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Evaluation of the Ache Inhibitory Capacity of a Hydroalcoholic Extract of Amazonian Grape (*Pourouma Cecropiifolia*)

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Abstract

Alzheimer's disease represents the leading cause of dementia, severely affecting millions of patients worldwide. Pharmacological treatment consists of acetylcholinesterase inhibitors, improving the cognitive symptoms associated with this pathology. Pourouma cecropiifolia has great nutritional value due to its high concentration of carbohydrates, minerals, and vitamins. The aim of the study was to evaluate the AChE inhibitory capacity of a hydroalcoholic extract of Pourouma cecropiifolia (EHPC). This was a cross-sectional and prospective study. To obtain the seeds of Pourouma cecropiifolia, its fruits were manually pulped, the seeds were dried at 40ºC ± 2 for 48h, then the seed was freed from its rind and crushed until a fine powder was obtained. To 8 g of seeds, a mixture of solvents, ethanol: water (3:1) in a 1:10 ratio, was added in an ultrasonic bath (Ultrasons HD, JP Selecta) with a fixed power of (180W) at 50ºC, for 20 minutes with periodic agitation. The filtrate was concentrated under vacuum until the total volume of the solvent was reduced, then freeze-dried and stored in dark bottles. Total phenols, determination of in vitro antioxidant activity (ABTS and DPPH) and evaluation of acetylcholinesterase inhibition were estimated. The evaluated extract showed high values of antioxidant activity, attributed to high concentration of phenolic compounds. The findings suggest that Pourouma cecropiifolia extract has great potential as a possible inhibitor of acetylcholinesterase enzymatic activity.

Keywords: *Alzheimer's disease, antioxidants, Pourouma cecropiifolia, acetylcholine esterase inhibitors, Amazon grape.*

1. Introduction

Alzheimer's disease is one of the leading causes of dementia worldwide, and is characterized by the accumulation of amyloid peptides β extracellular as well as intracellular neurofibrillary tangles, producing the progressive loss of cognitive functions, decreased thinking and the ability to perform daily activities independently (Knopman et al., 2021; Breijyeh & Karaman, 2020).

It is estimated that in the world, about 75% of people with dementia are not diagnosed with Alzheimer's and in 2019 it is estimated that about 55 million people live with dementia, and it is projected that they will reach 139 million by the year 2050 (Authier et al., 2022).

To date, only a restricted number of drugs for symptomatic treatment and with limited benefits, in mild and advanced stages such as Donepezil, Rivastigmine, Galantamine are approved by the different regulatory agencies, the Food and Drug Administration (FDA) in the United States and the European Medicines Agency (EMA), so the search and development of new and potential drugs that help counteract the effects of the disease become a priority (Authier et al., 2022; Vaz & Sylvester, 2020).

During the last years the use of different natural extracts shows positive effects in the treatment of Alzheimer's disease, and have become a potential alternative source for possible treatments (Breijyeh & Karaman, 2020).

The *Pourouma cecropiifolia*, known as uvilla, it is a native Amazonian species distributed by several countries in South America, such as Brazil, Colombia, Peru, Venezuela and Ecuador. It is described botanically as a tree between 5 to 10 meters high, which has fibrous spherical fruits, 2 to 4 centimeters in diameter, which change their hue depending on their stage of development, going from a green color in the immature state to a dark violet coloration when the fruit reaches all its organoleptic characteristics (Barrios et al., 2010). The fruits have white heart-shaped seeds that occupy 21% of the total composition and can be used as a substitute for coffee after a roasting process (Sánchez et al., 2005). However, the pulp is mainly consumed, since due to its semi-acidic sweet taste and its juicy and mucilaginous characteristics, it is used for the preparation of preserves, wines, juices, jams and nectars (Barrios et al., 2010).

