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ORIGINAL ARTICLE

The Anticholinesterase Properties of Plants from the Northeast of Brazil Selected by an Ethnopharmacological Study for Disorders Relating to the Nervous System

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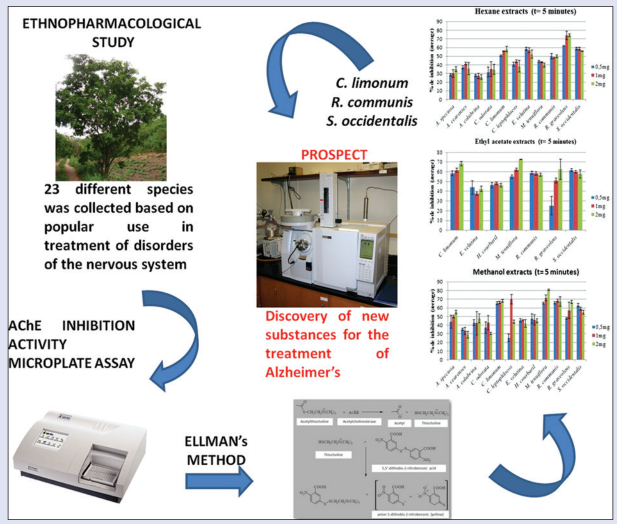
ABSTRACT

Background: Various factors may trigger Alzheimer's disease and the cholinergic hypothesis, which is one of the most widely accepted, argues damage to the brain nuclei, may reduce the production of the choline acetyltransferase enzyme, and cause a decline in the synthesis of acetylcholine (ACh). Studies have thus focused on discovering molecules that are capable of inhibiting the action of cholinesterase enzymes that degrade ACh, thereby preventing the evolution of the disease. **Objective:** The aim of the present study is to assess the anticholinesterase properties of extracts of medicinal plants in a semi-arid region of Northeast of Brazil. **Materials and Methods:** The species were selected by way of an ethnobotanical study and were collected if there were some indications that they are related to the nervous system. The plant samples were extracted using hexane, ethyl acetate, and methanol. Anticholinesterase activity *in vitro* was assessed by way of bioautography in thin layer chromatography and microassays in 96-well plates. **Results:** Twenty-three species of plant were collected, and 75 extracts were analyzed. The bioautography revealed that 26.7% of the samples showed inhibitory activity against the acetylcholinesterase (AChE) enzyme. After the test for false positives, 8% of the samples were found to inhibit AChE. Thirty samples were analyzed by microassay (500 μg/mL), on which 86.7% showed moderate to powerful anticholinesterase activity. **Conclusion:** Of the extracts tested, *Citrus limonum*, *Ricinus communis*, and *Senna occidentalis* stand out as was the most promising in terms of anticholinesterase activity and may serve as a guide for the discovery and development of new substances for the treatment of AD.

Key words: Acetylcholinesterase, Alzheimer's disease, Caatinga, dementia, semi-arid

SUMMARY

- The bioautography revealed that 26.7% of the samples showed inhibitory activity against the acetylcholinesterase enzyme
- Samples were analyzed by microassay (500 μg/mL), upon which 86.7% showed moderate to powerful anticholinesterase activity
- *Citrus limonum*, *Ricinus communis*, and *Senna occidentalis* stand out as being the most promising in terms of anticholinesterase activity
- *C. limonum*, *R. communis*, and *S. occidentalis* may serve as a guide for the discovery and development of new substances for the treatment of Alzheimer's disease.



