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Phytochemical profile and evaluation of antibacterial and cytotoxic activity of *Maytenus rigida* (Mart.) extracts and fractions

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ABSTRACT

Maytenus rigida (Mart.), is a caatinga plant species with wide distribution in Brazil. This study aimed to evaluate the phytochemical profile, and to determine antibacterial and cytotoxic activity of Maytenus rigida (Mart.). Phytochemical screening was performed with ethanolic crude extracts of leaf and stem bark. Antimicrobial activity was evaluated by Broth Microdilution Method to determine Minimum Inhibitory Concentration (MIC), against bacterial strains Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa and Salmonella enterica. Cytotoxic activity was obtained by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. In phytochemical screening, it was observed the presence of flavonoids, triterpenes, phenols, steroids, alkaloids, saponins and anthraquinones. The crude extracts of leaf and stem bark and acetate fraction presented inhibitory action front of S. enterica, S. aureus and S. epidermidis at tested concentrations. Ethanolic crude extract of leaf has proved being non-toxic at all, concentrations tested and ethanolic crude extract of stem bark presented toxicity with lethal significant effect front of J774 macrophages linage tested in the MTT assay, except at concentration of 1 µg/mL. Data obtained encourage new studies in order to isolate and test substances, which contribute to biological activity of this plant species, aiming a possible therapeutic application.

Keywords: *Maytenus rigida* (Mart.). Chemical composition. Cytotoxic activity. Antimicrobial activity.

INTRODUCTION

The use of plants for treatment of various diseases since the days of prehistory reveals that nature always constituted a major source of drugs for population [1]. In this context, plants are utilized in a traditional way due to population knowledge of certain plant properties by the use of plant species as a source of active molecules [2].

Experimental studies based on medicinal plants and other elements that act in the healing process and infection control are under development and this reaffirming the important attribution of nursing in the development of new technologies for treatment of wounds [3].

In the same form, plants that have antimicrobial activity are also of extreme importance due to the fact that many microorganisms present resistance, to not only already pre-set antibiotics, as well as the latest generation, causing an increasing problem of global public health [4, 5].

Caatinga is a biome with few studies in Brazil and a vegetation type of semiarid, occurring only in Brazil, almost exclusively in Northeast region [6]. Characteristic of Caatinga region, Celastraceae family has 88 genus and 1300 species distributed in tropical and subtropical weather [7].

Maytenus rigida (Mart.) is native from Brazil north-eastern and is found in Caatinga and savannah environments stands out from all other species of *Maytenus* for having a larger distribution throughout the Brazilian territory [8].

In the face of scientific evidence contained in literature on the presence of therapeutic compounds in this plant, it is appropriate to this study realization, with aim to perform phytochemical profile of ethanolic crude extracts and fractions of leaf, stem bark of *Maytenus rigida* (Mart.), and evaluate antibacterial and cytotoxic in vitro activity of these extracts.

EXPERIMENTAL SECTION

This was an experimental in vitro research, developed at Federal University of Alagoas, at Research Laboratory in Treatment of Wounds (LpTF), Laboratory of Medicinal Chemistry (LQM) and Laboratory of Pharmacology andImmunology (Lafi).

Vegetal Material

Maytenus rigida (Mart.) was selected according to ethnopharmacological criteria and leaves and stem bark were collected in August 2014 in a caatinga area of Olho d'Agua do Casado in Alagoas, Brazil. The plant material was identified by botanical Rosangela Pereira e Lyra Lemos and a sample is deposited in Herbarium Alagoas Institute of Environment (IMA-AL) under number of 45890.

Extract Preparation

After stem bark and leaves collection, they were desiccated at room temperature for 15 days and then grinded. The plant material was extracted with 96 % ethanol by macerating and dried in a rotary evaporator at a maximum temperature of 40 ° C with 120 rotations per minute, and then submitted to drying at room temperature, yielding crude ethanolic extract.

Fractionation of ethanolic crude extracts

It was utilized liquid-liquid partition for extraction of ethanolic crude extract and fractions of leaves and stem bark, consisting in the filtration of extracts on silica gel of increasingly by polar solvents [9].

For this process, an aliquot of 5 g of leaves and stem bark ethanolic crude extracts was partitioned into a chromatography column, utilizing as stationary phase silica gel and as the mobile phase, hexane (C_6H_{14}), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MeOH). The solutions obtained were concentrated in a rotary evaporator, resulting in four phases: hexane, chloroform, ethyl acetate and methanol.

