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Phytochemical screening of *Azadirachta indica* A. Juss for antimicrobial activity

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***Azadirachta indica* A. Juss, known as neem (Meliacea family), has insecticide and pesticide properties, and many studies have shown their efficacy as antifungal, anti-inflammatory, among others. Studies for the development of drugs from plants are rising due to several factors such as bacterial resistance, indiscriminate use and the adverse reactions of antibiotics. In this study, phytochemical triage and thin layer chromatography analysis were performed, with similar results as the presence of flavonoids, tannins and terpenes. The antimicrobial activity showed that the ethyl acetate extract and butanol fraction presented greater activity against *Streptococcus mutans* and *Streptococcus mitis* presenting a MIC = 50 µg/ml for these strains, and the strain *Enteroccocus faecalis*, the hydroethanolic extract and aqueous fraction were most promising samples with a MIC = 50 µg/ml and MIC = 25 µg/ml, respectively. Therefore, it encourages the continuation of studies, aiming at the development of cosmetics or toothpaste.**

Key words: Antimicrobial activity, *Azadirachta indica*, minimum inhibitory concentration, oral strains, phytochemistry.

INTRODUCTION

The widespread use of a limited number of antimicrobial agents concomitantly with the reduced arsenal of drugs with antimicrobial function, has led to the development of resistance to drugs that oppose both fungal and bacterial infections, which has been an increasing problem (Alexander and Perfect, 1997; Rex and Pfaller, 2002; Zida et al., 2016).

The need to find alternatives for microbial control has induced a lot of research in order to seek products that are effective and economical. Studies of natural products with biological activity offers credible alternatives for microbial control, particularly from bioactive products derived from plants with therapeutic properties of routine use (Albuquerque, 2001). Exotic plants well adapted to the Brazil climate have also shown several properties in traditional medicine, insecticides, among others, as in the

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case of *Azadirachta indica* A. Juss (neem). Originally from Southeast Asia, this plant is also found in tropical and semitropical regions like India, Bangladesh, Pakistan and Nepal. *A. indica* tree belongs to the family Meliaceae (Girish and Bhat, 2008; Alzohairy, 2016; Schmutterer, 2004) and its most important active constituent is azadirachtin (Hossain et al., 2011; Alzohairy, 2016). The most characteristic metabolites of this family are called limonoids, which are tetranortriterpenoides; which has considerable interest due to fascinating structural diversity and its broad biological activity (Tringali, 2001).

Study evidenced that plants fruits, oil, leaves, bark and other parts have important role in diseases prevention due to their rich source of antioxidant (Alzohairy, 2016). Quercetin and sitosterol, polyphenolic flavonoids, were purified from neem fresh leaves and were known to have antibacterial and antifungal properties (Govindachari et al., 1998; Alzohairy, 2016).

There are some available chromatographic methods and among these, the thin layer chromatography is the most widely used for their fast analysis, qualitative detection, and provision of semi-quantitative information of the most active constituent of the drug; thereby enabling an assessment of the drug quality (Wagner et al., 1996). According to previous study (Carneiro et al., 2012), the thin layer chromatography of the fractions hexane, dichloromethane and ethyl acetate showed a predominance of terpenes. The limonoids or tetranortriterpenoides which are very common on leaves and fruits of *A. indica* belong to this class of substances (Shumutterer, 2002; Roy et al., 2006).

The neem has a wide range of several therapeutic properties based on its characteristics, such as antifungal, antibacterial, antioxidant, antiviral, anti-inflammatory, analgesic, antipyretic, and immune stimulant activity (Subapriya and Nagini, 2005; Mustafa, 2016). The leaf extract is commonly used as an antibacterial agent. In addition, the neem has several applications, such as antiseptic, healing, anthelmintic; use in medicinal soaps, creams and toothpaste (Schmutterer, 2004; Dutta and Kundabala, 2005, 2013; Mustafa, 2016).

