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Antioxidant and antimicrobial properties of ethanolic extract of *Libidibia ferrea* pods

Propriedade antioxidante e antimicrobiana do extrato etanólico de vagens de *Libidibia ferrea*

[1]Patrícia L. A. Nascimento; [2]Talita C. E. S. Nascimento; [2]José E. G. Gomes; [2]Mitaliene D. S. Silva; [2]Silvana A. Souza; [3]Tania M. S. Silva; [4]Rosangela A. Falcão; [5*]Keila A. Moreira

[1] Faculty of Odontology, Caruaruense Association of Higher Education.

[2] Departments of Morphology and Animal Physiology, Federal Rural University of Pernambuco.

3 Department of Molecular Sciences, Federal Rural University of Pernambuco.

4 Department of Biological Sciences, University of Pernambuco.

5 Academic Unit of Garanhuns, Federal Rural University of Pernambuco.

* Corresponding author: moreirakeila@hotmail.com

Abstract

Libidibia ferrea is a typical plant of northern and north-east Brazil and has well-known medicinal properties that can be found in ethanolic extracts such as antipyretic, anti-inflammatory and anti-rheumatic compounds. This study seeks to evaluate the potential antioxidant and antimicrobial activity of ethanol extracts from *Libidibia ferrea* pods. The pods were used for the preparation of ethanolic extracts of *L. ferrea* which is used to determine biological activity, and measure their total phenolic content. For quantification of antioxidant methods of DPPH, ABTS and β-carotene were employed and the method of minimum inhibitory concentration (MIC) to measure the antimicrobial activity. The ethanolic extract of *L. ferrea* showed excellent antioxidant activity, as well as other similar substances commonly used for this purpose such as citric acid and trolox. In addition, it had antimicrobial activity against Gram-negative and Gram-positive bacteria.

Keywords: pau ferro; C*aesalpinia ferrea*; medicinal properties; antioxidant.

Resumo

Libidibia ferrea é uma planta típica do norte e nordeste do Brasil conhecida por suas propriedades medicinais que podem ser encontrados em extratos alcoólicos, possui compostos antipiréticos, anti-inflamatórios e antirreumáticos. Este estudo visa avaliar o potencial antioxidante e atividade antimicrobiana do extrato etanólico de vagens de *Libidibia ferrea*. As vagens foram empregadas na preparação de extrato etanólico de *L. férrea*, utilizado na determinação da atividade biológica, e para mensurar seu conteúdo fenólico total. Os métodos

DPPH, ABTS e β-caroteno foram utilizados para quantificar a atividade antioxidante, e o método da Concentração Inibitória Mínima (CIM) para avaliar a atividade antimicrobiana. O extrato etanólico de *L. ferrea* apresentou atividade antioxidante excelente, bem como outras substâncias compatíveis normalmente utilizadas para este fim, tais como ácido cítrico e trolox. Além disso, demonstrou atividade antimicrobiana contra bactérias Gram-negativas e Gram-positivas.

Palavras–chave: pau ferro; C*aesalpinia ferrea*; propriedades medicinais; antioxidante.

Introduction

The basionym of *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz (Leguminosae) is C*aesalpinia ferrea* Mart. ex Tul. (Caesalpiniaceae) according to the International Plant Names Index, 2009, (http://www.ipni.org/). It is a large tree that is found in the north and north east regions of Brazil, especially Pernambuco and Ceará states, where it is commonly known as juca or pau ferro (Peters et al., 2008).

Recent scientific studies have shown that *L. ferrea* contains compounds which have antimicrobial, anti-inflammatory, analgesic, antioxidant and hypoglycemic properties (Ferreira and Soares, 2015). It is used in folk medicine to treat diabetes, as antipyretic and antirheumatic, anti-inflammatory and antinociceptive activities have already been found in the ethanol extract of the fruits of this species (Vasconcelos et al., 2011; Lima et al., 2012).

The aqueous extract of the seeds of *C. ferrea* have cellulase, amylase, anticoagulant and larvicide activities against *A. aegypti* (Cavalheiro et al., 2009). In the Amazon region of Brazil, the fruits of *C. ferrea* (Brazilian ironwood) are widely used as an antimicrobial and healing medicine to treat many ailments including oral infections (Sampaio et al., 2009).