Phytochemical Screening

The phytochemical screening was performed according to methodology proposed by Matos [9], utilizing ferric chloride to phenols and tannins; PH variation (values between 3-12) to anthocyanins, flavonoids, anthocyanidins, leucoanthocyanidins, catechins, flavanones, flavonols, flavanonóis and xanthones; Liebermann-Buchard reagent for steroids and triterpenoids; mechanical agitation for saponins; Dragendorff's reagent for alkaloids; and 10% KOH solution for anthraquinones, coumarins and anthrones.

Broth Microdilution Method

The ethanolic crude extracts and fractions were tested against standardized bacteria by American Type Culture Collection - ATCC / Manassas - VA/USA: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 14942), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus epidermidis* (ATCC 149990).

The protocol followed was based on the Clinical Laboratory Standards Institute [10]. Samples solubilisation were performed by blending 0.05 mg / ml of plant ethanolic extract and fractions added to 1 ml of Cremophor or dimethyl sulfoxide (DMSO) 2% and diluted in 4,9 mL of Mueller Hinton Broth, obtaining concentrations of 10,000 mg / mL. All well in plate received 100 µL of Mueller Hinton Broth and in columns 1 to 9 of A line was destined for growth control, which was just added to the microbial inoculum; 11 for the negative control, with Cremophor or DMSO 2% and 12 for Sterility Control, which was only used Mueller Hinton Broth.

To determine the MIC, bacteria samples were solubilized in a solution of 1,5 x 10^8 CFU/mL, with concentration according to standard of 0.5 in McFarland scale and subsequently diluted in 1:10 (v/v) to obtain the standard concentration (10^4 CFU/mL). Each well received 5 µl of bacterial inoculum, resulting in a concentration of 10^4

CFU/mL. After this period, 20 uL of 2,3,5-Triphenyltetrazolium chloride at 5 % was added in each well, and the plates Were again stored in bacteriological oven at 35 °C for 3 hours. The wells, which had red colour, indicated bacterial growth, while the original colour indicated inhibition of bacterial growth.

Cell line employed

J774 macrophages lineage were used for in vitro assay to determine cell viability. These were maintained in culture bottles in 10 mL of Roswell Park Memorial Institute Medium (RPMI) supplemented with 10 % fetal bovine serum in CO_2 incubator. In the moment of use, cells were counted and adjusted in RPMI supplemented with 10% FBS.

Cell viability assay

For the evaluation of samples cytotoxicity, was performed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay, according to Mosmann [11]. Cells were treated with extract and fractions submitted to antibacterial in vitro tests at concentrations of 1000 and 100 µg/mL, 10 µg/mL and 1 µg/mL for 48h, and maintained in an incubator at 5 % of CO_2.

In the period of 1 hour before adding Metiltetrazolium (MTT), three wells containing cells were lysed by Triton 100X (2 µL), for comparison of cell death. Control wells consisted of dead cells as a positive control and cultured cells plus the diluent DMSO 0,1 % as negative control.

After the total period of incubation (48h), supernatant was discarded and to each well was added 100 µL of a MTT solution (500 µg/mL) and reincubated for 1 hour in an oven at 37 °C and 5 % of CO_2. After this period, the supernatant was discarded and the precipitate resuspended in 100 µL of DMSO.

To quantify the reduced formazan salt, the plates were read with assistance of a microplate reader at a wavelength of 550 nm. To obtained the results, data were expressed as absorbance mean ± SEM and statistical differences between the treated groups and the control were analysed by ANOVA and Dunnett test, where significance levels compared to the negative control group were identified by asterisk (*p<0,05; **p<0,01; ***p<0,001) in comparison to control groups.

RESULTS AND DISCUSSION

The results of phytochemical screening in both ethanolic extract from stem bark and leaves of *Maytenus rigida* (Mart.) (Table 1) indicated the presence of phenols, flavonoids and free steroids, and anthraquinones, alkaloids and saponins in ethanolic crude extract of leaves. In the stem bark, it was observed the presence of flavonoids, triterpenes and saponins, phenols, alkaloids and anthraquinones.

TABLE 1. Phytochemicals assays of ethanol extracts of stem bark and leaves of *Maytenus rigida* (Mart.)

Phytochemical Screening		
	Samples	
Secondary Metabolites	Leaves	Stem bark
Phenol	+	+
Tannin	-	-
Flavonols	+	+
Free steroids	+	-
Triterpenes	-	+
Free steroids	+	+
Alkaloids	+	+
Anthraquinones	+	+
Anthrones	-	-
Coumarins	-	-

Note: (-) Absent; (+) Present.

