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Antifungal and Antioxidant Activities of *Vernonia Chalybaea* Mart. ex DC. Essential Oil and their Major Constituent β-caryophyllene

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HIGHLIGHTS

- β-caryophyllene, bicyclogermacrene, caryophyllene oxide are major constituents.
- Essential oil and β-caryophyllene potentiated the effect of ketoconazole.
- The essential oil and major constituent showed antioxidant activities.
- Totally eleven sesquiterpenes were identified in *Vernonia chalybaea* essential oil.

Abstract: This article describes the chemical composition of *Vernonia chalybaea* essential oil, and investigates its antimicrobial, antioxidant and hemolytic activities. The evaluation of the antifungal activity was performed by the broth microdilution method using strains of yeasts and dermatophytic fungi. The checkerboard technique to find antimicrobial modulatory effects was performed using ketoconazole as standard drug. The antioxidant activity was evaluated by DPPH scavenging assay and β-carotene/linoleic-acid system. The toxicity was characterized by the brine shrimp lethality test and hemolysis bioassays. The essential oil was obtained by hydrodistillation and analyzed by GC-MS method, showing to be rich in the sesquiterpenes β-caryophyllene (39.06%) and bicyclogermacrene (19.69%), and also demonstrated a relevant antifungal activity against strains of *Trichophyton rubrum*. In the modulatory activity assay, the essential oil of *V. chalybaea* and β-caryophyllene demonstrated a synergistic interaction with ketoconazole, with increasing of its antifungal action. The antioxidant activity was evidenced mainly by β-carotene/linoleic acid system, with IC_{50} value of 35.87 ± 0.32 µg/mL. The results suggest that *V. chalybaea* essential oil and β-caryophyllene are valuable natural medicinal agents with antioxidant and antimicrobial activities.

Keywords: *Lepidaploa chalybaea*; *Trichophyton rubrum*; Sesquiterpene hydrocarbons; Antidermatophytic activity; Natural antioxidants.

INTRODUCTION

Vernonia species are found in tropical and sub-tropical areas, especially in South America, Asia and Africa. Several *Vernonia* species have been investigated revealing many properties, such as antiplasmodial, analgesic, anti-inflammatory, antimicrobial, anti-diabetes, antitumor, antioxidant and have been used as well for treatment of several microbial diseases, as snakebite antidote, and as food in some African regions [1]. Furthermore, the genus *Vernonia* has a commercial potential and included as new oilseed crop species [2].

Vernonia chalybaea Mart. ex DC. [syn. *Lepidaploa chalybaea* (Mart. ex DC.) H.Rob.] is a common species in South America, known in Brazil by many names, such as “cheira-bode”, “balaio” and “vassourinha”, endemic in Brazil with distribution in the Southeast and Northeast of the country, especially in regions of Caatinga (shrub lands) and rocky grasslands [3]. *V. chalybaea* is used to treat edema and liver disease in Latin-American folk medicine [4].

Previous studies reported the isolation of the sesquiterpene lactone glaucolide B from aerial parts of *V. chalybaea* [5] and investigated the antimicrobial activity against strains of Gram-positive and Gram-negative bacteria, yeasts and *Cladosporium cladosporioides* [6]. A study of Burim and coauthors [7] has shown that glaucolide B has cytotoxic and clastogenic activity.

The chemical investigation of the hexane and ethanol extracts from aerial parts of *V. chalybaea* conducted to the isolation and characterization of a new aliphatic tetrahydroxyl ether, along with a series of known compounds such the triterpenes 4α,10α-epoxyaromadendrane, friedelin, taraxasteryl acetate, pseudotaraxasteryl acetate, lupeyl acetate, lupeol, α-amiryn, β-amiryn; steroids as β-sitosterol, stigmasterol, and the flavonoids angophorol, angophorol-7-O-glucoside, angophorol-7-O-rutinoside, 3,7-dimethoxy-5,3',4'-trihydroxyflavone and acacetin [8]. These reports demonstrated a significant pharmacological and medicinal potential of *V. chalybaea* due to active compounds present in the plant extracts.

The therapeutic properties of many plants are supported by their chemical components, especially those isolated from extracts and essential oils, which often exhibit the ability to scavenge free radicals [9, 10].

