Research, Society and Development, v. 9, n. 9, e689997739, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i9.7739

Atividade inibitória da acetilcolinesterase sobre os extratos de folhas de *Pterodon pubescens* (Benth.) Benth. (*Leguminosae-Papilionoideae*)

Inhibitory activity of acetylcholinesterase by *Pterodon pubescens* (Benth.) Benth. (*Leguminosae-Papilionoideae*) leaf extracts

Actividad inhibidora de la acetilcolinesterasa en extractos de hojas de *Pterodon pubescens* (Benth.) Benth. (*Leguminosae-Papilionoideae*)

Received: 20/08/2020 | Reviewed: 29/08/2020 | Accept: 02/09/2020 | Published: 03/09/2020

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Resumo

Pterodon pubescens (Benth.) Benth. é uma espécie nativa do Cerrado brasileiro, popularmente conhecida como "sucupira-branca," e rica em metabólitos bioativos. O estudo teve como objetivo avaliar a atividade inibitória da enzima acetilcolinesterase do extrato das folhas de *P. pubescens*. Constituintes fenólicos e flavonoides foram analisados por LC- DAD e LCMS. A avaliação inibitória enzimática quantitativa foi realizada em microplaca de 96 poços em leitor ELISA. A análise estatística foi realizada com limite de significância $p \leq 0,05$ e aplicou-se o teste de *Tukey*. Os compostos bioativos detectados foram os terpenóides, ácidos fenólicos (ácido gálico, ferúlico e ácido rosmarínico) e flavonoides (caempferol, luteolina, apigenina, rutina e quercetina). Não houve diferença significativa de inibição entre o extrato bruto metanólico e acetato de etila, e entre suas respectivas fases hidroetanólica e acetato de etila na concentração de 80 µg mL^{-1}. O perfil fitoquímico identificado pelo LCMS foi relatado através do efeito inibitório pela acetilcolinesterase. A espécie apresenta grande importância para futuros estudos *in vivo*, de purificação e isolamento de moléculas com possível efeito biopesticida.

Palavras-chave: Compostos fenólicos; Flavonoides; Inibição da acetilcolinesterase; Cerrado brasileiro.

Abstract

Pterodon pubescens (Benth.) Benth is native species from the Brazilian Cerrado, popularly known as "sucupira-branca," and rich in bioactive metabolites. This study aimed to assess the inhibitory activity of the enzyme acetylcholinesterase by leaf extracts of *P. pubescens* Benth. Phenolic constituents and flavonoids were analyzed by LC-DAD and LCMS. The qualitative and quantitative enzymatic inhibitory profile was performed by 96-well microplate in ELISA reader. The statistical analysis was reached with a significance limit of $p \leq 0.05$ applied by the *Tukey's* test. Bioactive components detected were terpenoids, phenolic acids (gallic acid, ferulic acid, and rosmarinic acid), and flavonoids (kaempferol, luteolin, apigenin, rutin, and quercetin). There were no significant differences of inhibition between the crude methanolic and ethyl acetate extracts and between the hydroalcoholic and ethyl acetate phases at the concentration of 80 µg mL^{-1}. The phytochemical profile in LCMS are related to the acetylcholinesterase inhibitory effect. This species has an importance for future in vivo studies, purification, and isolation of molecules with a possible biopesticidal effect.

Keywords: Phenolic compounds; Flavonoids; Acetylcholinesterase inhibitors; Brazilian Cerrado.

Resumen

Pterodon pubescens (Benth.) Benth. es una especie nativa del *Cerrado* brasileño, conocida popularmente como "*sucupira-branca*" y rica en metabolitos bioactivos. El estudio tuvo como objetivo evaluar la actividad inhibidora de la enzima acetilcolinesterasa en el extracto de hojas de *P. pubescens* Benth. Los constituyentes fenólicos y flavonoides se analizaron mediante LC-DAD y LCMS. La evaluación inhibidora enzimática cuantitativa se realizó en una microplaca de 96 pocillos en un lector de ELISA. El análisis estadístico se realizó con un límite de significancia de $p \leq 0,05$ y se aplicó la prueba de *Tukey*. Los compuestos bioactivos detectados fueron terpenoides, ácidos fenólicos (ácidos gálico, ferúlico y rosmarínico) y flavonoides (caempferol, luteolina, apigenina, rutina y quercetina). No hubo diferencia significativa en la inhibición entre el extracto metanólico crudo y el acetato de etilo, y entre sus respectivas fases hidroetanólicas y el acetato de etilo a una concentración de 80 µg mL^{-1}. El perfil fitoquímico identificado por LCMS se informó a través del efecto inhibidor de la acetilcolinesterasa. La especie es de gran importancia para futuros estudios *in vivo*, de purificación y aislamiento de moléculas con posible efecto biopesticida.

Palabras clave: Compuestos fenólicos; Flavonoides; Inhibición de acetilcolinesterasa; *Cerrado* brasileño.

1. Introduction

The Cerrado is now considered a Neotropical domain with a rich number of species of Brazilian and world flora. More than 11.000 plant species are identified, of which 4.400 are endemic to this domain (Menezes Filho et al., 2019). Among the various botanical families, we can mention the *Leguminosae-Papilionoideae*, where the genus *Pterodon* is found. The species *Pterodon pubescens* (Benth.) Benth. (*Leguminosae-Papilionoideae*) is native to the Brazilian Cerrado and popularly known as "*sucupira-branca*, *fava-de-santo-inácio*, and *sucupira-lisa*".

Its bark and seeds are commonly used in folk medicine due to its pharmacological action. Some terpenes detected in this species have an allelopathic, insecticidal, and repellent effect, in addition to the anticholinesterase activity. Leaf extracts have revealed bioactive compounds such as flavonoids, saponins, tannins, triterpenes, and flavones with some biological properties (Negri et al., 2014).

