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EVALUATION OF CHEMICAL COMPOSITION AND IN VITRO ANTICHOLINESTERASIC ACTIVITY OF ESSENTIAL OILS FROM HYMENAEA CANGACEIRA AND EUGENIA GRACILLIMA

Natália Sônia de Sá Gomes Feitosa Rosa
Faculty of Integration of Sertão-FIS, Serra Talhada-PE, Brazil
http://lattes.cnpq.br/0320635223403287

Bruno José da Silva Bezerra
Federal University of Pernambuco, Department of Biochemistry, Recife-PE, Brazil
http://lattes.cnpq.br/5524038913919195

Layra Mirelly Feitosa Ferreira Diniz Ventura
Faculty of Integration of Sertão-FIS, Serra Talhada-PE, Brazil
http://lattes.cnpq.br/3496411924340327

Janiely Feitosa de Carvalho
Faculty of Integration of Sertão-FIS, Serra Talhada-PE, Brazil
http://lattes.cnpq.br/7607148797957841

Gabriela Pereira Calumbi
Faculty of Integration of Sertão-FIS, Serra Talhada-PE, Brazil
http://lattes.cnpq.br/4784625946115118

Estefani Pontes Simão
Sertão School of Medicine – FMS, Arcoverde-PE, Brazil
http://lattes.cnpq.br/5576357171256259

Bruno José do Nascimento
Rural Federal University of Pernambuco, Department of Animal Morphology and Physiology, Recife-PE, Brazil
http://lattes.cnpq.br/8213260513385508

Álvaro Aguiar Coelho Teixeira
Rural Federal University of Pernambuco, Department of Animal Morphology and Physiology, Recife-PE, Brazil
http://lattes.cnpq.br/1539131079574469

Valéria Wanderley Teixeira
Rural Federal University of Pernambuco, Department of Animal Morphology and Physiology, Recife-PE, Brazil
http://lattes.cnpq.br/4292195468804301

Maria Tereza dos Santos Correia
Federal University of Pernambuco, Department of Biochemistry, Recife-PE, Brazil
http://lattes.cnpq.br/7863845087003953

Bruno Oliveira de Veras
Federal University of Pernambuco, Department of Biochemistry, Recife-PE, Brazil
http://lattes.cnpq.br/7515860243444988

Fernanda Miguel de Andrade
Federal University of Pernambuco, Department of Biochemistry, Recife-PE, Brazil
Sertão School of Medicine – FMS, Arcoverde-PE, Brazil
http://lattes.cnpq.br/8915203118340030

Abstract: Plants have been manipulated for therapeutic purposes with the aim of preventing, treating and curing pathologies. Inhibition of cholinesterases contributes to increased bioavailability of the neurotransmitter acetylcholine in the synaptic cleft. The therapy for Alzheimer's disease (AD) consists of inhibiting cholinesterases, and natural products are rich sources of these inhibitors. This study aimed to evaluate the chemical composition and in vitro anticholinesterase activity of essential oils from Hymenaea cangaceira (OEHc) and Eugenia gracilima (OEEg). The essential oils were obtained by hydrodistillation and evaluated for composition in a gas chromatograph, coupled to a mass spectrometer (GC-MS) and flame ionization detector (GC-DIC). Cholinesterase inhibition was performed in in vitro colorimetric assays, using acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), the results being expressed in µg/mL of the concentration required for IC50. Neostigmine was used as a reference standard. The OEHc presented 15 compounds and the OEEg 42 compounds. In the evaluation of AChE inhibition, the OEHc presented an IC50 of 0.015µg/mL, being statistically lower than the OEEg (IC50 of 18.30µg/mL). The OEHc against the BChE enzyme presented an IC50 of 0.041µg/mL, being lower than the OEEg, which obtained an IC50 of 8.67µg/mL. OEHc obtained greater inhibitory activity on AChE and BChE when compared to OEEg. The results suggest that OEHc has potential for use in the clinical treatment of neurodegenerative diseases, since it decreases AChE and BChE activity in vitro.

