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CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF THE ESSENTIAL OIL OF *HYPTIS LEUCOCEPHALA*

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ABSTRACT - The paper reports chemical composition and antimicrobial activity of the essential oil of *Hyptis leucocephala* Mart ex Benth, Lamiaceae, isolated by hydrodistillation from leaves of plants collected in Casa Nova, semi-arid region of Northeastern Brazil. A total of ten compounds were identified, of which carvacrol as the main component (average 52.82%). This essential oil was also characterized by high level of biogenetic precursor of the phenols: p-cimene (18.13%) and y-terpinene (10.72%), and by *E-cariophyllene*, were the most abundant sesquiterpene hydrocarbons. Bioassay revealed a significant antibacterial activity of the essential oil against *Pseudomonas aeruginosa*, *Staphylococcus schleiferi*, *S. aureus*, *Salmonella typhi*, *Escherichia coli, Bacillus pumillus*, *Burkholderia cepacia*, *Klebsiella pneumoniae* with varying magnitudes. The minimal inhibition concentrations values extended from 50 to 250 µg mL^{-1}. The essential oil showed high antioxidant activity. The free radical scavenging capacity of the oil was determined with an EC_{50} value of 3.41 mg mL^{-1}. The data herein obtained show the relevant potential of *H. leucocephala* as a source of potent bioactivity compounds.

Keywords: antibacterial agent, antioxidant agent, caatinga biome, carvacrol, Lamiaceae

Composição química e atividade antibacteriana do óleo essencial de *Hyptis leucocephala*

RESUMO - O artigo relata composição química e atividade antimicrobiana do óleo essencial de *Hyptis leucocephala* Mart ex Benth, Lamiaceae, extraído pelo método de hidrodestilação das folhas, coletadas em Casa Nova, região de Caatinga do semi-árida do Nordeste do Brasil. Foram identificados dez compostos majoritários, dos quais carvacrol como o componente principal (média 52,82%). O óleo essencial também foi caracterizado por apresentar níveis elevados de precursores biogenéticos dos fenóis: p-cimeno (18,13%) e y-terpineno (10,72%), e por E-cariophyllene, como os mais abundantes hidrocarbonetos sesquiterpeno. Os ensaios biológicos revelaram que os compostos presentes neste óleo essencial apresentaram elevada atividade antibacteriana com valores da concentração inibitória mínima entre 50 a 250 µg mL^{-1}, principalmente contras as linhagens *Pseudomonas aeruginosa, Staphylococcus schleiferi*, *S. aureus*, *Salmonella typhi*, *E. coli*, *Bacillus pumillus*, *Burkholderia cepacia*, *Klebsiella pneumoniae*. Os constituintes do óleo essencial apresentaram alta eficiência na captura dos radicais livres, com elevada atividade antioxidante, com um valor de EC_{50} de 3,41 mg mL^{-1}. Os dados expostos reforçam o potencial biotecnológico do óleo essencial de *H. Leucocephala*, uma fonte alternativa para novos bioativos.

Palavras-chaves: agente antibacteriano, agente antioxidante, bioma caatinga, carvacrol, lamiaceae

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INTRODUCTION

In the last decade, invasive infections have become a public health problem. Infections remain the major direct cause of death in patients who are treated for malignant diseases, where the emergence of pan-resistant bacteria is also a prominent issue. Immunocompromised patients are mainly infected by *Mycobacterium tuberculosis, Klebsiella*, and *Acinetobacter* are often associated with serious invasive infections (Fortes et al. 2006, Santos et al. 2011a).

Moreover, the treatment of bacterial infections has been limited due to low drug effectiveness, antibiotic resistance and pharmacological safety in regards to the existing pharmaceutical drugs in the market, such as colistin, vancomycin, daptomycin, among others. These findings reinforce the growing demand for bioprospection of new drugs with specific antibacterial activity. Furthermore, increased demographic trends strongly suggest that the number of fungal infections are and will continue to increase, especially due to the aging of populations in developed countries (Anaissie 1992, Carrillo-Muñoz 2001, Odds 2005).

Natural products continue to play a major role in drug discovery and development, and medicinal plants have been a rich source of many compounds with large production of bioactive molecules and help to prevent the development of serious diseases (Strobel; Dayse 2003, Butler 2004, Santos et al. 2011b). Most of the natural aromas are obtained by extraction of essential oils from plants. Essential oils are volatile, natural and complex compounds characterized by a strong odor, produced by plants as secondary metabolites (Burt 2004, Russo et al. 2013). These compounds are versatile and they were mainly used for flavouring, confectioneries, perfume, soaps, cosmetics, household products and pharmaceutical industry (Botelho et al. 2007, Bitu et al. 2012).

