

## Antifungal activities of extract ethanolic from the barks of *Maytenus guianensis* klotzsch ex reissek on *Candida albicans*

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**ABSTRACT.** The present work aimed the evaluation of antifungal activity of the ethanolic extract of the barks of *Maytenus guianensis* against *Candida albicans* *in vitro*. Collected barks were properly dried and grinded, being subjected to Soxhlet extraction with ethanol p.a, after dilution with DMSO (2%). For the evaluation of biological activity against the fungus, agar well diffusion technique was used in the concentration of 1mg.mL<sup>-1</sup>, which was previously defined by the Minimum Inhibitory Concentration (MIC) technique performed. *Candida albicans* yeasts were cultured in BDA medium during 24 hours with turbidity absorbance. For negative control, only the BDA medium was used; Positive control was performed with the emulsifier chemical Kasumin®. The design was totally randomized, with three replicas per treatment. The evaluation consisted in assessing inhibition halos of fungal growth, each 24 hours, during five days. After 120 hours, the ethanolic extract from the barks of *M. guianensis* presented results of growth inhibition halos of 1,52 mm against *C. albicans*, demonstrating a larger inhibitory spectrum, if compared to negative control (inhibition halos of 2,11 mm). The results highlight the antimicrobial potential of *M. guianensis*, which may be promising for drug development research.

**Keywords:** Celastraceae; triterpenes; phytochemistry.

### Atividade antifúngica do extrato etanólico das cascas de *Maytenus guianensis* klotzsch ex reissek sobre *Candida albicans*

**RESUMO.** O presente trabalho teve como objetivo avaliar a atividade fungicida do extrato etanólico das cascas de *Maytenus guianensis* sobre *Candida albicans* *in vitro*. As cascas coletadas foram devidamente secas e trituradas, sendo submetidas à extração em aparelho de Soxhlet com etanol p.a., sendo posteriormente diluídas com DMSO a 2% e para avaliar o potencial biológico sobre o fungo, utilizou-se à técnica de difusão em ágar em poços na concentração de 1mg.mL<sup>-1</sup>, concentração esta já definida pela técnica de Menor Concentração Inibitória (MIC) realizada. Leveduras de *C. albicans* foram cultivadas em meio BDA durante 24 horas com a absorbância de turvação. Para o controle negativo, utilizou-se somente o meio BDA; controle positivo foi realizado com emulsificante Kasumin®. O delineamento foi inteiramente casualizado, com três repetições por tratamento. A avaliação consistiu em verificar os halos de inibição de crescimento fúngico, a cada 24 horas, durante cinco dias. Observou-se, que após 120 horas, o extrato etanólico das cascas de *M. guianensis* apresentou resultados dos halos de inibição sobre *C. albicans*, na qual os halos de inibição de crescimento foram de 1,52 mm do extrato etanólico, demonstrando maior espectro inibitório, se comparado com o controle negativo que foi de 2,87 mm, enquanto que no controle positivo o halo de inibição foi de 2,11 mm. Os resultados sinalizam o potencial antimicrobiano dessa planta, podendo ser promissoras para estudos de desenvolvimento de novos fármacos.

**Palavras-chave:** Celastraceae, triterpenos, fitoquímica.

### 1. Introduction

Medicinal plants have been increasingly used by industrialized societies, not only by its curative power, but also by its economic availability (DUTRA, 2009). Brazil has the greatest plant diversity of the world, with an estimated number of over 20% of the total species of the planet. With more than fifty thousand species described, corresponding to 22% of the total, this rich biodiversity is accompanied by a wide acceptance of medicinal plant use and traditional knowledge associated to. Approximately 48% of the medicines utilized in therapeutics are originated directly or indirectly from natural products, especially medicinal plants (CARVALHO et al., 2007).

Thereby, medicinal plant users of various parts of the world maintain the practice of phytotherapies consumption, making valid the therapeutic informations that were accumulated during centuries (LUBIAN et al., 2010).

Celastraceae family comprises about 98 genera and 1.264 species and can be found all over the Brazilian territory (FONSECA et al., 2007; OLIVEIRA et al., 2006).

There are many studies demonstrating the active compounds of biological interest are associated to flavonoids, sesquiterpenes, alkaloids and pentacyclic triterpenes (GONÇALVES et al., 2005; MICHELIN et al., 2005; OLIVEIRA et al., 2006).