Essential oils are frequently used in traditional medicine as antimicrobial agents, for their antiseptic action [11]. Many of these essential oils are rich in phenolic compounds, like phenylpropanoids and terpenoids, like eugenol and thymol, respectively of which antioxidant and antimicrobial activities are well documented [12, 13].

Then, the aim of this study was to investigate the biological potential of *V. chalybaea* essential oil (VCEO) and major constituent β-caryophyllene by analyzing the antioxidant and antifungal activities, including modulation activity assays with a standard antifungal drug and evaluates hemolytic action and toxicity against *Artemia salina*.

MATERIAL AND METHODS

Chemicals

All chemicals (if not stated otherwise) were obtained from Sigma–Aldrich Co (St. Louis, MO, USA), at the highest available grades.

Plant material

The botanical material used in this study (aerial parts) were collected in the flowering period in municipality of Alcântaras, in the Meruoca mountain region, Ceará, Brazil, in June 2014, in a semideciduous forest environment located around 800 m above sea level (latitude 3º 3' 3" S, longitude 40º 40' 40" W). A voucher specimen (No. 3106) was deposited in the Francisco José de Abreu Matos Herbarium (HUVA) and authenticated by Dr. Elnatan Bezerra de Souza of the Center for Agricultural Sciences and Biological Sciences, State University of Vale do Acaraú.

Isolation and analysis of the essential oil

Fresh aerial parts of *V. chalybaea* (640 g) were subjected to hydrodistillation for 4 h in a modified Clevenger-type apparatus [14]. The oil was dried over anhydrous Na2SO4 (~1 g), filtered and preserved in a sealed vial at 4 ºC prior to further analysis, with a yield of 0.1% (w/w). The chemical analysis of the essential oils constituents was performed with a Shimadzu QP-2010 Ultra instrument employing the following

conditions: column: Rtx-5MS (Crossbond 5%, diphenyl/95% dimethyl polysiloxane) with 30m x 0.25 mm x 0.25 µm df; carrier gas: He (24,2mL/min, in constant linear velocity mode); the injector temperature was 250 ºC, in split mode (1:100), and the detector temperature was 250ºC. The column temperature was programmed 35–180 ºC at 4 ºC/min then 180–280 ºC at 17 ºC/min, and at 280 ºC for 10 min; mass spectra: electron impact 70 eV. The sample was injected in volume of 1µL. The identity of the components was achieved from their GC retention times, calculated by linear interpolation relative to retention times of main compounds and by comparison of their mass spectra with those present in the computer data bank (NIST) and published literature [15].

Antifungal assay

The antifungal activity was determined by the broth microdilution method, in accordance to M27-A3 (yeasts) and M38-A (filamentous fungi) guidelines of Clinical and Laboratory Standards Institute [16, 17]. *Trichophyton rubrum* strains were obtained from the fungal collection of the Microbiology Laboratory, State University of Vale do Acaraú, and URM Culture Collection of the Department of Mycology, Federal University of Pernambuco, and *Candida* spp. strains from Hospital Santa Casa de Misericórdia de Sobral. A total of 4 strains of *T. rubrum*, 2 strains of *C. albicans*, 1 strain of *C. parapsilosis* and 1 strain of *C. tropicalis*. Standardized inocula (2.5–5 x 103 CFU mL-1 for *Candida* spp. and 5.0 x 104 CFU mL-1 for *T. rubrum*) were prepared by turbidimetry. Aliquot suspension were prepared in potato dextrose agar (Difco, Detroit, MI, USA), and then incubated at 28 ºC for 2 – 10 days. The suspensions were diluted to 1:2000 for *Candida* spp. and 1:500 for *T. rubrum*, both with RPMI 1640 medium with l-glutamine without sodium bicarbonate, and then buffered to pH 7.0 with 0.165 M MOPS. The minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) were determined according Fontenelle and coauthors [18] and CLSI guidelines document [16, 17]. The VCEO and β-caryophyllene were prepared in 100% mineral oil. Amphotericin B (AMB) and ketoconazole (KTC) were prepared in DMSO. Ketoconazole and amphotericin B (standard antifungals) were placed in concentrations ranging from 16 µg/mL to 0.125 µg/mL. For the antimicrobial activity, the essential oil samples were tested in concentrations ranging from 0.002 to 2.5 mg/mL. The microdilution test was performed in 96-well microdilution plates incubated at 37 ºC, and antifungal effect was analyzed visually after 2 days for *Candida* spp. and 5 days for *T. rubrum*. The MFC was determined by subculturing 100 µL of solution from wells without turbidity on potato dextrose, at 28 ºC. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after 2 days for *Candida* spp. and 5 days for *T. rubrum*.