A high content of phenolic compounds such as gallic acid, ferulic acid, ellagic acid, epicatechin, and catechins was isolated from the species *Dipteryx alata* Vogel.

(*Leguminosae*), and some of them exert potent antioxidant activity and enzymatic inhibitory effect (Menezes Filho et al., 2020; Menezes Filho et al., 2019; Lemos et al., 2012). Extracts of the species *Acacia nilotica* (L.) Willd. ex Delile (*Leguminosae*) revealed a high content of gallic acid (Sulaiman et al., 2013). Phytochemical tests of crude ethanolic extract from *Pterodon emarginatus* Vogel. barks detected the presence of flavonoids, saponins, and triterpenoids (Bustamante et al., 2010).

The enzyme acetylcholinesterase, when distributed in the central nervous system, has the role of hydrolyzing the acetylcholine, a neurotransmitter present in the synaptic clefts, and its inhibitors are used with insecticidal action (Hlila et al., 2015). Thus, this study aimed to assess the inhibitory activity of the acetylcholinesterase enzyme by leaf extracts of *P. pubescens*.

2. Materials and Methods

2.1 *Type of methodology used in the tests*

The study is of the qualitative-quantitative type, according to the approach of Alves et al. (2018) and Oliveira et al. (2020), performing in the first moment in the field, with the identification of the area, collection of biological material and in the second moment, in the laboratory where the tests were carried out.

2.2 Plant material and extract preparation

Plant leaves were collected in October 2015 and in the Cerrado areas of *Monte Alegre*, *Goiás* State, Brazil (16°46′59.1″ S and 51°7′24.2″). The species was deposited in the herbarium of the Goiano Federal Institute (IF Goiano), *Rio Verde* Campus, maintained in exsiccate-specimen under the Voucher 507, collected by Cinthia Alves Porfiro, and identified by Cristiana Vieira Teles.

Leaves of *P. pubescens* were dried in an oven at 40 °C for 72 h and then ground to a fine powder. For the preparation of leaf extract in ethyl acetate, 1000 g of previously ground leaves and 5 L of ethyl acetate were conditioned into glass vials with a capacity of 3 L. These samples were maintained at ambient temperature for 24 h. Subsequently, the samples were filtered and the obtained solutions were rotavaporated and the crude leaf extract in ethyl acetate was reserved in a glass vial. The remaining ethyl acetate from the rotavaporation

process was returned to each original Erlenmeyer flask. The resting, filtration, and rotavaporation processes were repeated two more times for each sample in order to obtain a greater amount of crude extract.

The ethyl acetate and methanolic leaf extracts were maintained in a gas exhaust hood until constant mass. Subsequently, the moisture was removed by lyophilization process and the extracts were stored in a refrigerator until its use in biochemical tests. Crude extracts were assessed by means of an LCMS (liquid chromatography coupled to a mass spectrometer).

2.3 Analysis of crude extracts by LCMS

Samples of ethyl acetate and methanolic leaf extracts were analyzed in an LCMS for the identification and quantification of gallic acid, ferulic acid, apigenin, rutin, kaempferol + luteolin, rosmarinic acid, and quercetin. The limits of quantification (LQ) considered as the first point of curve generating a signal/noise higher than 5 are gallic acid (25 ppb), ferulic acid (50 ppb), apigenin (25 ppb), kaempferol + luteolin (25 ppb), quercetin (250 ppb), rosmarinic acid (25 ppb), and rutin (250 ppb).

Compound solubilization was performed in triplicate by adding 3 mL of methanol or 4 mL of ethyl acetate in 200 mg of extract, obtaining the methanolic and ethyl acetate extracts, respectively. These extracts were filtered on a 0.45-micrometer nylon filter prior to analysis. To prepare the curve, standards with concentrations ranging from 25 to 3000 ppb were used. Compound separation was carried out in a 250 mm × 4.6 mm × 5 µm C18 reverse column and, for quantification, an SQ 300 MS mass spectrometer (Perkin Elmer®) was used. Solvents used in the elution were (A) water + acetic acid (0.1%) and (B) methanol.

The total running time was 32 minutes, with 2 minutes of equilibrium with 60% of solvent A and 40% of solvent B. Running was started with 60% of solvent A and 40% of solvent B, evolving to 100% of solvent B within 25 minutes. One hundred percent of solvent B remained for 5 minutes, returning to the initial condition (60% of solution A and 40% of solution B) for equilibrium for 2 minutes before the next injection.

2.4 Partition of methanolic extract

Initially, 20 g of lyophilized crude extract was dissolved in 50 mL of MeOH/H_2O in the proportion 8:2 (v/v). This solution was transferred to a separatory funnel and homogenized. For the hexane phase, 50 mL of hexane was added in the hydroalcoholic phase.

The formed solution was homogenized, being observed the formation of phases in the funnel. Hexane phase was rotavaporated and transferred to amber flasks. This procedure was repeated three times. The flask containing the hexane phase was maintained open in a gas exhaust hood to allow evaporating the solvent. After evaporation, these flasks were capped and stored.

In the previously used hydroalcoholic phase, 50 mL of ethyl acetate solution was added. This procedure was repeated two more times in the separatory funnel. The ethyl acetate and hydroalcoholic phases were obtained, being subsequently rotavaporated and transferred to amber flasks. These flasks were maintained in a gas exhaust hood and, after solvent evaporation, they were capped and stored. For the test of acetylcholinesterase inhibition in TLC described by Martson (2002), plates were eluted in the mobile phase 8:2 (v/v) chloroform/methanol and subsequently submitted to the 96 well microplate inhibition test.

2.5 Acetylcholinesterase inhibition test in 96-well microplate

This test was carried out by using an adaptation of the Fosberg (1984) method, differing in concentration and number of samples. The enzyme acetylcholinesterase (E.C. 3.1.1.7 electric ell code C3389) was purchased from Sigma Aldrich in 2 KU. The enzyme AChE was diluted in 30 mL buffer A 50 mM Tris-HCl pH 7.8, resulting in a concentration of 66.6 U mL^{-1}, being added 1% albumin for stabilization, aliquoted in 2 mL *Eppendorf*, and stored in a freezer.

