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Antimicrobial activity of extracts obtained from the seeds of *Vatairea guianensis* (Aublet)

[Actividad antimicrobiana de extractos obtenidos de las semillas de *Vatairea guianensis* (Aublet)]

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Abstract
Medicinal plants are widely and culturally used in an empiric way to treat several diseases in the Amazon. Here, *Vatairea guianensis*, which is used in traditional medicine to treat skin infections, such as cutaneous mycoses, was studied. The present work evaluated the *in vitro* antibacterial activity of hydroethanolic, hexanic, chloroformic, and methanolic extracts, obtained from the seeds of *V. guianensis* by the broth microdilution method, to measure their minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Pseudomonas aeruginosa* and *Salmonella* sp.) bacteria. All extracts showed antimicrobial activity against both Gram-positive and Gram-negative bacteria, with MICs ranging from 3.12 µg/mL to 50 µg/mL and MBCs ranging from 6.25 µg/mL to 100 µg/mL. Finally, these results on antimicrobial activity might contribute to the study of *V. guianensis* and increase the medical interest in this genus, which is still poorly studied.

Keywords: antimicrobial activity, medicinal plants, *Vatairea guianensis*

Resumen
Las plantas medicinales son ampliamente utilizados y culturalmente de forma empírica para tratar varias enfermedades en la Amazonía. Aquí, fue estudiado *Vatairea guianensis*, que se utiliza en la medicina tradicional para tratar infecciones de la piel, como las micosis cutáneas. El presente trabajo se evaluó la actividad *in vitro* antibacteriana de los extractos hidroetanólico, hexánico, clorofórmico y metanólico, obtenido de las semillas de *V. guianensis* por el método de microdilución en caldo, para medir su concentración inhibitoria mínima (CIM) y la concentración mínima bactericida (CMB) contra las bacterias Gram-positivas (*Staphylococcus aureus* y *Enterococcus faecalis*) y Gram-negativas (*Pseudomonas aeruginosa* y *Salmonella* sp.). Todos los extractos mostraron actividad antimicrobiana frente a bacterias Gram-positivas y Gram-negativas, con los CIM que van desde 3,12 µg / ml a 50 ug / mL y CMB que van de 6,25 µg / ml a 100 µg / ml. Por último, estos resultados sobre la actividad antimicrobiana podrían contribuir al estudio de *V. guianensis* y aumentar el interés médico en este género, que todavía está poco estudiada.

Palabras Clave: actividad antimicrobiana, plantas medicinales, *Vatairea guianensis*

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INTRODUCTION

Many plants used in Brazil are employed in the form of crude extracts, infusions, or plasters to treat common infections, without any scientific evidence of toxic potential to humans (Holetz *et al.*, 2002). Based on popular indication regarding the intermediate products obtained from these extracts, the tincture of the seeds of *Vatairea guianensis*, commonly known as faveira, is useful against skin infection caused by fungi and bacteria in Amapa, Brazil. The use of seeds is associated with the riverine population and traditional community knowledge.

Several Brazilian plants are used to treat infections caused by bacteria and fungi, but scientific studies supporting those activities are lacking (Alves *et al.*, 2000; Holetz *et al.*, 2002; Sartori *et al.*, 2003; Pretto *et al.*, 2004; De Campos *et al.*, 2005). Moreover, these plants should be studied not only to determine the properties underlying their popular traditional value but also to discover other, unknown activities based, for example, on their chemical composition.

The genus *Vatairea*, from the family Leguminosae, subfamily Faboideae, is exclusive to the neotropical belt, including only seven arboreal legume species, which are distributed from southern Mexico to southeastern Brazil, including Guatemala, Honduras, Costa Rica, Panama, Colombia, Venezuela, Guyana, French Guiana, Suriname, and Peru. The widest variety of this genus is located in the forest regions of central Amazonia. The genus name refers to a popular name used in Guyana (Cavada *et al.*, 1998; Barroso *et al.*, 1991; Lima, 1982).

The Amazonian forest species belonging to this genus include *V. guianensis*, *V. fusca*, *V. erythrocarpa*, *V. paraensis*, and *V. sericea*. Other species of the genus inhabit the hillside forests, such as *V. heteroptera.* In southern Mexico and Central America, the species *V. lundelli* predominates (Lima, 1982).

