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Determination of Antioxidant Activity by the D.P.P.H. and Dosage of Phenols and Flavonoids in Extracts of the Bark and Sap of *Hymenaea courbaril l* (Jatobá)

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Abstract

The existing biodiversity in Brazilian flora is gigantic, especially in the Amazon region. Among so many species of plants, stands out the genus Hymenaea with some studies demonstrating the antioxidant capacity and raising the hypothesis of potential healing aid. The aim of this study was to evaluate this potential in Hymenaea courbaril L sap and bark samples by the Radical 1,1-diphenyl-2-picrylhydrazine (DPPH) sweep assay method and Phenols and Flavonoids assay. The best result in the DPPH test was sap acetate and shell hexane with an IC50 average of 5.43 µg / ml (SD = 0.05) and 5.04 µg / ml (SD = 0.36). Respectively, approaching the standard sample of gallic acid and Trolox. Regarding the average dosage the best result was sap acetate with 77% (SD = 3.29) of Phenols and 13% (SD = 1.64) of Flavonoids. The extracts of the sap and bark of Hymenaea courbaril L obtained, from this study, a potential antioxidant effect, leading to suggest that this species can be used as an aid in wound healing response, acting in order to prevent cell damage caused by reactive species of oxygen.

Keywords*:* Phenolic compounds. Free radical. Dosage. Antioxidant capacity.

INTRODUCTION

Brazil is the bearer of one of the greatest biodiversity in the world. According to Flora Brasileira 2020 and the Reflora Virtual Herbarium, with the last update of the list in 2018, a total of 57,427 species were

catalogued. Faced with this, the Amazon stands out for the number of known species, as well as those that have not yet been discovered. The fragmented knowledge of Amazonian biodiversity brings with it the need for advances in research and studies on medicinal plants (Filardi et al*.,* 2018).

Medicinal plants are commonly used in traditional communities as home-made medicines and they represent the main medical raw material used by so-called traditional medicines in their therapeutic practices, with folk medicine using the largest number of different species for different types of treatment (Akbik et al*;*. 2014; Lima et al.,2012).

In recent decades, there has been a growing interest in the use of medicinal plants and their extracts in therapy, for the purpose of aiding primary health care and a therapeutic complement, compatible with conventional medicine. For this, there must be a guarantee of safety in relation to toxic effects and knowledge about secondary effects, interactions, contraindications, mutagenicity, among others. In addition to the proof through pharmacological and clinical trials that demonstrate the efficacy and safety of medicinal plants, their safe use (Maver et al., 2015).

The use of medicinal plants began empirically, but over the years it has been modernized and technologically improved. However, the potential of these raw materials is still little explored. There is an estimate that only 8% of Brazilian plant species were studied in search of bioactive molecules (Melo, Mendonça, Mendes, 2004).

Among so many known plant species, Hymenaea courbaril L or, popularly known as Jatobá, stands out. Its use as a medicinal plant is popularly known to treat various illnesses. The bark is used in folk medicine to treat flu, bronchitis, infections of the urogenital system, in addition to its use as an antiparasitic (Costa, Souza, Sousa, 2011).

It is also widely used in the treatment of inflammatory (Cecilio et al., 2012), infectious (Abdel-Kader et al., 2002) and rheumatic (Fernandes *et al.,* 2007) diseases. In addition, it is also popularly used to improve flu-like symptoms, worms, in the treatment of prostate cancer and epigastric pain (Di Stasi, Hiruma-Lima, 2002; Silva, Leite, Saba, 2012).

Regarding healing, anti-inflammatory, muscle relaxant, lipid peroxidation inhibitory and antioxidant action have been described in

products derived from Hymenaea courbaril L (Jayaprakasam et al., 2007, Bezerra et al., 2013).