Buffer B was prepared by using 0.067 M sodium phosphate at pH 6.85. Acetonitrile was the solvent used for preparing 1 mM *para*-nitrophenyl acetate substrate, which is insoluble above 3 mM. Concentrations of crude ethyl acetate and methanolic extracts and their respective hydroalcoholic and ethyl acetate phases were first prepared in DMSO at 40 mg mL^{-1} (stock solution) for further dilution and have a solution of 8 mg mL^{-1} to prepare serial dilutions. To optimize the inhibition test, the used concentrations were added in buffer B. In the first dilution, 979 µL of the buffer and 21 µL of the inhibitor were added to 8 mg mL^{-1} in DMSO, followed by serial dilutions in the factor of 2, totaling six concentrations.

The final highest concentration in the well was 160 µg mL^{-1} in a decreasing manner. In the first reaction step, 94 µL buffer B and 6 µL enzyme with 1% albumin were added for enzyme control. Then, 94 µL buffer B and 6 µL Tris-HCl buffer with 1% albumin were added to the blank of enzyme control to maintain enzyme conditions. After, 94 µL buffer B with inhibitor and 6 µL Tris-HCl buffer with 1% albumin were added to the blank of inhibitor.

Finally, 94 µL buffer B with inhibitor and 6 µL enzyme with 1% albumin at 2 U mL^{-1} were added to the inhibitor test.

Subsequently, it was conditioned in a BOD at 25 °C for 10 minutes to interact the inhibitor with the enzyme. In the second step, the reaction volume was completed with 98 µL of buffer B and 2 µL of 1 mM PNPA substrate, which was added every 20 seconds. After the reaction was completed with a volume of 200 µL, readings started after 2 minutes and 30 seconds on the Elisa plate reader every 20 seconds in a period of 5 minutes and at wavelengths of 405 nm. Yellow staining is the formed product para-nitrophenol, derived from the hydrolysis of nitrophenyl acetate by acetylcholinesterase.

2.6 Statistical analysis

The results presented here correspond to the average of three replications (n = 3) ± standard deviation of the mean. The significance limit for all statistical analyses was ($p \leq 0.05$), applied by the *Tukey's* test. All statistical analyses were performed using the software Sisvar (version 5.6 Build 86) and the graphs were built by using the software Sigma plot 12.

3. Results and Discussion

3.1 Analysis of extracts by comparative LCMS

The presence of phenolic acids and flavonoids was detected in extract samples of *P. pubescens* analyzed by comparative LCMS. These samples were compared with the standards gallic acid, ferulic acid, kaempferol + luteolin, quercetin, rosmarinic acid, rutin, and apigenin. This quantification (in µg g^{-1} of leaves) is shown in (Table 1).

Table 1. Quantification of phenolic compounds (µg g^{-1}) in leaves of *Pterodon pubescens*. **RT** – retention time; m z (**Da**) – mass/charge of the ion; **Nq** – non-quantified.

	MeOH extract		**AcOEt extract**			
	µg g^{-1}	**StdDev**	**µg g^{-1}**	**StdDev**	**m z (Da)**	**RT (min.)**
Gallic acid	2556.85	581.59	1863.79	594.91	168.7	13.5
Ferulic acid	5556.05	734.68	1057.34	391.16	191.1	14.75
Apigenin	nq		36.56	1.17	269.1	17.06
Kaempferol + luteolin	93.10	23.50	35.65	12.12	285.1	16.11
Quercetin	4308.75	3043.28	2045.60	300.72	301.1	14.66
Rosmarinic acid	202.92	93.75	736.20	858.88	359.4	14.29
Rutin	12953.38	8209.30	3773.13	3829.30	609.2	13.41

Source: by authors, (2020).

Gallic acid quantification in methanolic and ethyl acetate extract presented values of 2556.85 and 1863.79 µg g^{-1}, respectively (Table 1). Studies conducted by Muthukrishnan et al. (2016), with an ethanolic extract from leaves of *Erythrina variegata* L. (Leguminosae) detected gallic acid by LC-UV at a retention time of 5.858 min and a concentration of 2 ppm. According to Ceylan et al. (2016), a high gallic acid content was detected in the crude methanolic extract of *Cytisopsis dorycniifolia* Jaub. & Spach and *Ebenus hirsuta* Jaub. & Spach (*Leguminosae*), with a value of 13.89 and 22.69 µg g^{-1}, respectively. Total phenolic quantification was 76.03 and 46.57 mg of gallic acid 100 g^{-1} of extract, respectively. In this study, several phenolic compounds were detected and quantified. In addition, the antioxidant capacity and inhibitory effect in enzymatic activities were assessed. The anticholinesterase effect found in these species is related to the phenolic content. Nag and Bratati (2011), tested the anticholinesterase activity with methanolic extract of *Terminalia chebula* Retz., *Terminalia bellirica* (Gaertn.) Roxb., and *Emblica officinalis* Gaertn. The identified gallic acid and its properties are related to the inhibitory effect of the enzyme.

Ferulic acid was quantified in the methanolic and ethyl acetate extracts and presented values of 5556.05 and 1057.34 µg g^{-1}, respectively. Several phenolic acids were identified by LCMS in the ethanolic extract of *Genista tinctorial* L. (*Leguminosae*). The retention time detected for ferulic acid was 11.8 min, with a quantification value of 79.89 µg g^{-1} of plant. However, these compounds were not detected in *Genista sagittalis* L. (Hanganu et al., 2016). Ferulic acid (4-hydroxy-3-methoxycinnamic acid) was isolated in 1866 from *Ferula foetida*. Among the phenolic compounds, ferulic acid has several phytotherapeutic properties,

antioxidant activity, and anti-inflammatory agents (Sgarbossa et al., 2015). The major constituent detected in the hydroethanolic extract of leaves of *Santolina impressa* Hoffmanns. & Link (*Asteraceae*) was the ferulic acid. An in vitro test detected that 2 mg mL^{-1} of this extract presented 40% acetylcholinesterase inhibition (Tavares et al., 2012).