Recent evidence on *Pourouma cecropiifolia* It shows the great nutritional value of its fruits for its high concentration of carbohydrates, minerals such as: potassium, calcium, and phosphorus; water, vitamins such as: niacin, ascorbic acid and riboflavin, and to a lesser extent fats and fibers (Gonzales, 2002). In addition, it has been determined the presence of different types of anthocyanins, quercetin glycosides, catechins, aliphatic alcohols, flavonoids, tannins and coumarins, together with alkaloids and steroids in lower concentration (Lopes-Lutz et al., 2010; Arias, 2011) which demonstrates the great antioxidant potential of the pulp.

The diverse presence of antioxidant compounds is analyzed for various therapeutic uses. Thus, previous studies postulate that extracts of the pulp of uvilla have a cytotoxic action *in vitro* in cell lines of cervical (HeLa), breast (MCF-7), colon (HT-29) and human larynx (HEp-2) cancers; This possible effect as an anticancer agent is attributed to its high concentration of anthocyanins, flavonoids and polyphenols that neutralize the damage generated by free radicals (Barrios et al., 2010).

In the search for acetylcholinesterase inhibitors of natural origin, the objective of this study is to evaluate the AChE inhibitory capacity of a hydroalcoholic extract of *Pourouma cecropiifolia* (EHPC), given that inhibitors of this enzyme are proposed as the first-line pharmacological treatment in Alzheimer's disease.

2. Methods

It was a cross-sectional and prospective study.

Materials and reagents

The materials and reagents used in this study are as follows:

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Na2S2O8, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2′2-azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) diammonium salt (ABTS), Folin-Ciocalteu's reagent, gallic acid, quercetin, acetylcholinesterase (AChE), 5,5 0-dithiobis(2-nitro-benzoic acid) (DTNB), and acetylthiocholine iodide (TTIP) were purchased from

Sigma Aldrich (St Louis, MO, USA), Physostigmine free base was acquired from Tokyo Chemical Industry, ethanol, methanol (MERCK).

Plant material and preparation of extracts
The fruits of *Pourouma cecropiifolia* were acquired commercially, in the Putumayo canton, province of Sucumbíos, Ecuador, through local merchants.

Preparation of extracts
To obtain the seeds of *Pourouma cecropiifolia* its fruits were pulped manually, leaving the seed covered with its bark, the seeds were dried 40ºC ± 2 for 48h, then the seed was released from its bark and crushed until obtaining a fine powder. To 8 g of seeds were added a mixture of solvents, ethanol: water (3:1) in proportion 1:10, in an ultrasound bath (Ultrasons HD, JP Selecta) with a fixed power of (180W) at 50ºC, for 20 minutes with periodic stirring.

The filtrate was concentrated under vacuum, until the total volume of the solvent was reduced, then lyophilized and stored in dark bottles until further analysis. The extract obtained was 0.894g (11.18%).

Determination of total phenols
Total phenols were determined by the Folin Ciocalteu´s (Singleton & Rossi, 1965), with slight modifications for adaptation to a micro plate reader (Rea et al., 2020). 50 µL of extract were taken and mixed with 1250 µL of Folin Ciocalteu´s reagent dissolved in a 1:10 ratio with distilled water, and 1000 µL of sodium carbonate solution (7.5%).

It was allowed to incubate at room temperature for 30 minutes, and absorbance was measured at 750 nm. A standard gallic acid curve was used as a reference, and values were expressed as mg gallic acid equivalents (SAEs) per 100 mg sample.

In vitro *antioxidant activity*
DPPH radical uptake tests
The free radical scavenging assay was developed according to the protocol described in the literature and with slight modifications (Sánchez-Moreno et al., 1998). A solution of DPPH in ethanol (0.022%) was prepared and kept protected from sunlight and at 4ºC until use.