Keywords: Alzheimer's disease. Acetylcholinesterase. Butyrylcholinesterase. Enzyme Inhibition. Natural products.

INTRODUCTION

Plants have been manipulated for therapeutic purposes since antiquity with the aim of preventing, treating and curing many pathologies (DE CARVALHO et al., 2013). The progress of science has strengthened research on medicinal plants, associating their chemical composition with their effects, often attesting to their popular use (CAVALCANTE et al., 2013).

Natural products have attracted attention in major research centers around the world. Brazil has a wide biological variety and has numerous plant species with potential commercialization in the pharmaceutical, food and cosmetic sectors, among others (DA SILVA et al., 2013). Among plant species that are part of the biological diversity of Brazil are Hymenaea cangaceira and Eugenia gracillima.

Hymenaea cangaceira belongs to the Fabaceae family. It is popularly known as jatobá-do-cangaço/cangaceiro. It was recently described by Pinto (2017) and is found in the Caatinga region of Northeast Brazil, more specifically in the states of Bahia, Ceará and Pernambuco. The species of Eugenia gracillima belongs to the Myrtaceae family. Its popular name is described as myrtle/ guajuraia/pitanga-guabiroba. It is a native fruit tree in Chapada do Araripe, located in the state of Pernambuco (DE ARAÚJO et al., 2016). There are still no studies related to the two species regarding anticholinesterase activity.

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) are cholinesterase enzymes of the nervous system that catalyze the hydrolysis of acetylcholine (ACh) to acetic acid and choline in the synaptic cleft. The action of these enzymes reduces the levels of the neurotransmitter ACh and, consequently, the loss of communication between nerve cells, causing a decrease in brain function. Both enzymes have ACh as a substrate, however, BChE has a lower affinity for the substrate compared to AChE (COLOVIC et al., 2013; ZILBEYAZ et al., 2018).

The reduction of the neurotransmitters ACh, noradrenaline, serotonin, somatostatin and glutamate are associated with several diseases linked to the degeneration of cholinergic neurons, including Alzheimer's disease (AD). AD is considered a public health problem by the World Health Organization (WHO), as it is the most common reason for dementia, accounting for approximately 60% to 80% of cases, affecting thousands of elderly people worldwide. AD, which is a disturbing neurocognitive disorder recognized by a gradual reduction of cognitive functions and memory, leads patients to emotional stress, behavior and personality changes, and can reduce independent daily life (SIBERSKI, 2012; MAIER; BARNIKOL, 2014).; BONO, 2014; WHO, 2019).

Currently, therapy for AD consists of inhibiting AChE, as patients with this disease have low levels of ACh (FERRO; GUIMARÃES; GONÇALVES, 2019). AChE inhibitors, called anticholinesterases, increase the bioavailability of the neurotransmitter ACh in the synaptic cleft. This way, the inhibition of this enzyme contributes to the reestablishment of cell communication (ANAND; SINGH, 2013).

The importance of finding new therapeutic options for AD has motivated research aimed at evaluating in vitro and in vivo anticholinesterase activity of products obtained synthetically and/or naturally (natural products) (SILVA, 2018). Among the compounds that have shown enormous potential for application for the development of new drugs and, consecutively, the treatment of neurodegenerative diseases such as AD, are medicinal plants (NEVES et al., 2017).

Products obtained from medicinal plants, such as essential oils, have been reported as one of the innovative strategies for the development of new drugs, called herbal medicines, gaining considerable importance and visibility in recent decades (LOIZZO et al., 2009; JEON et al, 2011; MAJLESSI et al., 2012; CHAIYANA; OKONOGI, 2012). Based on this problem, the objective was to evaluate the chemical composition and in vitro anticholinesterase activity of the essential oils of Hymenaea cangaceira and Eugenia gracillima.

METHODOLOGY

PLANT MATERIAL

The leaves of Hymenaea cangaceira Pinto, Mansano & Azevedo and Eugenia gracillima Kiaersk were collected in April 2018, in Catimbau National Park, located in the municipality of Buíque, in the state of Pernambuco.