Microdilution checkerboard assay

The modulatory activity was determined on all *T. rubrum* strains by checkerboard technique [19, 20]. Interaction of the drugs was determined by calculating the Fractional Inhibitory Concentration Index (FICI). Fractional Inhibitory Concentration (FIC) was defined with the MIC values of each drug in the combination divided by the MIC of the drug alone. The turbidity of the fungal suspensions was adjusted to 0.5 McFarland standard (105 CFU/mL). The products were used at concentrations of their respective MICs. 50 µL of RPMI 1640 medium was added to the wells of 96 wells microdilution plate. Then 50 µL of each dilution of natural product was added in vertical orientation, with the concentrations ranging from 5 mg/mL to 0.03 mg/mL. In the horizontal orientation, 50 µL of ketoconazole (standard antifungal) was placed in concentrations ranging from 16 µg/mL to 0.125 µg/mL. 100 µL from suspension of *T. rubrum* (5.0 x 104 CFU mL-1) was added to all wells and incubated at 37 ºC for 5 days. Assays were performed in triplicate. The FICI was interpreted as synergistic effect at values ≤ 0.5, an indifferent effect at values > 0.5 or ≤ 4.0, and an antagonistic effect at values > 4.0 [19,21].

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

Concentrations of the samples in 10000 -1 µg/mL were prepared for the mixture of 0.1 mL aliquots with 3.9 mL of DPPH methanol solution (6.5 × 10−5 M) for 1h of reaction. The test was performed in triplicate and after 1h, the absorbance of the mixture was measured at 515 nm by spectrophotometry [22]. The medium inhibitory concentration (IC50) was calculated in the Origin 7.0 statistic program.

β-carotene/linoleic acid assay

This assay was described by Lopes-Lutz and coauthors [23] and Andrade and coauthors [24]. A stock solution of emulsion was prepared with 1 mg of β-carotene dissolved in 5 mL of chloroform (0.3 mg/mL), 200 µL of Tween 40 and 20 µL of linoleic acid. Chloroform was completely evaporated using a vacuum evaporator.

After, 100 mL of oxygenated distilled water was added and emulsified for 1 min in a sonificator to form emulsion. The solution was adjusted in the spectrophotometer (470 nm). The final emulsion had absorbance between 0.6 nm and 0.7 nm. Aliquots of 100 µl of oil sample were dissolved in methanol, at concentrations of 500 µg/mL to 25 µg/mL, and then we added 5-mL of β-carotene/linoleic acid emulsion. The sample absorbance was measured after 2 min, then the samples were subjected to oxidation by placing in an oven at 50 °C for 120 min, and a second reading was performed. The β-carotene/linoleic acid emulsion was used as negative control. As positive controls were used the oxygenated monoterpenes thymol and carvacrol (concentrations of 500 µg/mL to 25 µg/mL), present in many essential oils. Each assay was performed in triplicate. IC50 values were defined as concentration sufficient to obtain 50% of a maximum effect estimate in 100%, and were calculated by the linear regression equation of the concentration of the essential oil and β-caryophyllene.

Brine shrimp lethality bioassay

The bioassay using *Artemia salina* Leach larvae (Crustacea, Artemiidae) was modified from the assay performed according to Meyer and coauthor [25]. Brine shrimp eggs were incubated at room temperature (between 22-29 °C) in artificial sea water for 48 hours. The essential oil was dissolved in methanol, DMSO, and saline water in concentrations of 1000, 100, 10 and 1 µg/mL. Then 10 shrimps were added to test tubes containing 5 mL of each tested solution and negative and positive control solutions. Potassium dichromate ($K_2Cr_2O_7$) dissolved in saline solution and saline solution with DMSO were used as positive and negative controls, respectively in concentrations of 1000 µg/mL to 1 µg/mL. This assay was performed in triplicate and the number of dead larvae was counted after contact for 24 hours. The percentage mortality and LC50 values was obtained by linear regression using the GraphPad Prism software 5.0 program.

