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Volatile fraction from *Lippia alba* (Mill.) N. E. Brown: evaluation of its antioxidant property and identification of compounds by GC-MS/GC-FID

Fração volátil de Lippia alba (Mill.) N. E. Brown: avaliação de sua propriedade antioxidante e identificação de compostos por GC-MS/GC-FID

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In the municipality of Jequié, Bahia and in the Northeast of Brazil, *Lippia alba* is used as a compresses, baths or as alcoholic extract for the treatment of stomach disorders, respiratory diseases, to decrease hypertension and as a sedative. The objective of this study was to obtain the volatile fraction of *L. alba* cultivated in the medicinal plants garden of the Universidade Estadual do Sudoeste da Bahia, to evaluate its chemical constitution and its property as an antioxidant. The volatile fraction from *L. alba* was obtained using the simultaneous distillation-extraction process and analyzed by GC-MS/GC-FID. The antioxidant property of this fraction was evaluated through DPPH* method using gallic acid as standard reference. Compared to gallic acid, the volatile fraction shown low property to capture the DPPH* free radicals. Thirty-four constituents were identified, and the main monoterpene compounds were carvone (53.8%) and limonene (12.6%). Germacrene D (7.9%) was identified among the sesquiterpenes. The concentrations of carvone and limonene were equivalent to 2/3 of the total content of the volatile fraction, which gives it industrial value to this plant, mainly as flavoring agent. The small concentration of myrcene and the absence of citral in the composition of the volatile fraction of *L. alba* from Jequié confirmed the classification of this species as belonging to chemotype III (carvone-limonene), a variety that occurs in the Northeastern of Brazil. The volatile fraction of *L. alba* represents an economic potential due to its high carvone content, a monoterpene of high chemical and pharmaceutical value.
Keywords: Simultaneous distillation-extraction, carvone, limonene.

No município de Jequié, Bahia e no Nordeste do Brasil, *Lippia alba* é utilizada como compressas, banhos ou como extrato alcoólico para o tratamento de distúrbios gástricos, doenças respiratórias, para diminuir a hipertensão e como sedativo. O objetivo desse trabalho foi obter a fração volátil de *L. alba* cultivada no jardim de plantas medicinais da Universidade Estadual do Sudoeste da Bahia, avaliar a sua constituição química e a sua propriedade como antioxidante. A fração volátil foi obtida utilizando o processo de extração por destilação simultânea e analisada por CG-EM/CG-FID. A propriedade antioxidante dessa fração foi avaliada pelo método DPPH* utilizando ácido gálico como referencial padrão. Comparado ao ácido gálico, a fração volátil mostrou baixa propriedade para capturar os radicais livres DPPH*. Trinta e quatro constituintes foram identificados e os principais compostos foram os monoterenos carvona (53,8%) e o limoneno (12,6%). O germacreno D (7,9%) foi identificado entre os sesquiterpenos. As concentrações de carvona e limoneno foram equivalentes a 2/3 do teor total da fração volátil, o que confere valor industrial a essa planta, principalmente como agente flavorizante. A pequena concentração de mirceno e a ausência de citral na composição da fração volátil de *L. alba* de Jequié confirmaram a classificação desta espécie como pertencente ao quimiotipo III (carvona-limoneno), variedade que ocorre no Nordeste do Brasil. A fração volátil de *L. alba* representa um potencial econômico devido ao seu alto teor de carvona, um monoterpeno de alto valor químico e farmacêutico.
Palavras-chave: Extração por destilação simultanea, carvona, limoneno.

1. INTRODUCTION

The knowledge of the behavior of the species in relation to the factors influencing the quality of the plant material obtained enables the adoption of management techniques that ensure the production and maintenance of the therapeutic value of the plant. Another aspect involves the preservation of the plant material to be used in the production of phytotherapic drugs [1, 2].