The botanical identification of the species was performed using exsiccates. To prepare the exsiccates, the collected sample was pressed and dried (37º C for 72 h) (ORLANDA, 2011). Then, the exsiccates were sent to the Herbário Dárdano de Andrade Lima, of the Instituto Agronômico de Pernambuco (IPA), being deposited under the numbers 84888 and 78999, respectively.

EXTRACTION OF ESSENTIAL OILS

The leaves were submitted to mechanical separation for disposal of materials attacked by pests or diseases. Then, they were washed and crushed in a knife mill, and the material was later transferred to a hydrodistillation flask with water in a 1:10 proportion (m/v), and then heated for 5 hours at 100 °C.

Essential oils were obtained by condensing water vapor in the condenser together with volatile components. To remove the water, the drying agent sodium sulfate anhydrous was added to the mixture of essential oil and water. (Na_2SO_4). The oils were kept refrigerated at -4 °C in an amber bottle (DE VERAS et al., 2019).

ANALYSIS OF THE CHEMICAL COMPOSITION OF ESSENTIAL OILS

Essential oils were analyzed on a 5975C triple gas chromatograph (Palo Alto, CA, USA) equipped with an Agilent Technologies DB-5MS column (30 m x 0.25 mm x 0.25 μm). Aliquots of 1 mL in the 1:50 division of essential oils were injected in gas chromatography coupled to a mass spectrometer (CG-EM). Compounds were identified by comparing their mass spectra using MassFinder 4, NIST08 and Wiley Registry software. ™ 9th Edition, integrated with Agilent MSD Productivity ChemStation software (Agilent Technologies, Palo Alto, USA) and the Retention Index. The samples were quantified by gas chromatography with a flame ionization detector. (CG-DIC), under the same conditions as the GC-MS in triplicate, to calculate the standard deviation of the percentage of peak area for each compound in the chromatogram (DE VERAS et al., 2019).

ASSESSMENT OF CHOLINESTERASE INHIBITORY ACTIVITY

Obtaining the enzymes

It was used AChE de *Electrophorus electricus* (Type VI-S) obtained from Sigma-Aldrich. The BChE used was extracted from the gills of Crassostrea rhizophorae mangrove oysters, by homogenization of 20 mg of tissue per mL of 0.5 mol/L Tris-HCl pH 8.0 and centrifugation at 10,000 rpm (rotations per minute) for 10 minutes. The supernatant (crude extract) was collected and kept at -20°C for further analysis (DE SOUZA et al., 2018).

Enzyme inhibition assay

The enzyme inhibition assays were performed by incubating 200 µL of 0.25 mmol.L-1 DTNB (Ellman's Reagent, dissolved in 0.5 mol.L-1 of Tris-HCl buffer pH 7.4), 10 µL of the enzymes (20 mg / mL of buffer), with 10 µL of essential oils (increasing concentrations from 0.01 to 1000 µg/mL) for 60 minutes, according to Assis et al. (2012). The tests were carried out at room temperature (25ºC). The reaction started immediately after the addition of 62 mmol/L of S-acetyliocholine or S-butyrylthiocholine (20 µL). Reactions were performed in quadruplicate in 96-well microplates. The plates were read by spectrophotometry at 405 nm at 0 and 180 s. As a blank, a 0.5 mol/L solution of Tris-HCl pH 8.0 was used instead of the essential oils. Neostigmine was used as a reference standard, at the same concentrations as the essential oils.

Results were expressed as percent residual activity, mean ± SD (Standard Deviation), in contrast to inhibitor concentration in mg/mL. Neostigmine was used as the reference drug. Residual activity (%) was plotted against inhibitor concentration in µg/mL. Results were expressed as mean ± SD of four replicates and statistically analyzed by nonlinear regression fitted to sigmoidal decay models using Microcal ™ Origin® versão 8.0.

