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Atividades antioxidante e inibitória da acetil colinesterase do extrato das folhas de *Hymenaea rubriflora*

Antioxidant and acetylcholinesterase inhbition activity from *Hymenaea rubriflora* Ducke (Fabaceae) leaf extract

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ABSTRACT
Hymenaea rubriflora is an endemic and edible plant from tropical weather, especially present in Brazilian flora and have been reported as medicinal plant in ethnobotanical studies. There has been less previous evidence regarding its biological application, therefore, to overcome this problem the present work aimed to evaluate whether the metabolites of *Hymenaea rubriflora* host antioxidant and anti-acetylcholinesterase (AChE) activity. To investigate these activities a whole range of different approaches as phytochemical screening, lipidic peroxidation, DPPH scavenging and total antioxidant capacity, and acetylcholinesterase inhibition assay were used. The analysis found evidence for the presence of different secondary metabolites, among them: phenolic compounds (at 286.02 ± 1.75 GAE/mg·DW concentration), flavonoids (at 23.19 ± 2.38 QE/mg.DW concentration), known as natural antioxidants and highlighted its effectiveness as AChE inhibitor, being able to inhibit AChE activity in 96% on 10 mg/mL. In this study we provide insights of *H. rubriflora* extract as an alternative antioxidant and AchE inhibitor agent, relevant to the context of neurological disorders and cognitive processes.

Keywords: *Hymenaea rubriflora*, antioxidant activity, Acetylcholinesterase inhibitor.

RESUMO
Hymenaea rubriflora é uma planta endêmica e comestível de clima tropical da região brasileira, e a ela é atribuída o status de planta medicinal em estudos etnobotânicos. Existe poucos estudos no que diz respeitos a atividades biológicas dessa planta, portanto o presente trabalho objetivou investigar se os metabolitos secundários de *H. rubriflora* possuem atividade antioxidante e inibitória da acetilcolinesterase. Para investigar estas atividades, diferentes metodologias foram aplicadas, como triagem fitoquímica, peroxidação lipídica, ensaio de DPPH, capacidade antioxidante total e ensaio de inibição da enzima acetilcolinesterase. Os resultados das análises indicaram a presença de diferentes metabolitos secundários, dentre eles compostos fenólicos (na concentração de 286,02 286.02 ± 1.75 GAE/mg·DW) e flavonoides (na concentração de 23.19 ± 2.38 QE/mg.DW), que são classes de metabolitos com propriedades antioxidantes e potenciais inibidores da acetilcolinesterase, sendo capaz de inibir a atividade da acetilcolinestase em 96% na concentração de 10 mg/mL do extrato de *H. rubriflora*. No presente trabalho, nos apresentamos o extrato de *H. rubriflora* como um agente antioxidante e inibidor da acetilcolinestase, dentro do contexto desordens neurológicas e processos cognitivos.

Palavras-chave: *Hymenaea rubriflora*, atividade antioxidante, inibição da acetilcolinesterase.

1 INTRODUCTION

Plants are a source of natural products that are widely applied for treatment of diseases, especially on traditional medicine. Investigate those plants can promote the discovering of new compounds with important pharmacological activities and applicability (GONTIJO et al. 2018). A variety of pharmacological activity from *Hymenaea* genus (Fabaceae) is noticed in the literature. Studies have been shown a potent microbiological, antioxidant, myorelaxant and antinociceptive activity (BEZERRA et al., 2013; OLIVEIRA et al., 2016; BONIFACE et al., 2017; OLIVEIRA et al., 2019; SILVA et al., 2019; VERAS et al., 2020). *Hymenea rubriflora* Ducke, also known as "Jatobá" or "Tamarindo", is a botanical species endemic form Brazil, edible and used on traditional medicine, it occurs at Brazilian atlantic forest (LIMA & PINTO, 2015), recent studies proved it effectiveness as antimicrobial (SILVA et al., 2020).

Alzheimer's disease (AD) is related with several factors, one of them is the oxidative stress induced by free radicals, strongly discussed on Markesberry (1997) review. On AD, occurs a decrease on acetylcholine (Ach) concentrations, leading to a progressive memory and cognition loss (FOIDL *et al.*, 2016; AHMED et al., 2018). Thus, one alternative to AD treatment is the Acetylcholisnetase (AChE) inhibition, AChE is an enzyme responsible for the rapid hydrolysis of the neurotransmitter Ach, in the central and peripheral nervous systems (TOUGU, 2001). AChE inhibitors are considers as potent drugs to increase the expression of Ach in the synaptic cleft. This effect can modulate opposite cellular outcomes. Firstly, the hyperstimulation of nicotinic and muscarinic receptors may be reflected in cell toxicity. However, in another scenario, the increase of Ach is fundamental to the brain reorganization in cases of neuropathology such as dementias and Alzheimer's disease (COLOVIC et al., 2013). Thus, the study of molecules with the potential to act as an AChE inhibitor must shed light on new therapeutic tools to neurodegenerative disorders and normal aging commitment.