Abbreviations used: AChE: Acetilcolinesterase

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INTRODUCTION

Alzheimer's disease (AD) can be classified as sporadic or familial. The first type affects 90–95% of individuals, and most of these are aged over 60 years. The second type manifests itself in people under 60 years of age and is present in other family members, characterizing it as a disorder of genetic origin.[1] This makes the study of this disease even more important owing to the increased life expectancy of human beings. In Brazil, in the past decade, the population aged 60 years or above has grown 2.5 times more (36%) than the younger population (14%)[2] and the United Nations estimates that the elderly population, which currently exceeds 840 million, will triple by 2050.[3]

The principal anatomical and physiological characteristics of AD are degradation of the cholinergic neurons and reduction in acetylcholine (ACh), which together result in dementia, the main symptom of the disease. Histopathological studies show senile plaques formed by fragments of insoluble β-amyloid peptide and intracellular

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neurofibrillary tangles, which may be formed by disruption of the microtubule cytoskeleton, due to hyperphosphorylation of TAU protein, accompanied by massive loss of neurons.[4]

The production of ACh depends on the functioning of the base nuclei, especially Meynert's nucleus since this produces the enzyme that catalyzes the synthesis of ACh known as choline acetyltransferase (CAT). When these nuclei atrophy, there may be diminished production of CAT and hence, reduced production of the ACh neurotransmitter.[5]

The recycling of ACh in the synaptic cleft is carried out by the acetylcholinesterase enzyme (AChE) and butyrylcholinesterase enzyme, which are found in various organs and are capable of degrading ACh into choline and acetyl-CoA. The main drugs used to treat AD are therefore cholinesterase inhibitors (ChIs) which allow ACh to remain longer in the synaptic cleft.[6]

Starting out from the cholinergic hypothesis, various studies have looked for active substances based on natural sources. The alkaloids physostigmine, extracted from *Physostigma venenosum*, and galantamine, extracted from the *Galanthus* and *Narcissus* genera, have powerful anticholinesterase properties although their side effects include hepatotoxicity, gastrointestinal problems, and others relating to bioavailability.[7]

In view of this, various studies have been conducted to find new substances based on natural products, especially plants that are capable of inhibiting the action of AChE, in an attempt to mitigate the effects of AD, and have fewer side effects than the currently available drugs.[8-10]

Some studies are based species selection on traditional knowledge,[11] for example a triage with extracts from 18 traditionally used species related to the inhibition of AChE that showed promising candidates as *Jatropha gossypifolia* and *Senna alata*, which have a level of activity similar to the standard galantamine. Another study has evaluated the hydroalcoholic extract and fractions of *Bouvardia ternifolia* as ChIs and obtained moderately good results for the raw extract, and ethyl acetate and methanol-soluble fractions.[9]

The classes of compounds that have been most frequently isolated and identified in studies of anticholinesterase activity are alkaloids, followed by monoterpenes, coumarins, triterpenes, flavonoids, and benzenoids.[12]

The present study thus aims to evaluate the anticholinesterase activity of 75 plant extracts traditionally used to treat disorders of the nervous system in an attempt to find substances that may lead to new treatment options for AD.

MATERIALS AND METHODS

Selection of species

The plant samples were collected in a semi-arid part of the municipality of Altinho, in the Brazilian State of Pernambuco (08° 35'13.5"S and 36° 05'34.6"W). The selection of species was based on popular use in the treatment of disorders of the nervous system according to a data bank built up by the Laboratory of Applied Ethnobotany of the Federal Rural University of Pernambuco – PEUFR.

After this survey, species were excluded if they were bought in local markets and/or fairs or not easily available in the environment. Medicinal plants were selected for inclusion in the study if they were recommended by at least three informants for the treatment of some symptom or pathology related to the nervous system, such as headache, dizziness, insomnia, migraine, and forgetfulness, yielding 23 species. Of these, the bark and leaves were collected from *Erythrina velutina* and *Anadenanthera colubrina*, providing a total of 25 plant samples [Table 1].