RESULTS AND DISCUSSION

ANALYSIS OF THE CHEMICAL COMPOSITION OF ESSENTIAL OILS

The analysis of the chemical composition of Hymenaea cangaceira essential oil (OEHc) indicated the presence of 15 chemical components, corresponding to 85.37% of the total components, all of which are classified as sesquiterpene hydrocarbons. Among the major components, α-Copaene (6.34% ± 0.17), β-Elemene (7.05% ± 0.12), (E)-Caryophyllene (23.38% ± 0.58), α-Guaiene (9.75% ± 0.07), α -Humulene (4.65% ±0.12) and Germacrene D (14.66% ±0.14) (Table 1).

De Veras et al. (2020) reported that OEHc has α-Copaene, β-Elemene, (E)-Caryophyllene, α-Guaiene, α-Humulene and Germacrene D as main constituents, being present at the same concentrations as in the present study.

Several activities have been described with several compounds found in OEHc. α-Copaene has antimicrobial, anti-inflammatory and healing activity (BRITO et al., 2005). β-Elemene is presented as anticancer, antihypertensive and anti-inflammatory (LI et al., 2009; YVON et al., 2012; LI et al., 2013; CAI et al., 2013; ZOU et al., 2013; ; ZHANG et al., 2013; DONG; YIN; WANG, 2013). (E)-Caryophyllene has antimicrobial and antifungal activity (GARG; SIDDIQUI, 1992; FORMISANO et al., 2006). α-Guaiene is described as an inhibitor of cyclooxygenase, 5-lipoxygenase and acetylcholinesterase (IBRAHIM et al., 2016). Fernandes et al. (2007) described anti-inflammatory activity for α-Humulene. Germacrene D has been reported to be a potent antimicrobial for gram-positive bacteria (DUARTE, 2006).

A study carried out with essential oils of Piper anonifolium and Piper hispidum showed anticholinesterase activity, stating that the inhibition was due to the presence of sesquiterpene compounds found in their oils (DA SILVA et al., 2014). Some of the components were also described in the present work as: α-Copaene, β-Elemene, α-Humulene and Germacrene D.

The analysis of the chemical composition of Eugenia gracillima essential oil (OEEg) showed the presence of 42 compounds, corresponding to more than 99% of the total components. OEEg is mainly composed of 90.11% of sesquiterpene hydrocarbons and 9.57% are composed of oxygenated

Peak	Compound	RI Cal	RI Lit	Percentage (%)	D.P.
1	δ-Elemene	1341	1335	2.21	0.06
2	α-Copaene	**1380**	**1374**	**6.34**	**0.17**
3	β-Elemene	**1396**	**1389**	**7.05**	**0.12**
4	Cyperene	1403	1398	2.68	0.06
5	**(E)-Caryophyllene**	**1424**	**1417**	**23.38**	**0.58**
6	γ-Elemene	1438	1434	2.18	0.13
7	α-Guaiene	**1443**	**1437**	**9.75**	**0.07**
8	α-Humulene	**1458**	**1452**	**4.65**	**0.12**
9	γ-Muurolene	1481	1478	1.16	0.10
10	**Germacrene D**	**1486**	**1480**	**14.66**	**0.14**
11	δ-Selinene	1496	1492	1.14	0.04
12	Bicyclogermacrene	1501	1500	4.59	0.02
13	α-Bulnesene	1511	1509	2.84	0.04
14	δ-Cadinene	1529	1522	2.21	0.06
15	Germacrene B	1563	1559	0.53	0.02
	Total Others			85.37 14.63	

RICal- Relative experimental retention indices and RILit - literature retention indices; DP. Standard deviation.

Table 1. Chemical composition of OEHc, highlighted in bold the main components.

sesquiterpenes. The most abundant constituents were δ-Elemene (6.06% ± 0.08), Bergamotene (4.89% ± 0.06), γ-Muurolene (15.60% ± 0.22), Germacrene D (16.10% ± 0.38), Bicyclogermacrene (8, 53% ± 0.09), δ-Cadinene (6.23% ± 0.16) and Germacrene B (7.43% ± 0.62) (Table 2).

