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Phytochemical Screening and Evaluation of the Antimicrobial Activity of the Ethanolic Extract of the Leaves of *Morus alba L.*

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Abstract

The search for knowledge of biological activities and phytochemical screening of the species *Morus alba L.* is non- existent, however the present study came to explore this plant. To perform the phytochemical screening and the evaluation of the antimicrobial activity of the leaves of the species *Morus alba L.* Experimental analysis, with the collection of leaves of the species to obtain hydroalcoholic extract at 70% and from this extract the main secondary metabolites were identified. And after contributing to the systematic knowledge of the secondary metabolites of *Morus alba L.*, antimicrobial activity was tested from the concentrated extracts of the leaves of the species. Among the extracts tested, the one that presented the most inhibitory activity in S. aureus was the concentrate, then the aqueous and finally the alcoholic.

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In E. coli strains the extracts showed no effects. Phytochemical tests were performed using the concentrated extract using the methods of reference [1], where it presented the presence of reducing sugars, phenols and tannins, alkaloids and depsides and depsidones. The research emphasized the microbiological action of the species *Morus alba L.*, as it is a poorly studied plant and may help in the future in the development of new drugs. Phytochemical studies were necessary to confirm this microbiological action, as they were essential for the identification of natural and efficient antibacterial agents.

Keywords: *Morus alba L; Staphylococcus aureus*; Phytochemical; Antimicrobial.

1. Introduction

The use of medicinal plants for curative purposes comes from the beginning where literary data inform that about 50,000 years ago man already enjoyed [2]. The ethnopharmacological study defined as interdisciplinary scientific exploration of biologically active agents, traditionally employed or observed by man [3] has been carried out to collaborate with the validation of popular use once scientifically proven. With the advance in the pharmacological area, through chemical, biological and microbial studies, positive results have been highlighted and made possible the development of new chemical substances for humans. Because of this, medicinal plants have aroused some interest from researchers around the world for their therapeutic properties. The plant that drew our attention to carry out the present work is popularly known as white mulberry (*Morus alba L*), *moraceae* family already used in folk medicine to combat various diseases due to its anti-inflammatory, antimicrobial, analgesic, antineoplastic, among others. The federal government, through Presidential Decree No. 5,813, of June 22, 2006, approved the National Policy of Medic- inal plants and phytotherapeutics, which has as one of the strategies the promotion of research, technological development and innovation based on Brazilian biodiversity, covering native and exotic plant species adapted, prioritizing the epidemiological needs of the population, which reinforces the importance of conducting ethnobotanical and ethnopharmacological studies for further phytochemical studies. The *moraceae* family has about 50 genera and 1500 species, which predominate in tropical and subtropical climates. They are represented in Brazil by 27 genera with 250 species among these trees, shrubs, herbs or lianas [4]. The species of the *genus Morus* are predominantly in temperate or cold climates and can be found more in Central and South America, Mediterranean Basin, Africa and Asia [5]. The *genus Morus* covers the following species: *Morus alba L, Morus lhou Koidz, Morus bombycis Koidz, Morus nigra L.* and *Morus rubra L*., of which the first one stands out [6]. According to reference [5] *Morus leaves* are astringent and are used for antifebrile actions, externally for gargling such as thrush, inflammations of the throat, tonsils and pharyngitis, and purgative when made from bark tea. Mulberry leaves are used for treating fever, headache, beriberi, vomiting and stomach pain caused by the cholera agent. Also reinforcing, that the young part of the tree is intended for the treatment of hypertension and paralysis of arms and legs [7]. The mulberry to *Morus nigra L*. not so far from the white one exposes its popularly known beneficial activities: used as a hormonal repository in cases of hot flashes, besides having antioxidant, hyperglycemic, anti-inflammatory and antimicrobial activities [8]. Several biological activities already with different prenilated flavonoids were identified with cytotoxic activity [9], antioxidant [10] and inhibitor of certain enzymes, such as tyrosinase [11], cyclooxygenase and lipoxygenase [12]. In the present work, we chose to perform a phytochemical study and the *in vitro* evaluation of the antimicrobial activity of the leaves of the species *Morus alba L.* considering through

ethnopharmacological studies present in the scientific literature that this species has several biological properties, among which it stands out antimicrobial.