Recent publications about this genus have mostly focused on *V. guianensis* Aubl., *V. heteroptera* Ducke, *V. macrocarpa* Benth, *V. sericea* Ducke, and *V. paraensis* Ducke.

V. guianensis is popularly known in Amazonia as *faveira*, *fava de empigem*, *faveira de empigem*, *fava bolacha*, *fava mutum*, *faveiro*, and *Angelim do igapó* (Piedade, 1987). The Palikur Indian population in northern Amazonia, near the border of Brazil and French Guiana in the extreme north of Amapa state, refers to *V. guianensis* as *waru*. In Suriname it is known as *gales habbes*; in Peru as *anacapi* and *marupa del bajo*; in French Guiana as *graine i dartres*, *bois dartre*, and *maria congo*; and in Venezuela as *guaboa* (Grenand *et al.*, 1987).

V. guianensis is native to Amazonia, commonly found in seasonally flooded forest areas, such as the *igapo* and *varzea* forests in Amazonia, and is rarely found on solid ground, occurring throughout the region bounded by the Amazon River and its tributaries, reaching the borders of French Guiana, Venezuela, Colombia, Peru, and Suriname (Correa, 1986). In the State of Amapa, Brazil, the occurrence of these species is higher in the city of Mazagão, but they are also found in other cities in the state, such as Porto Grande and the Archipelago Bailique (Santos *et al.*, 2004).

Ethnopharmacological reports indicate that the juice from the fruit is used in Amazon traditional medicine to cure ringworm and to treat certain skin diseases in Brazil, Venezuela, Colombia, and French Guiana (Lima, 1982). In middle and lower Amazonia, the population uses the seeds of *V. guianensis* Aubl. against several kinds of superficial mycoses, in the form of alcoholic tincture or by direct application of the soaked seeds to the skin. Preparations of bark from the stem and roots are used against fungi while *V. guianensis* is not in the fruiting phase (Piedade and Filho, 1988; Revilla, 2003). Fructification occurs from June to January (Lima, 1982). The oil extracted from the seeds is used topically against patches dry skin, freckles, and dermatomycosis (Corrêa, 1986).

The aim of the present study was to evaluate the antimicrobial activities of hydroethanolic, hexanic, chloroformic, and methanolic extract, obtained from the seeds of *V. guianensis* by determining the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of each.

MATERIAL AND METHODS

Collecting and obtaining botanical material

The botanical material (fruits) from *V. guianensis* were collected in the region of the municipality of Porto Grande, 100 km away from Macapa, the capital of Amapa, Brazil, in July of 2009. After obtaining the material, exsiccates of the plant material were deposited in the Herbarium from the Amapa Institute for Scientific and Technological Research, identified by comparison by the botanist Benedito Rabelo, and registered with the number 1916.

Preparing the plant material and obtaining the extract

The fruits of *V. guianensis* were collected in their natural habitat. Seeds were removed, then subsequently washed in water and sprayed with 70% alcohol. Next, the seeds were dried in an oven with air circulation at 50°C for 2 consecutive days. Later they were ground in a Hammer mill to create a dark-colored powder.

The hydroalcoholic extract was obtained by maceration, and 500 g of the powder was added to a 1,520 mL solution of 75% hydroethanol in a glass container. Maceration occurred for 7 days, and the mixture was manually stirred for 3 min every day.

The hydroethanol extract was concentrated in a rotary evaporator, under reduced pressure, at 55°C. The material obtained after concentration was in the form of a soft, dark paste, and samples were taken for *in vitro* tests and kept in a -20°C freezer until use. The yield of crude extract was 30.68%, based on the initial mass of raw material.

The hexanic, chloroformic, and methanolic extracts were obtained directly from the powder by macerating seeds for 5 days, using 350 g of ground seeds added to 700 mL of solvents with increasing polarity, starting with hexane, then chloroform, and finally methanol. After macerating, the extracts were subjected to the process of concentration in a low-pressure rotary evaporator at 50° C until total evaporation of the solvent. After this procedure, the concentrate of each extract was subsequently weighed, thus obtaining the yield of each extract. Ultimately, we obtained a yield of 4.57% for the hexanic extract, 2.02% for the chloroformic extract, and 6.22% for the methanolic extract, based on the initial mass of raw material.