Several species belonging to the *Hyptis* (Lamiaceae) genus represent an important source of bioactive constituents, which are reputed for their wide range of antimicrobial, anticancer and insecticidal activities (McNeil et al. 2011). The volatile oils obtained from various parts of the *Hyptis* plants were found to be primarily composed of mono- and sesquiterpenes. Significant differences were observed in the percentage compositions of the major components, which allowed the differentiation among the species. Based on the dominant constituents, phylogenetic relationships were found to be common among some species: 1,8-cineole (*H. fruticosa*, *H. goyazensis*, *H. martiusii* and *H. suaveolens*); β-caryophyllene (*H. marrubioides*, *H. pectinata*, *H. spicigera* and *H. suaveolens*); eugenol (*H. recurvata* and *H. suaveolens*); γ-cadinene (*H. glomerata* and *H. ovalifolia*); p-cymene (*H. mutabilis* and *H. pectinata*); α-pinene (*H. crenata* and *H. emoryi*). The monoterpenes, α-pinene and p-cymene were detected at various concentrations in all the *Hyptis* oils investigated (Peerzada 1997, Falcão et al. 2003, McNeil et al. 2011).

Hyptis leucocephala Mart. ex Benth is an endemic specie to the Brazilian tropical dry forest, Northeastern Brazil and popularly known as “Alecrim do campo”. This specie is a fast-growing perennial herb found in dense clumps along roadsides, in over-grazed pastures and around stockyards in the tropics. The plant gives off a characteristic minty smell when crushed (Facey et al. 2005, Oliveira et al. 2011). This genus originally native to tropical America, is popularly used in the treatment of respiratory and gastrointestinal infections, indigestion, colds, pain, fever, cramps and skin diseases (Barbosa 1992, Kuhnt et al. 1995, Abagli; Alavo 2011). The leaves are used as an anticancer and antifertility (in females) agent, while their aqueous extract has showed antinociceptive and cardiovascular effects and acute toxicity (German 1971, Sheth et al. 1972, Kingston et al. 1979, Peerzada 1997, Santos et al. 2007). This study aimed to analyze the chemical composition and determine the antibacterial and antioxidant activity of the essential oil of *H. leucocephala*.

MATERIAL AND METHODS

Plant material

Fresh leaves of *Hyptis leucocephala* were collected from the Brazilian semi-arid region Casa Nova, S09°16'29' W41°19'24.2'', were collected and the voucher specimen were deposited at *Herbarium* of the State University of Feira de Santana, BA, Brazil under deposit number 52959.

Essential oil Extraction and Chemical composition

Essential oil was obtained by hydrodistilattion in a Clevenger-type apparatus for 3hs. The main components of essential oil was identified and quantified by gas chromatography (GC) coupled with mass spectrometry (MS), using GC/MS (Shimadzu QP5000), column chromatography silica capillary type - OV-5 (30m X 0.25m X 0.25μm). The GC analyses were carried out using split-mode injection with split rate of 1:30. The injector and detector temperature was 240°C and 230ºC, respectively. The flow rate for the carrier gas (Helium) was 1 mL/min. The initial column temperature was 60°C, held for 1 min, heating rate of 3°C/min up to 240 °C. The identification of components was performed comparing mass spectrum with library data (Nist 62 lib.) and Retention Index (RI) (Adams, 2007). The Kovats Index (KI) was obtained by co-injection of sample and n-Alkanes (C_9H_{20} - $C_{25}H_{52}$, Sigma – Aldrich, 99%), and using the equation of Van Den Dool and Kratz (Van Den Dool; Kratz 1963).

Antimicrobial activity

The essential oil from *H. leucocephala* (EOHL) was screened for its antibacterial activity using the agar diffusion method (Bauer et al. 1959, adapted by Santos et al. 2011a) against potentially pathogenic bacteria (obtained from the Culture Collection of Agriculture and Environmental Importance – CMAA, of Brazilian Agriculture Research Corporation - EMBRAPA, Jaguariúna, SP, Brazil).

