academicJournals

Vol. 10(16), pp. 346-355, 29 April, 2016
DOI: 10.5897/AJPP2016.4537
Article Number: 4485D6E58304
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Cytotoxicity, antifungal and antioxidant activities of the essential oil from *Eupatorium ballotifolium* Kunth (Asteraceae)

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Received 20 January, 2016, Accepted 18 March, 2016

This study aimed to characterize chemically and evaluate the cytotoxicity and antioxidant, antifungal and modulatory activities of the essential oil of *Eupatorium ballotifolium*, collected in the mountainous region of Meruoca in the state of Ceará. The antioxidant activity was investigated by the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and β-carotene/linoleic-acid oxidation model system. The lethality bioassay was performed using *Artemia salina* (brine shrimp). The evaluation of the antifungal activity *in vitro* was performed by broth microdilution using strains of dermatophyte fungi and yeasts. The modulatory activity assays were performed by the checkerboard technique using ketoconazole as standard. Chromatographic analysis associated with mass spectrometry showed the main constituents of *E. ballotifolium* essential oil were β-caryophyllene (23.59%), thymol methyl ether (12.28%), germacrene D (6.56%) and bicyclogermacrene (6.47%). The brine shrimp lethality assay demonstrated potential biological activity. The essential oil showed better antioxidant action by the β-carotene/linoleic acid assay, with IC50 value of 19.47 µg/mL for essential oil, 11.32 µg/mL for thymol and 22.83 µg/mL for carvacrol, used as test standards. The broth microdilution test demonstrated that the essential oil inhibited fungal growth of all *Trichophyton rubrum* strains. In the modulation activity assay against strains of *Trichophyton rubrum,* there was synergism of essential oil on the strains of dermatophyte fungi when combined with ketoconazole.

Key words: Compositae, *Lourteigia ballotaefolia*, *Trichophyton rubrum*, antioxidant activity, antimicrobial activity.

INTRODUCTION

The Asteraceae family contains about 10% of the world's flora, and includes around 24,000 described species, grouped in 1,600 to 1,700 genera distributed in 17 tribes and 3 subfamilies (Funk et al., 2009; Petacci et al., 2012). It is the largest family of the eudicotyledons, and together with Calyceraceae, Campanulaceae, Menyanthaceae and Goodeniaceae, it forms a clade, the Asterales order (Pozner et al., 2012).

The genus *Eupatorium* L. (tribe Eupatorieae, subtribe Eupatoriinae) is significant in the Asteraceae family, comprising around 1,200 species. It is widely distributed, especially in Europe, Asia, North America and South America (Albuquerque et al., 2010). Brazil has around 250 native species with distribution in all geographical regions (Souza, 2007). *Eupatorium* species have been used to treat many diseases in folk medicine in various places in the world (Albuquerque et al., 2010).

Eupatorium ballotifolium Kunth (syn. *Lourteigia ballotaefolia* (Kunth) R.M. King & H.Rob.) is a perennial herbaceous species whose vertical growth ranges to 40 to 80 cm. It is an aromatic plant with few branches and many flowers, with pubescence and pink color with purple tones. Its leaves typically have purplish edges that are rich in oleíferas glands. The plant spreads through seeds (Silveira and Pessoa, 2005). It is popularly known in Brazil by many names, such as "maria-preta", "maria-preta-verdadeira" and "picão-roxo" (Cardoso et al., 2013).

Previous studies have demonstrated that the essential oil of the aerial parts of *E. ballotifolium* have anticholinesterase activity (Albuquerque et al., 2004), and two flavonoids were isolated from the aerial parts of the plant, nepetin and quercetin-3-O-glucoside, both showed antimitotic activity (Militão et al., 2004).

The therapeutic potential of many plants used in folk medicine has not been scientifically proved (Desoti et al., 2011). Hence, there is a need for chemical studies of natural products used in folk medicine to complement the studies to develop synthetic organic chemicals (Suffredini et al., 2006). Research of plants with antioxidant activity contributes to the development of new therapeutic strategies for inflammation, aging and chronic degenerative diseases (Fabri et al., 2011). Likewise, plants with potential antimicrobial activity can be a therapeutic alternative against multiresistant microorganisms to antimicrobial drugs (Bekele et al., 2015).