The Northeastern Region of Brazil has an expressive diversity of native species recognized for its phytotherapeutic properties and which are therefore used in folk medicine [3, 4]. The Verbenaceae family, with tropical and subtropical distribution, is composed of approximately 90 genera, including *Lippia*.

Among the plants used in the Brazilian northeastern folk medicine species belonging to the family Verbenaceae are highlighted [5-7]. This family is composed of approximately 90 genera, including *Lippia* which have about 200 species abundantly present in Central America, South America and Africa. The *L. alba* is a medicinal plant native to South America, well adapted to the Brazilian soil and climate conditions [5-7].

In practically all regions of Brazil *L. alba* is cultivated, and is known by different popular names: in the Northern region (carmelita, erva-cidreira-do-campo or salvia-do-brasil), Northeastern (melissa, cedrilha, chá-do-tabuleiro, cidreira-brava or chá-da-terra), Southeast (falsa melissa) and in the South (alecrim-do-campo, cidrão, salsa-limão, sálvia, lípia, mal-me-quer-do-mato, malva, salvia-limão or tomilho-do-mato) [8]. In the municipality of Jequié, Bahia and in the Northeast of Brazil, *L. alba* leaves and roots are prepared as infusion, decoction or maceration and are used in compresses, baths or alcoholic extracts for the treatment of stomach disorders, respiratory diseases, to decrease hypertension and as a sedative [7]. *L. alba* is characterized as a shrub up to 1.50 m in height, thin branches, whitish and brittle [6]. The leaves are opposite, with serrated edges and sharp apex, and 3-6 cm long. The purplish-blue flowers are gathered in capituliform axillary inflorescences, with short axis and variable size. The fruits are pinkish-coloured, globular-shaped drupe [6, 9]. Throughout Brazilian regions, the traditional use of *L. alba* tea occurs both for its pleasant taste and for the soothing action [9].

Essential oil is a term applied for the product of extraction obtained by hydrodistillation, steam distillation or expression of plants [10, 11]. And, volatile fraction is a generic term used to refer to a sample obtained by other extraction techniques [11]. The product obtained in both cases is a complex mixture of compounds produced by different organs of the aromatic plants, most of them of terpenic nature, liquid, lipophilic and usually odoriferous [12].

To the essential oil of *Lippia* species are attributed pharmacological properties such as antimalarial, anti-inflammatory and sedative [13]. *Lippia*-aromatized oils are traditionally used for the treatment of colds, bronchitis, coughs, asthma, fever, digestive and hepatic problems, syphilis, diarrhea and dysentery, as well as mild antispasmodic action, antipyretic and diaphoretic activity [14, 15]. The inhibitory effect on *Staphylococcus aureus* growth was attributed to the oil obtained from the leaves of *L. alba* [16] and the activity against *Candida albicans* was induced by the ethanolic extract of roots [17]. In addition, insecticide activity was attributed to the essential oil of *L. alba* [18]. Therefore, knowledge of the chemical composition of *L. alba* volatile fraction is important, mainly due to its phytotherapeutic potential.

Generally, the vegetable oils are extracted by hydrodistillation. However, a problem inherent to this process is the loss of low molecular mass constituents, such as monoterpenes, sesquiterpenes, phenylpropanoids and others. By means of SDE, the compounds are extracted as a function of their affinities by the extracting organic solvent, with dichloromethane being one of the most used. The appropriate shelf-life for the isolation of plant oils has been empirically established in up to 48 hours of extraction-distillation. This time of extraction is considered adequate to obtain cinnamyl alcohol, 2-phenylethyl alcohol and other of high boiling point [19]. It is important to ensure that SDE extraction is not compromised by undesirable components due to oxidation processes, extraction with contaminated solvents and/or sample quality. Another important prevention measure is to degas the extractor solvent before submitting it to the SDE process. Using the SDE technique, a greater index of recovery of a variety of compounds present in a complex mixture is obtained. An advantage of SDE is the use of a low amount of solvent. In general about 1 mL of solvent is enough to reach high concentration factor, allowing a direct injection of the extract into

the gas chromatograph, without an additional concentration of the solution, which may cause changes in the proportions of components of the oil obtained [19]. Through the analysis of a volatile fraction by online coupling of gas chromatography (GC)-mass spectrometry (MS) and flame ionization detector (FID) as ways of cross-detector analysis GC-MS/GC-FID it is possible to know its chemical composition. This technique contributes to the evaluation of those constituents responsible for the volatile fraction activity against a pathogen.