Hemolysis assay

In this assay were used human red blood cells to determine cytotoxicity from essential oil in accordance as described to Ahmad and coauthors [26]. Blood samples were obtained from Sysmex (RocheTM) as hematological control sample from individuals with normal hematological parameters. An aliquot of 10 mL of fresh blood was collected in EDTA tubes, and then centrifuged at 2000 rpm for 10 m at 20 ºC. After plasma removal, the pellet containing the human erythrocytes was washed five times with PBS and then re-suspended in PBS to obtain an 8% (v/v) suspension. Then was added 100 µL of this suspension to different microcentrifuge tubes with 100 µL of 2-fold serial dilutions of essential oil, ranging from 0.005 to 2.5 mg/mL. Final concentrations were 4% (v/v) of erythrocyte suspension and the essential oil concentration range was 0.1-100 µM. The resulting suspensions were incubated with agitation for 60 min at 37 ºC. After incubation, the solutions were centrifuged for 2 min at 1000 g. The supernatants were transferred to 96-well plates and the hemoglobin release was measured by absorbance spectrophotometrically (540 nm). Triton X-100 at 1% and 4% (v/v) human erythrocytes in PBS with no essential oil (untreated) were used as positive and negative controls, respectively. Percentage hemolysis was determined as [(Abs540nm sample-treated – Abs540nm untreated)/ (Abs540nm1% Triton X-100 – Abs540nm untreated)] x 100, and experiments were carried out in triplicate.

Statistical analysis

All experiments were performed in triplicate. One-way ANOVA with the Tukey test was performed followed by multiple comparison testing where appropriate. IC50 values were performed with GraphPad Prism software 5.0 (GraphPad Software, San Diego, CA). Significance of difference was accepted at $p < 0.05$.

RESULTS

The essential oil yield of *V. chalybaea* (VCEO) was 0.1% (w/w - dry weight). Relative percentages of individual components of the VCEO in Table 1 were obtained from the GC-MS peak area-percent report. Chemical analysis of the essential oil revealed the presence of eleven sesquiterpenes, accounting for 91.94%. The main constituents were β-caryophyllene (39.06%), bicyclogermacrene (19.69%), caryophyllene oxide (8.69%) and β-elemene (8.25). Except for caryophyllene oxide all other constituents were sesquiterpene hydrocarbons.

Table 1. Chemical constituents of the essential oil from *Vernonia chalybaea* (VCEO)

Compound[a]	KI	VCEO
β-Elemene	1389	8.25
iso-Italicene	1392	1.26
cis-Bergamotene	1392	0.47
β-Caryophyllene	1427	39.06
epi-β-Santalene	1445	0.49
α-Humulene	1450	4.92
Bicyclogermacrene	1499	19.69
Spathulenol	1580	6.79
Caryophyllene oxide	1583	8.69
Globulol	1591	1.62
Humulene epoxide II	1610	0.70
Total identified compounds		91.94

[a] Order of elution on DB-5 capillary column. [b] Kovats retention index [15]

In the broth microdilution assay against dermatophytes and yeasts, the VCEO only inhibited the growth of dermatophytes, with MIC values of 1.25 mg/mL for all tested strains of *T. rubrum*. The results are summarized in Table 2. The tests showed that the VCEO did not significantly inhibit the growth of any of the tested yeasts (*C. albicans* LABMIC 0101, *C. albicans* LABMIC 0102, *C. tropicalis* LABMIC 0102 and *C. parapsilosis* LABMIC 0113).

Table 2. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of essential oils from *V. chalybaea* (VCEO) and β-caryophyllene against *Candida* spp and *T. rubrum*

Strains	VCEO		β-caryophyllene		Drug (µg/mL)
	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MIC (mg/mL)	Amphotericin B/Ketoconazole
***C. albicans* LABMIC 0101**	NI	NI	NI	NI	2.0
***C. albicans* LABMIC 0102**	NI	NI	NI	NI	2.0
***C. tropicalis* LABMIC 0102**	NI	NI	NI	NI	2.0
***C. parapsilosis* LABMIC 0113**	NI	NI	NI	NI	2.0
***T. rubrum* LABMIC 0201**	1.25	2.5	1.25	2.5	1.0
***T. rubrum* LABMIC 0202**	1.25	2.5	0.625	1.25	1.0
***T. rubrum* LABMIC 0203**	1.25	2.5	1.25	2.5	1.0
***T. rubrum* LABMIC 0204**	1.25	2.5	1.25	2.5	1.0
Geometric mean	1.25	2.5	1.05	2.1	-

NI, no inhibition of fungal growth. LABMIC, Microbiology Laboratory. Amphotericin B used for yeast and ketoconazole for dermatophytes.