Phytochemical studies have reported that aqueous extracts of *Caesalpinia ferrea* pods and barks consist of a mixture of different compounds with anti-inflammatory and analgesic actions, which confirms its popular use and ethnomedicinal value (Carvalho et al., 1996; Pereira et al., 2012).

The crude extract of *Caesalpinia ferrea* contains anthraquinones, alkaloids, depsides, depsidones, flavonoids, lactones, saponins, sugars, tannins, sesquiterpenes and triterpenes. Tannins are considered as the main component (Souza et al., 2006). Phenolic compounds, such as tannins and flavonoids, have antimicrobial and antioxidative properties and are involved in the defence against fungi and other microorganisms (Oliveira et al., 2010).

For centuries, extracts from plants have been used as folk remedies to treat various ailments, with many natural products leading to the development of clinically beneficial drugs (Siqueira et al., 2012). Organisms that belongs to the vegetable kingdom are those that have contributed most significantly to providing compounds which possess a large spectrum of biological properties (Cavalheiro et al., 2009).

The aim of this study is to evaluate the potential of both antioxidants, as the antimicrobial properties of ethanolic extracts obtained from the pods of *Libidibia ferrea*, are indicative of the presence of chemical compounds that may be used in various branches of industrial biology.

Materials and Methods

Plant material

The pods (peel and seeds) of *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz (Leguminosae) were collected from the town of Garanhuns, State of Pernambuco, in North-East Brazil. A sample of the collected material was classified and filed (Specimen Number 89749), at the Herbarium D´Ardano de Andrade Lima, Institute of Agronomy, Pernambuco - IPA, Pernambuco, Brazil. It was dried at 40°C in a Circulated Air Incubator for 48 hours, grounded in a grinder and stored to -20 °C.

Reagents

Tryptic Soy Broth -TSB (Acumedia, Lansing, USA), Müeller-Hinton Broth (HIMEDIA, Mumbai, India), dimethyl sulfoxide (FMaia, Cotia, Brazil), chloramphenicol (Ariston, São Paulo, Brazil) were used and all the solvents were HPLC commercial grade. The β-carotene, 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid, and the Folin–Ciocalteu reagent were purchased from Sigma–Aldrich (St. Louis, USA).

Preparation of the extract

The ethanolic extract of the *L. ferrea* pod was obtained by repeated soaking under agitation in ethanol P.A. at a ratio of 1:10, until the depletion of substances extractable by ethyl alcohol. For the concentration of the extract, ethanol was removed from the filtrate using a rotary evaporator at 40°C under reduced pressure.

Determination of the total phenolic content

The determination of the total phenolic content present in the ethanolic extract of *L. ferrea* pods was carried out by the Folin- Ciocalteu spectrophotometric method (Slinkard and Singleton, 1977) with modifications. Gallic acid was used as a standard. Briefly, 300 µl of a solution of the ethanolic extract (1 $mg.mL^{-1}$) was added to 60 µl of the Folin-Ciocalteu reagent and 2460 µl of distilled water, and this mixture was stirred for 1 minute. Following this, 180 µl of sodium carbonate (2%) was added to the mixture, which was then shaken for 30 seconds, and resulted in a final concentration of 100 µg/ml. After two hours of incubation, the absorbance of each sample was determined spectrophotometrically at 760 nm. The results were expressed as mg of gallic acid equivalents (GAE)/g of extract.

DPPH free radical scavenging assay

The free radical scavenging activities of the samples were determined by means of the 2,2,-diphenyl-2-picrylhydrazyl (DPPH) spectrophotometric method in accordance with the study of Silva and collaborators (2011). This method is often used to determine the ability of plant species to capture free radicals (Ghasemnezhad, Sherafati and Payvast, 2011).

A stock solution of the ethanolic extract of *L.ferrea* pod was prepared at 5.0 mg/ml. The appropriate amounts of an ethanolic solution of DPPH• (23.6 µg/ml) were added to samples to obtain final concentrations ranging from 100 to 500 µg/ml. The absorbance was measured at 517 nm after an incubation interval of 30 min with ultrasonication in the dark. The EC_{50} value is the sample concentration necessary to reduce the absorbance of DPPH by 50%; ascorbic acid was used as a standard.

