum at 24 h, but not at 48 h, indicating that evodiamine-induced autophagy in a dual-regulated manner.	Activating calcium/c-Jun N-terminal kinase and calcium/mitochondria-mediated pathways	[61, 62]
Panc-1 and SW1990	Pancreatic cancer	PANC-1 and SW1990 cells were treated with evodiamine (1.0, 5.0, and 10 μM) for 48 h. The exposure of cells to evodiamine led to a dose-dependent increase in apoptosis. Apoptosis rate: 2.5%–17.5% (Panc-1); 4%–18% (SW1990).	Inhibiting the PI3K-Akt and the MAPK/ERK signaling pathways	[63]
Murine Lewis lung carcinoma	Lung carcinoma	The cells exhibited an increase in MDC fluorescence within 1.5–6 h after evodiamine treatment, while the peak of autophagy activity was observed in 1.5 h treatment. In addition, evodiamine-treated cells displayed a greater number of distinct spots within the cytoplasm or perinuclear regions compared with controls.	Activating caspase-independent pathways	[64]

significantly increased the mitochondrial membrane depolarization, increased the cell apoptosis rate, and slightly decreased the Bcl-2/Bax ratio in human lung cancer A549 and H1299 cells. It also increased the release of cytochrome C from mitochondria into the cytoplasm, thereby activating the cascade of proapoptotic pathways, including caspases 3 and 9 [57]. In another study, evodiamine was found to promote the mitochondrial release of AIF into the nucleus in human leukemia U937 cells, leading to chromosome agglutination and the induction of cellular apoptosis [58]. In addition, evodiamine can also promote mitochondrial apoptosis of tumor cells by suppressing the expression of the inhibitor of apoptosis proteins. Mcl-1 is an apoptosis inhibitor protein located on the mitochondrial membrane. Sachita et al. found that evodiamine reduced the expression of Mcl-1 during transcriptional modification, and knockout of Mcl-1 significantly increased the expression of active Bax, thereby inducing apoptosis [59]. These studies have shown that evodiamine can effectively regulate the ratio of pro-apoptotic and antiapoptotic factors to promote changes in mitochondrial membrane that are conducive to apoptosis and promote the occurrence of mitochondria-dependent apoptosis.

Endoplasmic reticulum stress occurs when proteins are disrupted during transport from the endoplasmic reticulum (ER) to the Golgi apparatus. When newly synthesized or when the Ca^{2+} equilibrium state is broken, unfolded or misfolded proteins accumulate in large quantities in the ER. Fang et al. treated human small cell lung cancer H446 and H1688 cells with evodiamine and found excessive accumulation of reactive oxygen species (ROS), which caused endoplasmic reticulum stress and released a large amount of

Ca^{2+}. Furthermore, the upregulation of caspase-12 led to the upregulation of caspase-9 and caspase-3 expression, which in turn led to apoptosis [60]. Therefore, evodiamine can disrupt the Ca^{2+} balance in cells and induce ER stress by activating caspase-12 and other signaling pathways, thereby activating the ER-mediated apoptosis pathway (Table 3).

2.3. Induction of Autophagy of Tumor Cells. Autophagy is a process of lysosome phagocytosis and degradation of its own structure, which can remove damaged cellular structures and senile organelles. This process exists in most eukaryotic cells [65–67]. Autophagosome formation and processing of microtubule-associated protein 1 light 3 (LC3) are two major signs of autophagy.

The role of autophagy in tumors is controversial. In fact, it may play different roles at different stages of tumor genesis and development [68–73]. Evodiamine induces protective autophagy mediated by the activation of the JNK pathway following extracellular Ca^{2+} influx. Liu et al. found that evodiamine could induce autophagy in human glioblastoma U87-MG cells. The application of an extracellular calcium scavenger and an antagonist of transient receptor potential vanillin-1 (TRPV-1) reduced the percentage of cells undergoing autophagy. The same phenomenon was confirmed by knocking down the expression of TRPV-1 by small-interfering RNA technology. Moreover, the activation of c-Junn terminal kinase (JNK) by evodiamine was decreased by the TRPV1 antagonist, which further confirmed that evodiamine can promote autophagy through the Ca^{2+}-mediated JNK pathway [61].