In vitro antimicrobial activity evaluation of the hydroethanolic, hexanic, chloroformic and methanolic extracts of Vatairea guianensis

Extract dilution

To test their antimicrobial activities, the seed extracts of *V. guianensis* were diluted in a solution of 60% of dimethylsulfoxide (DMSO):distilled water and homogenized by vortex for 2 min.

Collection and maintenance of strains

The microorganisms used in the study were the Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212) and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 9027) and *Salmonella* sp. (clinical isolate). These strains (ATCC) were obtained from the INCQS/FIOCRUZ (National Institute for Health Quality Control, Brazil) and maintained in the Laboratory of Microbiology in the Pharmacy Faculty, Federal University of Pará/UFPA. The standard strains (ATCC) and the clinical isolate were kept in nutrient broth at room temperature.

For the tests, all strains were grown in Petri dishes containing media specific to each bacterium. For *S. aureus*, mannitol salt agar medium was used; for *E. faecalis*, nutrient agar was used; for *P. aeruginosa*, cetrimide agar was used, and for *Salmonella* sp., Salmonella-Shigella Agar was used. Then, all of the plates were incubated at 37°C for 24 h in a bacteriological incubator to induce the exponential growth after lag time.

Preparation of culture media

The culture media used were Mueller–Hinton broth (Merck, Germany), Mueller–Hinton agar (Merck), Cetrimide agar (Himedia, India), nutrient agar (Himedia), and mannitol salt agar (Himedia). These media were prepared from a dehydrated base commercially available and according to the manufacturer's instructions.

In vitro antimicrobial activity

Bacterial inoculum was prepared according to the recommendations to the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards, NCCLS). Briefly, 3 to 4 colonies of microorganisms, each 1 mm or more in diameter, from 24-hour at 25º C on subcultures were suspended in 1mL of Müller Hinton broth. The resulting suspension was mechanically mixed and the cell turbidity adjusted to correspond to a 0.5 McFarland standard. This procedure yielded a stock suspension containing 1×10^8 CFU/mL and then diluted until $1x10^3$.

The minimum inhibitory concentration (MIC) was evaluated for each of the above extracts according to the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards, NCCLS) microdilution protocol (M7-A6). For this, 180µl of bacterial inoculum was added in 96-wells plates (TPP/ISSO 9001/Switerland) with 20µl of the different extracts in concentration of 100µg/mL. Then, serial twofold dilutions were performed until the final concentration of 0,4µg/mL, and plates were incubated at 37 ° C for 24h. After this time, 20µL of 3 - (4,5-Dimethylthiazol-2-yl) -2,5-diphenytetrazolium bromide (MTT), a tetrazolium salt,

which is reduced by metabolically active cells to a coloured (Moismann, 1983), water-soluble formazan derivative, was added to the wells, to allow visual identification of metabolic activity. The final concentration of MTT after inoculation was 0.005% (v/v). After incubation, growth was indicated by the development of a blue colour and therefore the MIC was read as the lowest concentration of extracts at which no colour change occurred. After determination of the MICs, minimum bactericidal concentration (MBC) was determined by spreading 10 mL suspension from wells showing no growth on to Müller Hinton agar plates, which were then incubated as described previously and examined for 99.9% killing.

Control wells consisted of 180 µL the bacterial inoculum in 20µL of dimethylsulfoxide (DMSO), the solvent of the stock solutions of the four extracts to confirm the microbicide inactivity of the solvent in these solutions. Positive control was used for commercial antimicrobial chloramphenicol gram-positive bacteria, and gentamicin for gram-negative. The results were obtained for the visual inspection.

RESULTS

Antimicrobial activity evaluation of the hydroethanolic, hexanic, chloroformic and methanolic crude extracts from the seeds of Vatairea guianensis Aublet

To determine the MIC of each extract against each bacterium, the microdilution method with MTT (5 mg/mL) was used. The MIC results show that the hydroethanolic crude extract from the seeds of *V. guianensis* showed a MIC of 3.12 µg/mL and a MBC of 6.25 µg/mL against *S. aureus*.