This study describes the chemical composition of the essential oil of *Eupatorium ballotifolium* (EOEB), and reports the antioxidant and antifungal activities against dermatophytes and yeasts, as well as the cytotoxicity, based on modulatory activity assays with ketoconazole.

MATERIALS AND METHODS

Plant material

The aerial parts of Eupatorium ballotifolium were collected in the flowering period in Brazil, Ceará, municipality of Alcântaras, in the Meruoca mountain region, in June, 2014 in a semideciduous forest environment located around 800 m above sea level. This region is located in the middle reaches of the Acaraú River, about 250 km from Fortaleza, the state capital. A voucher specimen (No. 3105) was deposited in Francisco José de Abreu Matos Herbarium (HUVA) and authenticated by Dr. Elnatan Bezerra de Souza of the Center for Agricultural Sciences and Biological Sciences, Vale do Acaraú State University.

Isolation of essential oil

Fresh aerial parts of *E. ballotifolium* (680 g) were subjected to hydrodistillation for 2 h in a modified Clevenger-type apparatus. The oil was dried over anhydrous Na_2SO_4 (~1 g), filtered and preserved in a sealed vial at 4°C prior to further analysis, with a yield of 0.1% (w/w).

Analysis of essential oil

The essential oil was analyzed using a Hewlett-Packard 5971 GC/MS instrument under the following conditions: dimethylpolysiloxane DB-5 fused silica capillary column (30 m × 0.25 mm i.d., 0.1 µm film thickness); carrier gas: helium (1 mL/min); injector temperature: 250°C; detector temperature 200°C; column temperature: 35 to 180°C at 4°C/min, then 180 to 250°C at 10°C/min; and mass spectra: electronic impact 70 eV. The identity of the components was achieved from their GC retention times relative to known compounds, calculated by linear interpolation relative to retention times of a series of n-alkanes and by comparison of their mass spectra with those present in the computer data bank (NIST) and published spectra (Adams, 2012).

In vitro antifungal assay

Fungal strains

The strains were obtained from the fungal collection of the Specialized Medical Mycology Center (CEMM), Federal University of Ceará, the URM Culture Collection of the Department of Mycology, Federal University of Pernambuco, and Hospital Santa Casa de Misericórdia de Sobral. In all these collections, the strains were maintained in saline (0.9% NaCl) at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated onto potato dextrose agar (Difco, Detroit, MI, USA), and then incubated at 28°C for 2 to 10 days.

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A total of four strains of *Trichophyton rubrum*, two strains of *Candida albicans*, one strain of *Candida parapsilosis* and one strain of *Candida tropicalis* were included in this study. *Candida* spp. strains were clinical isolates obtained from Hospital Santa Casa de Misericórdia de Sobral.

Preparation of inocula

For the broth microdilution method, standardized inocula (2.5 to 5 x 10^3 CFU mL^{-1} for *Candida* spp. and 5.0 x 10^4 CFU mL^{-1} for *T. rubrum*) were prepared by turbidimetry. Stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *T. rubrum*, respectively, grown on potato dextrose agar at 28°C. Sterile saline solution (0.9%) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *T. rubrum* and *Candida* spp., respectively. The suspensions were diluted to 1:2000 for *Candida* spp. and 1:500 for *T. rubrum*, both with RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with l-glutamine without sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA), and then buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co.), to obtain inocula of 2.5 to 5 x 10^3 CFU mL^{-1} and 5.0 x 10^4 CFU mL^{-1}, respectively.

Broth microdilution method

The minimum inhibitory concentration (MIC) for *Candida* spp. was determined by the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute (CLSI M27-A3, 2008). The broth microdilution assay for *T. rubrum* was performed as previously described (Sousa et al., 2009) based on the M38-A document (CLSI M38-A2, 2008).

The minimum fungicidal concentrations (MFC) for both *Candida* spp. and *T. rubrum* were determined according to the study of Fontenelle et al (2008). The EOEB was prepared in 100% mineral oil. Amphotericin B (AMB) and ketoconazole (Sigma, Chemical Co., USA) were prepared in distilled water. For the susceptibility analysis, the essential oil samples were tested in concentrations ranging from 0.002 to 2.5 mg/mL.