The bacterial strains were: *Pseudomonas aeruginosa* (CMAA 120), *Staphylococcus schleiferi* (CMAA 192), *S. aureus* (CMAA 190), *Salmonella typhi* (CMAA 85), *Escherichia coli* (CMAA 104), *Bacillus pumillus* (CMAA 282), *Burkholderia cepacia* (CMAA 92) and *Klebsiella pneumonae* (CMAA 540). After incubation at 35°C for 24h the inhibition zones were observed, measured and record. Minimal inhibition concentrations (MICs) was determined by both microdilution assay method adapted from National Committee for Clinical Laboratory Standards (USA) (NCCLS, 2004) against bacteria. After incubation under appropriate conditions, the lowest concentration of extract that inhibited visible growth was recorded as the MICs. Chloramphenicol and tetracycline were used as antibacterial agent, and experiments were carried out in triplicate.

Antioxidant activity

The antioxidant activity of EOHL was analyzed using DPPH (2,2-diphenyl-1 picrylhydrazyl), which is a radical scavenging assay. This was carried out according to Milardovic et al. (2006) and Santos et al. (2011b). 100µL of intact cells or intracellular cell-free extract and 900µL of freshly prepared DPPH solution (0.2 mM in methanol) were mixed and allowed to react for 30 min. Blank samples contained either deionized water. The scavenged DPPH was then monitored by measuring the decrease in absorbance at 517 nm. Radical scavenging activity was expressed as percentage and was calculated by using the following formula: % scavenging = [(Acontrol - Asample)/Acontrol] x 100. For each sample, the result was presented as an EC_{50} (sample concentration that produced 50% scavenging of the DPPH radical). Trolox, quercetine and rutine were used as standard for this assay.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The yield of the essential oil extracted by hydrodistillation from dried leaves of *Hyptis leucocephala* Mart exBenth, Lamiaceae, collected at flowering was 5,59 ± 0,40%. The chemical composition of sample EOHL was investigated by means of GC-MS in order to determine their qualitative and quantitative profile. Table I shows the composition of the essential oil for sample by single compound.

According to the analyzes, phenols were the main components, exhibiting the highest percentage: Carvacrol with 52,83%, while thymol 4,10%, confirming that *H. leucocephala* is a carvacrol chemotype, according to literature data for this family (Azevedo et al. 2002) Biogenetic precursor of the phenols were present with 18.13% of p-cimene and 10,72% y-terpinene. According to Russo et al. (2013) the variations between the main compounds of essential oil can be explained by biosynthetic relationship between the two phenols.

The metabolic pathways for the carvacrol and thymol formation begins with the autoxidation of y-terpinene to p-cymene and the subsequent hydroxylation to thymol. Instead the carvacrol originates from unsaturation of γ-terpinene to p-cymene followed by the hydroxylation to C-2 aromatic ring. So, it is evident the key role played by γ-terpinene in the process of flavoring and by p-cymene as precursor of oxygenated compounds. The γ-terpinene originates in the biosynthetic chain which from acetil CoA leads to the synthesis of terpenoids through the formation of geranyl-pyrophosphate (Russo et al. 2013). Among the sesquiterpene hydrocarbons, E-cariophyllene was the most abundant, and ranged from 2.30% (Table 1).

Table 1. Quantitative and qualitative composition (% w/w) of the four *Hyptis leucocephala* essential oils studied

Components	*Hyptis leucocephala* (%)	RI*
α-thujene	1,18	925
β-myrcene	3,88	988
α –terpinene	1,98	1014
p-cymene	18,13	1020
1,8-cineole	1,58	1027
γ-terpinene	10,72	1055
3-metoxi-*p*-cimene	3,31	1228
Thymol	4,10	1286
Carvacrol	52,82	1298
E-cariophyllene	2,30	1414

*RI: relative retention indices calculated against n-alkanes (Adams, 2007). Percentage calculated from TIC data; tr: trace (<0.1%).

Antimicrobial activity

Many microorganisms which cause damage to human health, exhibit drug resistance due to inadequate use of antibiotics. Thus, there is a need for the discovery of new substances from natural sources, including plants. In this work, the antimicrobial activity of essential oils from aromatic species such as *H. leucocephala* was evaluated.