The hexanic extract had a MIC of 6.25 µg/mL and MBC of 12.5 µg/mL against *S. aureus*. The chloroformic extract was more effective against *S. aureus*, with a MIC of 3.12 µg/mL and MBC of 12.5 µg/mL. For the methanolic extract, the MIC against *S. aureus* was 6.25 µg/mL, and the MBC was 12.5 µg/mL.

Against *E. faecalis*, the hydroethanolic and hexanic extracts both showed MICs of 12.5 µg/mL and MBCs of 25 µg/mL. The chloroformic extract was the most effective, with a MIC of 3.12 µg/mL and MBC of 12.5 µg/mL. The methanolic extract was least effective against *E. faecalis*, showing a MIC of 12.5 µg/mL and MBC of 50 µg/mL.

Against *Pseudomonas aeruginosa*, all extracts had MICs of 25 µg/mL. The hydroethanolic, hexanic, and chloroformic extracts showed MBCs at 100 µg/mL. Only the methanolic extract showed a MBC of 50 µg/mL.

Regarding the *Salmonella* sp., only the chloroformic and methanolic extracts were active, showing MICs of 50 µg/mL and MBCs of 100 µg/mL. It was not possible to determine the MIC or MBC of the hydroethanolic or hexanic extracts in the present study. The antimicrobial activity results are summarized in Table 1.

Table 1

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of the extracts of Vatairea guianensis seeds against four bacteria were determined by the broth microdilution method.

	Microorganisms							
Extract	***S. aureus***		***E. faecalis***		***P. aeruginosa***		***Salmonella* sp.**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Hydroethanolic crude extract	3.12	6.25	12.5	25	25	100	n.d.	n.d.
Hexanic extract	6.25	12.5	12.5	25	25	100	n.d.	n.d.
Chloroformic extract	3.12	12.5	3.12	12.5	25	100	50	100
Methanolic extract	6.25	12.5	12.5	50	25	50	50	100
Chloramphenicol	8.31	15.21	11.24	24.80				
Gentamicin					18.33	30.24	32.80	38.58

The numbers are shown as µg/mL. n.d.= not determined. MICs and MBCs are shown in µg/mL.

DISCUSSION

The medicinal plant *V. guianensis* is frequently used by the Amazon population, but few scientific studies have been conducted on the genus *Vatairea* and the species *V. guianensis*. We aimed to evaluate the *in vitro* antibacterial activities of hydroethanolic, hexanic, chloroformic, and methanolic crude extracts of the seeds of *V. guianensis* against *S. aureus (*ATCC 29213), *E. faecalis* (ATCC 29212), *P. aeruginosa* (ATCC 9027), and *Salmonella* sp. (clinical isolate).

The selection of the solvent used is important in the extraction of active compounds from medicinal plants. In this study alcohol was chosen because in popular practice, treatments using plants often involve soaking them in wine, grain alcohol, and brandy (*cachaça*). The other solvents were selected by increasing order of polarity, to evaluate more specifically the behavior of these extracts against the selected microorganisms, taking into account that the substances responsible for the biological activity could be present in the extracts due to similarity of solubility. Thus, solvents of low, medium, and high polarity were chosen: hexane, chloroform, and methanol, respectively. We emphasize that even though these solvents are not used in popular medicine, several antimicrobial substances with biological activity have been isolated from these extracts (Otobelli, 2009; Piedade and Filho, 1988).

The MIC results show that all extracts prepared from the seeds of *V. guianensis* have antimicrobial activity against all of the bacterial species selected.

Every extract showed activity against *S. aureus*, and the hydroethanolic and chloroformic crude extracts were noticeably more active, both with a MIC of 3.12 µg/mL, being more effective than chloramphenicol which was 8.31 µg/mL. However, the hydroethanolic crude extract showed the most effective MBC against *S. aureus*, showing good potential to be used in the production of herbal medicines for topical application against skin infections caused by Gram-positive bacteria, particularly *S. aureus.* This result suggests that the inhibitory effects of these extracts can probably be associated with the presence of phenolic compounds, particularly anthraquinones, polar substances that have been isolated from extracts of medium and high polarity, such as the methanol extract of the bark stem of *V. guianensis*, from which the anthraquinones chrysophanol, emodin, and physcion have been isolated (Piedade and Filho, 1998), and from other species of the genus *Vatairea* (Formiga, 1975). The anthraquinone chrysophanol can be highlighted as one of the substances most commonly isolated from the genus *Vatairea*. Anthraquinones isolated from the methanol extract of other species belonging to other genera of plants native to Mexico have confirmed antimicrobial activity (Sousa *et al.*, 2006). Most studies on the chemical composition of the genus *Vatairea* refer to the isolation of anthraquinones (Piedade and Filho, 1998, Matos *et al.*, 1998).