The objective of this work was to obtain the volatile fraction of *L. alba* cultivated in the medicinal plants garden of the Universidade Estadual do Sudoeste da Bahia, to evaluate its chemical constitution and its property as an antioxidant.

2. MATERIAL E MÉTODOS

2.1 General procedures

Analyses by GC-FID were performed on an Agilent HP 5890 series II chromatograph. The following experimental conditions were used: capillary column of 5% phenyl, 95% dimethylpolysiloxane (DB-5) with 20m x 0.18 mm internal diameter, with 0.4 μm film thickness, nitrogen (1 $mL.min^{-1}$) as mobile phase, injector at 250 °C; flame ionization detector (FID) at 300 °C, and the column temperature programmed between 60 to 240 °C with a ratio of 3 $°C.min^{-1}$. Analyses by GC-MS were performed in an Agilent 6890 gas chromatograph coupled to the Agilent 5973 MSD mass detector, helium as mobile phase, the same column and the conditions described above were used. The temperature of the interface was maintained at 240 °C, ion source operated at 70 eV and a mass scanning range of 40 to 400 atomic mass units was adopted. The retention time was measured in minutes. The linear retention index was based on Van den Dool and Kratz (1963) [21] and Adams (2007) [22]. The data obtained were processed using the software HP ChemStation V-1.05. The mass spectra found were correlated with those of the WILEY 138K, NIST 2002 and QuadLib 2004 databases. For the identification by comparison with spectra available in these databases only matches above 90% were considered.

2.2 Plant material

Lippia alba (Mill.) N. E. Brown (Verbenaceae) (60 g) was collected in the herb garden of the Universidade Estadual do Sudoeste da Bahia (UESB), campus of Jequié, Bahia, Brazil. The plant was identified by Prof. Drª. Guadalupe Edilma Licona de Macedo and a voucher (N0 HUESB-8295) is deposited in the UESB Herbarium. The collection area climate is typical of the semi-arid region of the Brazilian Northeastern. For this reason, the collected material was maintained at a temperature below 10 ^{0}C and before 1 hour was submitted to extraction.

2.3 Volatile fraction extraction

The volatile fraction from *L. alba* (VFLa) was obtained through simultaneous distillation-extraction (SDE). The Likens-Nickerson apparatus [20] was used, with distillation flask containing the sample (100 °C), solvent flask containing 2 mL dichloromethane, during 4 hours. The percent yield of VFLa was measured in relation to the freshly plant mass.

2.4. Antioxidant activity

The qualitative and quantitative evaluation of the antioxidant activity of VFLa was done using the photochromic method based on the scavenging of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH*) [23]. For the qualitative analysis, VFLa samples were submitted to TLC in parallel with gallic acid used as a positive antioxidant control. The plates were eluted in $CHCl_3$/MeOH (9:1) and $CHCl_3$:MeOH:H_2O (65:30:5) and, after drying, were nebulized with 0.4 $mmol.L^{-1}$ MeOH solution of DPPH* radical and heated to 100 ^{0}C.5min^{-1}. Yellow spots on a purple background were considered a positive antioxidant activity. For the quantitative analysis of the antioxidant activity, samples (1.0 mg each) of VFLa were dissolved in methanol and transferred to