Vouchers of the selected species were identified at the Applied Ethnobotany Laboratory of Federal Rural University of Pernambuco (PEUFR) with the aid of the keys for botanical identification and specialized bibliography. The material collected was deposited in the Professor Vasconcelos Herbarium collection, Federal Rural University of Pernambuco (PEUFR), and copies were included in the Professor Geraldo Mariz Herbarium, collection Federal University of Pernambuco, Pernambuco, and the Agronomic Institute of Pernambuco.[13,14]

Table 1: Result of qualitative analysis of 75 extracts tested using the silica plate test for anticholinesterase activity of medicinal plants collected in a semi-arid part of the municipality of Altinho/PE

Scientific name/(voucher number)	Common name	Part used	Pretest	False positive test	Positive
Alpinia speciosa (Blume) D. Dietr./(-)	Colônia	Leaf	H	-	H
Amburana cearensis (Allemão) A.C. Sm./(50486 PEUFR)	Imburana de cheiro	Bark	H/A	H/A	-
Anadenanthera colubrina (Vell.) Brenan/(48714 PEUFR)	Angico	Bark	A	-	A
Anadenanthera colubrina (Vell.) Brenan/(48714 PEUFR)	Angico	Leaf	-	-	-
Calotropis procera (Aiton) W.T. Aiton/(-)	Algodão de seda	Leaf	-	-	-
Catharanthus roseus (L.) G. Don/(81229 IPA)	Boa noite	Flower	-	-	-
Cedrela odorata L./(54191 UFP)	Cedro	Bark	H	H	-
Citrus limonum Risso/(-)	Limão grande	Leaf	H	H/A	-
Commiphora leptophloeos (Mart.) J.B. Gillett	Imburana	Bark	H/A	H/A	-
Cymbopogon citratus (DC.) Stapf/(-)	Capim santo	Leaf	-	-	-
Erythrina velutina Willd./(46180 UFP)	Mulungu	Bark	H/A	H/A	-
Erythrina velutina Willd./(46180 UFP)	Mulungu	Leaf	-	-	-
Eucalyptus globulus Labill/(-)	Eucalipto	Leaf	-	-	-
Hymenaea courbaril L./(-)	Jatobá	Bark	H	H/A	-
Lippia alba (Mill.) N.E. Br. ex Britton & P. Wilson/(53504 UFP)	Cidreira	Leaf	-	-	-
Lippia sp./(81234 IPA)	Alecrim de caco	Twig/leaf	-	-	-
Mimosa tenuiflora (Willd.) Poir./(50871 PEUFR)	Jurema preta	Bark	H/A/M	M	H/A
Nicotiana glauca Graham/(81222 IPA)	Pára raio	Leaf	-	-	-
Ocimum basilicum L./(48670 PEUFR)	Manjericão	Leaf	-	-	-
Plectranthus barbatus Andrews/(-)	Hortelã miúda	Leaf	-	-	-
Prosopis juliflora (Sw.) DC./(-)	Algaroba	Fruit/seed	H	H/A	-
Ricinus communis L./(54257 UFP)	Mamona	Leaf	H/A	-	H/A
Ruta graveolens L./(-)	Arruda	Leaf	A	A	-
Senna occidentalis (L.) Link/(49615 PEUFR)	Manjiroba	Fruit/seed	H/A	H/A	-
Ziziphus joazeiro Mart/(46189 UFP)	Juazeiro	Leaf	-	-	-

(-) on voucher number: Crops or it was not possible to collect reproductive parts. H: Hexane extract; A: Ethyl acetate extract; M: Methanol extract

Obtaining the extracts

The samples collected were stabilized in a dryer at 40 ± 2°C for 3 days and then ground in a Willey type knife mill to obtain a 20 Mesh particle size. The powders (50 g) were extracted by maceration with 250 mL of hexane for 48 h and once filtered, the solvent was replenished 3 times. The plant residue was then extracted with ethyl acetate and methanol, respectively, as described previously. Hexane, ethyl acetate, and methanol extracts were produced for all 25 plant samples, giving a total of 75 extracts. The fluid extracts were pooled together and concentrated under reduced pressure at 40 ± 2°C to obtain the dry extracts.

Chemicals

The acetylcholinesterase was obtained from *Electrophorus electricus* (AChE, Type VI-S), bovine serum albumin (BSA), physostigmine, acetylthiocholine iodide (ATCi), and Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) from Sigma (St. Louis, MO, USA). The ethyl acetate, hexane, methanol, and other reagents were acquired from Vetec Química Fina, Brazil.