Apigenin was analyzed in the ethyl acetate and methanolic extracts, being detected only in the ethyl acetate extract, with a value of 36.56 µg g^{-1}. Apigenin was detected in crude ethyl acetate and *n*-butanolic extracts and in fractions of *Cynometra cauliflora* L. (*Leguminosae*), with a notorious inhibitory activity of the acetylcholinesterase enzyme. Aqueous fractions presented a better result when compared to hexane and dichloromethane fractions. The activity of these samples is due to the content of detected phenolic compounds (Ado et al., 2014).

The value of molecular ion at 285.1 refers to kaempferol and luteolin, which presented values of 93 and 35.65 µg g^{-1} in the methanolic and ethyl acetate extracts, respectively. Flavonoids were identified and isolated from an ethanolic extract of *Astragalus armatus* Willd. (*Leguminosae*), being related to several biological activities. The high antioxidant effect and low inhibitory activity of acetylcholinesterase are related to phytochemical constituents such as kaempferol (Labed et al., 2016). Luteolin (flavone) was detected in the methanolic extract of *Lavandula viridis* L'Hér. (*Lamiaceae*). In both ethanol and aqueous extracts, this compound obtained a percentage of acetylcholinesterase inhibition. The quantification of ethanol/water extract was higher when compared to the ethanolic extract (Costa et al., 2013). An inhibition of 60% of the enzyme was detected in the hydroethanolic extract of *Thymus capitellatus* Hoffmanns & Link (*Laminaceae*). Luteolin was the major constituent detected in this species (Tavares et al., 2012). According to the authors, both compounds presented inhibitory effect in different extracts from different species.

Quercetin was quantified and presented values of 4308.75 and 2045.60 µg g^{-1} in the methanol and ethyl acetate extracts, respectively. There is a correlation of phenolic compounds with an anticholinesterase effect. Studies were carried out with the fraction from the partition of crude ethanolic extract from *Aristotelia chilensis* (Molina) Stuntz and a high content of phenolic compounds such as quercetin, mycetin, and luteolin was detected. The required concentration of isolated quercetin to achieve the IC_{50} of acetylcholinesterase inhibition was 47.8 µg mL^{-1}, with a noticeable effect in relation to the other constituents (Cespedes et al., 2017). LCMS profile of flavonoids from the aqueous extract of *Eugenia dysenterica* was positively identified, with a retention time of 47 minutes similar to the tested standard. The inhibitory effect of quercetin was moderate considering the acetylcholinesterase (Gasca et al., 2017).

Rosmarinic acid was also detected and quantified in the methanolic and ethyl acetate

extracts, with values of 202 and 736.20 µg g^{-1}, respectively. In studies carried out with the profile of ten species from the Family *Lamiaceae*, different ethanolic extracts were investigated by means of LCMS. Rosmarinic acid and its influence on AChE inhibitory properties were detected. The inhibition effect is inversely proportional to extract concentration (Vladimir-Knežević et al. 2014). The methanolic extract of *Salvia trichoclada* Benth. (*Lamiaceae*) was purified and the rosmarinic acid was isolated and a potent anticholinesterase effect was observed.

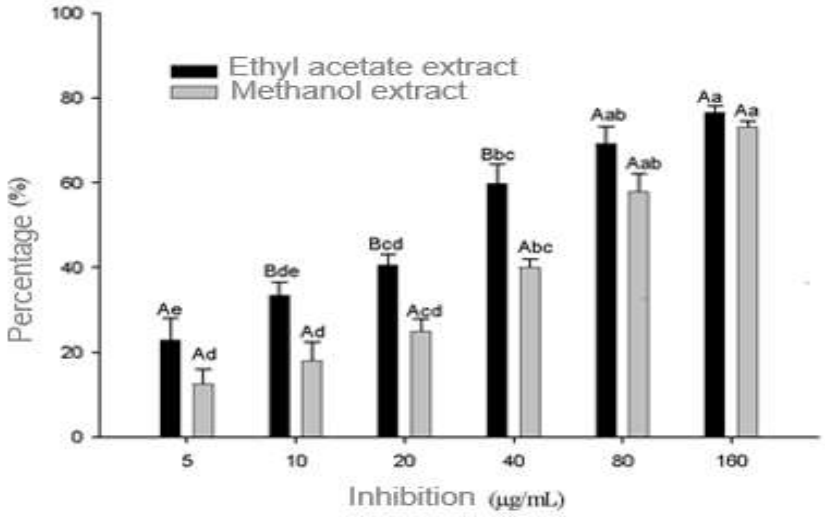
Rutin was the main constituent of ethyl acetate and methanol extracts, with a concentration of 3773.13 and 12953.38 µg g^{-1}, respectively. A high rutin content was detected in the methanolic extract of *Cytisopsis dorycniifolia* Jaub. & Spach and *Ebenus hirsuta* Jaub. & Spach (*Leguminosae*). The inhibitory action against for enzymes was detected. A moderate inhibition effect was found for AChE (Ceylan et al., 2016). LCMS analysis quantified some flavonoid derivatives in four ethanolic extracts from different species of the Rosaceae family. Rutin was the most detected flavonoid in the species *Sorbus umbelleta* Fritsch., with a moderate inhibitory activity for acetylcholinesterase (Ekin et al., 2016).

3.2 Acetylcholinesterase inhibition test

The inhibitory effect of the acetylcholinesterase enzyme was assessed by the Forsberg (1984), method by using the substrate acetate-nitrophenyl that, in the presence of this enzyme, leads to the hydrolysis and formation of 4-nitrophenol. The intensity of yellow staining determines the amount of product formed from the enzyme-substrate reaction confirmed by the absorbance readings at 405 nm.