ABTS radical cation assay

This test involves the generation of the ABTS chromophore •+ by oxidation of ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] with potassium persulfate. The test was performed according to the method of Re and collaborators (1999) with modifications. ABTS was dissolved in

water at a concentration of 7 mM. The ABTS radical cation (ABTS•+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark for 12–16 h before use. After this, the $ABTS^{\bullet+}$ solution was diluted with ethanol (approximately 1:100 v/v) until an absorbance of 0.7±0.05 nm was reached. A stock solution of the ethanolic extract was prepared with a concentration of 1.0 mg/ml. An appropriate amount of ABTS•+ (2700 µl) was added to each sample to give final concentrations ranging from 20 to 120 µg/ml, and the samples were ultrasonicated in the dark after a period of 10 minutes. Then, the absorbance of the samples was measured at 734 nm. Trolox (0.1 mg/ml) was used as a positive control.

The β-Carotene bleaching test

The level of antioxidant activity was determined by conducting the β-carotene bleaching test and employing the method of Bamoniri and collaborators (2010) with modifications. A solution of linoleic β-carotene/linoleic acid was prepared by adding an aliquot of 150 µl of β-carotene solution to 160 µl of linoleic acid and 660 µl of Tween 20. After this, 140 ml of oxygenated distilled water was added to the system. The absorbance of this emulsion at 470 nm was adjusted to 0.6 - 0.7 nm. Aliquots of the crude ethanolic extract of *L.ferrea* pod (16 µg/ml) were compared with the control (without an antioxidant) and to Trolox (16 µg/ml), which was used as a standard antioxidant. An initial reading of the absorbance was taken immediately after the samples and the standard were added to the system to determine the baseline. Subsequently, the absorbance was monitored every 20 minutes for 120 minutes. The samples were kept in a water bath at 40°C between the readings. The antioxidant capacity was expressed as the percentage inhibition of oxidation.

Determination of minimal inhibitory concentration

The microbial strains used belong to Gram positive families (*Staphylococcus aureus* UFPEDA 02*, Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* UFPEDA 86) and Gram negative families (*Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 29665, and *Pseudomonas aeruginosa* UFPEDA 416) bacteria, which were acquired from the Antibiotics Department of the Federal University of Pernambuco, Brazil.

The broth microdilution assay was performed in accordance with the CLSI reference method M7-A6, for bacteria (Clinical Laboratory Standards Institute, 2003). Ninety-six-well microplates were used to determine the MIC of the crude ethanolic extract of *Libidibia ferrea* pod. Briefly, the crude extract was dissolved in DMSO (1 mg/ml) and then diluted in water to achieve concentrations ranging from 500µg/ml to 25µg/ml. The inoculum size was adjusted to each microorganism to yield a cell concentration of 10^8 CFU ml. A final volume of 100 µl was achieved in each well. One well with a specific medium and microorganism was used as control of the growth, and one inoculated well was free of the test extract so that it could check the sterility of the media. The microplates were prepared in triplicate and incubated at 37°C for 24 h. The MIC was determined by measuring the absorbance of each well with a microplate reader (ASYS UVM 340, Cambridge, UK). The MIC was defined as the lowest sample concentration that inhibited bacterial growth in proportion to the growth of the controls. Chloramphenicol (50 µg/ml) was used as a positive control for all the bacterial strains.

Statistical analysis

All the samples were analysed in triplicate, and the results were pooled and expressed as the means ± standard error. A statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego CA, USA). The anti-free

radical activity was determined by conducting a linear regression analysis with a confidence interval of 95% ($p<0.05$). The results were expressed as EC_{50} ± SEM, which represents the concentration of the sample necessary to reduce the absorbance of DPPH or ABTS•+ by 50% compared with the negative control.

Results

In choosing a suitable assay to investigate the antioxidant activity, ethanolic extract of *Libidibia ferrea* pods was evaluated to determine their total phenolic content by means of the Folin-Ciocalteu method; the radical scavenging activity was assessed through DPPH and ABTS assays and their antioxidant potential was measured through the β-carotene bleaching test. **TABLE 1** shows the free radical scavenging activity (DPPH• and ABTS•+) of the ethanolic extract of the *L. ferrea* pod. The results demonstrated a high radical scavenging activity when compared with a control of antioxidant capacity (trolox and ascorbic acid).

TABLE 1. Free radical scavenging activity (DPPH• and ABTS•+) and total phenolic content of the ethanolic extract of *L. ferrea* pod.