10.0 mL volumetric flasks. From this, diluted solutions of 10.0 to 1.0 $\mu g.mL^{-1}$ were obtained. A solution of DPPH* (40.0 $\mu g.mL^{-1}$) was prepared in a volumetric flask wrapped in aluminum foil to avoid exposure to light. Aliquots of 750.0 µL of each of the following sample dilutions (1.0, 10.0 and 100.0 $\mu g.mL^{-1}$) were placed in a quartz cuvette of 1.0 cm optical path. Thereafter, 1.5 mL of the DPPH* solution was added. All analyses were carried out in a protected light environment. The absorbance (λ = 517nm) of the samples were performed 30 minutes after addition of the DPPH* solution. The absorbance was measured in a Varian Cary 50 UV-Visible spectrophotometer. The absorbance value of the solvent (white) was subtracted from the absorbance determined for each sample tested. Methanol (Merck®) was used to correct the spectrophotometer baseline. For comparison, the activity of capture of DPPH* radicals of gallic acid in solutions of similar concentrations to those of the samples was evaluated.

2.5 Statistical analysis

The Origin 6.0 software was used for analyses of antioxidant activity data, which were presented as the mean ± standard deviation for three independent experiments.

3. RESULTS AND DISCUSSION

Gas chromatographic retention data are adequate to study of volatile fractions from vegetables, due to the simplicity of analyses and data interpretation. In addition, through the retention index (RI) of compounds, the comparison with available retention data on literature in combination with mass spectrometry is an adequate approach to confirmation of a compound [24, 25].

In the present work, the volatile fraction of *L. alba* [VFLa, 0.56% yield (m/m)] from samples collected in the UESB Medicinal Plants Garden was compatible with the yield published by Tavares et al. (2011) [26]. Through the GC-FID chromatogram of the VFLa, 34 compounds were detected, which correspond to 98.4% of the analyzed sample. Based on the total ion chromatogram (Figure 1), the retention time and linear retention indices (LRI) of detected compounds were compared with those published by Adams (2007) [22] and showed in Table 1.

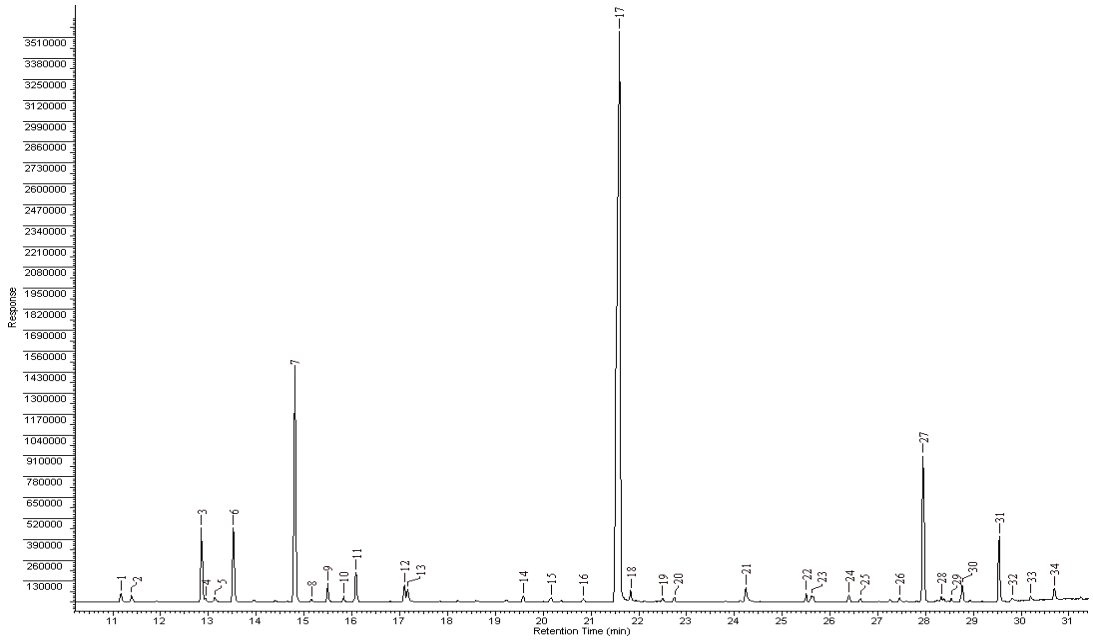

Figure 1. Total ion chromatogram obtained by GC-MS/GC-FID for volatile fraction extracted from Lippia alba by SDE.