In this context, we aimed to investigate *H. rubriflora* leaf extract effectiveness as antioxidant and acetylcholinesterase inhibition potential.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND LEAF EXTRACT PHYTOCHEMICAL SCREENING

Hymenaea rubriflora leaves were collected in Igarassu (State of Pernambuco, Brazil) (7°50'03.0"S 34°54'23.0"W) in September 2016. An exsiccate was deposited in the herbarium of the Agronomic Institute of Pernambuco (IPA) under number 78292. Leaves were washed and dried at 40 ºC, then 100 g of plant material were placed into a solvent accelerated extractor (Dionex Corporation, Sunnyvale, CA, EUA), at 40 ºC and 1700 psi, using methanol as solvent (HANWEN et al. 2012). The

extract chemical profile was performed by thin layer chromatography, according to Wagner & Bladt (1996) protocol.

2.2 ESTIMATION OF TOTAL FLAVONOIDS AND TOTAL PHENOLIC COMPOUNDS CONTENT

Total phenolic compound concentration was determined by Folin-Ciocalteu method, with gallic acid as standart, the results were expressed as GAE/mg.DW (gallic acid equivalent) (SINGLETON & ROSSI, 1965). As for total flavonoids determination, the aluminium chloride colorimetric method was used with quercetin as standart, and it was expressed as QE/mg DW (quercetin equivalent) (WOINSKY & SALATINO, 1998).

2.3 TOTAL ANTIOXIDANT CAPACITY (TAC)

The total antioxidant capacity assay was performed using 1 mL of extract (1 mg/mL) were mixed with the reagent solution (0.6M of sulfuric acid, 28 mM of sodium phosphate and 4 mM of ammonium molybdate), the tubes containing the solutions were incubated at 95 ºC for 90 minutes, then were placed at room temperature. When cooled, the absorbance of each sample was measured at 695 nm, the blank was 1 mL of reagent solution and 0.1 of methanol (PRIETO et al., 1999).

2.4 LIPIDIC PEROXIDATION

For this assay, the protocol of linoleic acid lipidic peroxidation was performed (KIKUZAKI & NAKATANI, 1993). We added 200 µL of sample (1 mg/mL) and mixed with 800 µL from reagent solution (200 µL of linoleic acid at 2.5 M, 400 µL of phosphate buffer at 20 mM, pH 7,0 and 200 µL of distilled water). The tubes were incubated at dark room for 24 hours at 40 ºC. After this period, 0.05 mL of the mixture were added to 0.05 mL of ethanol (75%), 0.05 mL of ammonium thiacianide solution (0.3 M) and 0.05 mL of ferrous chloride (20 mM). After 3 minutes of the insertion of compounds, the optic density was measured on spectrophotometer at 500 nm. Then, the original mixture was placed on an incubator, after the 24 h, the procedure was repeated until the positive control had achieved the maximum of absorbance. The lipidic peroxidation was expressed in percent (%), and calculated with the following formula:

$$I\ (\%): \frac{(Ac - As)}{Ac}\ x\ 100$$

Ac = control absorbance on the last day; Aa= sample absorbance on the last day.

2.5 2,2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH) SCAVENGING ACTIVITY

The DPPH free radical scavenging activity of the extract was performed according to Brand-Williams et al. (1995) protocol. A solution of DPPH at 200 µM in methanol was diluted in methanol to obtain an absorbance of 517 nm, between 0.6 - 0.7 concentration (work solution). Different concentrations of the extract (0.03 to 1 mg/mL) were mixed with work solution and incubated for 30 min in dark room; the absorbance was read at the same wavelength aforementioned. Then it was plotted a graph of DPPH scavenging activity against different concentrations of extracts to calculate the IC_{50} (extract concentration required to decrease the initial DPPH concentration in 50%). Gallic acid was used as standard. The measurements were triplicate, and their scavenging activities were calculated based on the percentage of DPPH scavenged.

2.6 ACETYLCOHLISNETERASE INIBITION ASSAY

Ellman et al. (1961) protocol was applied for the acetylcohlisneterase inhibition assay using Acetil colinesterase from Sigma-Aldrich (VI-S type). The reaction solutions consisted of 200µL of DTNB (0.25 mM), Tris-HCl [0.5 M, pH 7,4) and 10µL from AChE (1 µg/mL) and 10 µL of *H. rubriflora* extract (at concentration of 1 mg/mL). The solution was incubated for 60 minutes. Then, the reaction was started by adding 20 µL of acetylcoline (62 mM), and the absorbance was read at 405 nm from time 0 to 180s. The enzymatic activity was expressed in mean ± standard deviation, according to acetylcholine molar extinction coefficient.

2.7 STATISTICAL ANALYSIS

Values are expressed as means ± standard deviation of three replicates. Linear regression analysis and Pearson's correlation coefficient were calculated using Statistic 8.0.