Total Phenolic (mg GAE/g extract ±SD)*	EC50 DPPH (µg /ml ± SD)		EC50 ABTS (µg /ml ± SD)	
Ethanolic extract	Ethanolic extract	Ascorbic Acid	Ethanolic extract	Trolox
32.6 ± 0.0	4.4 ± 0.05	3.3 ± 0.02	2.5 ± 0.06	2.9 ± 0.05

*GAE/g extract - Gallic acid equivalent/g extract.

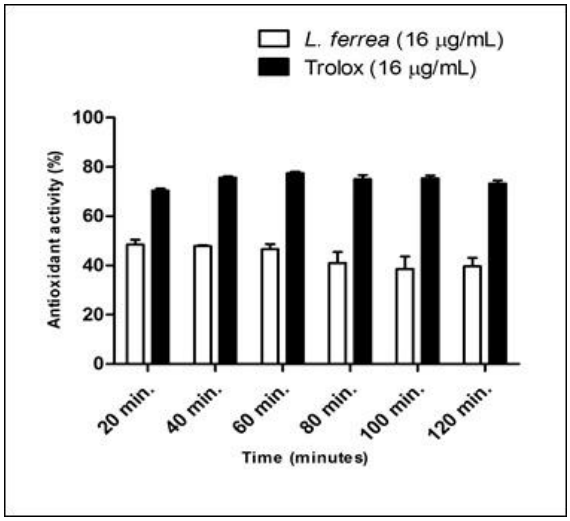

FIGURE 1. Antioxidant activity of the ethanolic extract of *L. ferrea* determined using the β-carotene bleaching test. Black columns – Trolox (16 µg/mL); white columns – *L. ferrea* pod ethanolic extract (16 µg/mL).

The antioxidant activity of the ethanolic extract of *L. ferrea* which was determined by using the β-carotene bleaching test is shown in **FIGURE 1**. When the extract activity is compared with the standard Trolox which is a vitamin E analogue, it can be observed that the antioxidant activity was excellent (46.63%).

The minimum inhibitory concentrations (MICs) in µg/ml for the ethanolic extract of *L. ferrea* pod, are shown in **TABLE 2**. Our results reveal that all the tested strains of Gram-positive and Gram-negative bacteria were inhibited by the ethanolic extract of the *Libidibia ferrea* pods.

TABLE 2. Minimal inhibitory concentrations (MIC) for the ethanolic extract of *Libidibia ferrea* pod tested on different microorganisms.

Microorganisms	Concentrations (µg.mL^{-1})
Gram positive bacteria	
Enterococcus faecalis	50
Bacillus subtillis	50
Staphylococcus aureus	125
Gram negative bactéria	
Escherichia coli	50
Klebsiella pneumoniae	125
Pseudomonas aeruginosa	50

Discussion

Antioxidant activity

Phenolic compounds are commonly found in both edible and inedible plants, and have multiple biological effects, including antioxidant activity (Silva et al., 2011).

The total phenolic content value of this study was higher than that found by Oliveira and collaborators (2010) who identified a phenolic content of 0.151 ± 0.005 mg GAE/g extract for *C. ferrea* wood extract. However Surveswaran and collaborators (2007) evaluated the total phenolic content of *Caesalpinia bonducella* (L.) Roxb. Seeds and *Caesalpinia sappan* L. heartwood and found 1.3 and 94.7 mg GAE/g dry weight. The nature of the species, parts of the tree and extraction techniques that were used may explain the differences between the studies.

A preliminary phytochemical analysis of the hydroalcoholic extracts of stem, bark and leaves of *C. Ferrea,* showed the presence of flavonoids, saponins, tannins, coumarins, sterols and phenolic compounds (Lima et al., 2012).

The free radical scavenging of DPPH forms the basis of a common antioxidant assay (Sharma and Bhat, 2009). The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This free radical, which is stable at room temperature, is reduced in size in the presence of an antioxidant molecule, and gives rise to a colourless ethanol solution. The use of the DPPH assay is an easy and fast way to evaluate antioxidants by spectrophotometry, so it might be a useful way of assessing different products at the same time (Huang, Ou and Prior, 2005). The extract was able to reduce the stable free radical DPPH$^{\cdot}$ to the yellow DPPH.