The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for the EOEB. The microplates were incubated at 37°C and read visually after 2 days for *Candida* spp. and 5 days for *T. rubrum*. The assay for the essential oil was run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 100% inhibition of visible fungal growth. The results were read visually as recommended by CLSI. The MFC was determined by subculturing 100 µL of solution from wells without turbidity on potato dextrose, at 28°C. 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* (Fontenelle et al., 2008).

Microdilution checkerboard assay

Assays were performed on all strains of *T. rubrum* according to the checkerboard technique (Johnson, 2004; Pyun and Shin, 2006), to determine the combined effect of the essential oil with the standard antifungal drugs. The interaction of the drugs was ascertained by calculating the fractional inhibitory concentration index (FICI). The FICI is calculated by adding the fractional inhibitory concentration (FIC) for each of the tested compounds, being defined as the addition of the MIC values of each drug in the combination divided by the MIC of the drug alone.

FIC^A = MIC of agent A in combination/MIC of agent A alone

FIC^B = MIC of agent B in combination /MIC of agent B alone

$FICI = FIC^A + FIC^B$

In the equations, A represents the EOEB and B the antifungal, ketoconazole. The turbidity of the fungal suspensions was adjusted to 0.5 McFarland standard (10^5 UFC/mL). In the solutions, the tested products were used at concentrations of their respective MICs. Initially, 50 µL of RPMI 1640 medium was added to all 96 wells of the microdilution plate. Then 50 µL of essential oil was added in the first column, in which serial dilutions were made in the plate until the 8th column, with the essential oil concentrations ranging from 5 to 0.03 mg/mL. In the vertical lines, 50 µL of standard antifungal ketoconazole was placed in concentrations ranging from 16 to 0.125 µg/mL.

Finally, 100 µL of inoculum was added to all wells. RPMI 1640 medium with inoculum was used as a negative control, while the antifungals and essential oil separately were used as positive controls at the respective MIC values. The microplates were incubated at 37°C and read visually after five days for dermatophytes. Assays were performed in triplicate. The FICI was interpreted as indicating a synergistic effect at values ≤ 0.5, an indifferent effect at values > 0.5 or ≤ 4.0, and an antagonistic effect at values > 4.0 (Odds, 2003; Johnson, 2004).

Determination of DPPH radical scavenging activity

To evaluate the antioxidant activity of the essential oil, the study used the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, according to Fenglin et al. (2004), with modifications. The activity was investigated by the ability to scavenge the DPPH radical through variation of absorbance obtained for a stoichiometric color loss of the radical solution in the presence of antioxidant substances present in the essential oil sample.

In a test tube, 3.9 mL of a methanol solution of free radical DPPH 6.5 x 10^{-5}M was combined with 0.1 mL of the methanol solution of essential oil in the concentrations to be tested. After 60 min, the absorbance was determined with an UV-VIS spectrophotometer at a wavelength of 515 nm. Assays were performed in triplicate. The inhibition of free radical DPPH was calculated in percent using the following equation:

IP% = Abs(DPPH) - Abs(sample) / Abs(DPPH) x 100

Where IP% is the inhibition percentage; Abs (DPPH) is the absorbance of the DPPH solution and Abs (sample) is the absorbance of the solution containing the essential oil at a particular concentration. IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated by the regression equation of the concentration of the essential oil, and percentage inhibition of free radical formation/percentage inhibition DPPH was calculated (Bajpai et al., 2009).

Determination of antioxidant activity using β-carotene/linoleic acid assay

The evaluation of oxidation inhibition by β-carotene/linoleic-acid oxidation system was performed by means of a spectrophotometric assay based on the discoloration of β-carotene due to oxidation induced by oxidative degradation products of linoleic acid (Silva et al., 1999; Alves et al., 2010). The antioxidant activity was determined by measuring the ability of the volatile organic compounds to inhibit the conjugated diene hydroperoxide formation from linoleic acid and β-carotene coupled oxidation in an emulsified aqueous system, which loses its orange color when reacting with the radicals (Alves et al., 2010; Lopes-Lutz et al., 2008).