Sampaio et al. (2019) reported that OEEg has δ-Elemene, γ-Muurolene, Germacrene D, Bicyclogermacrene and Germacrene B as main constituents, being present at the same concentrations as in the present study.

Several species of Eugenia spp. have great biological potential such as antimicrobial, antifungal, antitumor, among others. These can be attributed to the volatile chemical composition present in its essential oils (MAGINA, 2008). δ-Elemene exerts anticancer activity (LU et al., 2012). Bergamotene has antioxidant activity (DE MELO et al. 2011). γ-Muurolene has antimicrobial activity (SILVA et al., 2010; CHAIBUB et al., 2013). Germacrene D has insecticidal, antibacterial and fungicidal action (ALMEIDA; DELACHIAVE; MARQUES, 2005). Bicyclogermacrene has a high potential for antimicrobial action (CONSTANTIN et al., 2001; CYSNE et al., 2005; SOUSA et al., 2010). δ-Cadinene has antitumor and antimicrobial activity (SKALTSA et al., 2003; WRIGHT et al., 2007). Germacrene B has antifungal activity (COSTA et al., 2010).

Besides, in the study carried out by Da Silva et al. (2014), some OEEg compounds were also seen in the essential oils of Piper

Peak	Compound	RI Cal	RI Lit	Percentage (%)	D.P.
1	**δ-Elemene**	**1336**	**1335**	**6.06**	**0.08**
2	α-Cubebene	1345	1345	0.12	0.00
3	α-Ylangene	1376	1373	0.50	0.02
4	Isoledene	1375	1374	1.30	0.03
5	Copaene	1377	1374	0.60	0.00
6	Guaia-6,9-diene	1380	1379	0.68	0.04
7	β-Bourbonene	1387	1387	0.71	0.00
8	β-Cubebene	1388	1387	0.11	0.00
9	β-Elemene	1390	1389	1.41	0.08
10	Sativene	1392	1390	0.21	0.00
11	α-Gurjunene	1410	1409	4.03	0.02
12	β-Copaene	1432	1430	0.31	0.05
13	**Bergamotene**	**1433**	**1432**	**4.89**	**0.06**
14	γ-Elemene	1434	1434	0.73	0.00
15	α-Guaiene	1437	1437	1.80	0.08
16	Cadina-3,5-diene	1449	1448	0.26	0.09
17	α-Humulene	1454	1452	1.64	0.11
18	Alloaromadendrene	1458	1458	0.90	0.00
19	Caryophyllene-9-epi-(e)	1461	1464	0.40	0.00
20	Cumacrene	1470	1470	0.27	0.00
21	**γ-Muurolene**	**1478**	**1478**	**15.60**	**0.22**
22	γ-Amorphene	1482	1483	3.14	0.00
23	**Germacrene D**	**1483**	**1484**	**16.10**	**0.38**
24	β-Selinene	1490	1489	0.16	0.00
25	Bicyclogermacrene	1501	1500	8.53	0.09
26	α-Muurolene	1503	1500	0.73	0.00
27	β-Guaiene	1504	1502	0.84	0.01
28	**δ-Cadinene**	**1524**	**1522**	**6.23**	**0.16**
29	Zonarene	1528	1528	0.93	0.00
30	α-Cadinene	1537	1537	2.33	0.17
31	Selina-3,7(11)-diene	1548	1545	0.15	0.00

32	Elemol	1549	1548	0.61	0.00
33	**Germacrene B**	**1561**	**1559**	**7.43**	**0.62**
34	Spathulenol	1578	1577	0.67	0.00
35	Thujopsan-2β-ol	1589	1588	0.29	0.00
36	Globulol	1591	1590	1.03	0.05
37	1h-cycloprope-azulen-4-ol	1592	1592	1.01	0.10
38	Viridiflorol	1593	1592	0.42	0.00
39	Isospathulenol	1626	1625	1.25	0.09
40	Torreyol	1644	1644	1.27	0.12
41	Cubenol	1645	1645	3.58	0.19
42	α-Cadinol	1656	1654	0.45	0.00
	Total			99.68	
	Outros			0.32	

RICal- Relative experimental retention indices and RILit - literature retention indices; DP. Standard deviation.