The anticholinesterase activity was observed in both crude methanolic and ethyl acetate extract. Methanolic extract presented an inhibition activity of 73%, while the ethyl acetate presented an effect 77% on the enzyme, as shown in (Figure 1). In the phase ethyl acetate, this effect was 79% whereas, in the phase hydroalcoholic, this value reached 70%, as shown in (Figure 2). No significant difference was observed between extracts and phases at the highest concentration. The inhibition potential differs between different doses, i.e. from 5 to 160 µg mL^{-1}, in which the inhibitory activity is directly proportional to concentration. Moreover, no significant difference was observed between the doses 80 and 160 µg mL^{-1} for both extracts and phases, which present a relevant inhibition above 70% at these concentrations. A significant difference was observed when the initial doses were compared to 160 µg mL^{-1}.

Figure 1. Percentage inhibition of crude ethyl acetate and methanolic extract for the acetylcholinesterase enzyme. Means followed by the same letter (uppercase for extracts and lowercase for doses of the same extract) do not differ from each other by the *Tukey's* test at 5% probability.



Source: by authors, (2020).

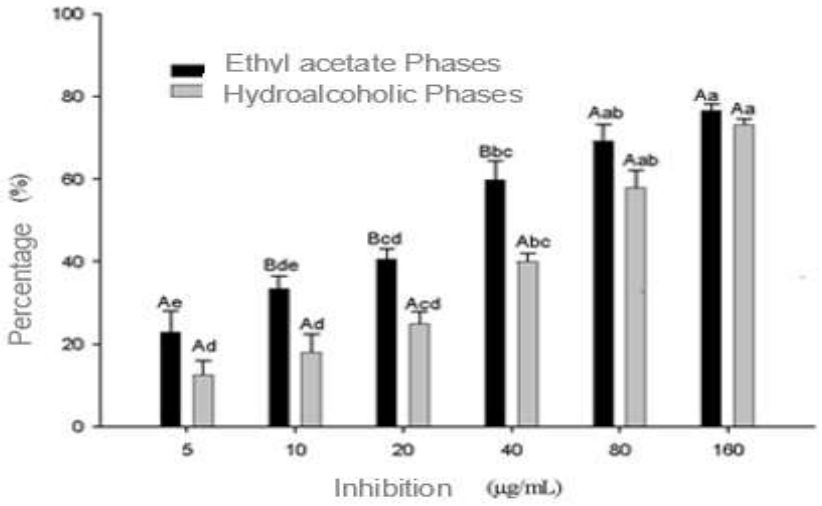
Previous studies with a crude hydroethanolic extract from leaves of *P. emarginatus* detected by LCMS different flavonoids such as luteolin, mycetin, apigenin, among others (Negri et al., 2014), and diterpenes 14,15-epoxygeranylgeraniol and some derivates, isolated in *Pterodon pubescens* (Coelho et al., 2005). In the Family *Leguminosae*, the crude methanolic extract from seeds of *Psoralea corylifolia* L. presented flavonoids and their derivatives, in addition to purify and isolate the 4-methoxy-flavone (Prasad et al., 2004). The genus *Desmodium* Desv., is composed of some alkaloids, flavonoids, phenylpropanoids, terpenoids, and flavones in its different species (Ma et al., 2011). Several flavonoids were isolated from leaves of *P. pubescens* such as kaempferol, luteolin, rutin, quercetin, among others (Miranda et al., 2014).

The literature reports different genera, families, and species with the previously mentioned metabolites, which have an anticholinesterase activity. In this context, the aqueous extract from *Ficus capensis* Thunb. (*Moraceae*) has an inhibitory effect for an acetylcholinesterase of 70% in 1150 µg mL^{-1}, with an IC_{50} of 579.63 µg mL^{-1}. Rutin, quercetin, and ellagic gallic acid were the major constituents quantified in this species (Akomolafe et al., 2016). The rosmarinic acid was the predominant compound in the ethanolic and hydroethanolic extracts in in vitro culture of *Lavandula viridis* L'Hér. (*Lamiaceae*). In this case, the ethanolic extract was more active, with an inhibition of 87%

when compared to the enzyme (Costa et al., 2013). However, an inhibitory response of 45% was observed by means of ferulic acid isolated from ethyl acetate extract of *Impatiens bicolor* Linn (*Balsaminaceae*) at a dominant concentration of 250 µg mL^{-1} (Shahwar et al., 2010).

Studies conducted with four extracts (petroleum ether, water, methanol, and ethyl acetate) from *Haplophyllum myrtifolium* Boiss (*Rutaceae*) were quantified regarding phenolic constituents and assessed regarding the anticholinesterase action. The gallic acid found presented values between 32.32 and 52.50 mg g^{-1} in the petroleum ether extract and ethyl acetate, respectively, and lower concentrations in the others. The apolar extracts petroleum ether and ethyl acetate reached a higher inhibition potential when compared to the others. Therefore, solvent polarity directly interferes with the extraction of bioactive compounds (Zengin et al., 2014).

Figure 2. Percentage inhibition of ethyl acetate and hydroalcoholic phases for the acetylcholinesterase enzyme. Means followed by the same letter (uppercase for extracts and lowercase for doses of the same extract) do not differ from each other, by the *Tukey's* test at 5% probability.



Source: by authors, (2020).

Polyphenolic compounds are important bioactive components in various medicinal plant extracts, which express different pharmacological properties. Several compounds were identified in the fractions of crude methanolic extract from *Cynometra cauliflora* L. (*Leguminosae*) such as kaempferol, vitexin, catechin, isovitexin, taxifolin, and apigenin, which exhibited anticholinesterase activity. More polar fractions showed a better effect when

compared to those less polar (Ado et al., 2014).