Dental caries and oral health/dental health are multifactorial diseases related to diet, oral microbiota, hygiene, salivary characteristics, and are inseparable part of general health, which can lead to considerable pain and suffering. It has an impact on a person's speech, selection of food, quality of life, and general well-being. In view of the prevalence of oral diseases, their impact on individuals and society and the expense of treatment, may be considered a major public health problem and they are listed among the most common of the chronic diseases that affect mankind. Oral diseases are the fourth most expensive diseases to treat in certain countries (Chandra Shekar et al., 2015; Sheiham, 2005). *A. indica* was tested as herbal alternatives to endodontic irrigants in comparison with sodium hypochlorite (standard irrigant), and this study showed the zones of inhibition of leaf extracts had antimicrobial properties with significant greater zones of inhibition than 3% sodium hypochlorite (Alzohairy, 2016; Honmode et al., 2013).

According to the World Health Organization (WHO) report, dental caries, though exhibiting a declining trend in many parts of the industrialized world; is still an important public health concern in many developing countries. The statistics suggest that dental caries affect 60 to 90% of school going children in developing countries (Chandra Shekar et al., 2015; Petersen, 2003).

From a microbiological point of view, the appearance of lesions of the disease is linked to the complex structure of the dental biofilm, involving the participation of several micro-organisms, such as *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus sanguis,* which colonize the surface of the teeth shortly after their outbreak in the oral cavity (Kawashima et al., 2003; Svensater et al., 2003; Lindquist et al., 2004; Marsh, 2004; Seki et al., 2006).

MATERIALS AND METHODS

Collection and preparation of plant material

The collection of *A. indica* A. Juss sheets was held in the Garden of Medicinal Plants and Toxin of the University of Pharmaceutical Sciences of UNESP, Araraquara, situated at geographic coordinates 21°48'52.44"S and 48°12'07.13". It was also collected copy for the preparation of voucher specimen, which was sent to tipping in the Herbarium of São José do Rio Preto under SJRP31236 number.

The extracts were obtained from the leaves, which were washed with sodium hypochlorite (0.2%) and deionized water, and dried in an air circulating oven at 40°C for three days, followed by grinding the dried leaves in knife mill, and then the material was passed at 0.42 mm mesh sieve in order to reduce and standardize the particle size. The hydroethanolic extract was obtained by percolation of *Azadirachta indica* A. Juss. using 70% ethanol as solvent at a flow rate controlled to 10 drops/min until exhaustion of the drug. The volume of the solution extractive obtained was reduced on a rotary evaporator (pressure and temperature) and then, the drying of the residue was completed in petri dishes in an oven at 40°C. The ethyl acetate extract used as extraction liquid was obtained by maceration in an attempt to achieve antimicrobial activity of different compounds extracted by this solvent. In order, 100 g of the plant drug moistened with 200 ml of the solvent was placed, gradually adding more 200 ml. After that, it was put in contact with the heating mantle at 30°C, stirring every 1 h in 1 h for 3 h. The extraction solution was first dried on rotaevaporator, and then dried in an oven at 40°C. Fractions to be tested were obtained by solubilizing up 2.00 g of the dry hydroethanolic extract in 100.00 ml of distilled water, using separator funnel of 300.00 ml. Then the liquid / liquid partition with increasingly polar solvents began (n-hexane, ethyl acetate and n-butanol) in similar proportions of organic solvent and aqueous solution until complete separation of the respective phases (with an average duration 15 min). The obtained fractions were concentrated to dryness in chapel.

Phytochemical screening

To characterize the major groups of secondary metabolites such as

Table 1. Mobile phase and reagents used for determination of secondary metabolites using technique of thin layer chromatography.

Secundary metabolite	Mobile phase	Reagent
Flavonoids	*n*-butanol:acetic acid: water (63:33:4)	NP-PEG+UV
Alkaloids	*n*-butanol:acetic acid: water (70:28:2)	Dragendorff
Terpenes	*n*-hexano:ethyl acetate:isopropanol (70:28:2)	Anisaldehyde sulfuric
Tannins	Ethyl acetate:formic acid:water (90:10:10)	Ethanolic solution of ferric chloride 1%

Table 2. Phytochemical screening of the plant from *Azadirachta indica*.

Secundary metabolite	Results
Saponins	Positive
Tannins	Gelatin 2.5% - Negative; Ferric chloride 1% - Negative
Flavonoids	Positive
Anthraquinone	Negative
Cardiotonics glycosides	Negative
Alkaloids	Negative

tannins, flavonoid, glycoside cardiotonics, saponins, anthraquinones and alkaloids, chemical reactions were performed. The development of the color and / or characteristic precipitated was observed (Costa, 2001).