The stock solution and serial solutions dissolved with ethanol (0.001 – 0.1 mg mL-1) were prepared. Briefly, 100 µL of the different concentrated solutions were added to a multiwell plate, followed by 100 µL of absolute ethanol and 50 µL of DPPH solution. The mixture was incubated for 30 minutes in darkness and stirring. Absorbance was determined at 515 nm in a microplate reader. Inhibition percentages were calculated using the following formula:

$$\% \text{ Inhibición} = [1 - \frac{(A - A')}{A^0}] \, x \, 100$$

Where:

- ✓ ***A*** is the absorbance of the sample
- ✓ ***A´*** is the absorbance of the sample without solution DPPH
- ✓ ***A0*** is the absorbance of the DPPH solution

IC50 values were obtained by linear regression analysis interpolation. Quercetin was used as a reference antioxidant standard and the results were expressed as µM of Trolox Equivalent (ET) per gram of extract.

ABTS radical uptake assays

Free radicals were generated according to the literature described (Re et al., 1999). Briefly, 50 µL of extract at different concentrations (0.001 – 0.1 mg mL^{-1}) were added to a multiwell plate, followed by 250 µL of ABTS solution and incubated at room temperature protected from light and with constant stirring for 6 minutes. The absorbance was determined at 740 nm and the results were expressed as µM of Trolox Equivalent (ET) per gram of extract.

Acetylcholinesterase (AChE) inhibition

The determination of AChE inhibition was developed following the protocol described by Ellman (15), with slight modifications described in the bibliography (Rea et al., 2020). Instantly, 100 µL of 0.1 M phosphate buffer solution (pH 7.8) were added to a multiwell plate, followed by 20 µL of acetylcholinesterase solution (0.5 U/mL dissolved in phosphate buffer, pH 7.8) and 20 µL of sample at different concentrations were added, mixed and incubated at 37°C for 15 minutes.

After the incubation time, 40 µL of a 0.75 mM DNTB solution (prepared in phosphate buffer, pH 7.8), and 20 µL of TTIP solution were added to initiate the reaction. Absorbance was determined at 405 nm after 6 minutes. The percentage of inhibition (%) was calculated according to the formula described above. Physostigmine an AChE inhibitor was used as a positive control.

Statistical analysis

All results were expressed as the mean ± standard error of the results obtained.

The data were processed by *GraphPad Prism* 6.01 software. IC50 values were calculated by linear regression for DPPH and ABTS, and by nonlinear log (inhibitory) vs. response regression for AChE.

3. Results and Discussion

The results obtained in this study are summarized in Table 1 and Figure 1.

Table 1. Total phenols expressed as mg EAG/100 mg extract, IC50 values of DPPH, ABTS with ET values (Trolox equivalent) and IC50 of AChE, from hydroalcoholic extract of *Pourouma cecropiifolia* (EHPC) and reference standards.

Sample	Total phenol content (mg/100 mg extract)	IC50 (µg mL-1)		ET (µM ET $^{g-1}$ extract)		IC50 (µg mL-1)
		ABTS	DPPH	ABTS	DPPH	Ache
EHPC	5.72±0.05	4.76±0.17	21.65 ± 2.14	3245.81 ± 123.74	2179.12 ± 236.38	18.92±1.03
Quercetin	-	2.16±0.02	6.85±0.33	-	-	-
Trolox	-	3.84±0.11	11.12 ± 0.56	-	-	-
Physostigmine	-	-	-	-	-	0.003

Quercetin and physostigmine were used as positive controls. The values of IC50, ET_{DPPH}, $_{ABTS}$ and AChE are expressed as mean ± error of three different experiments. The IC50 values for DPPH and ABTS were calculated by linear regression, and by non-linear log regression (inhibitory) vs response for AChE.

In this study the seed components of *Pourouma cecropiifolia* were extracted with a mixture of aqueous solvent, ethanol:water (3:1) along with the addition of sonication and temperature as detailed above. The extract obtained was evaluated for its antioxidant capacity in DPPH and ABTS assays (Figure 1).