The β-carotene (Sigma, St. Louis, MO) was dissolved in 5 ml of chloroform (0.3 mg/mL), followed by adding 20 µL of linoleic acid (Sigma, St. Louis, MO) and 200 µL of Tween 40 (Sigma, St. Louis, MO). Chloroform was completely evaporated using a vacuum evaporator. After removal of $CHCl_3$, 100 ml of distilled water saturated with oxygen under constant agitation was added to form an emulsion. The solution was adjusted in the spectrophotometer to a wavelength of 470 nm. The final emulsion had absorbance between 0.6 and 0.7 nm. Then 5 ml aliquots of the emulsion were placed in test tubes followed by 100 µl of dilutions of previously prepared methanol solutions of the essential oil, at concentrations of 500 to 25 mg/ml. Sample readings were taken 2 min after contact of the methanol solutions having varied concentrations with the emulsion. Then the samples were put in a water bath at 50°C for 120 min, and a second reading was performed. The negative control consisted of 5 ml of emulsion alone (Andrade et al., 2012). The percentage inhibition was calculated from the data with the formula:

$$IP\% = [(Abs_{sample(0)} - Abs_{sample(120)}) / (Abs_{system(0)} - Abs_{system(120)})] \times 100$$

$$\% \text{ Protection} = 100 - IP\%$$

Where IP% is the inhibition percentage; $Abs_{sample(0)}$ is the absorbance of the essential oil at t=0 min; $Abs_{sample(120)}$ is the absorbance of the essential oil at t=120 min; $Abs_{system(0)}$ is the absorbance of the system at t=0 min and $Abs_{system(120)}$ is the absorbance of the system at t=120 min. Thymol and carvacrol, oxygenated monoterpenes present in many essential oils, were used as positive controls. Samples were read against a blank containing the emulsion minus beta-carotene. Each assay was repeated three times and the IC_{50} values (concentration sufficient to obtain 50% of a maximum effect estimate in 100%) were calculated by the regression equation of the concentration of the essential oil, and percentage of protection (Lopes-Lutz et al., 2008; Andrade et al., 2012).

Brine shrimp lethality bioassay

The lethality assay against *Artemia salina* Leach (Crustacea, Artemiidae) was performed according to the method proposed by Meyer et al. (1982) with adaptations. The eggs of *A. salina* were incubated at room temperature (between 22 to 29°C) in artificial brine consisting of 23 g/L of sea salt and 0.7 g/L of sodium bicarbonate in distilled water for a period of 48 h in a tank fitted with a dark compartment and another clear one.

Using a light source, the nauplii were attracted to the light, collected with a Pasteur pipette and transferred to a beaker with saline water. Extraction solutions were prepared with the solvents methanol, DMSO and saline water in concentrations of 10,000 to 1 µg/mL. The positive control was prepared with potassium dichromate ($K_2Cr_2O_7$) and saline solution, and the negative control with saline solution and DMSO. Then 10 larvae were added to test tubes containing 5 ml of each tested solution and negative and positive control solutions (Costa et al., 2009).

Assays were performed in triplicate and the number of dead larvae was counted after contact for 24 h with the solutions. For counting the number of nauplii, the study considered those that remained immobile for more than 10 s after gentle agitation of the tubes (Lhullier et al., 2006).

Hemolysis assay

Fresh blood (10 ml) was collected in EDTA tubes and centrifuged at 1000 g for 10 min at 4°C. After plasma removal, the pellet containing the red blood cells (RBCs) was washed five times with PBS and then re-suspended in PBS to obtain an 8% (v/v) suspension. Then 100 µL of this suspension was added 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 to 100 µM. The resulting suspensions were incubated with agitation for 60 min at 37°C. After incubation, the samples were centrifuged for 2 min at 1000 g. The supernatants were transferred to 96-well plates and the hemoglobin release was measured by absorbance at 540 nm, using the Biotek Synergy HT multiplate reader. Triton X-100 at 1% and 4% (v/v) RBCs in PBS with no essential oil (untreated) were used as positive and negative controls, respectively. Percentage hemolysis was determined as [(Abs_{540nm} sample-treated – Abs_{540nm} untreated)/ (Abs_{540nm}1% Triton X-100 – Abs_{540nm} untreated)] x 100, and experiments were carried out in triplicate (Ahmad et al., 2010).