The mass spectrum of each constituent of VFLa was compared to the spectra of the WILEY 138K, NIST 2002 and QuadLib 2004 databases. The monoterpenes carvone (53.8%), limonene

(12.6%) and the sesquiterpene germacrene D (7.8%) were the main compounds detected in the volatile fraction from *L. alba*, grown in UESB campus at Jequié, Bahia (Table 1).

Table 1: Retention time and linear retention indices (LRI) of compounds identified through GC-MS/GC-FID in volatile fraction of Lippia alba.

Peak	Compound	RT	Area (%)[a]	LRI	
				Exp.	Lit.[b]
1	α-Thujene[a]	11.168	0.6	917	924
2	α-Pinene[a]	11.391	0.4	924	932
3	Sabinene[a]	12.860	4.0	965	969
4	β-pinene[a]	12.945	0.2	967	974
5	a-Octen-3-ol[a]	13.128	0.3	982	974
6	β-Mircene[a]	13.528	3.9	994	988
7	Limonene[a]	14.803	12.6	1020	1024
8	*cis*-Ocimene[a]	15.151	0.2	1040	1032
9	*trans*-β-ocimene[a]	15.494	1.0	1051	1044
10	γ-Terpinene[a]	15.825	0.3	1060	1054
11	*cis*-Sabinene hydrate[a]	16.088	1.6	1060	1065
12	*trans*-Sabinene hydrate[a]	17.105	1.0	1091	1098
13	Linalool[a]	17.168	0.9	1096	1095
14	Terpinen-4-ol[a]	19.591	0.3	1171	1174
15	Myrtenol[a]	20.174	0.3	1187	1194
16	*trans*-Carveol[a]	20.843	0.2	1211	1215
17	Carvone[a]	21.597	53.8	1239	1239
18	Piperitone[a]	21.831	0.6	1248	1249
19	*cis*-carvone oxide[a]	22.500	0.2	1262	1259
20	Isobornyl acetate[a]	22.751	0.2	1281	1283
21	(2*E*.4*E)* -Decadienal[a]	24.243	1.1	1320	1315
22	β-Bourbonene[a]	25.506	0.5	1381	1387
23	β-Cubebene[a]	25.626	0.6	1391	1387
24	β-Caryophyllene[a]	26.408	0.4	1416	1417
25	Alloaromadendrene[a]	26.649	0.2	1432	1439
26	Bicyclosesquiphellandrene[b]	27.460	0.3	1478	1481
27	Germacrene D[b]	27.957	7.9	1488	1484
28	(E)-β-Ionone[b]	28.334	0.5	1493	1492
29	Germacrene A[b]	28.551	0.2	1502	1508
30	Cubebol[b]	28.774	0.9	1513	1514
31	Elemol	29.546	3.5	1547	1548
32	*cis*-Nerolidol[b]	29.826	0.3	1558	1561
33	Germacrene D-4-ol[b]	30.209	0.3	1567	1574
34	Guaiol[b]	30.706	0.6	1596	1600

RT = retention time in minutes, [a]GC-FID analysis; [b]Adams (2007) [22].
[a] = Monoterpene, [b] = Sesquiterpene.

Due to its higher concentration in VFLa and based on the data of its mass spectrum [Intensity versus m/z (mass-to-charge ratio) plot] (Figure 3A), it was suggested a fragmentation for the

carvone structure (Figure 3B). The proposed fragmentation is in accordance with that suggested by Castilho et al. (2014) [27].