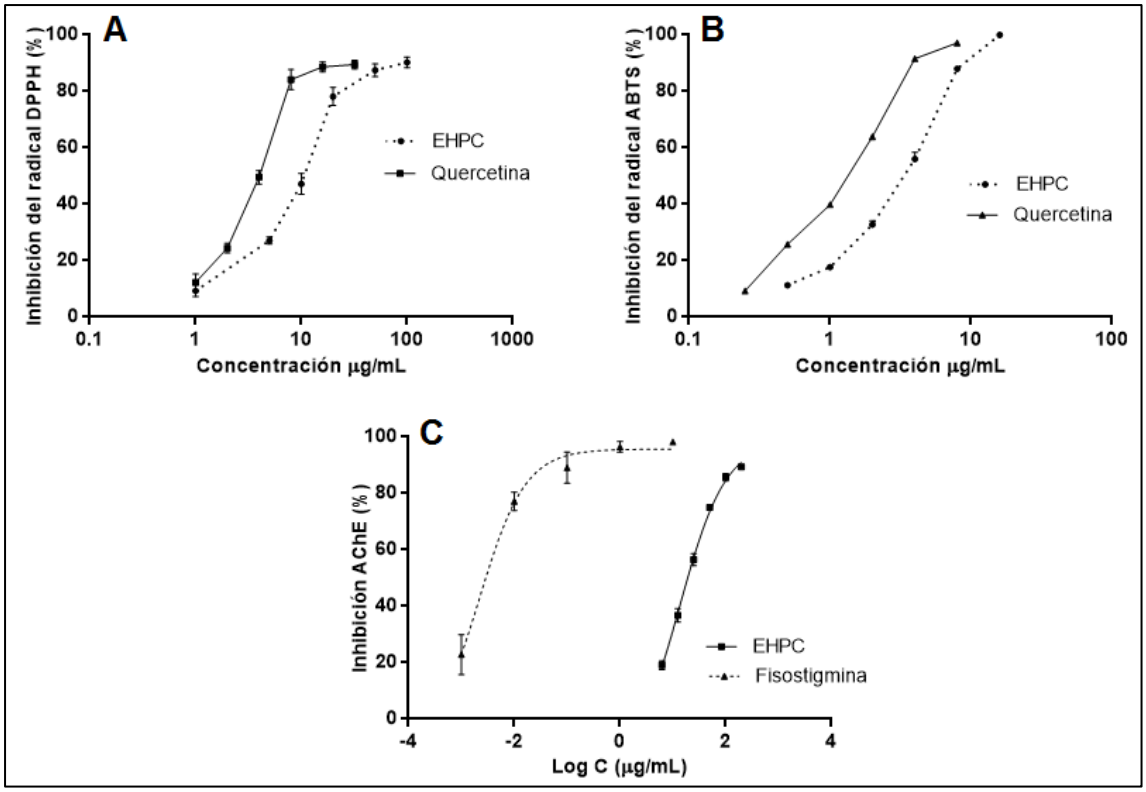

Figure 1. Free radical scavenging capacity DPPH and ABTS (0.5 – 100 µg mL^{-1}) and AChE inhibitory capacity (6.25 – 200 µg mL^{-1}) of the hydroalcoholic extract of seeds of Pourouma cecropiifolia. (A) DPPH radical inhibition, (B) ABTS radical inhibition and (C) acetylcholinesterase (AChE) inhibition. Quercetin (0.25 – 32 µg mL-1) and physostigmine (0.001 – 10 µg mL-1) were used as reference standards. Each value represents the mean ± error of three repetitions.

Quantification of total phenols

The content of total phenols in seeds of *Pourouma cecropiifolia* is 5.72±0.05 mg/100mg extract. Similar studies report values of 9.07±0.16 mg/100g extract, in solvent mixtures water: methanol (50:50) (Ordoñez et al., 2019), indicating that they possess an appreciable amount of phenolic compounds. In addition to the seed, in skin and pulp appreciable amounts of phenolic compounds are determined 84.66 ±1.22 mg / 100g fresh skin and 8.85 ±3.74 mg / 100 g fresh pulp respectively (Lopes-Lutz et al., 2010), which shows that a higher content of phenolic compounds are found in the seeds of *Pourouma cecropiifolia*. The use of different aqueous solvents together with temperature and sonication show an increase in the extraction of phenols in seeds such as hemp (Liang et al., 2018).