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). One-way ANOVA with the Tukey test was performed followed by multiple comparisons testing where appropriate. LC_{50} values were obtained using statistical package for social sciences (SPSS) 14.0 for Windows (SPSS Inc. Chicago, USA). Significance of difference was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

The essential oil yield was 0.1% (w/w) dry weight. The chemical compositions of the volatile constituents of the EOEB and the respective percentages are reported in Table 1. The essential oil contained 25 components, accounting for 93.84%, among them mono- and sesquiterpenes, with a prevalence of sesquiterpenes. The main constituents were β-caryophyllene (23.59%), thymol methyl ether (12.28%), germacrene D (6.56%) and bicyclogermacrene (6.47%). Among these main constituents, only one monoterpene was found, thymol methyl ether. Sesquiterpene hydrocarbons predominate in the chemical composition.

A previous study of the chemical composition of the aerial parts of EOEB identified 27 components, representing 91.2% of the total content (Albuquerque et al. 2001). In this study, the EOEB showed a high content of monoterpenes, limonene (15.3%), (E)-β-ocimene (10.5%), followed by β-caryophyllene sesquiterpene (7.5%) and myrcene monoterpene (7.3%) (Albuquerque et al., 2001). Variations in the chemical composition of essential oils, secondary metabolism products of the plant, occur due to changes in abiotic factors, as well in the geographic location of the plant, season of the year and even time of day when the plants are sampled (Dudareva et al., 2004; Gobbo-Neto; Lopes, 2007). In other Eupatorium species, the chemical analysis showed a corresponding composition. In *E. capillifolium* (Lam.) Small. ex Porter & Britton, the chemical composition of the essential oil of the aerial parts contained thymol methyl ether (36.3%) as the main constituent (Tabanca et al., 2010).

Table 1. Chemical composition of the aerial parts of the essential oil from *E. ballotifolium* (EOEB).

Compound[a]	RI Lit[b]	RI[c]	EOEB
Sabinene	975	975	1.88
β-Myrcene	990	992	0.51
α-Phellandrene	1002	1004	1.32
p-Cymene	1024	1025	3.41
Limonene	1029	1023	0.88
(Z)-β-Ocimene	1037	1039	1.40
(E)-β-Ocimene	1050	1050	5.05
Terpinen-4-ol	1177	1186	2.13
Thymol methyl ether	1245	1235	12.28
δ-Elemene	1338	1350	1.87
α-Copaene	1376	1373	2.29
β-Elemene	1390	1389	5.47
β-Caryophyllene	1419	1427	23.59
α-Humulene	1454	1450	1.58
epi-Caryophyllene	1466	1471	0.76
Germacrene D	1481	1490	6.56
Bicyclogermacrene	1500	1499	6.47
Germacrene A	1509	1513	1.08
δ-Cadinene	1523	1529	5.54
Germacrene B	1561	1560	0.97
Caryophyllene oxide	1583	1583	1.38
Globulol	1590	1591	2.85
1,10-de-*epi*-Cubenol	1619	1621	0.73
epi-α-Muurolol	1642	1634	2.40
α-Cadinol	1654	1644	1.44
Total identified	-	-	93.84

[a] Order of elution on DB-5 capillary column. [b] RI_{lit} refers to the retention index taken from Adams, 2012. [c] RI_{ca} refers to the retention index experimentally calculated using C_7–C_{26} n-alkanes.

Regarding the *in vitro* antifungal activity of the EOEB, the results are summarized in Table 2. The essential oil showed no significant activity when tested against strains of *Candida* spp (*C. albicans* LABMIC 0201, *C. albicans* LABMIC 0202, *C. parapsilosis* LABMIC 0301 and *C. tropicalis* LABMIC 0401). However, the results showed MIC values ranging from 2.5 to 1.25 mg/ml for the strains of dermatophytes (*T. rubrum* LABMIC 0101, *T. rubrum* LABMIC 0102, *T. rubrum* CEMM 05-1-08 and *T. rubrum* CEMM 05-1-034). Ketoconazole was used as positive control.