In the crude ethanolic extract from seeds of *Vigna radiata* (L.) R. Wilczek (*Leguminosae*), vitexin was the dominant flavonoid, followed by isovitexin, and phenolic acids (gallic acid, synapic acid, and ferulic acid) quantified by HPLC, with values of 15.36, 10.05, 7.56, 8.21, and 11.28 mg g^{-1}, respectively. A concentration of 9.6 µg/mL of gallic acid elicited moderate AChE inhibition. These compounds are strongly related to the biological response found (Basha et al., 2017). Studies carried out by Gocer et al. (2016) showed the inhibitory effect of taxifolin for the AChE enzyme. Considered as a flavonol (dihydroquercetin), the hydroxyl group and its ability to sequester free radicals are related to antioxidant effect and interaction with enzymes, specifically acetylcholinesterase.

Fractions from an ethanolic extract from *Ocotea aciphylla* Nees (*Lauraceae*), expressed a significant inhibition of AChE. The prevailing constituents were terpenoids, vitexin, and phenylpropanoids, which are related to the activity found (Conceição et al. 2016). The compounds gallic acid and quercetin, isolated from the ethyl acetate extract from *Caesalpinia mimosoides* (*Leguminosae*), in 100 µg mL^{-1}, expressed an anticholinesterase effect of 73% and 80.94% inhibition, respectively, with the flavonoid quercetin expressing a greater activity considering the enzyme (Tangsaengvit et al., 2013). The flavonoid quercetin was found in the methanol extract from *Phyllanthus acidus* (L.) Skeels (*Phyllanthaceae*), presenting an inhibitory response to acetylcholinesterase, with a value of IC_{50} of 1009.87 µg mL^{-1} of the extract (Moniruzzaman et al., 2015).

The quantification of methanolic extract in LCMS overlapped the ethyl acetate extract in the main compounds. Only rosmarinic acid and apigenin showed a higher concentration of ethyl acetate extract. Crude ethyl acetate and methanolic extracts and ethyl acetate and hydroalcoholic phases from *P. pubescequens* are classified as potent inhibitors of acetylcholinesterase, which are related to the presence of phenolic acids, flavonoids, and terpenoids. According to Vinutha et al. (2007), several species with an inhibitory action higher than 50% are classified as potent inhibitors of acetylcholinesterase, between 30 and 50% they present a moderate inhibitory effect, and values lower than 30% are assessed as presenting a low inhibitory activity. The mentioned phytochemical constituents exert several biological activities, specifically an anticholinesterase action, as approached by several authors.

4. Conclusions

Our result show that the potent inhibitory action of acetylcholinesterase from crude

methanolic and ethyl acetate extracts and hydroalcoholic and ethyl acetate phases from leaves of *Pterodon pubescens* may be due to phenolic compounds such as gallic acid, ferulic acid, rosmarinic acid, and the flavonoids kaempferol, luteolin, apigenin, rutin, and quercetin quantified by LCMS and the 96 well quantitative microplate test confirmed the inhibitory response. No significant difference was observed between crude extracts and phases, which showed an inhibition higher than 70%. Partitioning process to obtain the phases does not interfere with the biological response achieved for the enzyme when compared to the crude extract.

The recommended dose is 80 µg mL^{-1}, which expresses a potent enzymatic inhibition. Inhibition of AChE in the central nervous system causes changes in the insect's motor system, leading to paralysis and possible death. This species is promising for future in vivo studies, isolation and purification of bioactive molecules, which could act as bio-pesticides. The information obtained in this study contributes to the knowledge of this species and benefit for the current science.

Acknowledgements

We thank the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), the National Council for Scientific and Technological Development (CNPq), the Goiás Research Foundation (FAPEG), the Federal Institute of Education, Science and Technology Goiano (IF Goiano) for their support.

References

Ado, M. A., Abas, F., Ismail, I. S., Mghazali, H., & Shaari, K. (2014) Chemical profile and antiacetylcholinesterase, antityrosinase, antioxidant and α-glucosidase inhibitory activity of *Cynometra cauliflora* L. leaves. *Journal of the Science of Food and Agriculture*, 95(3), 635-642. DOI: 10.1002/jsfa.6832

Akomolafe, S. F., Oboh, G., Oyeleye, S. I., & Boligon, A. A. (2016). Aqueous extract from *Ficus capensis* leaves inhibits key enzymes linked to erectile dysfunction and prevent oxidative stress in rats' penile tissue. *NFS Journal*, 4,15-21. https://doi.org/10.1016/j.nfs.2016.06.001

Basha, S. A., Maheswaraiah, A., & Rao, U. J. S. P. (2017). Antioxidant profile, acetylcholinesterase inhibition and platelet aggregation of polyphenols and proteins from germinating green gram (*Vigna radiata*). *International Journal of Food Properties*, 20(1), 1-33. https://doi.org/10.1080/10942912.2017.1325899

Bustamante, K. G. L., Lima, A. D. F., Soares, M. L., Fiuza, T. S., Tresvenzol, L. M. F., Bara, M. T. F., Pimenta, F. C., & Paula, J. R. (2010). Avaliação da atividade antimicrobiana do extrato etanólico bruto da casca da sucupira branca (*Pterodon emarginatus* Vogel) - *Fabaceae*. *Revista Brasileira de Plantas Medicinais*, 12(3), 341-345. https://doi.org/10.1590/S1516-05722010000300012

Cespedes, C. L., Balbontin, C., Avila, J. G., Dominguez, M., Alarcon, J., Paz, C., Burgos, V., Ortiz, L., Penaloza-Castro, I., Seigler, D. S., & Kubo, I. (2017). Inhibition on cholinesterase and tyrosinase by alkaloids and phenolics from *Aristotelia chilensis* leaves. *Food and Chemical Toxicology*, 109(2), 1-12. doi: 10.1016/j.fct.2017.05.009

Ceylan, R., Katani, J., Zengin, G., Mati, S., Aktumsek, A., Boroja, T., Stani, S., Mihailovi, V., Guler, G. O., Boga, M., & Yilmaz, M. A. (2016). Chemical and biological fingerprints of two *Fabaceae* species (*Cytisopsis dorycniifolia* and *Ebenus hirsuta*): Are they novel sources of natural agents for pharmaceutical and food formulations? *Industrial Crops and Products*, 84, 254-262. https://doi.org/10.1016/j.indcrop.2016.02.019

Coelho, L. P., Reis, P. A., De astro, F. L., Gayer, C. R. M., Lopes, C. S., Da Costa e Silva, M. C., Sabino, K. C. C., Todeschini, A. R. & Coelho, M. G. P. (2005). Antinociceptive properties of ethanolic extract and fractions of *Pterodon pubescens* Benth. seeds. *Journal of Ethnopharmacology*, 98, 109-116.