2. Materials and methods

Plant material: The leaves of *M. alba* were collected in the city of Gurupi/TO, in the period of January 2012, for the preparation of exsiccates, and sent them to the herbarium of the Federal University of Tocantins, in Porto Nacional. Extract preparation: Leaves of the species *Morus alba* were collected in February 2012, taken to the Pharmacognosy Laboratory of the Unirg University Center and dried in an oven at 40ºC until they reached constant weight. The material was sprayed in a Marconi MA 048 knife mill, which after this grinding process, 50g of the powder suspended in 500 ml of 70% ethanol was used, then stored in an amber bottle, protected from light, where it was left at rest for 7 days at room temperature. From the filtered solution (500ml), they were separated for 100ml phytochemical tests and for 400ml antimicrobial tests, where it was concentrated at 60º, in a water bath, until the complete evaporation of the alcoholic solvent. Then the extract to perform phytochemical tests the extract containing 100ml was submitted to a temperature of 70ºC in the oven for 1 hour, for the removal of water. The concentrate was subsequently weighed, obtaining 9.65g, representing the yield of 19.3%.

2.1. Preliminary Tests for the Identification of Secondary Metabolites

The hydroalcoholic extract 70% once concentrated was submitted to preliminary phytochemical analysis, where specific reagents were used for each chemical group, according to the methodology proposed by reference [1]. Saponins, reducing sugars, polysaccharides, tannins, flavonoids, proteins and amino acids, depsides and depsidones, alkaloids, purines and an- thraquinones were studied.

2.1.1. Test for saponins

A few milligrams of the dry extract were dissolved in 5ml of distilled water. Then diluted to 15ml and then stirred vigorously for 2 minutes in a closed tube.

2.1.2. Test for reducing sugars

A few milligrams of the dry extract were dissolved in 5ml of distilled water. 2ml of FEHLING A reactive (prepared with 34.65g of copper sulfate in distilled water and supplemented to the volume of 500ml) was added to 2ml of the reactive FEHLING B (dissolved with 173g of sodium and potassium tartrate and 125g of potassium permanganate in distilled water diluted to 500ml).

2.1.3. Test for polysaccharides

Dissolved a few milligrams of dry extract in 5ml distilled water and added two drops of lugol.

2.1.4. Test for proteins and amino acids (nihidrin reaction)

A few milligrams of the alcoholic extract were dissolved in 3ml of distilled water and 0.5ml of the nihhydrin

aqueous solution was added at 1%, until it reached boiling.

2.1.5. Test for phenols and tannins

A few milligrams of dry extract were dissolved in 5ml distilled water, and 2 drops of 1% ferric chloride alcoholic solution was added.

2.1.6. Test for Flavonoids (general)

Dissolved a few milligrams of dry extract, in 10ml of methanol, added 5 drops of concentrated hydrochloric acid and magnesium zest.

2.1.7. Test for Flavonoids (by classes)

A few milligrams of the dry extract were dissolved in 20ml of distilled water. It was transferred to three test tubes, 3ml of the solution (for each tube). A tube with pH 3 was acidulated, and the other two were alkalized one to pH 8 and the other pH 11.

2.1.8. Leucoanthocyanidins, catechins and flavanones

In two 3ml tubes of the solution already prepared previously, the first was acidified with hydrochloric acid solution at pH 1 – 3 and alkalized the other to pH 11 with sodium hydroxide solution and heated with the aid of an alcohol lamp for 2-3 minutes, carefully.

2.1.9. Flavonols, flavones, flavanonols and xanthones

Transferred to a test tube, 3ml of the same extractive used in the previous test and added a few milligrams of magnesium in scrapings, and 0.5ml of concentrated hydrochloric acid.