Acetylcholinesterase inhibition assay

Bioautography of Acetylcholinesterase inhibition

This assay was divided into two stages using a modified version of the method described by Rhee *et al*.[15] A pretest was first carried out with the raw extracts (10 mg/mL) and positive standard physostigmine (0.1 mM) which were analyzed using thin layer chromatography (TLC) F_{254} silica gel plates from Merck (Darmstadt, Germany). Aliquots of 2.5 µL of the samples and standard were applied to the plate and sprayed with a solution of Ellman's reagent (1 mM) and ATCi (1 mM) diluted in a Tris/HCl buffer (50 mM, pH 8). After drying, the plates were sprayed with the AChE solution (3 U/mL) diluted in a Tris/HCl buffer (50 mM, pH 8) containing 0.1% BSA. After 5 min, white inhibition haloes were visualized on the yellow coloring of the plate.

A "false positive" test was then carried out to confirm inhibition of the enzyme.[15] Another plate of TLC was prepared in a similar manner although first sprayed with Ellman's reagent and, after drying, the ATCi and AChE solution (preheated to 37°C for 10 min). In a few minutes, the yellow coloring appeared on the plate, and it was checked for the presence of white haloes. The extracts that did not form haloes on the "false positive" test even though these were present in the pretest were considered positive.[16] To confirm the results obtained in the qualitative tests, the species that exhibited activity in the pretest, regardless of the polarity of the extract, was tested quantitatively to compare the two methodologies and to check the significance and authenticity of the false-positive test.

Acetylcholinesterase inhibition micro-assay

The AChEI activity microplate assay was based on Ellman's method (1961)[17] as modified by Rhee *et al*.[15] Readings were taken using a Thermo-Plate automatic 96-well microplate reader (Mod. TP-Reader). The raw extracts (500–2000 µg/mL) were diluted in a Tris/HCl buffer (50 mM, pH 8) containing 0.1 M of NaCl and 0.02 M of $MgCl_2$, and the positive standard physostigmine (0.10–1.35 mg/mL) was diluted with methanol. The negative control was methanol in a Tris/HCl buffer (50 mM, pH 8) in place of the sample/standard.

Aliquots of 25 µL of ATCi (15 mM) diluted in a Tris/HCl buffer (50 mM, pH 8), 125 µL of Ellman's reagent (3 mM) diluted in a Tris/HCl buffer (50 mM, pH 8), 50 µL of Tris/HCl buffer (50 mM, pH 8) containing 0.1% BSA, and 25 µL of the sample were placed in the wells. The absorbance of the wells was determined over a period of 5 min at 412 nm. Finally, 25 µL of AChE enzyme (0.22 U/mL) diluted in Tris/HCl buffer (50 mM, pH 8) containing 0.1% BSA was added. The absorbance was again measured over a period of 5 min. All analyses were carried out in triplicate.

The percentage of inhibition was calculated by comparing the absorbance of the samples compared to the blank (Tris/HCl buffer at 50 mM and pH 8) according to the following equation:[18]

$$I(\%) = 1 - \frac{ABS_{sample}}{ABS_{blank}} \times 100$$

Data analysis

All the data were expressed in terms of mean ± mean standard error. The comparison of inhibition data was carried out using ANOVA analysis of variance, followed by the Tukey test for comparing the % inhibition of anticholinesterase between species and concentrations of extract ($P < 0.05$). These analyses were carried out using Bio Estat 5.0 (Mamiraua Institute) (Ayres et al. 2007). The graphs were plotted using GraphPad Prism5 (GraphPad Software, Inc.).

A scale, adapted for microplate assays, was used to classify anticholinesterase activity, according to which acetylcholinesterase inhibition (AChEI) ≤30% are classified as weak inhibitors, 30–50% moderate inhibitors, and ≥50% strong inhibitors and candidates for future fractioning.[18]

RESULTS AND DISCUSSION

Anticholinesterase bioautography

The false-positive and positive results from the pretests are presented in Table 1. Of the 75 samples examined, 20 extracts (26.7%) produced a white halo on the yellow background in the pretest for AChE enzyme inhibition activity. When the false-positive test was carried out, six extracts from four species exhibited activity, representing 8% of the total number of samples analyzed.

