ARTIGO ORIGINAL

Chemical composition and anticholinesterase evaluation of aerial parts of *Crotalaria retusa*

Layana Karine Farias Lima[1], Maria do Carmo Gomes Lustosa[1], Mahendra Rai[2], Wanessa Sales de Almeida[3], Nilza Campos de Andrade[1], Chistiane Mendes Feitosa[1], Sidney Gonçalo de Lima[1*]

[1] *Programa de Pós-Graduação em Química and Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Piauí, 64049-550, Teresina, Brazil*

[2] *Department of Biotechnology SGB Amravati University, Amravati, Maharashtra, India*

[3] *Programa de Pós-Graduação em Ciências e Engenharia de Materiais, Universidade Federal do Piauí, 64049-550, Teresina, Brazil*

**Corresponding author: sidney@ufpi.edu.br*

ABSTRACT: Acetylcholinesterase (AChE) inhibition is considered as a promising strategy for the treatment of neurological disorders. The aim of this research was to determine the chemical composition of the aerial parts of *Crotalaria retusa* by gas chromatography coupled to mass spectrometry (GC-MS), and evaluate their anticholinesterase and cytotoxic potential against *Artemia salina*. The main compounds found were *trans*-hex-2-enal (66.67%) in fresh leaves; in dry leaves benzene acetaldehyde (23.90%), and in fresh flowers 3-ethyl-4-methyl-pent-3-en-2-one (42.80%). The volatile constituents of fresh leaves, hexane and ethanolic extracts, decoction and dichloromethane fraction showed inhibition against AChE. The extracts were found to be nontoxic against *Artemia salina*, which increases the perspectives of study of the species about new biological activities.
Keywords: *Crotalaria retusa*; volatile constituents; acetylcholinesterase; chemical composition.

RESUMO

Composição química e avaliação anticolinesterase de partes aéreas de *Crotalaria retusa*. A inibição da acetilcolinesterase (AChE) é considerada uma estratégia promissora para o tratamento de distúrbios neurológicos. O objetivo desta pesquisa foi determinar a composição química das partes aéreas da *Crotalaria retusa* por cromatografia gasosa acoplada à espectrometria de massas (CG-EM), e avaliar seu potencial citotóxico e atividade anticolinesterase. Os principais compostos identificados foram *trans*-hex-2-enal (66,67%) nas folhas frescas; fenilacetaldeído (23,90%) nas folhas secas, e nas flores frescas 3-etil-4-metilpent-3-en-2-ona (42,80%). Os constituintes voláteis das folhas frescas, hexano e extratos etanólicos, decocção e fração diclorometano apresentaram inibição da AChE. Os extratos foram encontrados não tóxicos contra *Artemia salina*, o que aumenta as perspectivas de estudo da espécie sobre novas atividades biológicas.
Palavras-chave: *Crotalaria retusa*, Óleo essencial, Composição química, acetilcolinesterase.

INTRODUCTION

The genus *Crotalaria* L. belongs to the family Leguminosae, with more than 600 known species. Africa, India, Mexico and Brazil are the main centres of plant diversity of this genus, and in the latter, there are more than 40 species known as “xique-xique”, “guizo de cascavel”, and “chocalho de cascavel” (Honório et al. 2010). A large number of species of the genus, distributed in different regions of the world, are toxic to animals, the most important are *Crotalaria spectabilis* Roth*, C. crispate* Benth.*, C. retusa* L.*, C. dura* J.M.Wood & M.S.Evans*,* and *C. globifera* E.Mey. (Nobre et al. 2004).

These plants contain high levels of pyrrolizidine alkaloids (PAs) that are natural toxins present in more than 6000 vegetable plant species, in different genera and families, and affect humans and animals (Honório et al. 2010; Lucena et al. 2010).

Received: 24/05/2018
Accepted after revision:20/07/2022
Published on line: 28/07/2022
ISSN 1983-084X

Compounds extracted from medicinal plants present a high potential in the treatment of neuropsychiatric and/or neurodegenerative diseases (Kumar and Khanum 2012). Plants are naturally a major source of compounds with anticholinesterase potential, such as the alkaloid galantamine (Colovic et al. 2013), a natural product isolated from several plant species of the family Amaryllidaceae, with therapeutic effects that last even after the end of treatment, thus serving as a base to develop new anticholinesterase drugs (Viegas Junior et al. 2004).

AChE is an enzyme that makes a crucial role in the cholinergic mechanism and is responsible for the hydrolysis of acetylcholine (ACh) in the transmission of the nerve impulse (Colovic et al. 2013). The degradation of this neurotransmitter by AChE must occur before the beginning of a new nervous impulse, thus preventing its generation in a continuous way. In the absence of this regulation mechanism, behavioral changes such as hyperactivity, asphyxia and death occur (Roex et al. 2003).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, affecting in great numbers the old people. The main symptoms of this disease are memory loss, speech deficit, depression, behavioral changes, restlessness, mood swings and psychosis (Barbosa Filho et al. 2006). Increasing the level of ACh in the brain through the inhibition of AChE is an alternative for the treatment of AD (Dohi et al. 2009). This elevation of ACh levels may be useful to improve one of the signs of disease, learning disability (Zhao and Zhao 2013).