2.1.10. Test for alkaloids

A few milligrams of the dry extract were dissolved in 5ml of 5% hydrochloric acid solution, where it was separated into two 1ml portions in test tubes, and added drops of the reagents below:

A) Dragendorff reactive

For the preparation of this reactive, two solutions were prepared: - Solution A dissolved 8g of Bismuth Subnitrate in 20ml of acetic acid. - Solution B where 27.2g of potassium iodide was dissolved in 50ml of distilled water. Solution A was gradually added to solution B.

B) Mayer reactive

For the preparation of this reactive, two solutions were prepared: - Solution A which were dissolved 1.36g of

mercury chloride in 60ml of distilled water. - Solution B dissolved 5g of potassium iodide in 20ml of distilled water. After the preparation of solutions A and B, they were mixed and diluted to 100ml.

2.1.11. Test for purines

In a porcelain capsule, a few milligrams of the dry extract were added with 3 drops of 6N hy- drochloric acid solution and two drops of concentrated hydrogen peroxide 30%. It was submitted to evaporation in a water bath and was mixed 3 drops of ammonium hydroxide 6N.

2.1.12. Test for depsides and depsidones

A few milligrams of the dry extract were dissolved in 5ml of ethyl ether where the ether evaporated in the Maria bath and then joined the residue 3ml of methanol. It stirred and 3 drops of ferric chloride solution was added to 1%.

2.1.13. Test for anthraquidons

A few milligrams of the dry extract were dissolved in 5ml of toluene. 2ml of 10% NH_4OH solution was added, it stirred gently.

2.2. Antimicrobial Activity ssessment

2.2.1. Microorganisms

For the present work, the microorganisms to be tested were NEWP standard strains recommended for antimicrobial susceptibility tests, namely, gram-positive bacteria Streptococcus aureus (NEWP 0023) and gram-negative bacteria Escherichia coli (NEWP 0022).

2.2.2. Means of cultivation

500mL of dehydrated Mueller-Hinton Agar was prepared with a ratio of 19g to 500mL according to the manufacturer's specifications. Then, the agar was submitted to sterilization by wet heat in autoclave at 122ºC for 20 minutes. After autoclavation, it was allowed to cool down to 45-50ºC and Mueller-Hinton broth was dispensed in 150 x 10 mm petri dishes.

2.2.3. Standardization of Inoculum and Sowing

Gram-positive and Gram-negative microorganisms were prepared in sterile TSB (triptych soy broth) medium, until reaching turbidity corresponding to tube 0.5 of the Mac-Farland scale correspond- ing to approximately 1.0-2.0 x 10^5 CFU/mL of each strain, after reactivation and turbidity, sowing was performed taking 3 to 4 colonies of the strain isolated in Mueller-Hinton agar.

2.2.4. Preliminary Evaluation of Antibacterial Activity

The inhibitory effect of plant extracts was evaluated by the agar diffusion technique, as described by reference [13] and reference [14]. Plates containing 10ml of agar were prepared. On these plates, 9ml of semi-solid agar, inoculated with about 10^6 cells/ml, of bacterial suspensions were added. Aseptically, 3 wells/plate, 6 mm in diameter, were made only in the upper layer. 10μl of the extracts were applied individually in each season. The control was made, consisting of the solvents used in the preparation of each extract, that is, two plates of each bacterium, where in each one was made only one wells, and dispensed water in one (negative control) and alcohol at 70% in the other (negative control). Then, the plates were incubated at 35˚C for 24 h and the readings of the bacterial growth inhibition halo were made, in mm, formed around the discs containing the concentrated extract. For the final result of the extract, halo equal to or above 8 mm in diameter was considered as susceptible according to Parekh's criteria; [15,16].