A. colubrina (angico), *A. speciosa* (shell plant), *Mimosa tenuiflora* (jurema preta), and *Ricinus communis* (castor oil plant) were the species that tested positive. In the case of *Mimosa tenuiflora*, the test was positive for the hexane and ethyl acetate extracts, whereas the methanol extract produced a false positive.

The two silica gel plate tests were capable of identifying spots indicating false positive results, which may be attributed to compounds such as aldehydes and amines present in the extracts that inhibit the reaction between Ellman's reagent and the substrate originating from hydrolysis of acetylthiocholine by AChE.[16]

The ethyl acetate extracts of *Prosopis juliflora*, *Hymenaea courbaril,* and *Citrus limonum*, which did not produce spots in the pretest, did produce white spots in the false positive test.

Acetylcholinesterase enzyme inhibition micro-assay

Thirty-nine samples from 13 species that produced inhibition spots in silica gel plates in the pretest were selected for the microplate assay, with the exception of the extract of *P. juliflora* and the hexane extract of *H. courbaril*, for reason of the low yield and the impossibility of collecting more samples for the preparation of further extracts without impairing the ability to undertake faithful comparisons, owing to the different time of the year and the effect of this on such comparisons. It is known that such factors are responsible for qualitative and quantitative modifications of the secondary metabolites.[19] It should also be noted that only the bark of *A. colubrina* and *E. velutina* tested positive in silica plates and were submitted to the quantitative test,;the leaves of these species were not used for the microplate assay.

Of the 35 samples tested quantitatively, the ethyl acetate extracts of *A. speciosa, Amburana cearenses*, *A. colubrina*, *Cedrela odorata*, and *Commiphora leptophloeos* produced results that were negative and/or close to zero and considered nondetectable (ND), leaving 30 samples for statistical analysis.

The AChEI percentages (t = 5 min) of the extracts at three concentrations (500, 1000, and 2000 µg/mL), on the last reading of the microplate, were analyzed statistically to ascertain whether there was a significant difference between the concentrations used [Table 2], since the lower the quantity of extract used in relation to activity, the more promising the extract for drug development. A lower concentration of physostigmine (0.10 mg/mL) showed 92.87% inhibition.

Among hexane extracts, *R. graveolens, S. occidentalis* and *E. velutina* showed higher percentage of anticholinesterase inhibition on the basis of the lowest concentration tested [Table 2].

In general terms, the hexane fraction of *R. graveolens* at a concentration of 1000 µg/mL can be considered more promising since it yielded a higher inhibition percentage (74.14%) compared to the other species, not differing statistically from the concentration of 2000 µg/mL (74.37%).This is an interesting finding since it suggests that it would not be necessary to use higher concentrations to obtain a satisfactory result. However, as the literature and studies of *R. graveolens* have shown extracts of this species to produce toxic effects, more attention needs to be paid to this aspect.[20]

In a study of the anticholinesterase activity of methanolic and aqueous extracts of *R. graveolens*, 39% and 22% inhibition of AchE, respectively, were found for a concentration of 0.1 mg/mL.[21] Another study has used the same Ellman's method to investigate methanolic and hexane extracts of *R. graveolens,* finding 59% and 95% inhibition, respectively, at a concentration of 0.4 mg/mL.[22]

In the case of ethyl acetate extracts, *S. occidentalis*, *R. communis*, *C. limonum,* and *Mimosa tenuiflora* exhibited the highest percentage of anticholinesterase activity, at the lowest concentration tested [Table 2]. In the case of *S. Ocidentalis* and *R. communis*, there was no significant difference between the three concentrations tested. With *M. tenuiflora* and *C. limonum*, an increased concentration contributed positively to an increase in inhibitory action although the concentration of 2000 µg/ml was the most efficient for these extracts of these species.