Conceição, R. S., Carneiro, M. M. A. A., Reis, I. M. A., Branco, A., Vieira, I. J. C., Braz-Filho, R., & Botura, M. B. (2016). *In vitro* acaricide activity of *Ocotea aciphylla* (Nees) Mez. (Lauraceae) extracts and identification of the compounds from the active fractions Actions. *Ticks and Tick-Borne Diseases*, 8(2), 275-282. doi: 10.1016/j.ttbdis.2016.11.013

Costa, P., Gonçalves, S., Valentão, P., Andrade, P. B., & Romano, A. (2013). Accumulation of phenolic compounds in in vitro cultures and wild plants of *Lavandula viridis* L'Hér and

their antioxidant and anti-cholinesterase potential. *Food and Chemical Toxicology*, 57, 69-74. doi: 10.1016/j.fct.2013.03.00.

Ekin, H. N., Gokbulut, A., Aydin, Z. U., Donmez, A. A., & Orhan, I. E. (2016). Insight into anticholinesterase and antioxidant potential of thirty-four Rosaceae samples and phenolic characterization of the active extracts by HPLC. *Industrial Crops and Products*, 91, 104-113. doi: 10.1016/j.indcrop.2016.06.029

Forsberg, A., & Puu, G. (1984). Kinetics for the inhibition of acetylcholinesterase from the electric eel by some organophosphates and carbamates. *European Journal of Biochemistry*, 140(1), 153-156. doi: 10.1111/j.1432-1033.1984.tb08079.x

Gasca, C. A., Castillo, W. O., Takahashi, C. S., Fagg, C. W., Magalhães, P. O., Fonseca-Bazzo, Y. M., & Silveira, D. (2017). Assessment of anticholinesterase activity and cytotoxicity of cagaita (*Eugenia dysenterica*) leaves. *Food and Chemical Toxicology*, 109(2), 996-1002. doi: 10.1016/j.fct.2017.02.032

Gocer, H., Topal, F., Topal, M., Küçük, M., Teke, D., Gülçin, İ., Alwasel, S. H., & Supuran, C. T. (2016). Acetylcholinesterase and carbonic anhydrase isoenzymes I and II inhibition profiles of taxifolin. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 31(3), 441-447. doi: 10.3109/14756366.2015.1036051.

Hanganu, D., Olah, N. K., Benedec, D., Mocan, A., Crisan, G., Vlase, L., Popica, I., & Oniga, I. (2016). Comparative polyphenolic content and antioxidant activities of *Genista tinctoria* L. and *Genistella sagittalis* (L.) Gams (*Fabaceae*). *Pakistan Journal of Pharmaceutical Sciences*, 29(1), 301-307.

Labed. A., Ferhat, M., Labed-Zouad, I., Kaplaner, E., Zerizer, S., Voutquenne-Nazabadioko, L., Alabdul Magid, A., Semra, Z., Kabouche, A., Kabouche, Z., & Öztürk, M. (2016). Compounds from the pods of *Astragalus armatus* with antioxidant, anticholinesterase, antibacterial and phagocytic activities. *Pharmaceutical Biology*, 54(12), 3026-3032. https://doi.org/10.1080/13880209.2016.1200632

Lemos, M. R. B., Siqueira, E. M. A., Arruda, S. F., & Zambiazi, R. C. (2012). The effect of

roasting on the phenolic compounds and antioxidant potential of baru nuts [*Dipteryx alata* Vog.]. *Food Research International*, 48(2), 592-597. doi: 10.1016/j.foodres.2012.05.027

López, M. D., & Pascual-Villalobos, M. J. (2010). Mode of inhibition of acetylcholinesterase by monoterpenoids and implications for pest control. *Industrial Crops and Products*, 31(2), 284-288. doi: 10.1016/j.indcrop.2009.11.005

Ma, X., Zheng, C., Changling, H., Rahman, K., & Qin, L. (2011). The genus *Desmodium* (*Fabaceae*) – traditional uses in Chinese medicine, phytochemistry and pharmacology. *Journal of Ethnopharmacology*, 138(2), 314-332. doi: 10.1016/j.jep.2011.09.053.

Marston, A., Kissling, J., & Hostettmann, K. A. (2002). A rapid CCD bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. *Phytochemical Analysis*, 13(1), 51-54.

Menezes Filho, A. C. P., Sousa, W. C., Castro, C. F. S., & De Souza, L. F. (2019). Composição química do óleo essencial das flores de *Myrcia guianensis* (Aubl.) DC. *Revista Cubana de Plantas Medicinales*, 24(4), e892.

Menezes Filho, A. C. P., Romão, L. T. G., Dos Reis, E. B., Malaquias, K. S., Castro, C. F. S., Marcionilio, S. M. L. O., & Oliveira, M. S. (2020). Chromatographic analysis and physicochemical evaluation of the essential oil of *Bauhinia monandra* Kurz flowers. *Research, Society and Development*, 9(8), 454985979.

Menezes Filho, A. C. P., Oliveira Filho, J. G., Christofoli, M., & Castro, C. F. S. (2019). Atividade antioxidante e compostos bioativos em espécies de um fragmento de Cerrado goiano tipo cerradão. *Colloquium Agrariae*, 15(1), 1-8.