In this way, an alternative hypothesis is formulated for the origin of AD based on the failure of the cholinergic function of the brain by the degradation of acetylcholine, which when degraded loses its functions of motor control, memory and cognition (Mufson et al. 2008; Giordani et al. 2008). At the cellular level, when there is a decrease in ACh levels in the synaptic process occurs the loss of neurons responsible for cognitive functions which decreases cortical cholinergic neurotransmission (Zhao and Zhao 2013).

The anticholinesterases provide improvements in the cognitive, behavioral and functional symptoms related to hypocholinergic dementias, where AD is the main agent (Gomes and Koszuoski 2005). The treatment with anticholinesterases provides improvements and reduces the progression of AD in approximately 30 to 40% of patients in the mild and moderate stage (Sereniki and Vital 2008).

The great structural variety of anticholinesterases known today, and the possibility of exploring different modes of action, has stimulated the phytochemical study of several plant species and microorganisms that will provide new models of anticholinesterase substances. Because of their popular use or ethnobotanical data, several examples of biodiversity have been studied (Colovic et al. 2013). The present study aimed to determine the chemical composition and to evaluate the anticholinesterase potential and toxicity against *Artemia salina* L. of aerial parts of *C. retusa*.

MATERIALS AND METHODS

Plant Material

The leaves and flowers of *C. retusa* (Leguminosae - Faboideae) were collected in the municipality of Teresina-PI in May 2012. The specimens were identified and a voucher was deposited at the Graziela Barroso herbarium of the Federal University of Piauí under the number TEPB 27955.

Extraction of the volatile constituents

Fresh crushed leaves (273 g), dried crushed leaves (180.5 g) and fresh flowers (450 g) were subjected to hydrodistillation for two hours using a modified Clevenger system. From each extraction, 500 ml of the hydrosols was collected, which was subjected to liquid-liquid extraction with dichloromethane three times (170 ml). The organic phases were concentrated on rotary evaporator under reduced pressure. The obtained materials were analysed by gas chromatography coupled to mass spectrometry (GC-MS) and submitted to inhibition test with acetylcholinesterase.

During the extraction from the fresh leaves, a yellowish material adhered to the Clevenger surface was observed. Which was collected with dichloromethane, dried with anhydrous sodium sulfate, filtered, concentrated and subjected to GC-MS analysis and later to an Inhibition test with acetyl cholinesterase. The decoction was collected (450 ml), lyophilized and submitted to enzymatic testing and toxicity test against *A. salina*.

Analysis by gas chromatography coupled to mass spectrometry

Analysis by gas chromatography was performed on SHIMADZU GC-17A equipment coupled to a GC-MS-QP5050A mass spectrometer. Chromatography of the components was carried out using the DB-5HT column of the J & W Scientific brand, with 30 m x 0.25 mm, internal film thickness of 0.10 µm. Helium was used as the carrier gas at a flow rate of 1.0 ml/min, the column temperature was programmed kept at 50 °C for 0.50 min, followed by a 5 °C/min increase until 180 °C while maintaining this temperature for 4 minutes, then at 10 °C until reaching 260 °C keeping this temperature constant

for 10 min. The injector temperature was 250 °C and detector or interface temperature 270 °C that was injected with a volume of 1.0 µl of dichloromethane. The conditions of the mass spectrometer (MS) were: detector of quadruple type ions operating by electronic impact and electron impact energy of 70 eV and fragments detected in the range of 40 to 500 Da. The Identification of the respective components was done by comparing the mass spectra patterns of internal library (Wiley 229) to experimental retention index, calculated from an *n*-alkane series (C_8 to C_{20} - Sigma Aldrich), and by comparison with literal data (Adams 2007) and also the through sites like http://www.webbook.nist.gov; http: //www.pherobase.com and https://pubchem.ncbi.nlm.nih.gov

Preparation of extracts

The leaves of *C. retusa* were dried at room temperature, grinded in a knife mill to obtain 114.5 g. Then extracted by maceration, three times with *n*-hexane, followed by extraction with ethanol, each extraction lasted for 72 h. After filtration of the material, the solvent was evaporated to give hexane (hex) extracts, and ethanolic (EtOH) extracts. The Hex and EtOH extracts were submitted to toxicity activities, using *A. salina* larvae as toxicity model, and enzymatic test against acetylcholinesterase.

Fractionating column

A 100 mg sample of the ethanolic extract was fractionated using classical chromatography. Hexane (200 ml) was used to remove the fatty acids, obtaining the hexane fraction, and afterwards dichloromethane was used to collect three fractions: DCM1, DCM2 and DCM3, which were subjected to enzyme activity test against acetylcholinesterase.