It is necessary the characterization, identification and isolation of the compounds involved in the composition of the seed of *Pourouma cecropiifolia*, through the application of more sensitive and specific techniques such as HPLC, HPLC-MS / MS, to elucidate the compounds present in the seed.

In vitro *antioxidant activity*

The results indicate a significant capacity to capture DPPH and ABTS radicals at the concentrations evaluated. The IC50 values represented in the Table 1 show CI $values_{50}$ 21.65±2.14 µg mL^{-1} for DPPH and 4.76±0.17 µg mL^{-1} for ABTS respectively. Other studies report HF $values_{50}$ 1.62 ± 0.01 mg mL^{-1} DPPH and 1.72 ± 0.01 mg mL^{-1} ABTS for seed *Pourouma cecropiifolia* (Ordoñez et al., 2019) indicating that they have a high antioxidant capacity.

Previous studies indicate a higher concentration of anthocyanins, flavonoids and chlorogenic acids in the skin of the Amazonian grape (*Pourouma cecropiifolia)* (Barrios et al., 2010), it is also determined that the concentration of phenolic compounds are found mainly in the seed > skin > pulp in different grape varieties (*Vitis vinifera*) that are analyzed (Pantelić et al., 2016), as well as its antioxidant activity,

is higher in seeds than grape skin and pulp, and has a value that is directly related to the content of phenolic compounds present (Kupe et al., 2021).

Acetylcholinesterase inhibition

For the symptomatic treatment of Alzheimer's a limited number of drugs, known as acetylcholinesterase inhibitors (AChEIs) are approved during the last decades (Vaz & Sylvester, 2020), based on the cholinergic hypothesis of Alzheimer's disease, an increase in cholinergic activity with the help of drugs, would improve cognitive symptoms that are impaired by the disease (Authier et al., 2022).

The results obtained from EHPC show a high inhibitory activity of acetylcholinesterase with IC values$_{50}$ 18.92±1.03 μg mL^{-1} (Table 1). Different studies show promising values in the enzymatic inhibition of acetylcholinesterase with IC50 values 0.08 – 18.9 μg mL^{-1} in different plant species (Taqui et al., 2022), as well as compounds isolated from safflower seeds (*Carthamus tinctorius* L.*)*, charters A and B with CI values$_{50}$ 17.96 and 66.83 μM respectively (Peng et al., 2017) or from alkaloids isolated from seeds of *Peganum nigellastrum* Bunge (Wang et al., 2009).

This is the first study of *Pourouma cecropiifolia* seeds that reports the inhibitory effect of acetylcholinesterase, in which the inhibition of enzyme activity is the therapeutic strategy in the search for drugs for the treatment of Alzheimer's.

4. Conclusions

In summary, it has been evaluated the scavenging action of free radicals DPPH and ABTS, in addition to the inhibitory capacity of the enzyme acetylcholine esterase of a hydroalcoholic extract of seeds of Amazonian grape (*Pourouma cecropiifolia*). The results of this research show that there is a higher concentration of phenolic compounds in the extract obtained from the seeds, which is directly related to the high antioxidant activity. In addition, EHPC proved to be a potent AChE inhibitor, and future possible candidate applicable to neurodegenerative disorders. Although the present study provides a contribution on a new inhibitor of AChE, it is necessary to characterize the seed of *Pourouma cecropiifolia* in order to elucidate the compounds involved in the activity evaluated, as well as preclinical trials to know the real contribution of the extract, in the search for new therapeutic alternatives against Alzheimer's disease.

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