The ABTS assay is based on the generation of a blue/green ABTS$^{\cdot+}$ that can be reduced by antioxidants, and is applicable to both hydrophilic and lipophilic antioxidant systems. The ABTS assay is superior to the DPPH assay when applied to a variety of plant foods containing hydrophilic, lipophilic, and high-pigmented antioxidant compounds, whereas the DPPH assay uses a radical dissolved in organic media and is, therefore, applicable to hydrophobic systems (Floegel et al., 2011).

Surveswaran and collaborators (2007) evaluated all the antioxidant capacities of *Caesalpinia bonducella* (L.) Roxb. Seeds and *Caesalpinia sappan* L. heartwood by ABTS and DPPH and their results were 0.61 and 0.13 mmol trolox/100g dry weight for *C. bonducella* and 34.65 and 28.33 mmol trolox/100 g dry weights respectively.

The health effects of medicinal plants can partly be attributed to the presence of phenolic compound, which may have an effect as a result of their antioxidant properties (Kaur, Arora and Singh, 2008).

Tannins and flavonoids have a therapeutic value due to their anti-inflammatory, anti-fungal, antioxidant and healing properties (Araújo et al., 2008).

With regard to its antioxidant activity, the use of the β-carotene is due to the bleaching of this compound during the autoxidation of linoleic acid in aqueous emulsion where the decay is monitored by its absorbance in the visible region. The addition of an antioxidant-containing sample, individual antioxidants or natural extracts assists in retarding the β-carotene decay (Roginsky and Lissi, 2005). Antioxidants are able to scavenge radicals, hydrogen peroxide and other peroxides and prevent the formation of radicals (Martysiak-Żurowska and Wenta, 2012).

Antimicrobial activity

Generally, owing to their extra protective outer membrane and other particular features, Gram-negative bacteria are considerably more resistant to antibacterial agents than Gram-positive bacteria (Bamoniri et al., 2010). However, in this study, a higher concentration is only needed for *S. aureus* e *K. pneumoniae* (125 µg/ml). The MIC values obtained in this study meet the stringent endpoint criteria adopted by some authors (Cos et al., 2006; Mbosso et al., 2010), which considers concentrations of up to 1 mg/ml for extracts or 0.1 mg/ml for isolated compounds when displaying antimicrobial activities.

Sampaio and collaborators (2009) found minimum inhibitory concentration values of the crude extract of *C. ferrea* fruits for *Candida albicans*, *Streptococcus mutans*, *Streptococcus salivarius, Streptococcus oralis* and *Lactobacillus casei* of 25.0, 40.0, 66.0, 100.0, 66.0 µg/ml, respectively. Cavalheiro and collaborators (2009) observed that the aqueous extract of seeds do not inhibit the growth of any bacterial strains analyzed, which demonstrates the presence of distinct compounds between the pods and seeds of this plant species.

Interestingly, our current findings show a remarkable antimicrobial activity against Gram negative and Gram positive bacteria, ranging from 50 to125 µg/ml. To put these values into context, extracts with MICs ≤ 100 µg/ml and isolated compounds with MICs ≤ 10 µg/ml can be considered to be of great value (Cos et al., 2006). Significantly, since our results were an ethanolic extract, they how remarkable activity against most Gram negative and Gram positive bacteria.

A phytochemical investigation of the hydroalcoholic extract of the stem bark and leaves of *Caesalpinia ferrea* has revealed flavonoids, saponins, tannins, coumarins, steroids and other phenolic compounds (Vasconcelos et al., 2011). Phenolic compounds, such as tannins and flavonoids, have antimicrobial and antioxidative properties and are involved in the defence against fungi and other microorganisms (Boudet, 2007). The antimicrobial activity of this extract may be on account of the presence of tannins, flavonoids and terpenoids in its composition (Siqueira et al., 2012). Nakamura and collaborators (2002) study identified gallic acid among the active constituents of Juca fruits. Gallic acid has been found to have antibacterial, antiviral and antifungal activities, and also anti-inflammatory, anti-tumour, antianaphylactic, antimutagenic, chloretic and bronchodilator activities (Nakamura et al., 2002).

Conclusion

In this study, it has been shown that ethanolic extract of *L. ferrea* pod is capable of inhibiting the growth of Gram positive and Gram negative microorganisms. Moreover, antioxidant and free radical scavenging activity was found. It is expected that further studies currently in progress will enable us to understand the nature of the precise compounds and mechanisms involved.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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