This same group of plants also presented the best inhibition percentage results for the most polar fraction. In particular, an increase in concentration of the extract of *S. occidentalis* decreased inhibitory activity. In the case of the methanol extracts of *R. comunins* and *C. limonum*, the concentration of 500 µg/mL did not differ statistically from higher concentrations. Of these species, *M. tenuiflora* showed important activity in terms of inhibition and as with the ethyl acetate extracts, an increase in concentration positively influenced AchE inhibition, varying from 65.49% to 80.77%.

It can be seen that 66.7% of samples showed no significant difference as to the concentration used for the extract. However, those which did show some difference included species such as *C. limonum*, *M. tenuiflora,* and *R. communis*, which produced the best results.

It should be noted that ethnobotanical studies and plant diversity are important for AChEI research, as these provide a wide variety of metabolites conducive to phytochemical studies.[23] Although most anticholinesterase studies concentrate on the search for alkaloids, in view of the discovery of physostigmine in *P. venenosum* and more than 35 alkaloids reported to be active in inhibiting acetylcholinesterase, an increasing number of other classes of the compound have been related to such activity, including terpenoids, glycosides, and coumarins.[24]

Flavonoids, essential oils, coumarins, and pectins[25] are abundant in species of the genus Citrus. Leaves of *Citrus aurantifolia* (Christm.). Swingle were

Table 2: Statistical analysis of acetylcholinesterase enzyme inhibitors percentages (t=5 min) for hexane, ethyl acetate and methanol extracts at 500, 1000, and 2000 µg/mL

	Scientific name	Percentage of de inhibition (AChEI)		
		500 µg/mL	1000 µg/mL	2000 µg/mL
Hexane	*Alpinia speciosa*	28.30±1.17Aa	30.04±4.53Aa	35.14±2.75ABa
	Amburana cearensis	37.20±1.83CFa	41.48±1.52CEa	35.71±7.18ABa
	Anadenanthera colubrina	27.98±1.35Aa	27.22±2.13Aa	25.98±2.52Ba
	Cedrela odorata	31.75±5.74AFa	35.04±8.66AEa	34.82±5.77ABa
	Citrus limonum	50.70±0.77Ea	56.08±0.19Db	58.21±3.10Db
	Commiphora leptophloeos	40.97±2.26CDa	44.11±1.44CEa	38.43±6.83AEa
	Erythrina velutina	58.49±1.69Ba	56.94±2.84Da	52.38±4.55Da
	Mimosa tenuiflora	44.14±0.90DEa	43.19±0.71CEab	39.70±2.47AEb
	Ricinus communis	50.03±3.02Ea	48.35±0.29CDa	49.75±1.61DEa
	Ruta graveolens	61.72±0.49Ba	74.14±4.76Bb	74.37±1.52Cb
	Senna occidentalis	58.78±1.41Ba	58.56±1.62Da	55.86±0.47Da
Ethyl acetate	*Citrus limonum*	58.52±2.62Aba	61.65±2.20Ba	68.57±2.30ACb
	Erythrina velutina	43.92±6.96Ca	37.74±1.69Ca	42.40±2.68Ba
	Hymenaea courbaril	46.32±2.72Aca	48.07±1.58Aa	46.29±1.87BCa
	Mimosa tenuiflora	55.04±1.78ABCa	62.17±1.56Bb	72.66±0.22ACc
	Ricinus communis	59.06±1.64Aba	58.40±1.87Ba	57.09±1.80CDa
	Ruta graveolens	25.32±9.52Da	50.98±2.58Ab	62.71±10.36ACb
	Senna occidentalis	61.72±1.59Ba	59.95±1.48Ba	57.83±4.09CDa
Methanol	*Alpinia speciosa*	44.11±7.49Aca	49.84±1.70Aa	55.29±2.17Aa
	Amburana cearensis	35.33±1.31BCa	33.37±4.23Ca	29.02±3.96Ca
	Anadenanthera colubrina	42.90±3.27Aca	42.17±13.40ACa	47.94±5.23ADa
	Cedrela odorata	37.33±6.57Aca	42.74±8.14ACa	30.48±1.10Ca
	Citrus limonum	65.40±1.67Da	66.38±1.18BDa	68.06±1.44Ba
	Commiphora leptophloeos	25.51±4.38Ba	69.84±5.33Bb	43.79±1.77Dc
	Erythrina velutina	45.69±1.84Aca	45.79±0.73Aa	42.02±4.52Da
	Hymenaea courbaril	47.56±5.31Aa	45.94±5.91ACa	45.06±2.53Da
	Mimosa tenuiflora	65.49±1.07Da	71.58±3.36Bb	80.77±0.98Ec
	Ricinus communis	65.56±1.27Da	68.41±1.64Ba	67.46±5.49Ba
	Ruta graveolens	47.88±1.11Aa	57.19±9.54ABab	66.76±1.82Bb
	Senna occidentalis	62.83±2.22Da	59.03±1.96ABab	54.94±2.24Ab