There are no reports in the literature of antimicrobial activities of the *E. ballotifolium* species, so this study is groundbreaking in investigating the antifungal activity against dermatophytes, which are medically important fungi responsible for superficial skin infections, with *T. rubrum* being the species most often found to cause dermatophytosis (Cafarchia et al., 2013).

Previous studies with some of the major constituents in the composition of EOEB report antifungal activity for β-caryophyllene (Tampieri et al., 2005; Skaltsa et al., 2003; Bougatsos et al., 2004) and bicyclogermacrene (Silva et al., 2007), both sesquiterpene hydrocarbons. These results indicate that the antifungal activity found in our study may represent a combined effect of these main EOEB constituents. For the *Eupatorium* genus, numerous studies have been conducted of the antimicrobial activity, mainly involving extracts and essential oils. The data are still incipient, although *Eupatorium* has been found to be one of the largest genera of the family Asteraceae (Garcia-Sanchez et al., 2011; Roque and Bautista, 2008).

In testing the modulatory activity, the study used the strains of *T. rubrum* LABMIC 0101 and *T. rubrum* LABMIC 0102. The results (Table 3) demonstrate that the combination of ketoconazole with EOEB reduced the MICs for both strains of *T. rubrum* (Table 3). Mutual synergistic potentiation of antifungal activity of EOEB and ketoconazole occurred, with a significant reduction in the MIC of ketoconazole of 1.0 to 0.125 µg/mL on the strain *T. rubrum* LABMIC 0102. The most significant reduction occurred in the tests with strain LABMIC 0102, whose fractional inhibitory concentration index (FICI) was low

Table 2. Minimum inhibitory concentration of essential oils from *Eupatorium ballotifolium* against *Candida* spp and *Trichophyton rubrum.*

Strains	Essential oil of *E. ballotifolium*		Drug (µg/mL)	
	MIC (mg/mL)	MFC (mg/mL)	Amphotericin B	ketoconazole
C. albicans LABMIC 0201	NI	NI	2.0	–
C. albicans LABMIC 0202	NI	NI	1.0	–
C. parapsilosis LABMIC 0301	NI	NI	4.0	–
C. tropicalis LABMIC 0401	NI	NI	2.0	–
T. rubrum LABMIC 0101	1.25	–	–	1.0
T. rubrum LABMIC 0102	2.5	–	–	1.0
T. rubrum CEMM 05-1-08	2.5	–	–	1.0
T. rubrum CEMM 05-1-034	2.5	–	–	1.0
Geometric mean of *T. rubrum*	2.69	–	–	–

LABMIC, Microbiology Laboratory; CEMM, Specialized Centre of Medical Mycology.

Table 3. MIC of the ketoconazole in the presence and absence of essential oil from *Eupatorium ballotifolium* against *Trichophyton rubrum.*

Plant essential oil/Drug	*T. rubrum* LABMIC 0102			*T. rubrum* LABMIC 0101		
	MIC (µg/ml) alone	MIC (µg/ml) combined	FIC indexa	MIC (µg/ml) alone	MIC (µg/mL) Combined	FIC index
E. ballotifolium	5000	39	0.1	2500	39	1.0
ketoconazole	1.0	0.125		1.0	1.0	

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

Table 4. Antioxidant activity of the essential oil from *E. ballotifolium* and the thymol and carvacrol, tested standard.

Methods	β-carotene / linoleic acid	DPPH
Compound	IC_{50} (µg/mL)	IC_{50} (µg/mL)
E. ballotifolium	19.47	222.17
Thymol	11.32	21.71
Carvacrol	22.83	25.5

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

(0.1) compared with strain LABMIC 0101, whose FICI value was 1.0. So, for LABMIC 0102 the modulatory activity was synergistic and for LABMIC 0101 the activity was indifferent.

The mechanisms of inhibiting microbial growth may be related to the hydrophobic nature of the constituents of the essential oil. Such compounds can act on the plasma membrane, causing it to become more permeable to antifungal agents, affecting the mitochondrial respiratory chain and cellular energy production. Thus, this mechanism can occur due to combined action of the antifungals with natural products at subinhibitory concentrations (Nogueira et al., 2014; Tintino et al., 2014). This is the first report of modulatory activity of a standard antifungal drug combined with EOEB.