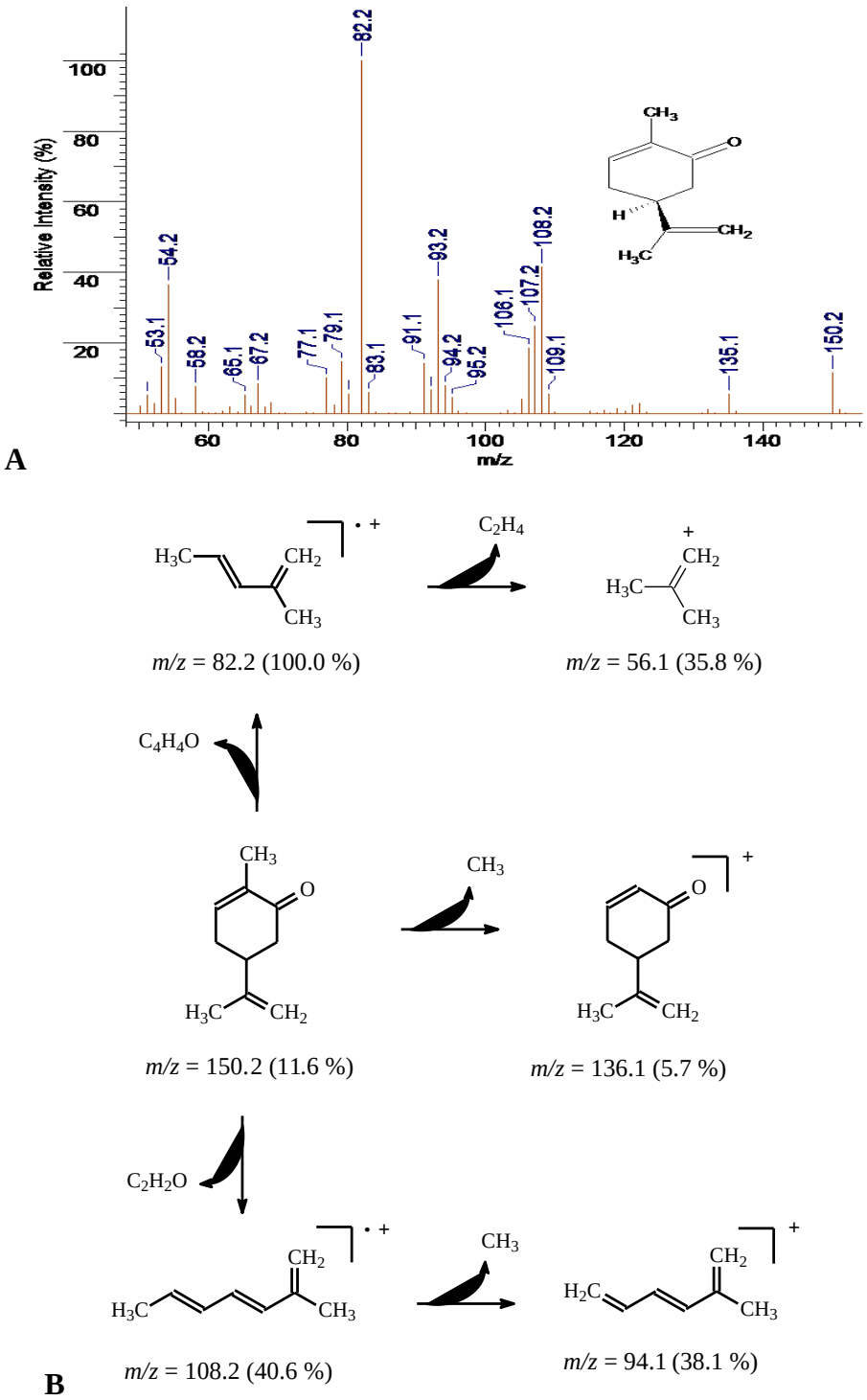

Figure 3: A: Mass spectrum of carvone (RT = 21.60 min) detected in the volatile fraction of Lippia alba, through GC-MS/GC-FID, using DB-5 Column. B: Fragmentation suggestion of the carvone structure, based on its mass spectrum.