In previous studies, Estevam et al. [12] traced phytochemical profile of *Maytenus rigida* bast, which revealed presence of triterpenes, but also other classes of substances as catechin, quinones, steroids, saponins, flavonoids, and phenolic compounds. However, there were no substances class as alkaloids, tannins, glycosides, resins, chalcones, fixed acids and leucoanthocyanidins.

Maytenus genus is chemically characterized by presence of triterpenes, alkaloids, flavonoids and tannins [13, 14]. According to Pessuto [15], *Maytenus ilicifolia* leaves have tannins. In a study conducted by Tiberti et al. [16], *Maytenus ilicifolia* and *Maytenus aquifolium*, revealed the presence of flavonoids in their leaves.

With regard to flavonoids class, which was observed in leaves and stem bark are present flavones, flavonols, xanthones, flavononas and flavanonóis (Table 2). In this way, flavonoids such as, for example, flavones, flavonols, flavonones, and isoflavones, as well as some of its derivatives have demonstrated antibacterial activity [17].

TABLE 2. Class of flavonoids present in the ethanolic extracts of *Maytenus rigida* (Mart.) stem bark and leaves

Flavonoid class	Ph	Leaves	Stem bark
Anthocyanins/Anthocyanidins	3	-	-
	8,5	-	-
	11	-	-
Flavones/Flavonols/Xanthones	11	+	+
Flavanoids	11	+	+
Leucoanthocyanidins	3	-	-
Catechins	3	-	-
Flavonones	11	+	+

Note: (-) Absent; (+) Present.

Antimicrobial activity of plant extracts can be assessed by determination of a substance small amount necessary to inhibit microorganism growth under test MIC [18].

In presented study, the results of antimicrobial activity evaluation of studied plant related to ethanolic crude extract of leaf and stem bark and ethyl acetate fraction of stem bark, front of standard strains *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 14990), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella enterica* (ATCC 14028) are in Table 3.

TABLE 3. Determination of Minimum Inhibitory Concentration - MIC (µg/mL) of ethanolic crude extracts front tested microorganisms

Minimum Inhibitory Concentration - MIC **(µg/mL)**			
Bacterial Strains	**Ethanolic crude extract of leaf**	**Ethanolic crude extract of stem bark**	**Ethyl acetate fraction of stem bark**
S. aureus	NI	NI	2.500µg/mL
S. epidermidis	5.000µg/mL	2.500µg/mL	1.250µg/mL
P. aeruginosa	NI	NI	NI
S. entérica	NI	2.500µg/mL	2.500µg/mL

Note: NI: not inhibited; S. aureus: Staphylococcus aureus; S. Epidermidis: Staphylococcus epidermidis; P. aeruginosa: Pseudomonas aeruginosa; S. enterica: Salmonella enterica; µg: microgram; mL: milliliter.

According to tested bacteria, *Maytenus rigida* ethanolic crude extract of leaves has not established inhibitory action in front of the strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella enterica* at 10.000 µg/mL. Front of *Staphylococcus epidermidis*, ethanolic crude extract of leaves showed inhibitory action with a MIC of 5.000 µg/mL.

This result diverges from the study of Ahmed [19] suggesting that these crude extracts and fractions of different polarities of *Maytenus senegalensis*, *Maytenus peduncularis*, *Maytenus undata* and *Maytenus procumbens* tested exhibited antimicrobial activity front of *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli.*

Stem Bark ethanolic crude extract has not provided inhibitory action front of the strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In the face of strains of *Staphylococcus epidermidis* and *Salmonella enterica*, stem Bark ethanolic crude extract had inhibitory action with a MIC of 2.500 µg/mL for both bacteria.

Based on results of the present study about antimicrobial potential of *Maytenus rigida*, ethanolic crude extract of the stem bark and leaf was submitted to liquid-liquid partition process in order to obtain semi-purified fractions for a better evaluation of activity antimicrobial.

However, fractions of hexane, chloroform, ethyl acetate and methanol of *Maytenus rigida* leaves and stem bark did not present an antimicrobial activity against all the tested strains, except for ethyl acetate fraction of stem bark, which showed an MIC of 2.500 µg/mL for strains of *S. enterica* and *S. aureus*, and MIC of 1.250 µg/mL for *S. epidermidis.*

The activity presented by ethyl acetate fraction front *S. aureus* corroborates with Santos [20] in a recent study, so ethyl acetate phase of stem bark of *Maytenus rigida* had activity against strain of *Staphylococcus aureus*. However, this same sample presented no activity against strains of *E. coli*, *P. aeruginosa* and *Salmonella* sp.