Thin layer chromatography

The thin layer chromatography was performed with different and specific mobile phase and reagents for each secondary metabolite (Table 1).

Bacterial samples

In this study, *Streptococcus mitis* ATCC 49456, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556 and *Enterococcus faecalis* ATCC 4082 were used, and the extracts and fractions were prepared in stock solution of 1600 µg / ml using solvent DMSO 20%.

Determination of antimicrobial activity - minimum inhibitory concentration (MIC) of the extract and fractions of *A. indica* A. Juss leaves

The Minimum Inhibitory Concentration (MIC) was determined using the dilution method on microplates according to standard M7-A6 Clinical and Laboratory Standards Institute (CLSI), with modifications.

Bacterial strains were maintained on Mueller-Hinton broth plus 50% glycerol and held at -20°C. After that, the bacterial strains were transplanted in 2 ml of Mueller-Hinton broth, incubated for 24 h at 37°C and were subsequently subcultured on solid medium (Blood Agar Base), incubated for 24 h at 37°C and maintained in refrigerator.

The bacterial suspensions were standardized by adding a culture prepared from the bacterial growth at 24 h elapsed MH broth at 37°C and subsequently, after stirring, a suspension aliquot was transferred to another tube containing sterile saline (PBS) until turbidity comparable to McFarland scale suspension corresponding to 0.5 tube unit (approximately 1.5×10^8 CFU/ml).

Next, the spectrophotometric reading was performed at 620 nm absorbance ranging from 0.10 to 0.15, which correspond to 1.5×10^8 CFU/ml. Subsequently, it was diluted on tube with sterile saline (PBS), to obtain an inoculum of bacteria *S. mutans, S. mitis, S. sanguinis* and *E. faecalis* the concentration of 5×10^5 CFU / ml used in the tests (Kawashima et al., 2003).

The plate's holes (96 wells) were filled with 80 µl of Mueller-Hinton broth. Then, 100 µl of solutions of plant extractives were added and serial dilutions carried out at 400 to 0.19 µg/ml for the concentration of 1600 µg/ml. Additionally**,** 20 µl of micro-organisms suspensions were distributed in each well of microplate. As a positive control was used chlorhexidine to 59 µg/ml for *S. mutans, S. sanguinis* and *S. mitis* and 14.75 µg/ ml for *E. faecalis*, and the negative control DMSO 20%.

Control of the culture medium, bacterial growth and plant extractives were also performed. The microplates were incubated at 37°C for 24 h. In each microplate two plant extractives were tested in duplicate. All tests were performed in triplicate.

RESULTS

Phytochemical screening

The results obtained on the phytochemical screening are presented in Table 2.

Thin layer chromatography

The results were positive for terpenes in the ethyl acetate extract; and the fractions resulting from the hydroethanol extract, which are hexane and ethyl acetate fraction; flavonoids in the ethyl acetate extract, hexane hydroethanolic extract and its fractions, n-butanol, ethyl

Table 3. Thin layer chromatography.

Extracts and fractions	Secundary metabolites
Hydroethanol extract	Flavonoids
Ethyl acetate extract	Flavonoids and terpenes
Hexane fraction	Flavonoids and terpenes
Ethyl acetate fraction	Flavonoids and terpenes
Water fraction	Flavonoids
n-Butanol fraction	Flavonoids

Table 4. Determination of the antibacterial activity and the minimal inhibitory concentration (MIC) in µg/ml for *S. mitis*, *S. sanguinis*, *S. mutans* and *E. faecalis* by dilution technique in microplate using resazurin as developer.

	Micro-organism	*S. mitis*	*S. sanguinis*	*S. mutans*	*E. faecalis*
MIC (µg/ml)	Hydroethanolic extract	400	≥400	≥400	50
	Hexane fraction	200	≥400	400	>400
	Ethyl acetate fraction	**100**	400	400	>400
	N-butanol Fraction	**50**	**100**	**50**	>400
	Aqueous fraction	400	≥400	≥400	**25**
	Extract ethyl acetate	**50**	**100**	**50**	>400
	Ampicillin	-	-	-	-
	Chlorhexidine	3.688	3.688	19.530	14.75

acetate and aqueous. Table 3 shows these results.