Miranda, M. L. D., Garceza, F. R., Abotb, A. R., & Garceza, W. S. (2014). Sesquiterpenes and other constituents from leaves of *Pterodon pubescens* Benth. (*Leguminosae*). *Química Nova*, 37(3), 473-476. doi: /10.5935/0100-4042.20140065

Moniruzzaman, M. M., Asaduzzaman, M., Hossain, M. S., Sarker, J., Rahman, S. M. A., Rashid, M., & Rahman, M. M. (2015). *In vitro* antioxidant and cholinesterase inhibitory

activities of methanolic fruit extract of *Phyllanthus acidus*. *BMC Complementary and Alternative Medicine*, 15, 403-413. doi: 10.1186/s12906-015-0930-y

Muthukrishnan, S., Palanisamy, S., Subramanian, S., Selvaraj, S., Mari, K. R., & Kuppulingam, R. (2016). Phytochemical Profile of *Erythrina variegata* by Using High-Performance Liquid Chromatography and Gas Chromatography-Mass Spectroscopy Analyses. *Journal of Acupuncture and Meridian Studies*, 9(4), 207-212. doi: 10.1016/j.jams.2016.06.001

Nag, G., & De Bratati. (2011) Acetylcholinesterase inhibitory activity of *Terminalia chebula*, *Terminalia bellerica* and *Emblica officinalis* and some phenolic compounds. International *Journal of Pharmacy and Pharmaceutical Sciences*, 3(3), 121-124.

Negri, G., Mattei, R., & Mendes, F. R. (2014). Antinociceptive activity of the HPLC- and MS-standardized hydroethanolic extract of *Pterodon emarginatus* Vogel leaves. *Phytomedicine*, 21(8-9), 1062-1069. doi: 10.1016/j.phymed.2014.04.009.

Oliveira, L. M. N., Silva, L. M. R., Lima, A. C. S., Almeida, R. R., Ricardo, N. M. P. S., Figueiredo, E. A. T., & Figueiredo, R. W. (2020). Caracterização de rutina, compostos fenólicos e capacidade antioxidante de polpas e subprodutos de frutas tropicais. *Research, Society and Development*, *9*(4), e42942812. https://doi.org/10.33448/rsd-v9i4.2812

Prasad, N. R., Anandi, C., Balasubramanian, S., & Pugalendi, K. V. (2004). Antidermatophytic activity of extracts from *Psoralea corylifolia* (*Fabaceae*) correlated with the presence of a flavonoid compound. *Journal of Ethnopharmacology*, 91(1), 21-24. https://doi.org/10.1016/j.jep.2003.11.010

Pereira, A. S., Shitsuka, D. M., Parreira, F. J. & Shitsuka, R. Metodologia da Pesquisa Científica. UAB/NTE/UFSM, Universidade Federal de Santa Maria, Santa Maria-RS, 2018. Recuperado de https://repositorio.ufsm.br/bitstream/handle/1/15824/Lic_Computacao_Metodologia-Pesquisa-Cientifica.pdf?sequence=1.

Sgarbossa, A., Giacomazza, D., & Di Carlo, M. (2015). Ferulic Acid: A hope for Alzheimer's disease therapy from plants. *Nutrients*, 7(7), 5764-5782. doi: 10.3390/nu7075246

Shahwar, D., Rehman, S. U., & Raza, M. A. (2010). Acetyl cholinesterase inhibition potential and antioxidant activities of ferulic acid isolated from *Impatiens bicolor* Linn. *Journal of Medicine Plant Research*, 4, 260-266.

Sulaiman, C. T., George, S., Goplakrishnan, V. K., & Balachandran, I. (2013). Chromatographic Studies and *in vitro* screening for acetyl cholinesterase inhibition and antioxidant activity of three *Acacia* species from South India. *Analytical Chemistry Letters 3*, 111-118. doi: 10.1080/22297928.2013.806405

Tangsaengvit, N., Kitphati, W., Tadtong, S., Bunyapraphatsara, N., & Nukoolkarn, V. (2013). Neurite outgrowth and neuroprotective effects of quercetin from *Caesalpinia mimosoides* Lamk. on cultured P19-derived neurons. *Evidence-Based Complementary and Alternative Medicine*, 2013, 1-7. doi: 10.1155/2013/838051

Tavares, L., Fortalezas, S., Tyagi, M., Barata, D., Serra, A. T., Duarte, C. M., Duarte, R. O., Feliciano, R. P., Bronze, M. R., Espírito-Santo, M. D., Ferreira, R. B., & Santos, C. N. (2012). Bioactive compounds from endemic plants of Southwest Portugal: Inhibition of acetylcholinesterase and radical scavenging activities. *Pharmaceutical Biology*, 50(2), 239-246. doi: 10.3109/13880209.2011.596209.

Vinutha, B., Prashanth, D., Salma, K., Sreeja, S. L., Pratiti, D., Padmaja, R., Radhika, S., Amit, A., Venkateshwarlu, K., & Deepak, M. (2007). Screening of selected Indian medicinal plants for acetylcholinesterase inhibitory activity. *Journal of Ethnopharmacology*, 109(2), 359-363.

Vladimir-Knežević, S., Blažeković, B., Kindl, M., Vladić, J., Lower-Nedza, A. D., Brantner, A. H. (2014). Acetylcholinesterase inhibitory, antioxidant and phytochemical properties of selected medicinal plants of the *Lamiaceae* Family. *Molecules*, 19(1), 767-782. doi: 10.3390/molecules19010767.

Zengin, G., Sarikurkcu, C., Aktumsek, A., Ceylan, R., & Ceylan, O. (2014). A comprehensive study on phytochemical characterization of *Haplophyllum myrtifolium* Boiss. endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes. *Industrial Crops and Products*, 53, 244-251. doi:

10.1016/j.indcrop.2013.12.04.

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