Toxicity assay

Preparation of larvae

The toxicity against *A. salina* (Artemiidae) was according to Meyer's methodology (Meyer 1982), with modifications. The samples used for the bioassay were the hexane extracts, ethanolic extracts and decoct from the leaves of *C. retusa*.

The eggs of *A. salina* were hatched in a glass mini-aquarium (12 cm x 10 cm x 8 cm), previously filled with filtered sea water. This container had a divider glass with a small aperture in order to form two unequal compartments. The eggs were kept in a larger container and dark environment for a period of 24 h. *A. salina* larvae were attracted by phototropism towards the smaller compartment, an environment illuminated with the aid of a light source.

Preparation of the samples

The samples were prepared by dissolving 100 mg of each extract in 10.0 ml of Tween 40 solution (1%) obtaining a stock solution of 10.0 mg/ml. From this solution, aliquots of 500, 425, 350, 325, and 200 µl were transferred into test tubes, followed by the addition of 1.0 ml of seawater and ten *A. saline* larvae. Then, the volume was adjusted to 5.0 ml. The final concentrations of the samples were obtained as: 1000, 850, 700, 650, and 400 *µg*/ml, respectively. The control was maintained with saline water and 1.0 ml of the Tween 40 solution (1%), under the same conditions as the extracts, the test was performed in triplicate.

Inhibitory activity of AChE enzyme

Test in thin-layer chromatography (TLC)

The AChE enzyme inhibition detection assay was performed with the plant samples dissolved in methanol obtaining a concentration of 10 mg/ml, then 1.5-2.5 µl of the solutions of each sample were applied to a silica plate and eluted in chloroform methanol (9:1). After plate development, the inhibitory activity was detected using revealer based on the Ellman method (Ellman et al. 1961). The plate was sprayed with DTNB (5,5'-dithiobis-[2-nitrobenzoic acid])/TCE (acetylthiocholine iodide) 1 mM DTNB and 1 mM ATCI in buffer A (tris-HCl, pH 8.0 in water). After drying (5 min) it was sprayed with 3 units/ml of lyophilized acetylcholinesterase enzyme type VI-s, 292 U/mg solid, 394 U/mg protein (Sigma Chemical Co.) and after 10 min a yellow color was observed, and the inhibition was visualized by the observation of white halos. The color disappears in approximately 15 to 30 min. Caffeine was used as the standard substance (Ingkaninan et al. 1999; Ingkaninan et al. 2000; Rhee et al. 2001).

RESULTS AND DISCUSSION

Hydrolates are called hydrosols, floral waters or aromatic waters, and even with a low content of volatile oil that make up in the solution, they are sufficient to confer to them a strong aroma. Its applications occur in several sectors such as: perfumery, cooking and therapy, such as aromatherapy, some hydrolates may present an unpleasant smell even if volatile oil does not contain it (Souza et al. 2007).

Chemical constituents of volatile oil from fresh leaves

Table 1 shows the chemical constituents identified by GC-MS analysis of the volatile oil of *C. retusa* fresh leaves.

Table 1. Chemical constituents of volatile oil from fresh leaves of *Crotalaria retusa*.

Compounds	IK_{exp}	Área (%)
trans-hex-2-enal	846	66.67
trans-hex-2-enol	854	19.48
Cyclohexanol	886	13.85

Trans-hex-2-enal is a widely distributed constituent in fresh leaves of vegetables and fruits, and alongside other compounds present in these leaves are responsible for its characteristic smell, insect excretions may also occur (Hatanaka and Harada 1973). The isomerization process of cis-hex-3-enal for the formation of trans-hex-2-enal occurs through enzyme action in the leaves and in the presence of oxygen in isolated chloroplasts. When added to the isolated chloroplasts, linoleic acid is converted to cis-hex-3-enal and trans-hex-2-enal. These two constituents are the only volatile products in isolated chloroplasts (Hatanaka et al. 1976). Figure 2 shows the mass spectrum of trans-hex-2-enal.

Chemical constituents of the material adhered to the Clevenger surface

Chromatographic analysis of the yellowish material adhered to the Clevenger surface identified two constituents, palmitic acid and neofitadiene (Table 2), being palmitic acid the predominant component (97.36%), as shown in Figure 3.

Table 2. Chemical constituents of the material adhered to the Clevenger surface.