3. Results and Discussion

3.1. Phytochemical Approach

Table 1: Preliminary phytochemical screening of concentrated ethanol extract of the species *Morus alba L.*

RESEARCH	**REACTION**
Reducing sugars	+++
Polysaccharides	-
Proteins and Amino Acids	-
Flavonoids	-
Flavones, Flavonols and xanthones	-
Favanonodes	-
Depsids and Depeptides	+++
Derivatives of Cuumarin	-
Anthraquinonas	-
Alkaloids **(RD)** **(RM)**	 ++ ++
Purinas	-
Caption: (-) Negative Result (+) Weakly Positive Result (++) Moderately Positive Result (+++) Strongly Positive Result	
DR: Dragendorff Reactive **MR: Mayer Reactive**	

The preliminary phytochemical screening of the concentrated ethanol extract indicated the presence of different groups of metabolites in the leaves of the species of *Morus alba L*. In the analysis the presence of saponins, polysaccharides, proteins and amino acids, flavonoids, purines, coumarins and anthraquinones were not detected. However, the extract showed positive results for reducing sugars, phenols and tannins, alkaloids and depsides and depsidones as shown in Table 1.

3.2. Microbiological Approach

The analysis of antibacterial activity was performed with three types of extracts, Crude Alcoholic Extract (CALE), Crude Con- centrated Extract (CCE) and Crude Aqueous Extract (CAE), demonstrating the antibacterial potential of these extracts on gram- positive bacteria (S. aureus). The CCE reached an inhibition halo of 20mm, followed by the CAE which reached a halo of 14mm and finally the CALE that corresponded to a halo of 12mm (Table 2). In gram-negative bacteria (E. coli) the extracts did not obtain antimicrobial results. Table 2. Inhibition of bacterial growth with Crude Concentrated Extract (CCE), Crude Aqueous Extract (CAE) and Gross Alcoholic Extract (CALE) of *Morus alba L*.

Table 2: Inhibition of bacterial growth with Crude Concentrated Extract (CCE), Crude Aqueous Extract (CAE) and Gross Alcoholic Extract (CALE) of *Morus alba L*.

Growth inhibition zone (mm diameter)			
Extract	**Dose (µl)**	***S. aureus***	***E. coli***
CCE	10 µL	20mm	-
CAE	10 µL	14mm	-
CALE	10 µL	12mm	-

The species studied in this work is *Morus alba L.*, better known popularly as white mulberry [5]. According to reference [17] there are investigations on the *genus Morus* regarding different groups of chemical compounds, these being alkaloids, coumarins, triterpenes and steroids. From the results of the present study, it was observed conclusively through phytochemical tests the presence of alkaloids, and the absence of coumarins on the species *Morus alba*. According to reference [7] mulberry leaves get treatment on aggressor effects on the human organism caused by the cholera agent. According to reference [5] morusem leaves are generally astringent and have various biological activities. Focusing on this fact of reference [18] developed a research and concluded that astringency occurs by the presence of tannins, occurring due to the precipitation of salivary glucoproteins, where lubricating power is lost. Reference research [19] emphasized the results of biological activities where tannins showed important action against certain microorganisms. The present research presented positive results regarding phytophymic tests for phenols and tannins, thus confirming its presence in the leaves of the *genus Morus* and more focused on the species *Morus alba L.* Reference [9] reported that several biological activities, such as cytotoxic, have already been identified among others in different prenilated flavonoids, thus evidencing according to Reference [20] that such flavonoids have biological activities being antioxidant and anti-inflammatory, also stating that it constitutes an important class of natural polyphenolic compounds. Although in this study it did not detect flavonoids and their derivatives, it was positive for phenols, probably suggesting actions similar to those already reported. Reference [21] also recently demonstrated that mulberry (*Morus alba*) showed anti-arterosclerotic activity for rats deficient in LDL receptors. In addition, reference [22] they added that the root bark fractions exhibited a potent antioxidant activity by the inhibition mechanism of lipid