Although there have been many reports that evodiamine can promote autophagy in tumors, some studies have shown that evodiamine-related induction of autophagy in specific cells antagonizes apoptosis. This observation illustrates the complexity of the antitumor mechanism of evodiamine. Hong et al. found that evodiamine-induced apoptosis of prostate cancer (PC) cells by inhibiting the PI3K-Akt and MAPK/ERK signaling pathways in human pancreatic cancer Panc-1 and SW1990 cells, and by inhibiting the phosphorylation of signal transduction and transcriptional activator 3 to inhibit autophagy [63]. Tu et al. found that evodiamine could improve the expression level of the autophagy-specific genes (Atgs) in Lewis lung cancer cells, accelerate the transformation from LC3-I to LC3-II, and thus promote the formation of autophagosomes (Table 3). Moreover, when combined with the autophagy inhibitor 3-methyladenine (3-MA), autophagy induced by evodiamine was significantly reduced, while apoptosis was increased. This suggests that autophagy induced by evodiamine may have a protective effect on tumor cells and that the inhibition of autophagy can promote the occurrence of apoptosis [64]. Liu et al. further found that evodiamine could induce autophagy mediated by calcium/JNK signals in glioma cells, while treatment with Ca^{2+} scavenger BAPTA-AM significantly inhibited the activation of the intracellular JNK pathway and decreased autophagy, while apoptosis was increased [62]. These results confirm that evodiamine can promote Ca^{2+}-mediated autophagy in the JNK pathway and that the inhibition of autophagy induced by this pathway can reduce the activity of tumor cells and promote apoptosis.

2.4. Inhibition of Tumor Microangiogenesis and Tumor Migration. Vascular endothelial growth factor (VEGF), one of the most important growth factors, plays a key role in promoting angiogenesis (Table 4). It can promote mitosis and has antiapoptotic effect on endothelial cells; moreover, it increases vascular permeability and promotes cell migration. Because of these effects, VEGF actively regulates the angiogenesis of normal and pathological blood vessels [78–80]. VEGF and VEGF receptors (VEGFRs) are expressed on both endothelial and nonendothelial cells [81, 82]. Currently, the use of anti-VEGF and anti-VEGF receptor therapies to block angiogenesis in cancer or other pathological processes is considered extremely important.

Hepatocellular carcinoma (HCC) is a highly vascularized tumor, with high microvascular density and high levels of circulating VEGF. Shi et al. found that after the intervention with evodiamine in the subcutaneous H22 cell xenograft model, tumor growth was inhibited, and serum tumor markers, β-catenin, and VEGFa levels were significantly reduced. Evodiamine also blocked angiogenesis in the matrigel plug assay by inhibiting VEGF. In addition, evodiamine inhibited tumor growth and reduced the expression levels of various angiogenesis biomarkers, β-catenin, and VEGFa in the SMMC-7721 HCC cell xenograft model. Evodiamine was shown to have an antitumor effect on HCCs by inhibiting β-catenin, interacting with VEGFa, and reducing VEGFa expression, thereby inhibiting angiogenesis [74]. These results suggest that evodiamine can inhibit cell invasion and migration, block angiogenesis, and act as a potential therapeutic agent for HCC.

Signal transduction and activator of transcription (STAT) is activated in many human tumor cell lines and primary tumors. STAT3 plays an important role in angiogenesis in the tumor microenvironment and can regulate the expression of VEGF, matrix metallopeptidases (MMP), and other factors [83–86]. Hwang et al. found that treatment with evodiamine in PC-3 and DU145 cells significantly inhibited cell proliferation, reduced HGF-regulated c-Met/Src/STAT3 phosphorylation, and disrupted nuclear transfer of STAT3 protein. That study also revealed that evodiamine downregulated the expression of several carcinogenic markers, including COX-2, VEGF, and MMP-9. In addition, reduced Src/STAT3 activation was observed in PC-3 and DU145 cells transfected with c-Met small-interfering RNA (siRNA), resulting in reduced evodiamine-induced apoptosis [75] (Table 4). These results indicate that evodiamine can markedly inhibit the activation of the c-Met/Src/STAT3 signaling pathway, thereby inhibiting tumor cell survival, proliferation, and angiogenesis.