In another study conducted by Estevam et al. [12], ethanolic crude extract of *Maytenus rigida* bast presented antimicrobial activity for both Gram positive bacterium *Staphylococcus aureus* and for Gram-negative *E. coli.*

Some species of *Maytenus* as *Maytenus krukovii* did not demonstrate significant antimicrobial activity [21]. While others, such as *Maytenus macrocarpa*, exhibit activity against Gram positive and Gram-negative bacteria [22]. The variation for the presence of antimicrobial activity may be related not only to plant characteristics, as well as to characteristics of the tested strains and analytical methodology used [20].

Although the plants possess many therapeutic practices, which are popularly, known by people, human being unaware the fact that they can cause toxicity both to man and animals [23, 24].

In this form, the toxicological approach becomes an important issue with regard to natural products, so should not be considered a medicinal plant or herbal medicine offers immediate effect and easily correlated with their intake, but also effects that are installed in long-term and asymptomatically, such as carcinogenic, hepatotoxic and nephrotoxic [25].

Before approving any new compound for testing in humans, toxicity tests are performed in vitro and in vivo systems in several animal species. These bioassay techniques assess and determine the concentration of a substance, which is able to produce a biological response [26].

Cytotoxicity of ethanolic crude extract of leaves and stem bark of *Maytenus rigida* was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay, front of J774 macrophages at concentrations of 1000 µg/mL, 100 µg/mL, 10 µg/mL and 1 µg/mL, as presented in Table 4.

TABLE 4. Cytotoxicity determination of ethanolic crude extract of leaf and stem bark of *Maytenus rigida* through the means of absorbance compared to control group front J774 macrophages (MTT assay)

	Cell viability by colorimetric test			
	Concentrations			
Samples	1000µg/mL	100µg/mL	10µg/mL	1µg/mL
DMSO	1.112±0,008	1.607 ±0,23	1.607±0,23	1.607±0,23
Medium	1.115 ±0,19	1.195 ±0,04	1.195±0,04	1.195±0,04
Leaf	1.218 ±0,01	1.115±0,31	0.822±0,01	1.671±0,58
Stem bark	0***	0.592±0,43*	0.407±0,20**	2.170±0,06

*Note: DMSO: Dimethyl sulfoxide. Results are expressed as means ± standard error of absorbance; *P<0,05 ; ** P <0,01; *** P <0,001 compared with control group.*

While analysing the average absorbance, the standard error and the significance level of cytotoxicity when compared to negative control (DMSO control group), ethanolic crude extract of leaf presented no lethal effect for J774 macrophages in all concentrations tested.

However, ethanolic crude extract of stem bark presented statistically significant differences with * p <0,05 ** p <0,01; *** P <0,001, demonstrating cytotoxic activity at all tested concentrations, except at concentration of 1 µg/mL. After reading in a spectrophotometer, the optical density value when compared with the control at concentration of 1000 µg/mL was zero. At this concentration, no metabolization of MTT was observed due to death of all cells death when in contact with the sample in question.

From the results, it is possible to verify that ethanolic crude extracts of stem bark present detrimental effect in relatively low dosage, while ethanolic extract of leaf may be a safe therapeutic source in respect to cytotoxicity.

Several species of *Maytenus* have proved potentially cytotoxic when tested against cell lines of human tumours, such as *Maytenus retusa* [27], *Maytenus chiapensis* and *Maytenus cuzcoina* [28] and *Maytenus ilicifolia* [29]. This result corroborates with the findings about cytotoxicity of ethanolic extracts of stem bark in this study.

Over the centuries, plants with toxic effects played an important role as a source of active substances, able to provide molecular models for the development of new drugs. [25]. However, it is essential to know the plants cytotoxic effect, especially those that are used by the population, in order to prevent poisoning, and significant harm to public health.

CONCLUSION

The presented study related to *Maytenus rigida* (Mart.) demonstrated that this plant species is rich in secondary metabolites, which contribute to antimicrobial activity presented by crude extracts of leaf and stem bark and ethyl acetate fraction of stem bark against *S. aureus* and crude extracts of stem bark and ethyl acetate of bark against *S. enterica* and cytotoxic activity presented by the crude extract of stem bark in all tested concentrations except 1 µg/mL. Data obtained encourage new studies with *Maytenus rigida* (Mart.) to determine the substances, which contribute to biological activity and understand its mechanism of action, targeting a possible pharmaceutical application.

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