Compounds	IK_{exp}	Área (%)
Neophytadiene	1825	97.36
Hexadecanoic acid	1960	2.64

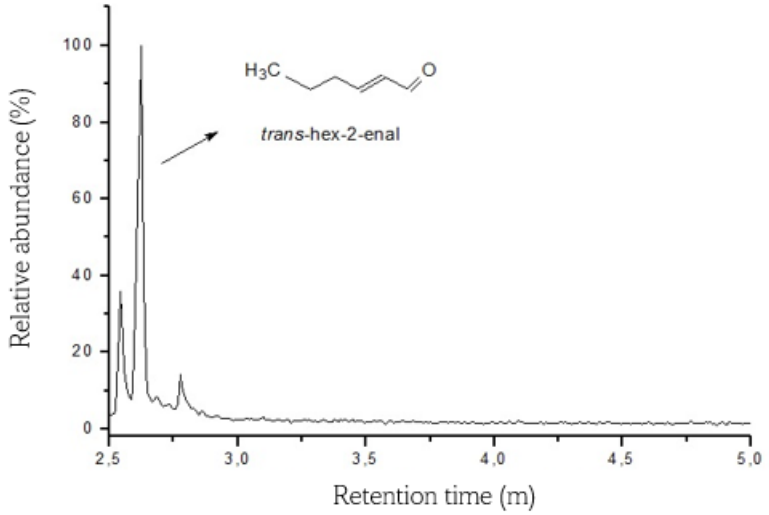

Figure 1. Chromatogram of total ions of volatile oil of fresh leaves of *Crotalaria retusa*.

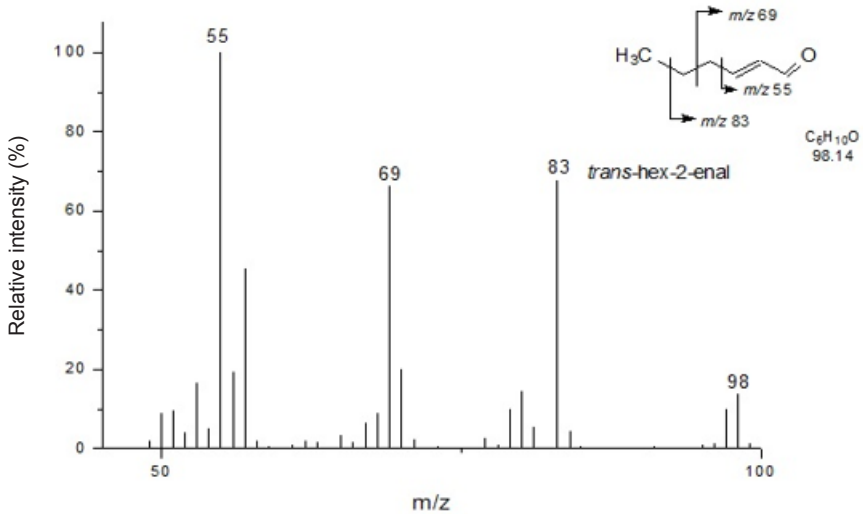

Figure 2. Mass spectra of *trans*-hex-2-enal (*m/z* 98) presents in the volatile oil of fresh leaves of *Crotalaria retusa*.

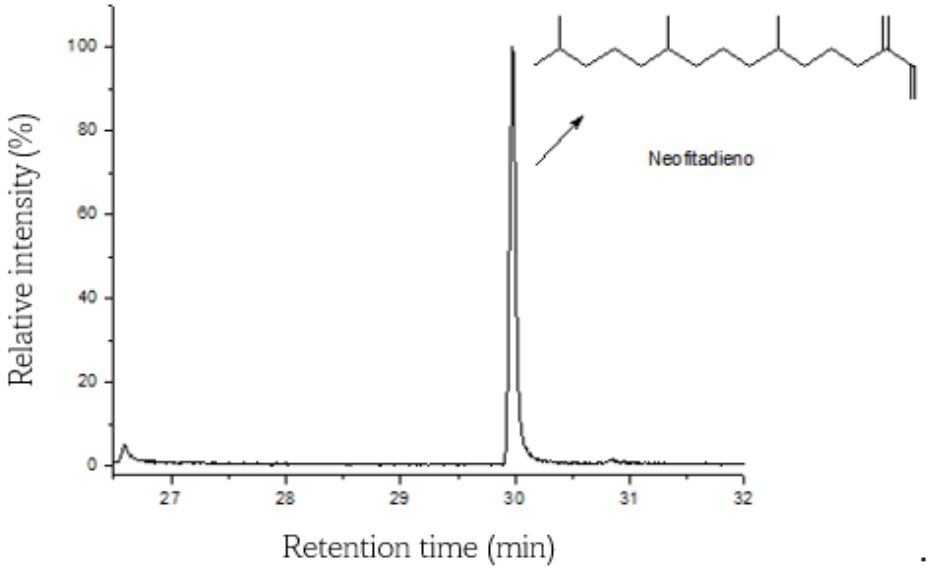

Figure 3. Chromatogram of total ions of the material adhered to the Clevenger surface.

The identification of the constituents was carried out by comparing the retention index (RI) and the pattern of fragmentation of the mass spectra with literature data and with the database of the analysis system. Kovat's Index theoretical. nd: not detected or low relative abundance.

The neofitadiene (Figure 4) is a diterpene with an antimicrobial activity. Peres (Peres et al. 2012) reported the activity of neofitadiene against fungi *Aspergillus niger, Cladosporium cladosporioides* and *C. sphaerospermum*.