3 RESULTS AND DISCUSSION

Phytochemical screening from *Hymenaea rubriflora* leaf extract revealed the presence of phenolic compounds, coumarins, terpenes, tannins, among others secondary metabolites, as displayed on table 1. Phytochemical screening promotes a guidance of potent biological activities present in biomolecules (AZMIR et al., 2013). A similar phytochemical profile was found by Bezerra et al. (2013) on *H. coubaril* extract testing positive for the same metabolites class, as also for *H. eriogyne and H. martiana*, except for tannins (OLIVEIRA et al., 2019; SILVA et al. 2019).

Table 1 - *Hymenaeae rubriflora* phytochemical screening by thin layer chromatography

Secondary metabolite class	Positive/negative
Alkaloids	-
Anthocyanins	-
Anthraquinones	-
Phenolic compound	+
Coumarines	++
Anthracene derivates	++
Lignans	-
Terpenes	+++
Naphthoquinones	++
Saponins	-
Tanins	+
Xanthines	-

(-) Not detected; (+) weak presence; (++) moderate presence; (+++) strong presence.

The concentration of phenolic compound and flavonoids were 286.02 ± 1.75 GAE/mg.DW and 23.19 ± 2.38 QE/mg.DW, respectively. Since flavonoids are a phenolic compounds subclass, we expected lower concentrations of flavonoids when compared to phenolic compounds. To note, phenolic compounds are well abundant compounds found in plants. It holds several metabolic function and pharmacological application pointing them as famous class of natural antioxidants and biological tool to prevent physiological disturbance (APAK et al., 2016)

The antioxidant activity assay results are disposed on table 2. More than one method is recommended to assess the plant antioxidant potential. Here we have used three methodologies to state the antioxidant capacity of *H. rubriflora* extracts, the total Antioxidant Capacity (TAC), DPPH and Lipidic peroxidation. TAC expresses the potential of the product of interest reduces the reactiveness of molybdenum in solution (PRIETO et al., 1999). DPPH quantifies the capability of *H. rubriflora* sequestrate free radicals and lipidic peroxidation assay evaluates the product capacity in inhibit free radicals from oxidize biomolecules from cell membrane (KIKUZAKI & NAKATANI, 1993).

Our results stated that *H. rubriflora* extract is an efficient antioxidant in all systems represented and its effectiveness is probably possibly due to the antioxidant capacity of its secondary metabolites present on extract. As discussed by Patro et al. (2016), plants rich in phenolic have neuroprotective effect due to an antioxidant potential by decreasing the peroxidation and enhancing antioxidant enzymes in mice brain. Once oxidative stress is responsible for trigger several disruptors process like inflammation, neuronal excitotoxicity, nucleic acids damage, which can further induce several diseases, thus the use of antioxidant agents prevents those disruptions (TIWARI & PAL, 2017).

Table 2 – Effect of *Hymenaea rubriflora* methanolic extract in different antioxidant models

Sample	DPPH (IC_{50} µg/mL)	Lipidic peroxidation inhibition (%)	Total Antioxidant Capacity (%)
***Hymenaea rubriflora* leaf extract**	198.3 ± 21.87	60.30 ± 2.71	49.35 ± 4.39

The inhibition of AChE assay demonstrated that 10 mg/mL of *H. rubriflora* extract was able to inhibit the enzyme activity in 96.17 ± 3.48 %. According to Vinhuta et al. (2007), molecules or products able to inhibit the AChE activity in 50% or more are considered strong enzymatic inhibitors. One line to follow is that the inhibition occurred due to phenolic compounds and terpenes present on the extract as assumed by literature (MURRAY et al., 2013; SANTOS et al., 2018). Computational simulation studies suggest that phenolic compounds and derivates, such flavonoids, binds to the AChE's active site, inhibiting enzyme activity and competing with acetylcholine (ROSEIRO et al., 2012; MONTEIRO et al., 2018). The same type of interaction is proposed for terpenes (WOJTUNIK-KULESZA et al. 2017), both class of metabolites was detected on *H. rubriflora extract*. The strong inhibitory effect verified on the present study is due to the mixture of compounds present on the extract. Thus, further investigations are required to better characterize the effect and function of specific metabolites.

Therefore, the data indicates *H. rubriflora* extract as notable choice for situations which requires increased inhibition of AChE. The potential of the compound must be further explored totreatment and prevention in cases of neurological alterations such as cognitive decline, Alzheimer's disease, aging processes and dementias.

4 CONCLUSION

Taken together, this study showed promising findings on *Hymenea rubriflora* leaf extract and its metabolites as antioxidant agent and AChE inhibitors. These findings prompted us to hypothesize that further studies involving *in vivo* models of cognitive declines and Alzheimer's disease might insert *Hymenea rubriflora* as an alternative on treatment and/or prevention of neurological alterations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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