In testing the modulatory activity, four *T. rubrum* strains were used. The results are shown in Table 3. The combination of VCEO with ketoconazole showed that there was a significant reduction in MIC values for essential oil against both strains, as well as significant reduction in the MIC of ketoconazole, from 1.0 to 0.25, for the strain *T. rubrum* LABMIC 0202. From these values, we calculated the fractional inhibitory concentration index (FICI), which showed a synergistic effect on the modulatory activity of strain *T. rubrum* LABMIC 0202 with FICI value of 0.3 and an indifferent effect in the tests with strain *T. rubrum* LABMIC 0201, whose FICI value was 1.0.

Table 3. MIC of the ketoconazole in the presence and absence of essential oil from *Vernonia chalybaea* (VCEO) and β-caryophyllene against dermatophyte strains.

Natural product/Drug	MIC (µg/mL) Alone	MIC (µg/mL) Combined	FIC index
	LABMIC 0201 (*T. rubrum*)		
VCEO	1250	39	0.5
Ketoconazole	1.0	0.5	
β-caryophyllene	1250	19.5	0.5
Ketoconazole	0.25	0.125	
	LABMIC 0202 (*T. rubrum*)		
VCEO	1250	78	0.3
Ketoconazole	1.0	0.25	
β-caryophyllene	625	19.5	0.5
Ketoconazole	0.25	0.125	
	LABMIC 0203 (*T. rubrum*)		
β-caryophyllene	1250	19.5	0.2
Ketoconazole	2	0.5	
	LABMIC 0204 (*T. rubrum*)		
β-caryophyllene	1250	19.5	0.2
Ketoconazole	1	0.25	

[a] FIC index, index fractional inhibitory concentration (FICI)

β-caryophyllene demonstrated capacity of modulatory activity against all dermatophytes strains, especially for the *T. rubrum* LABMIC 0203 and *T. rubrum* LABMIC 0204, whose FICI value was 0.2. These results obtained showed significant synergistic antifungal activity when combined β-caryophyllene with ketoconazole on the growth inhibition of dermatophytes.

The antioxidant potentials of VCEO and β-caryophyllene were investigated by the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay and β-carotene/linoleic-acid oxidation model system. IC50 values are shown in Table 4.

Table 4. Antioxidant activity of the essential oil from *Vernonia chalybaea* (VCEO), β-caryophyllene and the thymol and carvacrol, tested standards

Methods/ Compound	β-carotene / linoleic acid	DPPH
	IC_{50} (µg/mL)	IC_{50} (µg/mL)
VCEO	35.87 ± 0.32	249.68 ± 1.01
β-Caryophyllene	42.99 ± 0.88	98.74 ± 0.78
Thymol	11.32 ± 0.55	21.71 ± 0.16
Carvacrol	22.83 ± 0.98	25.5 ± 0.58

IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%.

The essential oil exhibited weak antioxidant capacity in the DPPH radical scavenging assay, with IC50 value of 249.68 ± 1.01 µg/mL, well above the standards, which were the oxygenated monoterpene thymol, whose IC50 was 21.71 ± 0.16 µg/mL and carvacrol (25.5± 0.58 µg/mL). However, the results were favorable for antioxidant effect in the β-carotene/linoleic-acid assay, preventing the oxidation of linoleic acid. In this assay, the IC50 of the essential oil was 35.87 µg/mL while that of thymol was 11.32 ± 0.55 µg/mL and carvacrol was 22.83 ± 0.98 µg/mL. These results characterize the hydrocarbon terpenic nature of compounds with apolar interactions similarity to β-carotene.

In the lethality assay of the VCEO with *A. salina* nauplii, the concentration required to kill 50% (LC50) was 29.96 ± 0.77 µg/mL. *In vitro* hemolytic activity assay of VCEO at concentrations 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 mg/mL showed the percentage of hemolysis ranged from 0.1% to 4.89% (Figure 1). Based on these results, and by extrapolation of hemolysis values the IC50 of 69.01 ± 0.78 mg/mL was estimated for the essential oil. Regarding hemolytic activity, the statistical analysis showed a significant difference at the level p =0.05 when compared to the positive control Triton X-100 with the VCEO concentrations used.