Chemical constituents of volatile oil of dry leaves of *C. retusa*

The analysis of the fresh leaves oil showed the presence of few constituents. To obtain a more complete characterization of the chemical composition of the leaves the same extraction process (hydrodistillation) using dry leaves was carried out.

Sodré (2012) emphasizes that the production of volatile oils in herbaceous and aromatic plants may be influenced by internal and external factors, such as the extraction method, temperature and temperature to dry the plant material, among others.

The hydrodistillation of crushed dry leaves allowed a greater volatilization of the constituents present in the leaves of *C. retusa*, which was due to the increase of the surface contact between vegetal material and water. In the volatile oil of the dry leaves was identified 13 main constituents, as shown in Table 3.

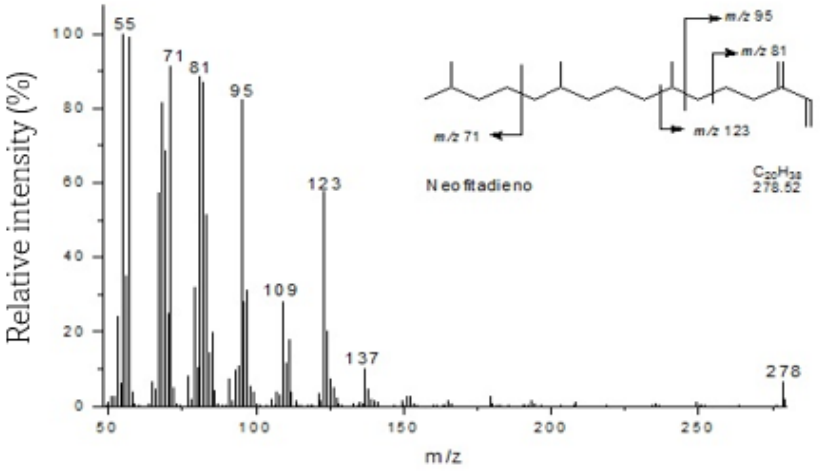

Figure 4. Mass spectrum of neofitadiene (*m/z* 278), present in the yellowish material adhered to the Clevenger surface.

Table 3. Chemical constituents of volatile oil of dry leaves of *Crotalaria retusa*.

Compounds	IK cal.	Área(%)
Benzaldehyde	942	3.6
Propilcyclohexane	1012	2.9
Benzenoacetaldehyde	1027	23.9
Linalool	1086	6.4
2,6-dimetilciclohexanane	1090	3.4
Benzeneethanol	1101	9.7
Isophorone	1105	3.4
ceto isoforone	1127	19.7
3-etil-4-metil- pent-3-en-2-one	1131	21.8
cis-2-metil-dec-3-ene	1150	3.3
Safranal	1180	1.8

The identification of the constituents was carried out by comparing the retention index (RI) and the pattern of fragmentation of the mass spectra with literature data and with the database of the analysis system. Ko v a t's Index theoretical. nd: not detected or low relative abundance.

The main constituent present in the volatile oil of dry leaves were benzene acetaldehyde (23.90%), followed by 3-ethyl-4-methyl-pent-3-en-2-one (21.85%) and keto isophorone (19.74%). Figure 5 shows the chromatogram of total ions of volatile oil of dry leaves and in Figure 6 the mass spectrum of benzene acetaldehyde.

Chemical constituents of the volatile oil of *C. retusa* flowers

The chemical constituents identified by GC-MS for the volatile oil of the *C. retusa* flowers are shown in Table 4 and Figure 7 shown the chromatrogram of total ions present.

The volatile oil of the flowers showed the presence of bioactive compounds in their chemical composition already reported in the literature. The monoterpene linalool, for example, is a constituent of numerous volatile oils with active anti-inflammatory, antinociceptive and antimicrobial properties, as well as being of industrial importance in cosmetology and