Thus, medicinal plants have been an effective alternative for the treatment of some pathologies, including skin wounds, which significantly affect the quality of life of patients. These wounds are also considered as one of the main causes of physical disabilities, regardless of their etiology, and which can be considered a serious public health problem, as they are related to significant morbidity and mortality rates (Fracassetti et al., 2013; Kajdžanoska, Gjamovsk, Stefova, 2010). The healing process is influenced by several factors, whether due to comorbidities inherent to the patient, such as diabetes mellitus, as well as factors intrinsic to the injury site, such as infections and production of the so-called reactive oxygen species (ROS). The latter promote biomolecular oxidation, and eventually make them inactive (Kumar, Sharma, Vasudeva, 2010).

The injury, whether surgical or not, increases the production of ROS and the deficit of certain nutrients can promote an oxidative stress that alters the regenerative capacity of the tissue healing (Brown, Phillips, 2010).

Therefore, the present study aimed to evaluate this potential in samples of the sap and bark of *Hymenaea courbaril L* by the method of scanning Radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and determination of Phenols and Flavonoids.

METHODOLOGY

Plant material

After authorization from the Chico Mendes Institute for Biodiversity Conservation – ICMBIO and the Brazilian Institute for the Environment IBAMA under protocol No. 57286-1, samples of the sap and bark of the species *H. courbaril* L were collected in the Chico Mendes extractive reserve located in the Western Amazon. The structures of the collected plants were exsiccated under the following GPS coordinates - 9º59.50.67º59.2.2ws, as well as were identified at Escola da Floresta and deposited in the herbarium of the Federal University of Acre (UFAC) with listing number UFACPZ 20025.

Extractive methods

The production process of the *H. Coubaril L* sap extract started with the washing of 500 ml of the sap with 500 ml of hexane using a glass apparatus of the Soxhlet type. After exhaustion extraction, the yield was washed with 500 ml of ethyl acetate. Then, using a rotoevaporator, the ethyl acetate was evaporated under vacuum and the residue obtained was stored in a desiccator until use. The extraction yield was approximately 17.5%.

The initial sample weighing 4,771g of *H. Coubaril L* bark was separated and stored in an oven until the material had completely dried. Then, it was taken to the Soxhlet apparatus, applying a new solvent to each cycle, namely Hexane, Acetate and Methanol.

After obtaining the bark and sap extracts, all samples were weighed in the proportion of 10 mg. Then, it was diluted individually in 1 ml of DMSO (dimethylsulfoxide). Finally, 100 mcl was removed from the 1 ml and supplemented with 900 mcl of DMSO. The final total sample concentration for carrying out the experiment was 1 mg/ml. The extracts were submitted to the method described by Brand-Willians (1995) with some modifications to use 96-well microplates. The Radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scanning assay was applied to all samples that were able to obtain extraction.

Initially, a DPPH solution (0.8 mmol/L) was prepared, then the solution was diluted, obtaining its absorbance close to 1,000±0.1nm. After this preparation, 30 μL of the extracts prepared in 270 μL of the DPPH solution were conditioned, in triplicate. In the control samples, the same volume mentioned above was used. Then, the microplates were incubated at room temperature and in the absence of light for half an hour. Finally, absorbances (ABS) were measured in a microplate reader (DTX800, Beckman) at a wavelength of 517 nm. This same reader was used throughout the work.

In obtaining the minimum inhibitory concentration of 50% of DPPH radicals (IC50), a curve was made and the equation of the straight line was formed. The results of this equation were in μmol/mL. The standard sample and extract sample solutions were in eight different concentrations (0.781 to 100 μg/mL) and the dilutions were from 1:1 to the 8th dilution and the last volume was discarded. The standard sample used in this assay was gallic acid and Trolox.

From the results by the DPPH method, the inhibition calculation was performed based on the formula:

% Inhibition = 100 × [(ABS2 sample – ABS control) /ABS control]

The quantification of total phenols was performed based on the description by Singleton & Rossi, in 1965, with some modifications, and firstly, 10 µL samples of each prepared extract (1 mg/mL) were mixed with 50 µL of the Folin solution Ciocalteu in the proportion of 1:10. Then, they were conditioned in microplates and placed in an incubator for 8 minutes, after which sodium carbonate was added at a concentration of 0.4% and incubated again for 3 minutes. Finally, the absorbance was measured at a value of 620 nm.