Metastasis is one of the main hazards and characteristics of malignant tumors, and is a key factor affecting the prognosis and survival status of patients. Matrix metalloproteinases are a large class of zinc-containing endopeptidases that have important biological functions in

Table 4: Inhibition of microangiogenesis and migration by evodiamine in cancer cell lines in vitro or in vivo.

Cell line	Cell type/animal models	Specific results	Mode of action	References
HepG2, SMMC-7721, H22	Hepatocellular carcinoma BALB/c nude mice	(1) To evaluate the effect of evodiamine on VEGF-induced angiogenesis, Matrigel plug assays were performed with C57BL/6 mice. The addition of evodiamine (200 μg) to the plugs containing VEGF inhibited vascular formation. These plugs displayed a paler appearance, and fewer blood vessels were observed. (2) HepG2 cells and SMMC-7721 cells were exposed to 0, 5, 10, or 20 μmol/L of evodiamine for 24 h. Representative images of cell migration and invasion were assessed with Transwells.	Inhibiting β-catenin and reducing VEGFa expression	[74]
PC-3 and DU145	Prostate cancer	PC-3 and DU145 cells were treated with 5 μM evodiamine for 24, 36, and 48 h. Evodiamine substantially downregulated the expression of VEGF.	Inhibiting the c-Met/Src/STAT3 signaling pathway	[75]
HT-29 and HCT-116	Colorectal cancer BALB/c nude mice	After treatment with different concentrations of evodiamine (0 or 1.5 μmol/L) for 24 h, the migration rates were detected. Wound-healing assay showed that treatment with evodiamine (1.5 μmol/L) significantly repressed the migration of HT-29 cells and HCT-116 cells.	Activating Sirt1	[76]
HCT-116	Colorectal cancer	Migration potential was assessed by Transwell assay after the cells had been treated by agents. The cells were seeded in the upper well of a Transwell insert, incubated with 10 μg/mL PGI/AMF and with 10 μg/mL PGI/AMF +6 μmol/L evodiamine for 28 h. Normal cells served as the control group. Evodiamine significantly inhibited PGI-induced migration in HCT-116 cells.	Inactivating the JAK2/STAT3 pathway through the downregulation of PGI	[77]

various events, including metastasis of cancer cells [87–90]. Zhou et al. found that evodiamine inhibited the expression of MMP-9 in vitro and in vivo by inhibiting the acetyl-NF-κB p65 subunit induced by Sirt1 in colorectal cancer HT-29 and HCT-116 cells, and thus inhibited the invasion and metastasis of the tumor cells [76]. Zhao et al. also found that evodiamine inactivated the JAK2/STAT3 signaling pathway by downregulating the expression of phosphoglucose isomerase (PGI), thereby downregulating the expression of MMP3 to inhibit the migration of human colorectal cancer HCT-116 cells [77] (Table 4). According to these studies, evodiamine has a significant inhibitory effect on MMPs, the key regulatory enzymes essential for tumor migration, which makes it suitable for further study as a lead compound for the inhibition of tumor migration.

2.5. Antitumor Immunotherapeutic Mechanism of Evodiamine. Inhibitory receptors, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD-1), play crucial roles in the inactivation of immune cells in the tumor microenvironment. The activation, amplification, and effector function of CD8+ T cells are significantly inhibited by the interaction between PD-1 and programmed death ligand 1 (PD-L1), which also assists in the immune escape of cancer cells [91–93]. In addition, the interaction between the microenvironment and immune regulation plays a critical role in clinical treatment. It is widely believed that activated CD8+ T cells have anticancer immunity in a variety of tumors and have a significant positive prognostic effect. Furthermore, the survival status of cancer patients is significantly affected by tumor-infiltrating CD8+ lymphocytes (TILs) in the tumor environment, further supporting the strong link between immune escape and the tumor microenvironment [94]. MUC1-C is highly expressed in a variety of tumor cells and regulates a variety of genes, including PD-L1, that are important for the immune escape of tumor cells. Jiang et al. found that, after acting on non-small cell lung cancer cells, evodiamine downregulated the expression of the MUC1-C/PD-L1 signaling pathway at both the transcription and protein levels, reduced the apoptosis of T cells, and significantly increased the level of CD8+ T cells. Evodiamine may therefore be a potential targeted therapy suitable as an adjuvant for immunotherapy [94] (Table 5). By acting on PD-L1 and other inhibitors that suppress T-cell activation, evodiamine improves the tumor microenvironment, increases the activation level of CD8+ T cells, and effectively inhibits immune escape, which has the potential to promote the efficacy of immunotherapy.