The fact that the hydroethanolic crude extract showed a stronger inhibitory effect against *S. aureus* partly justifies the reports of the popular use of *V. guianensis* seeds as antimicrobials to treat skin infections, as the seeds are popularly used macerated in alcohol, or "*in natura*" on the skin. Additionally, the results suggest that although the crude extract is a complex mixture of substances, the synergism among the substances is relevant to the antimicrobial activity. This finding is fully justified, because the therapeutic effects of many medicinal plant species are not only the result of isolated actions of a single chemical compound but the complex action of synergisms and antagonisms of various substances (Vasconcellos *et al.*, 2002).

The results of this study in relation to *S. aureus* are similar to the only study of antibacterial activity reported in the literature, but that study examined another species, *V. macrocarpa*. Acetone extract of *V. macrocarpa* showed marked antibacterial activity against *S. aureus* (Matos *et al.*,1998). Our data thus confirm those obtained by Matos et al. (1998) and suggest that the genus V*atairea* may have the antimicrobial potential against Gram-positive bacteria.

The hexanic and methanolic extracts also showed activity against *S. aureus*. Interestingly, only one report was found that used hexanic extract isolated from the bark of the fruit of *V. guianensis*, which contains two active compounds (in the process of patenting) that exhibit marked leishmanicidal activity against *Leishmania amazonensis* (Otobelli, 2009). Therefore, these results demonstrate that this medicinal species may be promising as antibacterials and antiparasiticals, taking into consideration that the active compounds isolated from the bark fruit by Otobelli et al. (2009) may also be present in seeds, opening the possibility for further studies that can identify and/or confirm such substances.

In general, the results in this study suggest that the antimicrobial activity of extracts of medium and high polarity in the genus *Vatairea* is due to high-polarity substances, such as phenolic anthraquinones.

The MIC test of the different extracts of *V. guianensis* against *Enterococcus faecalis* revealed that all extracts showed activity. However, the chloroform extract exhibited better performance than the others (Table 1). This result may suggest that the antibacterial activity of *V. guianensis* against *E. faecalis* can be partly attributed to phenolic compounds, probably anthraquinones extracted specifically by chloroform.

It is important to emphasize that the chloroform extract exhibited the same behavior against both Gram-positive microorganisms in the present study, which indicates that these microorganisms were more sensitive to the chloroform extract by the broth microdilution method. A significant amount of the chloroform extract's activity might also be related to the constitution of the cell wall of this class of bacteria, which is chemically less complex, with thick layers of peptidoglycans that are porous enough to permit the diffusion of products to the cytoplasmic membrane (Trabulsi *et al.*,1999).

Taken together, the results obtained in this study with different extracts against the Gram-positive microorganisms (which cause skin and nosocomial infections) should open new paths of research on this medicinal species in several areas, which could contribute to the potential clinical value of *V. guianensis* as a phytotherapeutic.

The results of the MIC tests against the Gram-negative bacteria *P. aeruginosa* and *Salmonella* sp. show that every extract inhibited the growth of these microorganisms. However, the methanolic extract performed better than the other extracts against *P. aeruginosa* and gentamicin was effective. Regarding *Salmonella* sp., it was possible to evaluate only the chloroformic and methanolic extracts, which showed significant activity.

This result is relevant because it relates to clinically important bacteria that cause serious nosocomial infections due to their high abundance in this environment and the facts that they possess high resistance to antibiotics and are typically opportunistic (Trabulsi *et al.* 1999). It is important to emphasize that the literature shows no records of antimicrobial activity of *V. guianensis*, or other species from this genus, against *P. aeruginosa* and *Salmonella*, indicating the unprecedented nature of this result.

Finally, the present results on *V. guianensis* antimicrobial activity might contribute to the study of this species and increase the interest in the genus, which remains poorly studied.

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