The results presented here corroborate with other studies involving essential oils of *Thymus vulgaris* L. and *Cinnamomum cassia* L. (Pekmezovic et al., 2015), *Ocimum sanctum* L. (Amber et al., 2010), *Myrtus communis* L. (Mahboubi and Bidgoli, 2010), *Melaleuca alternifolia* (Maiden & Betche) Cheel and *Lavandula angustifolia* Mill. (Cassella et al., 2002). These studies demonstrate the ability of essential oils used to modify the antibiotic activity of drugs through the checkerboard technique. IC_{50} values of the EOEB obtained from the antioxidant assays investigated by the free radical DPPH (1.1-diphenyl-2-picrylhydrazyl) scavenging assay and β-carotene/linoleic-acid oxidation model system are shown in Table 4.

In the DPPH scavenging assay, the IC_{50} value was 222.17 µg/mL. This result indicates that the essential oil exhibited weak antioxidant capacity when compared to the positive controls: thymol, whose IC_{50} was 21.71 µg/mL and carvacrol, with IC_{50} of 25.5 µg/mL. In the test

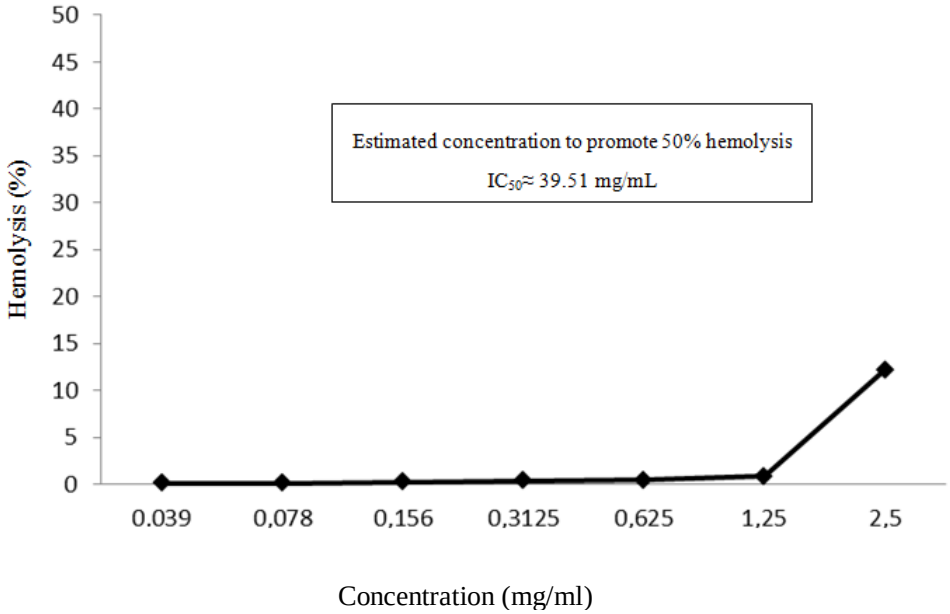

Figure 1. Hemolytic activity of the essential oil from *Eupatorium ballotifolium* (EOEB).

with β-carotene/linoleic-acid, the IC_{50} of the essential oil was 19.47 µg/mL, an excellent antioxidant action, while the IC_{50} of thymol was 11.32 µg/mL and carvacrol was 22.83 µg/mL.

The discrepant results between the two tests can be explained by the purpose of each method. The DPPH scavenging assay is based on the ability of the tested substance to sequester DPPH free radicals, which are reduced to the hydrazine compound and are routinely used to evaluate the antioxidant activity in plant extracts and pure substances such as terpenoids and flavonoids (David et al., 2007; Alves et al., 2010). The β-carotene/linoleic-acid assay is employed specifically to investigate the ability of a sample to minimize the complete oxidation of linoleic acid and β-carotene in an aqueous-lipid system which loses its orange color when it reacts with radicals produced by oxidative degradation of fatty acids (Alves et al., 2010). This method is used to investigate the antioxidant capacity of lipophilic substances, such as essential oils (Kulisic et al., 2004). Another property that makes it a useful method to test essential oils is that it does not require high temperatures, which allows determination of the antioxidant activity of thermo-sensitive substances such as essential oils (Silva et al., 1999).