The result of the absorbances were compared to a study standard using gallic acid and the results were applied to the following formula:

% phenols = (ABS sample /ABS Standard) *100

Total flavonoids were quantified according to the method described in 1999 by Zhishen, Mengcheng and Jianming, adding some modifications. First, 30 µL of the respective extract sample (1 mg/ml), 90 µL of ethanol, 6 µL of aluminum chloride at a concentration of 10% and 6 µL of potassium acetate were mixed in a microplate. These samples were incubated for a predetermined time of 30 minutes. Finally, the final mixtures with a wavelength of 510 nm were analyzed to verify the absorbance of each sample.

For final comparison and calculations, quercetin was used as a standard sample, applying the following formula:

Total flavonoids= ABS sample X 100 ABS Standard

The preparation of the sap extracts resulted in an amount of 3,493 g of Acetate and 8,358 g of Spray dry. However, it was not possible to extract the Hexane and Methanol extract. In relation to the bark extracts, at the end of this process, the following components were obtained: Bark hexane 0.211 g; Peel acetate 0.211 g; Bark methanol 2.403 g and Bark Spray dry 3.050 g. All yields were used in the determination of phenols and flavonoids and the DPPH scanning assay.

RESULTS AND DISCUSSION

The results presented in this work were produced to generate in vitro knowledge about the antioxidant activities of the species Hymenaea courbaril L (Jatobá), with potential for the healing process.

The result of the DPPH test can be viewed from Table 1.

Table 1 – Consolidation of DPPH scanning test results.

	Inhibition em %		CI50 em µg/ml	
SAMPLE	Average	DP	Average	DP
Bark Hex	28,31	1,32	5,04	0,36
Shell acetate	68,35	2,97	25,26	0,37
Sap acetate	72,07	1,98	5,43	0,05
Shell methanol	66,91	1,02	11,80	0,62
Dry peel spray	74,93	0,48	12,66	0,20
Sap dry spray	75,02	0,09	42,60	1,45
Gallic acid	86,22	0,08	2,86	0,05
Trolox	84,33	0,33	4,36	0,23

Among the tools analyzed, a better performance was observed in relation to the oxidation capacity of the acetate extract from the sap and hexane from the bark, as they dissipate an inhibitory concentration value of 50% (IC50) of 5.43 µg / ml and 5.04 µg / ml both and both with acceptable standard deviation. Results were found similar to the Gallic acid and Trolox standards which were 2.86 µg/ml and 4.36 µg/ml respectively. The least favorable result in those analyzed for the Spray dry of the sap with an IC50 value of 42.6 µg / ml with a standard deviation of 1.45.

The antioxidation capacity is inversely proportional to the inhibitory concentration value of the sample, this is due to the fact that less plant quantity is needed to reduce 50% of the free radical. Based on this, it can be inferred that the greater the antioxidant capacity of a substance, the lower the cellular damage propagated by reactive oxygen species (ROS), which may suggest that the acetate extract from the sap and hexane from the Jatobá bark have potential for the healing response of damaged tissue (Barbosa et al., 2010).

The DPPH assay is not used for any specific antioxidant component, thus applying to the overall antioxidant capacity of the

analyzed sample. Thus, it is not possible to infer which is the main agent intrinsic to the extract responsible for this effect however, this antioxidant activity has already been attributed, in other studies, to the lipid fraction of seeds produced by Hymenaea courbaril L. In addition, this component itself oxidative stability due to the oleic acid present in 46.09% of unsaturated fatty acids in the pulp of this plant (Ramalho, Jorge, 2006)

Phenolic compounds are structures characterized by having an aromatic ring unit with one or more hydroxyl groups in its composition, a fact that contributes to their acting as antioxidants (Saranraj, Behera, Ray, 2019).