Values on the lines followed by the same lower-case letter indicate that there was no significant difference when α=0.05. Values on the columns followed by the same uppercase letter, for each extractor liquid, indicates that there was no significant difference when α=0.05. AchEI: Acetylcholinesterase enzyme inhibitors tested for *in vivo* AChE inhibition[26] and presented positive results. Good results were also obtained *in vitro* and *in vivo* for a mixture of coumarins from the ethyl acetate extract of *C. limonum*, suggesting a possible application for this mixture in the treatment of neurodegenerative disorders such as AD.[27]

Pharmacological tests have been carried out *in vivo* with ricinine alkaloid isolated from *R. communis* to analyze its stimulant effect on the central nervous system. This study found a number of side effects, although at lower doses, there was an improvement in cognition, and the authors thus propose the hypothesis that substances similar to ricinine might emerge as a new class of drugs for treating diseases such as AD.[28]

A survey covering 175 references to natural products with anticholinesterase activity, citing 309 species and 260 substances isolated from plants has shown different results for the genus Mimosa. The study of *Mimosa acustipula* found 39% AchE inhibition in a microplate test using a concentration of 1.8 mg/mL and another study involving *Mimosa pudica* at a concentration of 0.1 mg/mL found it to be inactive.[12] Yet, another study cites various compounds that have been isolated from *Mimosa hostilis* and tested a group of isolated flavonoids for anticholinesterase activity, some of which exhibited positive activity ranging from 9.34% to 27.63% although this is well below the positive standard physostigmine which inhibited 94.83% of the AchE enzyme.[29]

Qualitative × quantitative analysis

One interesting detail of the results is that the qualitative analysis filter for the choice of extracts for the quantitative test showed that one of the four extracts that presented a positive result (the ethyl acetate extract of *Anadenanthera colubrine*) exhibited no detectable inhibition activity.

Another quantitative result that appears controversial when using the qualitative test as a criterion for selection of extracts for positive tests is that for *Mimosa tenuiflora*, which, in principle, corroborated the results of the (qualitative) silica gel plate test, showing moderate to strong inhibition, ranging from 44.1% to 80.7% for the hexane and methanol extracts, respectively. The latter, however, produced a false-positive result in the qualitative test, showing that the qualitative analysis may also have presented a false-negative and suggesting that it might be useful to carry out a microplate test even for false positives.

In a study similar to the present one, CCD analysis of extracts of medicinal plants found that some samples produced stains in both positive and false-positive tests and others only in the false positive test, corroborating our results. This same study suggested that active compounds may bond with the silica to produce lower activity.[11]

On the other hand, another study has reported that given that extracts with an enzyme percentage inhibition percentage of less than 30% were false-positive, the CCD tests were more than 80% reliable in comparing them with the microplate tests and thus conclude that this is a reliable methodology to guide future analyses.[30]

Enzyme availability behavior profile

The AChE enzyme inhibition activity of extracts during 5 min of reaction is presented in Figure 1. The profile is similar for practically all the species, with a notable reduction in AChEI, represented by the increase in the percentage of AChE available on the curve over time, suggesting a reversible bonding of anticholinesterase substances with the enzyme. The ethyl acetate extract of *R. graveolens* behaved differently, with only 50% of the acetylcholinesterase available for reaction present after only the 1st min of reaction time.