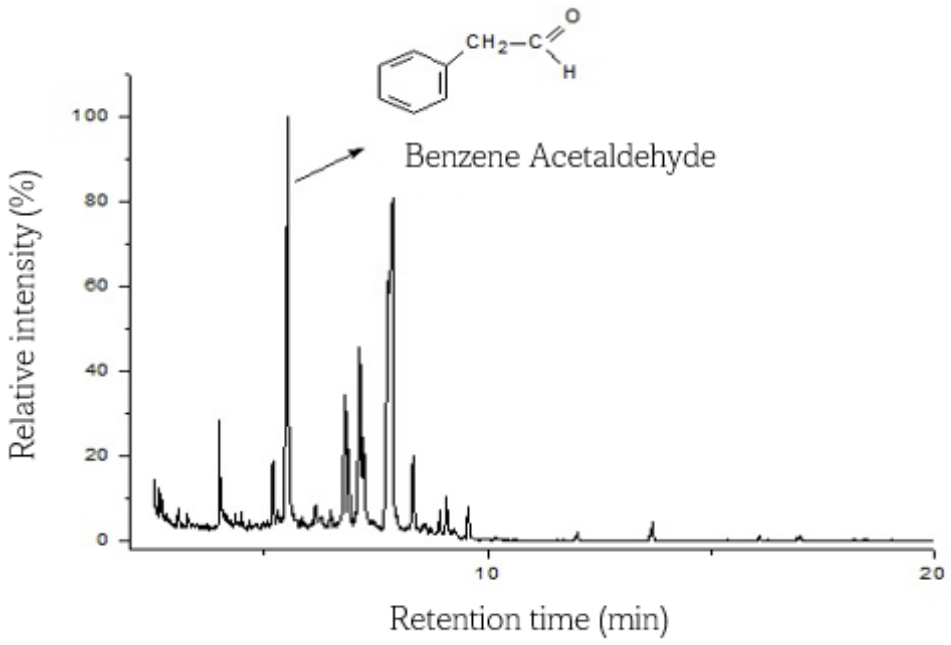

Figure 5. Chromatogram of total ions of volatile oil of dry leaves of *Crotalaria retusa*.

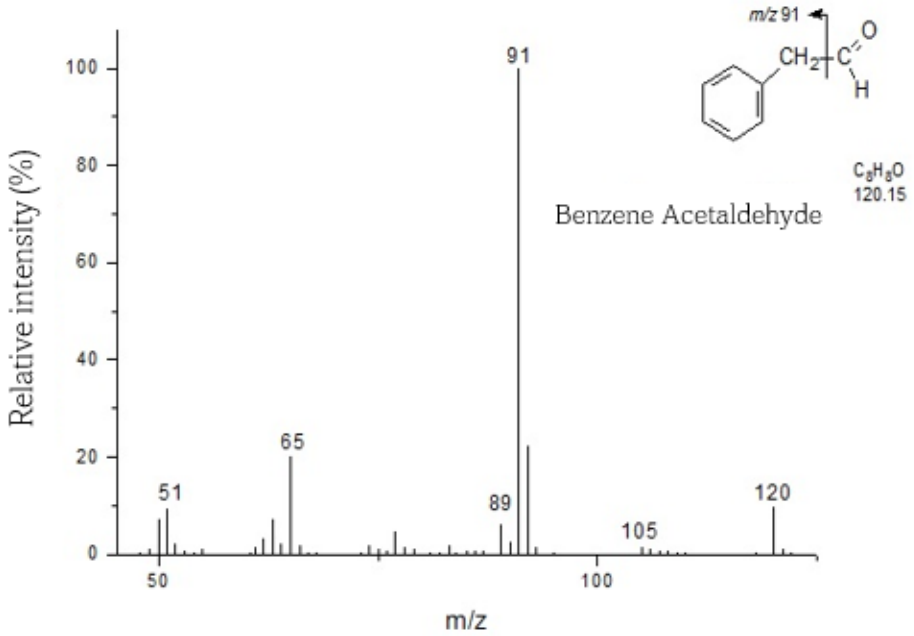

Figure 6: Mass spectrum of benzene acetaldehyde (m/z 120) present in the volatile oil of the dry leaves.

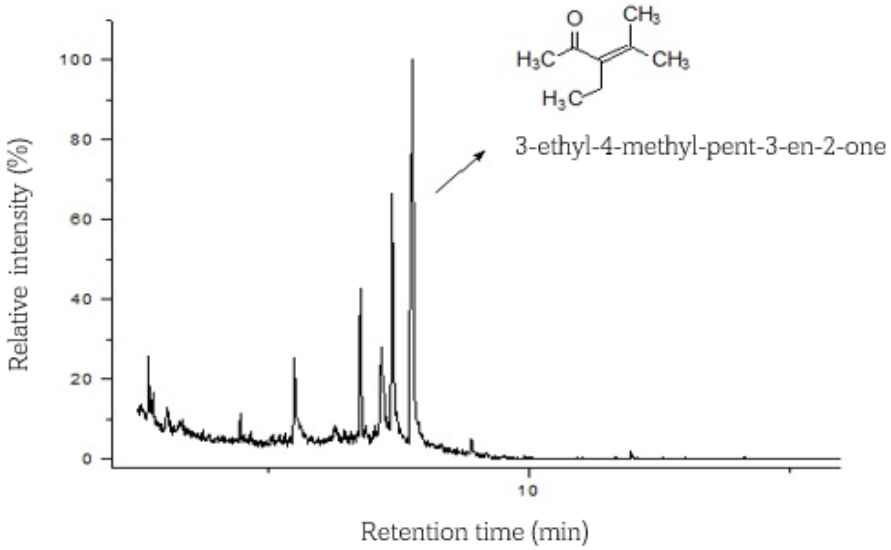

Figure 7. Chromatogram of total ions of volatile oil of fresh leaves of *Crotalaria retusa*.