According to Lorenzi and Matos (2008) [9], the composition of the oil from *Lippia* species is classified into three main chemotypes: “citral and myrcene”, “citral and limonene”, and “carvone and limonene”. It is proposed that the differences in the amount of the chemical compounds present

in these chemotypes are due to the genotypic variations of the *Lippia* sp. Thus, these chemotypes represent qualitative and quantitative variations of the main compounds found in the oil from the *Lippia* variations.

Tavares et al. (2011, 2004, 2005) [26, 28, 29] reported that citral (55.1%), β-myrcene (10.5%) and limonene (1.5%) were detected in the chemotype I in the analysis of *L. alba* essential oil collected in Northeast Brazil; citral (63.0%) and limonene (23.2%) in the chemotype II; carvone (54.7%) and limonene (12.1%) in chemotype III. Tavares et. al. (2005) [29] studying the chemotype II carvone, found that the 26 compounds identified represent 89.1% of the essential oil.

The main constituents identified in VFLa belong to the class of terpenoids, being 25 monoterpenes (73%) and 9 sesquiterpenes (27%) (Table 1). It was also observed that the chemical constitution of VFLa oil is rich in carvone and limonene (Table 1). Due to this fact and according to the classification established by Matos et al. (2000) [6] *L. alba* cultivated in Jequié, Bahia belongs to chemo-type III, also called carvone-limonene chemotype. The absence of citral and the low concentration of β-myrcene allowed us to infer that the studied plant cannot be classified as one of the other two chemotypes (I and II) that occur in northeastern Brazil. Tavares et al. (2005) [29], studying the chemotype III of *L. alba* identified 26 compounds in the material obtained using hydrodistillation extraction technique. In the present work, the use of SDE provided an essential oil containing a greater number of constituents of *L. alba* chemotype III (34 compounds), corroborating to the results (26 compounds) found by Tavares et al. (2004, 2005) [28, 29]. It was possible to conclude that SDE represents one of the better processes to obtain volatile fraction of *L. alba*.

It is believed that oxidative processes are associated with chronic degenerative diseases such as Alzheimer's disease, Parkinson's disease, atherosclerosis, diabetes mellitus, early ageing and other [31]. Genetic characteristics, environmental factors such as UV radiation and specific intrinsic properties of some cell lines may increase oxidative damage or decrease the ability of cells to degrade aggressive oxidizing agents [32]. The effects of oxidative stress vary according to their intensity and the type of cell affected. According to Halliwell and Gutteridge (2007) [33], the main effects of oxidative stress occur in the cell proliferation phase, in the processes of adaptation, after cell damage, during senescence and in cell death.

In the human organism, there are integrated antioxidant systems, which involves liposoluble (vitamin E, carotenoids), water-soluble (ascorbic acid, glutathione) and enzymatic (glutathione peroxidase; superoxide dismutase; catalase) compounds [34]. These systems are influenced by reactive oxygen species (ROS) which are chemically reactive species containing oxygen, such as peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen. ROS act on translation, gene transcription, and regulation of guanylate cyclase activity in cells [35]. Antioxidant compounds interact with free radicals inhibiting initiation or disrupting the ROS-induced chain of oxidative reactions [36]. In this context, the identification of substances with antioxidant properties is important for obtaining medicines for the treatment of the diseases mentioned above.

The principal methods for the evaluation of the antioxidant properties of natural substances are ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), ferric reducing antioxidant (FRAP) assay, ORAC for Oxygen Radical Absorbance Capacity and DPPH* [37]. The DPPH* method has been used to evaluate the antioxidant properties of isolated natural extracts and compounds, having a maximum UV-VIS absorption around λ = 515 nm, associated to the yellow color of diphenyl-picryl-hydrazine produced [38]. The DPPH* method is also effective in quantifying the antioxidant property of volatile fractions obtained from plants. And, TLC combined with DPPH radical detection of antioxidants *in situ* have been used for the screening of antioxidant substances, mainly those present in plant extracts [23, 39-41].