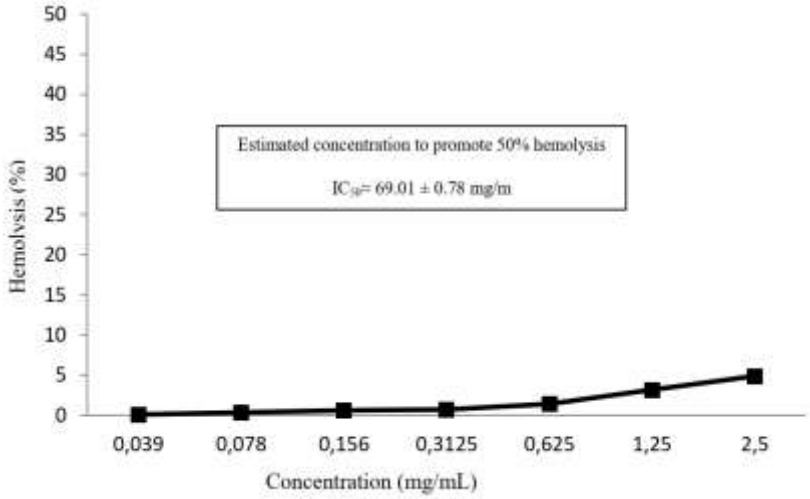

Figure 1. Hemolytic activity of the essential oil from *Vernonia chalybaea*. (**VCEO)**

DISCUSSION

A previous study of the chemical composition of the essential oil of the aerial parts of *V. chalybaea* [27] identified 23 components, representing 87.3% of the total content identified, with the presence of mono- and sesquiterpenes in similar proportions (46.8% and 40.5%), the main constituents being β-pinene (30.6%) and β-caryophyllene (12.1%). The difference in the essential oil composition in relation the previous study can be explained by many variables such as daily variations caused by the circadian cycle of the plant or the seasonal changes due to weather differences [28], water availability [29], ultraviolet radiation [30], nutrients [31], altitude [32] and mechanical stimuli [33]. Although the collections were carried out in the same region, they were collected at different points and at different times of the year.

Other studies have reported a biological potential of some of the major components found in the VCEO. Pharmacological trials demonstrated analgesic action [34] and antispasmodic action [35] of β-caryophyllene. Another constituent, bicyclogermacrene, has antimicrobial activity against Gram-positive and Gram-negative bacteria [36] and deuteromycete fungus *Cladosporium sphaerospermum* [37], which is one of the most commonly isolated airborne contaminants, responsible for the casuistry of cerebral and cutaneous phaehyphomycosis [38].

β-caryophyllene, major constituent of VCEO, is a bicyclic sesquiterpene with a rare 1,1-dimethylcyclobutane ring, commonly found in many essential oils from several plants, such as *Zingiber nimmonii* (J.Graham) Dalzell [39], *Copaifera reticulata* Ducke [40] and *Eupatorium ballotifolium* Kunth [41]. β-caryophyllene has been commonly used as a fragrance and flavouring agent in citrus flavors, soaps, spice blends, creams and lotions, detergents, and also in a variety of beverages and food products [42]. The antibacterial activity has been reported for several bacterial strains [43-45], thus increasing their antimicrobial potential.

There are no reports on the antimicrobial activity of *V. chalybaea* species. Therefore, this work describes for first time the antifungal activity *in vitro* of its essential oil against the dermatophyte *T. rubrum*. This fungus is responsible for clinical skin infections, dermatophytosis. It has high affinity for keratin protein and is one of the most prevalent fungi in the etiology of these contagious infections in humans [46], accounting for 80% of cases of onychomycosis [47].

Antifungal activity of β-caryophyllene [48-50], bicyclogermacrene [37] and caryophyllene oxide [48, 51] were reported in previous studies. These results indicate that the antifungal activity found in our study may represent a combined effect of these sesquiterpenes of the VCEO.