Table 4. Chemical constituents of the volatile oil of *Crotalaria retusa* flowers.

Compounds	**IK cal.**	**IK lit.**	Área%
Ethylbenzene	-		2.92
o-Xylene	-	875	2.49
Benzeneacetaldehyde	1026	1036	7.49
Linalool	1084	1095	9.65
Maltol	1102	11.06	11.89
1-phenyl-propan-2-one	1111	-	22.76
3-ethyl-4-methyl-pent-3-en-2-one	1127	-	42.80

The majority constituent for the volatile oil of flowers was 3-ethyl-4-methyl-pent-3-en-2-one (Figure 8), with a percentage area corresponding to 42.80%.

food where is used as a fixative of fragrances (da Silva et al. 2016).

Evaluation of anticholinesterase activity

The volatile oil of the fresh leaves and decoction, yellowish material adhered to the surface of the Clevenger presented inhibition potential of acetylcholinesterase. Inhibition of the enzyme responsible for substrate hydrolysis was detected by visualization of white halos around the sample points. The *n*-hexane and ethanol extracts also showed activity, but less expressive due to the strong greenish coloration as a result of the high content of chlorophyll present in the extracts, which makes it difficult to see the inhibition points of acetylcholinesterase. In the tests realized in CCD, caffeine was used as a positive control for being a noncompetitive inhibitor of acetylcholinesterase (Oboh et al. 2014).

Studies have pointed out that the drugs originated from natural products are effective in the treatment of AD. One of the approaches in recent research is the cholinergic treatment of the disease and the method that demonstrates greater clinical efficacy is the use of direct inhibitors of the enzyme AChE. This method is now the most appropriate therapy to improve cholinergic function in patients with AD (Trevisan et al. 2003; Mota et al. 2012).

The increase in the number of cases and the effects that AD causes to those affected has encouraged the search for natural products inhibiting AchE. Among these potential products are the essential oils, which have grown in numbers of publications that evaluate their potentials as AChE inhibitors (Kiendrebeogo et al. 2011; Mota et al. 2012).

The evaluation of the inhibition of AChE by volatile oil of flowers, dried leaves and dichloromethane fractions showed only a positive test, the DCM2, which due to the characteristic greenish coloration of the chlorophyll, made it difficult to see a richer view of the enzyme inhibition.

Most of the phytochemicals with potential AChE inhibitory activity are alkaloids followed by terpenes, sterols, flavonoids and glycosides, isolated from member of Buxaceae, Amaryllidaceae, Lycopodiaceae, Lamiaceae, Chenopodiaceae, Papaveraceae, and Apocynaceae (Ahmed et al. 2013). The drugs currently approved and marketed for AD, such as galantamine and rivastigmine, are alkaloids obtained from plant metabolism, offer only symptomatic relief without preventing the progression of the disease. In addition to Alzheimer's treatment inhibition of AChE is also a promising therapeutic strategy for other types of dementia, myasthenia gravis, glaucoma and Parkinson's disease (Mathew and Subramanian 2014; Owokotomo et al. 2015).

The yellowish material collected from Clevenger showed an inhibition halo, considered positive for enzyme inhibition, and its major constituent is a diterpene (Neophythadiene). This result corroborates with findings in the literature of terpenes with anticholinesterase activity such as the diterpene niloticane isolated from the *Acacia nilotica* subsp. *kraussiana* (Benth.) Brenan (Fabaceae) used in traditional African medicine, as well as new diterpenes isolated from *Leonurus heterophyllus* Sweet (Lamiaceae) (Murray et al. 2013).

In ethnopharmacological studies, humans to improve cognitive function and attenuate cognitive decline have used extracts of species of the genus Salvia. In experiments realized by Kennedy (2011) it was observed that *Salvia lavandula* Alain essential oil composed basically of monoterpenes, exhibited a

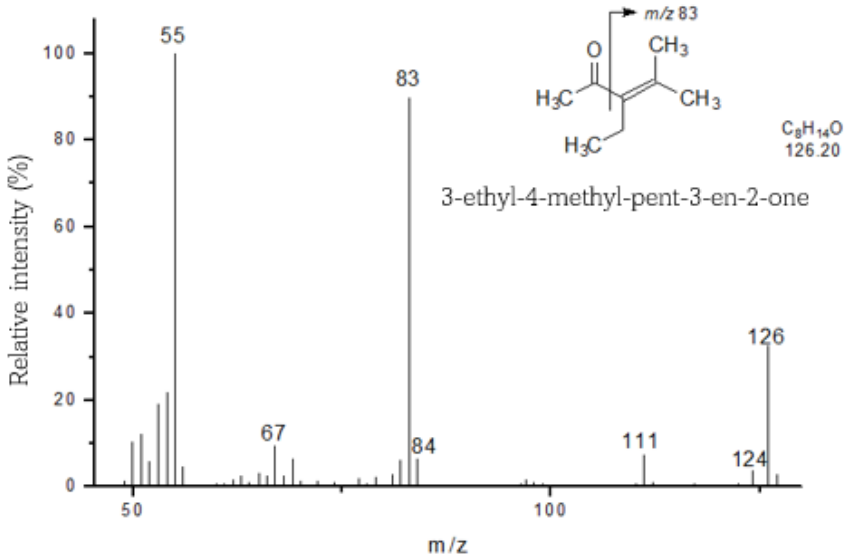

Figure 8. Mass spectrum of 3-ethyl-4-methyl-pent-3-en-2-one (*m/z* 126) present in the volatile oil of flowers.