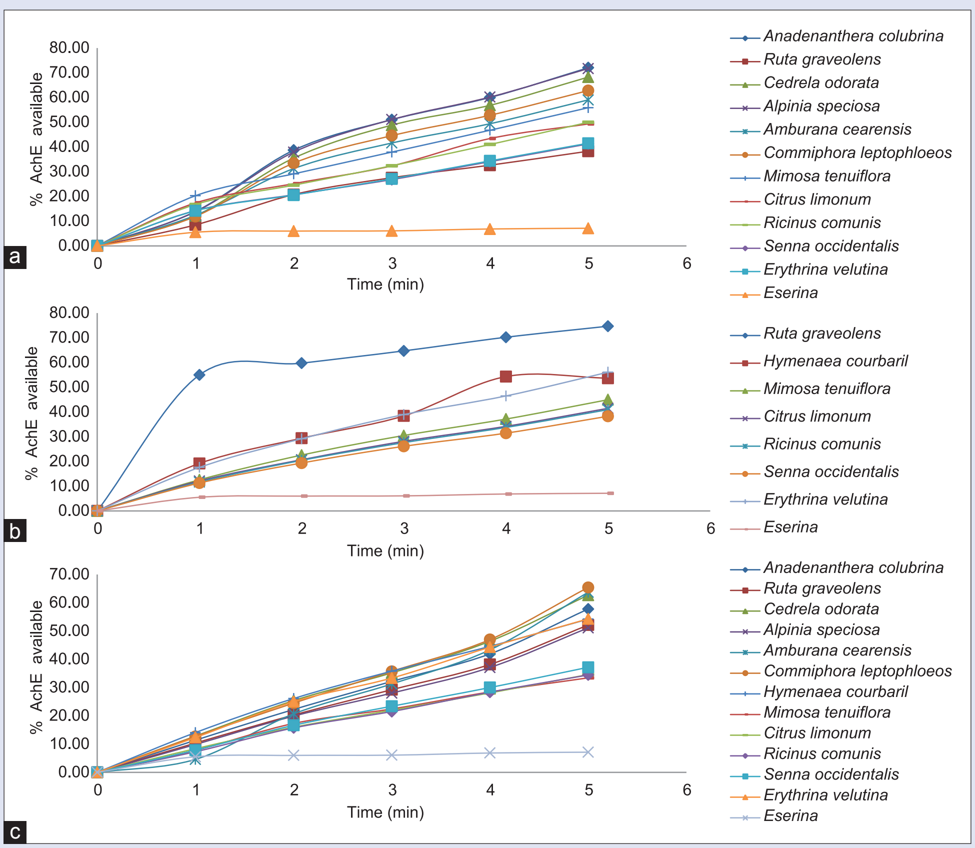

Figure 1: Enzymatic behavior of acetylcholinesterase enzyme over 5 min of reaction time of hexane (a), ethyl acetate (b), and methanol extracts (c) at a concentration of 500 μg/mL

It should be noted that the behavior of standard serine (100 μg/mL) differs from that of the extracts, in so far as the powerful anticholinesterase activity is represented on the curve only by the small quantity of AChE available (<10%) throughout the 5-min reaction time.

CONCLUSION

The results obtained suggest the conclusion that ethnobotanical studies have much to contribute to research into bioactive substances since 86.7% of the samples analyzed in the microplate assay exhibited moderate to strong AChEI activity at the lowest concentration of 500 μg/mL.

C. limonum, *R. communis*, and *S. occidentalis* possess anticholinesterase potential, and a biologically guided study may favor the isolation and identification of active molecules, contributing to the arsenal of treatments available for AD.

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Conflicts of interest

There are no conflicts of interest.

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