Sesquiterpenes were the constituents identified in greatest quantities in the study essential oil, and the literature indicates antioxidant potential of plant-derived sesquiterpenes (Xu et al., 2008; Sghaier et al., 2011). β-caryophyllene, the main constituent found in the study (23.59%), was previously shown to have antioxidant activity and protective effect on liver fibrosis and the ability to inhibit activation of hepatic stellate cells (Calleja et al., 2013). The isolated action of β-caryophyllene or the synergism between the main constituents of the essential oil can be also be related to the antioxidant action (Candan et al., 2003). These results of antioxidant activity of the EOEB, detected by two different methods, corroborate previous studies that have indicated antioxidant potential of the genus *Eupatorium*, with activity for many species, such as *E. adenophorum* (Ahluwalia et al., 2014), *E. odoratum* (Raman et al., 2012; Chakraborty et al., 2010), *E. polystachyum* DC. (Souza et al., 2007) and *E. triplinerve* Vahl (Melo et al., 2013).

In the brine shrimp lethality bioassay, mortality rates to the EOEB varied between 0 and 100%, and the concentration required to kill 50% of the larvae (LC_{50}) was 28.89 µg/mL. LC_{50} values less than 1000 µg/mL indicate a possible spectrum of biological activities of some of the constituents, combined and/or isolated (Meyer et al., 1982). The anticholinesterase activity has been investigated of the EOEB from leaves and bark (Albuquerque et al., 2010). Lethality bioassays with A. salina have also been used in prospecting studies to screen plants with possible pharmacological activity, in Brazil (Quignard et al., 2004), India (Krishnaraju et al., 2006) and Nicaragua (Coe et al., 2010).

The tests to determine the in vitro hemolytic activity of EOEB showed that at concentrations of 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 mg/mL respectively the percentage of hemolysis ranged from 0.1 to 12.16% (Figure 1). Based on these results, the study can extrapolate hemolysis values and estimate the IC50 of 39.51 mg/mL 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 EOEB concentrations used.

Methods to measure the hemolytic activity *in vitro* enable determining the cytotoxic profile of the studied substance, and consist of checking for potential damage caused by the substances present in essential oils to the membranes of erythrocytes, which when undergoing lysis release hemoglobin (Miyazaki et al., 2013). At the essential oils' MIC values, the study observed hemolysis percentages between 0.8 and 12.2%. Comparison of the IC_{50} value of hemolytic activity with the MIC indicated that the oil concentration responsible for the fungistatic activity is lower than the concentration required to damage red blood cells by rupture of their membrane. However, according to the test of hemolytic activity, the EOEB showed low cytotoxic effect at the concentrations that inhibited microbial growth.

Conclusion

Chemical analysis of the EOEB identified 25 components, accounting for 93.84% of the substances, with predominance of sesquiterpene hydrocarbons. The brine shrimp lethality bioassay (*A. salina*) used as for screening allowed establishing a correlation with other potential biological activities, and the hemolytic activity assay showed that the essential oil has low cytotoxicity.

The essential oil showed *in vitro* antifungal activity against the dermatophyte *T. rubrum*, and when tested in combination with ketoconazole, the EOEB interacted synergistically, increasing its antifungal action. Antioxidant activity was evidenced by the DPPH scavenging assay, with IC_{50} of 222.17 µg/mL, while the IC_{50} value was 19.47 µg/mL in the test with β-carotene/linoleic-acid. The antioxidant potential can be related to the high content of sesquiterpenes and especially β-caryophyllene, germacrene D and bicyclogermacrene, the main constituents found in this study.

It is necessary to investigate the mechanism of action in the fungal cells and perform tests to identify the components responsible for the biological activities. It is also important to carry out tests with isolated constituents of EOEB against strains of dermatophyte fungi, modulatory activity assays with other antifungal drugs used in antifungal therapy and to investigate new toxicological aspects of the essential oil.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to the Postgraduate Program in Resources of State University of Ceará, the financial support of CAPES, the Laboratory of Natural Products Chemistry of State University of Ceará and to the Microbiology Laboratory of State University of Vale do Acaraú.

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