2.6. Other Effects of Evodiamine. In recent years, the development of antitumor drugs has focused on the development of bioactive molecules that can effectively and selectively act on a single molecular target. However, due to the complexity

Table 5: Antitumor immunotherapeutic mechanisms and antitumor drug combinations involving evodiamine.

Cell line	Cell type/animal models	Specific results	Mode of action	References
H1650 and H1975	Non-small cell lung cancer	Evodiamine (0, 2, 4, 8, 16 μM; 24 h) suppressed IFN-γ-induced PD-L1 expression in H1975 and H1650 cells. MUC1-C mRNA and protein expression were decreased by evodiamine (0, 2, 4, 8, 16 μM; 24 h) in NSCLC cells as well. Evodiamine (0, 2, 4, 8, 16 μM; 24 h) downregulated the PD-L1 expression and diminished the apoptosis of T cells. It inhibited MUC1-C expression and potentiated CD8+ T-cell effector function.	Elevating CD8+ T cells and downregulating the MUC1-C/PD-L1 signaling pathway	[94]
A549, H460, H1299, and H1650	Non-small cell lung cancer	The cells were treated with evodiamine (3 μM), erlotinib (20 μM), or the combination for 48 h. Combining evodiamine with erlotinib successfully inhibited cell proliferation and survival in wild-type EGFR NSCLC cells, characterized as erlotinib-resistant. In addition, evodiamine plus erlotinib significantly increased the apoptotic rate (61.86%) of NSCLC cells, as compared to single-agent treatment alone (evodiamine ~33.71%; erlotinib ~21.92%).	mTOR/S6K1- mediated downregulation of Mcl-1	[95]
Caco-2 and HT-29	Colorectal adenocarcinoma	Evodiamine (10 μM) enhanced the effect of berberine (0, 2.5, 5, 10, 20, 40 μM) on cell viability with IC_{50} values ranging from 38.85 μM to 15.82 μM in Caco-2 cells at 24 h, but evodiamine did not have this effect in HT-29 cells.	Attenuating the overexpression of P-gp gene	[96]

of various regulatory mechanisms in tumor cells, it is difficult for many single-target drugs to achieve lasting and effective control of tumors in the actual development process [97–100]. Therefore, the use of drug combinations is an advanced way to overcome these limitations.

Li et al. found that evodiamine combined with erlotinib can successfully inhibit the proliferation and survival of wild-type EGFR non-small cell lung cancer (NSCLC) cells with erlotinib resistance. Furthermore, the combination of evodiamine and erlotinib promoted apoptosis in NSCLC cells more significantly than any single-dose therapy. That study suggested that the combination of evodiamine and erlotinib may downregulate the expression of MCL-1 by regulating the mTOR/S6K1 pathway. Thus, evodiamine can significantly increase the sensitivity of tumor cells to erlotinib, and the combination of erlotinib and evodiamine could be an alternative solution for the problem of erlotinib resistance [95] (Table 5). In addition, chemotherapy can lead to inevitable side effects. Guan et al. found that evodiamine significantly inhibited the overexpression of the p-glycoprotein gene of P-glycoprotein-positive colorectal cancer cells through the synergistic effect with berberine, thus achieving an excellent anticancer effect. However, this synergistic effect was not associated with cell cycle arrest and apoptosis. The same study found that the synergistic effect of evodiamine and berberine also reduced the cardiotoxicity caused by evodiamine, which was closely related to berberine's regulation of exogenous apoptosis in NRF2-dependent and ROS-independent pathways. Therefore, the combination regimen of berberine and evodiamine is considered to have a better antitumor effect, while it significantly reduces side effects by targeting specific cells [96] (Table 5). The above research provides strong evidence for the effects of evodiamine in antitumor combination drugs and further supports the rationality of its application in tumor chemotherapy.