The modulatory activity results show that the combined use the natural product VCEO and β-caryophyllene with ketoconazole reduced the MIC of this antifungal agent. This result indicates that these natural products have an effect on strains of the dermatophyte *T. rubrum*, suggesting that modulatory activity tests should be conducted with other filamentous fungi that are human pathogens. A possible mechanism for the inhibition of microbial growth can be the hydrophobic nature of the constituents of natural products, which can act on the plasma membrane, making it more permeable to antifungal agents and thereby affecting the biochemical apparatus of the respiratory chain and of energy production [52]. β-caryophyllene is a hydrophobic molecule, which presents low molecular weight; these properties facilitate the permeability in the cellular membrane of the fungi, being able to be involved in the mechanism of action of inhibition of

microbial growth. This work is the first investigating modulatory activity of VCEO and β-caryophyllene combination with and potentiation on antifungal agent.

The β-carotene/linoleic-acid oxidation system is an assay based on the bleaching of β-carotene due to oxidation induced by oxidative degradation products of linoleic acid. This assay is employed mainly to investigate the antioxidant capacity of lipophilic substances, such as essential oils [53, 54]. This method is also suitable to determine the antioxidant activity of thermo-sensitive substances such as essential oils, since it does not require high temperatures [55].

Studies involving antioxidant activity of compounds isolated from plants have shown the antioxidant action of sesquiterpenes [56,57]. A study of the essential oil from aerial parts of *Eupatorium ballotifolium* identified 25 components, while the β-caryophyllene was the main constituent (23.59%). It was demonstrated the antioxidant and antifungal activities of the essential oil, and suggested that the high level of sesquiterpene hydrocarbons, β-caryophyllene (23.59%), germacrene D (6.56%) and bicyclogermacrene (6.47%), is related to the biological activities [41].

This can explain the antioxidant action found in VCEO, considering that its essential oil's chemical composition was mainly sesquiterpenes (76.93%). β-caryophyllene, the main constituent found, was previously shown to have antioxidant activity and protective effect on liver fibrosis and ability to inhibit activation of hepatic stellate cells [58]. The antioxidant action reported can be related with the synergistic effect between sesquiterpene hydrocarbons, main constituents from essential oil [59]. The *Vernonia* genus presents great biotechnological potential, due to the antioxidant properties reported in this work and in other species, such as *V. amygdalina* Delile [60,61], *V. patula* Mart. ex Baker [62], *V. cinerea* (L.) Less. [63] and *V. condensata* Baker [64].

The β-caryophyllene in the DPPH scavenging assay exhibited a moderated antioxidant capacity, however when compared to the VCEO, the major constituent demonstrated a better result. In the β-carotene / linoleic acid test, β-caryophyllene showed a better antioxidant capacity to protect the emulsion between β-carotene and linoleic acid in the system. The DPPH radical scavenging assay is commonly used to evaluate the antioxidant activity of more polar compounds such as phenols and flavonoids [53,65].

Studies involving antioxidant properties of natural products are relevant when interpreted together with antimicrobial assays, since antioxidant substances are efficient free radical stabilizers, helping to strengthen the immune system, an important feature in the development of bioproducts with antimicrobial action.

Bioassays with *A.* salina are largely used due to the fast results that correlate with potential biological activities and therefore indicate biological activity [66]. Toxicity tests with A. salina present good correlation with antimicrobial activity [67, 68]. The LC50 value (less than 1000 µg/mL) of the VCEO indicates that it has significant biological activity [25], as demonstrated in this work.

The determination of hemolytic activity of a substance helps determine its cytotoxic effect. Methods to determine the hemolytic activity in vitro consist of checking for potential damage of substances present in essential oils on the membranes of erythrocytes, in particular to cause lysis, releasing hemoglobin in the medium [69]. A percentage of 4.89% hemolysis was observed at the MIC values of the essential oil. Based on antifungal activity results, the essential oil concentration responsible for the bioactivity is less when compared to that needed to damage erythrocytes by membrane rupture. Therefore, the VCEO showed low cytotoxic action according to the test of hemolytic activity.

CONCLUSION

The essential oil from *Vernonia chalybaea* contains only sesquiterpenes, which can be responsible for its antioxidant and antifungal properties. This interaction was synergistic, reducing the MIC values of both substances tested, modulating their antifungal actions against strains of dermatophytes. These results indicate that the essential oil and main constituent β-caryophyllene potentiate the antifungal action of ketoconazole suggesting a possible utilization of these compounds in addition to antifungal drugs for the treatment of mycoses. Hence, this study of chemical and biological characterization of the essential oil of *V. chalybaea* points out the biotechnological potential of this plant species, corroborating with popular medicine and ethnomedicinal uses in skin infections.

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