Samples of the VFLa were submitted to the DPPH* method to evaluate its antioxidant property. The qualitative test performed by TLC of VFLa (100.0 $\mu g.mL^{-1}$) showed yellowish spots (RF = 0.8) in a purple background, characterizing a positive antioxidant activity test. The results of the quantitative DPPH* (100.0, 10.0 and 1.0 $\mu g.mL^{-1}$) free radical capture assay were 8 ± 1.54%, 5 ± 1.65% and 2 ± 0.98%, respectively (Figure 4).

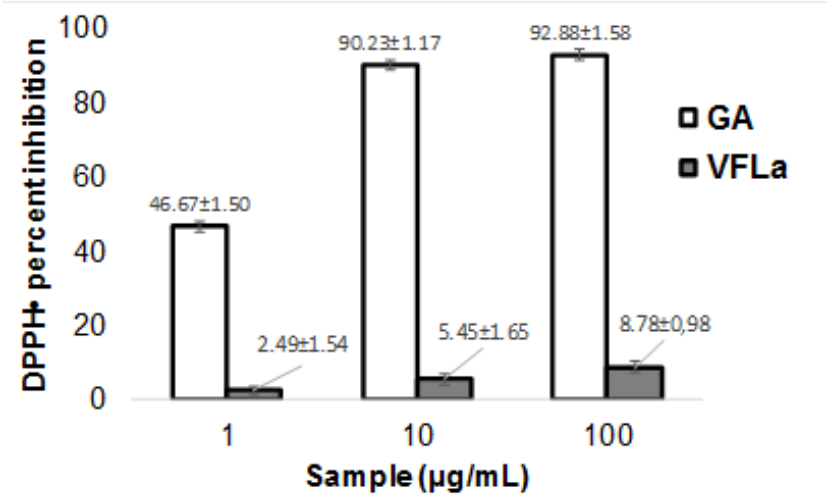

Figure 4: Percent inhibition of DPPH induced by the volatile fraction of Lippia alba (VFLa) extracted by SDE, compared to the gallic acid (GA) used as standard.*

In general, expressive antioxidant activities are observed in polar extracts of plants due to the presence of flavonoids. Thus, the identification of the antioxidant property of VFLa, even being small, is interesting. The smaller capacity to capture DPPH* was attributed to the fact that VFLa is a complex mixture of compounds and to the absence of phenylpropanoids, such as eugenol, which have phenolic groups in the molecular backbone. Phenylpropanoids are efficient in capturing and stabilizing the electrical charge of free radicals due to the double conjugated bonds that are in resonance. Gallic acid used as positive control is a phenolic compound that has an expressive property of sequestering DPPH*.

In relation to the chemotype III of *L. alba*, reports on the evaluation of the antioxidant property of the VFLa by means of the DPPH* method were not found in the literature. It is therefore considered that the results found here to contribute to further chemotaxonomic studies of *L. alba*.

4. CONCLUSION

In relation to gallic acid, VFLa showed low property to capture the free radical DPPH*, which was attributed to the absence of phenylpropanoids in its chemical composition. By GC-MS/GC-FID, 34 compounds were identified in the VFLa obtained by SDE from *L. alba* collected in Jequié, Bahia, Brazil, with carvone (53.8%) and limonene (12.6%) being its main constituents. Based on the data obtained it was concluded that the *L. alba* studied is of the chemotype III. The volatile fraction of this subspecies of *L. alba* represents an economic potential since half of its composition is constituted by carvone, a monoterpene of high chemical and pharmaceutical value. However, it is important to highlight that the potentialities of this plant don't mean that the people should stop medical treatments. This study allows us to infer that the *L. alba* is a potential source of volatile fraction rich in carvone. Moreover, the economic value attributed to carvone-rich essential oils, and their applications in the pharmaceutical, food and cosmetics industries, need to be realized as an agribusiness opportunity for the Brazilian Northeastern.

5. ACKNOWLEDGMENTS

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