2.7. Structure-Activity Analysis of the Effects of Evodiamine-Related Compounds on the Antitumor Mechanism of Evodiamine. Structure-activity analysis of evodiamine, evodiamine-2, evodiamine-4, evodiamine-7, evodiamine-8, and evodiamine-12 showed that these compounds have the ability to induce DNA ladder formation in some cell lines. The results showed that adding an alkyl group, such as methyl or butyl, to the 14th position of quinazoline was essential for evodiamine to induce apoptosis [48]. According to another report, the researchers performed docking and molecular dynamics simulations on the homology model of TRPV1 to better understand the possible binding mode of evodiamine in the TRPV1 binding pocket [101]. The pharmacodynamic gene model further provided confidence in the effectiveness of docking research. This study revealed for the first time the structural determinants required for the interaction between TRPV1 and evodiamine, and provided new suggestions for the rational design of new TRPV1 ligands.

Evodiamine has a free amine group with a medium molecular weight, which is easily converted into active derivatives with medicinal properties [102]. This structure, with a free N-H group and a scaffold with a molecular weight of approximately 350, can be included in structure-based virtual screening (SBVS) studies. In addition, evodiamine shares an "L-shaped" conformation at the active site of the topo1-DNA

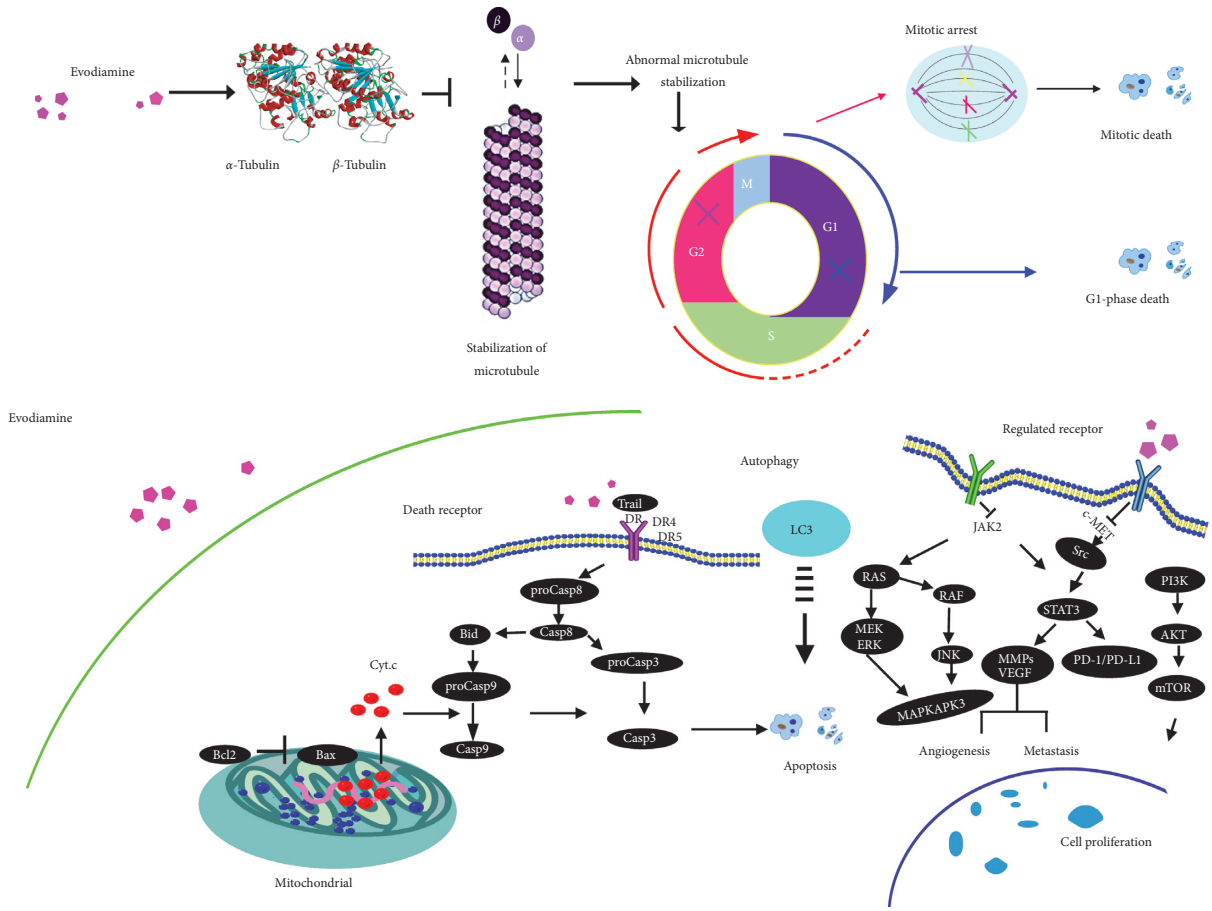

Figure 2: Probable molecular mechanisms by which evodiamine kills tumor cells.

cleavable complex. Its free indole N-H function is a suitable site for chemical derivatization. Proper functional group transformation to maintain hydrogen bond interaction is an effective method to maintain the binding affinity. When indoleamine is transferred to the amide, its carbonyl group can also form a hydrogen bond with Arg364. In this way, the key N-H group of evodiamine can undergo further chemical reactions and can be converted into active derivatives.

2.8. Cardiovascular Side Effects of Evodiamine. The effects of evodiamine on primary cultured neonatal rat cardiomyocytes have been studied in vitro, and the effects of evodiamine on zebrafish have been studied in vivo [103]. In vitro experiments have shown that evodiamine at a concentration of 28.44 μg/mL cocultured with cells for 24 h resulted in a 50% inhibition rate of cell viability. In vivo results have shown that evodiamine at a concentration of 354 ng/mL caused cardiac dysfunction. These findings suggest that cardiac function should be monitored during evodiamine therapy.

3. Conclusions and Prospects