peroxidation in rats submitted to a cholesterol-rich diet. Studies such as reference [23] have shown that butanolic extract from the leaves of the mulberry plant inhibits the increase in serum cholesterol, and consequently prevents arterosclerosis. For reference [17] the substance chal- comoracin, isolated from *Morus* species, presents antimicrobial activity with specificity to methicillin-resistant *Staphylococcus aureus*, and its efficacy in inhibitory activity against microbial growth compared to vancomycin. By reference [24] found that the methanolextract to 85% of the dried leaves of Morus alba has properties to promote skin whitening due to the presence of the compound called oxyresveratrol through the mechanimsum of repression of the action of tyrosinase, an enzyme that contains copper, and catalyzes the oxidation of phenols, activating melanins. From the isolated component of the bark and leaves of mulberry performed by reference [25], these being the alkaloids 1-deoxynojirimicicin, concluded that some of them presented suppressive activity on α-glycosidases of mamiferos. For reference [26] isolated 1-deoxynojirimcin, called moranoline, from the bark of mulberry root, based on the knowledge that the extracts were able to prevent increased blood glucose. According to references [27] and [28] prenilated flavonoids have been isolated from the barks of Morus root and some of them have been notified for cytotoxic activities. In studies conducted by reference [25] demonstrated that they were isolated from the bark of the root and leaves of mulberry, alkaloids that include 1- deoxynojirimin. Through the research of reference [29] by HPLC, they verified the detection of two active compounds, chalcomoracin a natural type of Diels-Alder that develops an antibacterial activity, and another was moracin N, considered a precursor of chalcomoracin. The reference [30] analyzed mulberry leaves, and identified by HPLC the presence of at least four flavonoids, consisting of two of rutin, a bioflavonoid, which offers capillary antifragility and prevents edemas in the legs, and quercetin, a natural flavonoid that has pharmacological characteristics, such as anti-inflammatory, anticarcinogenic, antiviral, besides influencing the inhibition of cataracts in diabetics. There are other mulberry sides presenting analgesic, antiasthmatic, anti-rheumatic, antitussive, astringent, sweating, diuretic, emollient and expectorant activities [31]. The plant extract has been shown to have antioxidant, hypoglycemic and potentially antioxidant activity of some phenolic compounds, being flavonoids, stilbenose 2-arylbenzofurans [32]. Reference [33] developed a research, analyzing the nutrients and secondary metabolites in Morus alba leaves for the feeding of silkworms. From this research, they were able to demonstrate the total phenolic quantity ranging from 3.22 to 4.56%. The moracetine compound, a hormone present in the plant, was isolated from the leaves of *M. alba*, in addition to phenolic compounds obtained through root bark and others such as rutin, isochextrin and astragaline [24]. Several studies have demonstrated antibacterial properties from proven plant products through intensive research worldwide [16,34,35]. Reference [8] had already reported antimicrobial activity of the species *Morus nigra L.*, and may consider this statement valid also for the species *Morus alba L.*, confirmed the activity in the present study. To test the antibacterial activity with the leaves of this species, we chose to obtain three different extracts to perform comparisons. Thus, it was demonstrated that the concentrated ethanol extract presented a higher inhibition halo than the other extracts, which proves that this extract has a more expressive concentration of secondary metabolites when compared to the others. The results indicate that Gram-positive bacteria, specifically E. coli, are relatively inhibited by the components of *Morus alba*. The phytochemical profile of selectivity against Gram positives is not restricted to plant compound, but is a general phenomenon observed among many antibiotics [36], suggesting other more specific studies to deepen the antibacterial activity found preliminary in this research.

4. Conclusion

The present research presented results of great value, because it emphasized the microbiological action of the species *Morus alba L*., a plant little studied, which in the future may help in the development of new drugs. The importance of such a plant is due to the presence of antimicrobial action on a very common bacterium, however villain of many pathologies (such as toxic shock syndrome, staphylococcal gastroenteritis, staphylococcal scalded skin syndrome and impetigo), being it S. *aureus*. It is also noted that phytochemistry work is essential for the identification of efficient natural antibacterial agents, which can contribute to an improvement in health services to the population, mainly due to a probable reduction in drug costs, by offering another treatment option with natural products provided that all their tests are validated.

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