The EOHL (5 mg disc^{-1}) was effective against all tested bacterial strains, measured by the disc agar diffusion method (Table 2). The essential oil showed antimicrobial activity but differences in microbial susceptibility were registered and induced a significant growth inhibition. In particular, it showed significant antimicrobial power against *B. pumillus* inhibiton zone of 45 mm, *E. coli* (40 mm), *Staphylococcus schleiferi* (37 mm). *S. typhi* was the most resistant strain (30 mm). The broth microdilution method showed MICs between 50 a 250 µg mL^{-1}. The most active antibacterial activity was against *B. pumillus*, *E. coli* and *S. aureus* (50 µg mL^{-1}), and *K. pneumonae* and *P. aeruginosa* with the smallest inhibitory concentration at 230 µg mL^{-1}. The correlation between the two different screening methods was examined and generally larger zones of inhibition correlated with lower MIC values.

Table 2. Bioassay antibacterial activity of essential oil of *Hyptis leucocephala*. The correlation between the two different screening methods was examined and generally larger zones of inhibition correlated with lower MIC values

Pathogen bacteria	**DD - Diameter of inhibition zone (mm)***			**MIC (µg mL^{-1})**		
	Essential oil (5mg.disc^{-1})	Standard 30 µg/disc (mm)		Essential oil (µg mL^{-1})	Standard	
		CLO	TET		CLO	TET
Pseudomonas aeruginosa	35 mm	15	20	230	≤7	≤3
Burkholderia cepaceae	35 mm	15	2	110	≤7	≤3
Staphylococcus schleiferi	37 mm	12	25	110	≤7	≤3
Staphylococcus aureus	35 mm	12	25	50	≤7	≤3
Escherichia coli	40 mm	15	25	50	≤7	≤3
Salmonella typhi	30 mm	15	10	110	≤7	≤3
Bacillus pumillus	45 mm	20	30	50	≤7	≤3
Klebsiella pneumonae	30 mm	20	25	230	≤7	≤3

*The diameter of the zone of inhibition is expressed in millimeter including the disc (6 mm).CLO: Chloramphenicol and TET: Tetracicline (30 µg/disc, CECON), served as controls for bacteria. DD: disc diffusion, MIC: minimum inhibitory concentration.

Some variation for *P. aeruginosa*, *S. aureus* and *S. typhi*, did occur, where the MIC value is lower than what would be expected when observing inhibition zones. This variation between methods (Janssen et al. 1987, Remmal et al. 1993, Pattnail et al. 1996, Chalchat; Garry 1997) can be attributed mainly to the variation of neat essential oil on disc, disc size, agar composition as well as the volatility of oil in an open air system (Viljoen et al. 2003).

Free radical scavenging activity

In the DPPH assay the radical scavenging ability of the oil and the positive controls (Trolox, Quercetine and Rutine) was measured spectrophotometrically (Table 3). In general, the oil was able to reduce the stable radical DPPH to the yellow coloured DPPH-H with an EC_{50} value of 3.41 mg mL^{-1}, and R^2 0,966. Combining the results obtained with antioxidant activity of the oil we could suggest that the free radical scavenging capacity of the oil may in part be attributed to the presence of γ-terpinene and/or also other phenolic and alcoholic components which constituted 82% of the total oil, as reported in the study of Tepe et al. (2004) and Burits and Bucar (2000).

Table 3. Antioxidant activity of the essential oil of *H. leucocephala* and positive controls (Trolox, Quercetine and Rutine) on DPPH assay

Samples	**R^2**	**EC_{50} (mg mL^{-1})**
Trolox	0,914	0,042
Quercitine	0,866	0,048
Rutine	0,919	0,082

*EOLHL	0,965	3,410

*EOLHP (Essential oil from leaves of *H. leucocephala*)

The action mechanisms of the essential oil constituents (phenolic and terpenes) have not been completely elucidated. Prindle and Wright (1977) mentioned that the effect of phenolic compounds is concentration dependent, what was also seen in this study. At low concentrations, phenols affect enzyme activity, especially of those enzymes associated with energy production; at greater concentrations, they cause protein denaturation. Phenolic compounds have also the ability to alter microbial cell permeability, permitting the loss of macromolecules from the interior.

Conner and Beuchat (1984) suggested that the antioxidant and antimicrobial activity of the essential oils of herbs and species or their constituents such as thymol, carvacrol, eugenol etc., could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds. In conclusion, our results demonstrated that the essential oil extracted from *Hyptis leucocephala* was rich in carvacrol, p-cymene and γ-terpinene and other components, with positive roles against microbial infection and also has an interesting potential for cosmetic industry for different applications in perfumes, creams as antioxidant activity properties. The essential oil also showed a wide spectrum of antimicrobial activity against the tested microorganisms suggesting that this essential oil would be a natural flavor additive substituting chemicals in food preservation.

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