In recent years, a large number of experiments in vivo and in vitro have shown that evodiamine can inhibit the activity, proliferation, and cell cycle progression in various tumor cells. Evodiamine also promotes apoptosis and induces autophagy in tumor cells, inhibits the formation and migration of tumor microvessels, and participates in the regulation of tumor immunotherapy. In addition, several signaling pathways are modulated by evodiamine, including PI3K/Akt, mTOR, and NF-κB cascades. Moreover, the excellent effect of the combination of other anticancer drugs with evodiamine in antitumor studies has further indicated its value for medicinal development (Figure 2).

It is predicted that efforts in using evodiamine as a multitarget antitumor drug in the following studies will be directed as follows. Its solubility, stability, and bioavailability need to be further improved so that it can exert its biological activity to a greater extent and more efficiently while reducing adverse reactions. In addition, further studies on the antitumor mechanism of evodiamine need to be conducted. For example, Src nonreceptor tyrosine kinases are important oncogenes that play an important role as an intermediate hub for phosphorylation and regulation of a variety of cytoplasmic downstream signals. In addition, STAT family transcriptional coactivators are important targets of Src. Furthermore, Src mediates the regulatory effects of extracellular signals on cell proliferation, migration, and apoptosis by participating in the transduction of various cellular signaling pathways. Current studies have shown that evodiamine interacts with the c-Met/Src/STAT3 signaling

pathway and inhibits tumor cell angiogenesis. However, the molecular target characteristics of its interaction with upstream Src need to be further studied. In addition, the existing experimental results are still limited to in vitro cell and in vivo animal experiments. The lack of clinical research reports related to evodiamine and the adverse reactions to evodiamine need to be further studied in depth.[103]

Abbreviations

TRPV-1: Transient receptor potential vanillin-1
HIF-1α: Hypoxia inducible factor-1α
ROS: Reactive oxygen species
TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand
JNK: c-Junn terminal kinase
VEGF: Vascular endothelial growth factor
STAT: Signal transduction and activator of transcription
MMP: Matrix metallopeptidases
CTLA4: Cytotoxic T lymphocyte-associated protein 4
PD-1: Programmed cell death protein 1
PD-L1: Programmed death ligand 1.

Data Availability

The datasets used in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Wenxue Sun and Shulong Jiang contributed to the conception of the review. Luning Li and Cunxin Zhang wrote the manuscript with support from Wenxue Sun and Shulong Jiang. Chen Huang and Xinchen Tian collected the literature. All authors have read and approved the final version of the manuscript. Luning Li and Cunxin Zhang contributed equally to this work.

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