Antimicrobial activity

According to tests, the bacterial strains were sensitive to extracts and fractions of *A. indica* A. Juss leaves. The results with the MIC values are shown in Table 4.

DISCUSSION

More and more people in developed countries apply traditional medicine for most health care (Houghton, 1995). The use of medicinal plants for the treatment of various diseases including bacterial and fungal infections is common in Brazil (Carvalho et al., 2009; Pereira et al., 2007; Schubert et al., 2007). Worldwide, many search groups that use plants detect secondary metabolites with antimicrobial properties in an attempt to find new antimycobacterial or antifungals compounds (Cos et al., 2006; Soberón et al., 2007; Rangasamy et al., 2007).

There are several factors that can interfere qualitatively and quantitatively in the results, in the metabolites presence, in the antimicrobial activity, and one of them can be seasonal factor, which may be considered as the period of leaves collection. Therefore, the results can be related to low production of active metabolites against these microorganisms, thus, it is suggested further study and could start the study conducting the tests in all seasons.

The results of the phytochemical screening and thin layer chromatography confirms the presence of flavonoids and saponins, which justifies this plant's biological activities as anti-inflammatory, antimicrobial, antioxidant, antiviral, among others.

Observing the composition of the extracts and fractions, it can be said that the ethyl acetate extract, hydroethanol extract, the hexane fraction and ethyl acetate extract from hydroethanol extract showed terpenes and flavonoids in the composition. The n-butanol and aqueous fraction obtained positive result only for flavonoid, thus observed the difference in composition of the extracts and fractions tested. This result corroborates with the study of Mossini et al. (2005), which describes the presence of terpenes and phenolic compounds.

According to Gibbons (2004) and Van Vuuren (2008), crude extracts of natural products with an MIC lower than 1000 µg/ml are considered relevant and extracts with a MIC below 100 µg/ml are considered promising as potential antimicrobial agents. Therefore, based on these studies, it can be said that the n-butanol fraction and the ethyl acetate extract achieved a promising results with a MIC of 50 µg/ml to the strains *S. mitis* and *S. mutans*. These same samples showed MIC = 100 µg/ml for *S. sanguinis*, being also regarded as promising potential. For the *S. mitis*, the hexane fraction showed MIC = 200 µg/ml, which may be considered relevant, the hydroethanolic extract and aqueous fraction showed an

MIC = 400 µg/ml, also showing a considerable result.

Regarding the *S. sanguinis*, the ethyl acetate fraction showed MIC = 400 µg / ml, hydroethanolic extract, hexane fraction and aqueous fraction showed MIC ≥ 400 ug/ml, and can also be considered as relevant antimicrobial agents. For *S. mutans* the hexane fraction and ethyl acetate fraction showed a MIC = 400 µg/ml, hydroethanolic extract and aqueous fraction MIC ≥ 400 µg/ml, considered as an important result.

The *E. faecalis* results regarding the hydroethanolic extract and the aqueous fraction samples were promising, because it had, respectively, a value of MIC of 50 µg/ml, and MIC = 25 µg/ml, highlighting the activities of these vegetable samples for this strain. The remaining samples presented an MIC> 400 µg/ml, which is considered relevant.

Thus, the results corroborate with studies (Patel et al., 1988) which reports that the *A. indica* extract is a powerful inhibitor agent against the increase and establishment of micro-organisms that cause infectious diseases in the oral cavity, wherein *S. mutans* is the main causative agent of caries.

The activity of the aqueous fraction and hydroethanolic extract against *E. faecalis* are highlighted, with MIC of 25 µg/ml and MIC of 50 µg/ml, respectively, emphasizing these growth inhibition concentration values.

Conclusion

The positive antimicrobial activity of neem against the oral strains studied could be due to the presence of flavonoids and saponins, which justifies this species has biological activities as anti-inflammatory, antimicrobial, antioxidant, antiviral, among others. Considering the relevant MIC values of n-butanol fraction and ethyl acetate extract, especially for *S. mitis* and *S. mutans,* it was possible to observe that these samples have some active compounds that caused inhibition of their growth. These results encourage the continuation of the studies further isolation of this substance for a future development of herbal medicine.

Conflicts of Interests

The authors have not declared any conflict of interests.

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