Based on their chemical structures, they can be divided into different subgroups: phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stylets and curcuminoids (Neilson, Goodrich, Ferruzzi, 2017).

Therefore, research into the presence of these compounds in samples of extracts derived from Hymenaea courbaril L is of fundamental importance in establishing the factor responsible for the antioxidant effect proven in the DPPH test.

The flavonoids are well-studied phytochemicals due to their beneficial relationship to health. These compounds are already recognized as substances capable of neutralizing free radicals. However, there is a downside to these phytochemicals which is the low systemic bioavailability, thus hindering the production of medicines. However, despite this fact, many of these compounds appear to be effective in preventing or ameliorating chronic diseases and wounds in vivo, even at low doses (Li et al., 2019).

The highest concentration was obtained in the sap acetate extract with 77.55% (SD: 3.29) of Phenols and 13.16% (SD: 1.64) of Flavonoids. The sample with the lowest contents was the bark hexane extract with quantification of 11.94% (SD: 0.55) of Phenols and 0.23% (SD: 0.15) of Flavonoids.

The results of the full assay of phenols and flavonoids are shown in Table 2.

Table 2 – Consolidation of the results of the determination of Phenols and Flavonoids.

PHENOLS				
	%Phenols		µg eq Ac Gallic	
	Average	DP	Average	DP
Bark Hex	11,94	0,56	79,05	3,86
Shell acetate	33,02	3,92	225,42	27,22
Sap acetate	77,56	3,29	534,77	22,88
Shell methanol	32,30	0,55	220,41	3,80
Dry peel spray	14,34	0,18	95,70	1,28
Sap dry spray	31,71	3,29	216,30	22,84
FLAVONOIDS				
	% Flavonoids		µg eq Quercetin	
	Média	DP	Média	DP
Bark Hex	0,24	0,16	0,82	0,85
Shell acetate	12,81	0,50	63,52	2,70
Sap acetate	13,16	1,65	65,43	8,88
Shell methanol	3,88	0,46	15,39	2,47
Dry peel spray	1,70	0,31	3,61	1,67
Sap dry spray	3,52	0,02	13,44	0,11

Other structures already known to be present in Hymenaea courbaril L are Terpenes, Tannins and Glycosides (Jayaprakasam et al., 2007). Such substances allow the use of this plant as a fungicide and repellent against various agricultural pests (Schwartz, 2018).

The processing of medicinal plants as raw material in the pharmaceutical industry is recognized and financially sustainable. Preliminary knowledge about the active pharmacological constitution and bioactivity of plants is of fundamental importance for the process to be developed. The species *Hymenaea courbaril* L from this study demonstrated an antioxidant effect and, therefore, its potential for the wound healing process is suggested, given the findings of a study carried out with the aim of demonstrating the importance of using substances with Antioxidant potential, identified that phenolic compounds are one of the main groups responsible for this property and among these are the flavonoids, which can be found in leaves, seeds, bark and flowers of several plants (Heim, Tagliaferro, Bobilya, 2002)

The biochemical and pharmacological effects of flavonoids are vast, among which the antioxidant, anti-inflammatory, antiplatelet, vasodilatory and antimicrobial actions stand out, in addition to antiallergenic effects, and the higher the content of phenolic compounds, the greater their antioxidant potential (Zhao et al, 2010), which justifies that these compounds can be explored by the pharmaceutical industry to formulate new compounds with potential for wound healing

CONCLUSION

The results of this study corroborate the literature regarding the presence of the antioxidant effect of Hymenaea courbaril L. As the presence of phenols and flavonoids is notorious, especially in sap extracts, this demonstrates the antioxidant capacity of the species, confirmed by the DPPH test in the same sample best result. This antioxidant effect brings with it the plant's potential to aid in the healing of skin wounds. Anyway, further studies are needed for clinical application of these extracts. However, there is the hypothesis of using this vegetable as a component for the production of pharmacological products, encouraging more research on this theme.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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