Table 2. Chemical composition of OEEg, the main components highlighted in bold.

Sample	**Inhibition of the activity of AChE** **IC_{50}** (μg/mL)	Inhibition of the activity of BChE **IC_{50}** (μg/mL)
OEHc	0.015 ± 0.000^{3}a	0.041 ± 0.005^{a}
OEEg	18.30 ± 0.0041^{c}	8.67 ± 0.0012^{c}
Neostigmine	0.16 ± 0.0^{1}b	1.9 ± 0.0006^{b}

Data represent the mean ± SD. [abc] Means followed by different letters in the column are significantly different ($p < 0,05$) by the test of Tukey.

Table 3. AChE and BChE inhibitory activity by OEHc and OEEg.

anonifolium and Piper hispidum that also have anticholinesterase activity, such as: δ-Elemene, γ-Muurolene, Germacrene D and Bicyclogermacrene. The chemical compositions found need to be isolated to understand whether the anticholinesterase activity acted synergistically, or is performed by some specific compounds alone. Because the biological actions of essential oils are not exactly associated with the amount of the main components, but can also be related to interactions between the largest and smallest compositions, which generate synergies and/or antagonistic responses (AAZZA; LYOUSSI; MIGUEL, 2011; TAK; JOVEL; ISMAN, 2016).

ENZYME INHIBITION

For the enzyme inhibition assay, the inhibitory concentration IC50 was used. IC50 values represent the average inhibition of a compound (50% inhibition), that is, it corresponds to the dose capable of inhibiting 50% of the enzyme. Thus, the lower the IC50, the more active a compound is.

The OEHc showed the highest inhibition of AChE statistically, presenting an IC50 value of 0.015±0.0003 µg/mL. OEEg, despite having shown inhibition of AChE (IC50 18.30±0.0041 µg/mL), had lower activity compared to OEHc. The drug Neostigmine, used as a standard, had an IC50 of 0.16±0.01 µg/mL(Table 3).

In the evaluation of the inhibitory activity against the BChE enzyme, it was observed that OEHc resulted in a high inhibitory activity with an IC50 of 0.041 ± 0.005 µg/mL. The Neostigmine standard presented an IC50 of 1.9 ± 0.0006 µg/mL. The OEEg obtained inhibitory activity with an IC50 of 8.67 ± 0.0012 µg/mL, the inhibitory action being not as significant as compared to the OEHc (Table 3).

A study already carried out with *Hymenaea cangaceira* relatou que o óleo essencial da planta possui potencial antioxidante, atividade antimicrobiana e atividade antinociceptive (DE VERAS et al., 2020).

Regarding anticholinesterase activity, this is the first study carried out in OEHc and OEEg. Several studies with essential oils have demonstrated their potential to inhibit key enzymes in the control of diseases such as AD, such as the essential oil of Citrus sinensis, which had an AChE enzyme inhibitory activity of IC_{50} = 63 µg/mL (SÁ et al., 2012). A study carried out with the species of Salvia lavandulaefolia showed inhibitory activity against the AChE enzyme in the value of IC_{50} = 50 µg/ml (SAVELEV et al., 2003). Another research, with the essential oils of Eucalyptus camaldulensis and Ocimum canum, showed inhibition of AChE with IC_{50} value = 18.98 µg/ml and IC_{50} = 36.16 µg/ml, respectively (KIENDREBEOGO et al., 2011). The OEHc and the OEEg in the present study showed superior anticholinesterase activity, in relation to the essential oils of other species already mentioned, confirming their effectiveness and better inhibition of the enzyme, revealing the importance of researching new therapies for AD.

CONCLUSION

OEHc obtained greater inhibitory activity on AChE and BChE enzymes compared to OEEg. The results suggest that OEHc has potential for use in the clinical treatment of neurodegenerative diseases, such as AD, since it significantly inhibited AChE and BChE activity in vitro. Therefore, these results are seen as innovative strategies for the development of new drugs/herbal medicines.

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