considerably greater inhibition of human erythrocyte AChE than the extract. This intense inhibition occurs through the synergistic interaction between the monoterpenes -penen and 1,8-cineol (Ustun et al. 2012).

The monoterpene linalool present in the aerial parts of *C. retusa,* was tested in previous studies showed AchE inhibition. In evaluations made by Owokotomo (2015) the presence of monoterpenes in essential oils of *Ocimum gratissimum* L. exceeded the AchE inhibition, at lower concentrations, the reference inhibitor. A possible explanation may be attributed to its very high pinene content. In addition to its lipophilicity, the presence of the hydrocarbon skeleton contributes to AchE activity of the monoterpenes, due to the susceptibility of hydrophobic interactions in the active site of the enzyme (Mukherjee et al. 2007).

The current enzymatic inhibitors used in the symptomatic treatment of AD such donepezil, tacrine, rivastigmine and galantamine, increase the availability and interaction time between Ach and cholinergic receptors of cells. However, adverse side-effects have been reported as elevations of hepatic transaminases and gastrointestinal complaints (Mukherjee et al. 2007; Feitosa et al. 2011; Oboh et al. 2014; Owokotomo et al. 2015). Natural products are potential sources of new bioactive compounds and have a long history of therapeutic utility since the establishment of the human era (Feitosa et al. 2011), in addition to the fact that their isolated compounds show better penetration of the blood-brain barrier than pharmaceutical options and better specificity for human type cells (Oboh et al. 2014).

The hexane and ethanol extracts of *C. retusa* also demonstrated white halos on the plaque. The investigation of various extracts of medicinal plants showed promising results in the inhibition of AChE, such as those of *Jatropha gossypiifolia* L.*, Kalanchoe brasiliensis* Cambess., and *Senna alata* (L.) Roxb. whose extracts showed higher inhibitory activity compared to the drug galantamine (Feitosa et al. 2011). The inhibition results obtained with the use of this extract can be justified by the possible simultaneous synergistic interactions of dozens of bioactive compounds in the extract. In this case, isolation of bioactive compounds and use of a single molecule for the treatment of disease requires an understanding of mechanism of action (Ali et al. 2016).

Toxicity activity

The toxicity of extracts, fractions and chemical constituents are often evaluated by the lethality test of microcrustacean *A. salina* because it is considered as an efficient, safe, fast, inexpensive and reproducible procedure for the detection of the biological potential of new compounds (Dias et al. 2013).

According to the classification proposed by Meyer (1982), the degree of toxicity and the average lethal dose (LD_{50}), for extracts and fractions of plants against *A. salina* are: $LD_{50} \geq 1000$ ppm are nontoxic, between $500 \leq LD_{50} \leq 1000$ ppm low toxicity and $LD_{50} < 500$ ppm highly toxic. In this way, the extracts of the leaves of *C. retusa*, were nontoxic, because they presented $DL_{50} > 1000$ ppm.

CONCLUSION

The oil of the fresh leaves presented constituents with smaller molecular weights when compared to the constituents present in the dry leaves and just only the oil of the fresh leaves inhibited acetylcholinesterase.

The hexane, ethanol and decoct extracts of *C. retusa* leaves demonstrated potential inhibition of AChE. These extracts were to be non-toxic to larvae of *A. salina*, which confirms their importance, increasing the prospects of further study of the species for the discovery of new biological activities.

The volatile oils of the leaves of *C. retusa* have not been reported in the literature as acetylcholinesterase inhibitors. Thus, these expressive results in this study is of great contribution in the research for new natural anticholinesterase drugs.

ACKNOWLEDGEMENTS

The authors thanks to the UFPI, Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, and Laboratório de Geoquímica Orgânica (LAGO – UFPI). There is no governmental or private source for financial support for this project.

DECLARATION OF CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTION

L.K.F. Lima was responsible for formal analysis, methodology, and writing-original draft; M.C.G. Lustosa was responsible for formal analysis, methodology, project administration, and writing-original draft; W.S. Almeida for formal analysis and writing review; M. Rai responsible for writing review, data interpretation, and data curation; C.M. Feitosa was responsible for the supervision, orientation for methodology and visualization; N.C. Andrade

was responsible for data curation; S.G. Lima for investigation, project administration, supervision, writing-review, and visualization.

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