The genus *Maytenus*, is the biggest genus of the family Celastraceae, comprising about 80 species (OLIVEIRA et al., 2006) and among the biological activities attributed to these species we can cite antinociceptive, antifungal (CUNICO et al., 2006), antioxidant (MAGALHÃES et al., 2011), anticytotoxic and antimutagenic (MENEQUETTI et al., 2014), antiplasmodial activity (BAY-HURTADO, 2013) and genotoxicity (MENEQUETTI et al., 2015).

Among the species with medicinal properties, *Maytenus guianensis* is a small tree endemic of Amazon, and popularly known as chichuá, xixuá and (SOUZA; LORENZI, 2008).

Its roots and stems are used as analgesic, anti-inflammatory, afrodisiac, muscular relaxant, antirheumatic and antidiarrheal, being also indicated in the treatment of arthritis, impotence, chills, bronchitis, haemorrhoidae,

helminthiases, lumbago, external ulcerations and gynecological uses and it is used as a cosmetic in cutaneous eruptions and prevents skin cancer (DUKE; VÁSQUEZ, 1994; REVILLA, 2002; BORRÁS, 2003).

The secondary metabolism of Celastraceae family presents as one of the most versatile of the known botanical families, wherein these metabolites accumulated by species of this family of plants that are characterized by its mixed biosynthetic origin (chiquimate/mevalonate), resulting in the production of amides or aromatic compounds essentially phenylpropanoidics of the lignane type and neolignanes; they are also characterized by the occurrence of terpenes, flavonoids and other classes of natural products (FAZOLIN et al., 2006).

Secondary metabolites, due to its roles on the interactions between organisms, will most often possess biological activities. Many of these compounds have a great significance in the pharmaceutical area, because they represent a promising source for the discovery of new molecules that are useful to humans (GOBBO-NETO; LOPES, 2007).

The genus *Candida* are normally found constituting the human normal microbiota, in which it is present in the mouth and digestive tract mucosas of healthy individuals, being capable of initiate an onset of infections, which are called candidiases, principally in people presenting factors that predispose them to this infection, like hormonal factors and low immune resistance (KHAN et al., 2012; KIRAZ; YASEMIN, 2011).

*Candida albicans* is the most common pathogen in cutaneous and oropharyngeal candidiasis, however the non-*albicans* species has raised in numbers and in importance in the vaginal and sistemic candidiases (KHAN et al., 2012).

Due to the occurrence of unwanted effects, such as the resistance of some strains to the conventional drugs principally in immunosuppressed individuals and the presence of toxic effects related to these drugs, the study of plants with therapeutic properties, embracing those species with antifungal activity has been increasingly growing, so, such study is justified not only by the possibility of a determined plant species constitutes an alternative resource, but also due to the perspectives related to the isolation of substances that presents significant efficacy, with fewer indexes of disadvantages (ARAÚJO et al., 2004).

Considering this reality, due to the shortage of works related to this area, this work aimed to assess the presence of secondary metabolites of the barks of *Maytenus guianensis*, that could be used for pharmaceutical purposes. In order to assess the presence of metabolites, a phytochemical analysis of the ethanolic extract was done, with the purpose of evaluate its antifungal potential against *C. albicans in vitro*.

## 2. Materials and Methods

The phytochemical study of the barks of *Maytenus guianensis* was performed at Laboratório de Pesquisa em Química de Produtos Naturais (LPQPN) of Universidade Federal de Rondônia (UNIR), on Porto Velho-RO.

Barks (2,3 kg) of *M. guianensis* were collected at Reserva

Florestal Adolpho Ducke, located at km 26 of Estrada Manaus-Itacoatiara (AM-010) on Manaus, state of Amazonas. The botanical identification was made by the dispatch of an voucher specimen (record number 188.48) at Herbarium of Institute National of research in Amazon (INPA).

Barks properly dried and grinded (300g) were subjected to extraction in a Soxhlet extractor using hexane (MGFH), chloroform (MGFC), ethyl acetate (MGFAcEt), methanol (MGFE) and ethanol (MGFE). After the evaporation of the solvent, the extract yielded 12,84g of MGFH, 11,25g of MGFC, 18,75g of MGFAcEt, 20g of MGFM, and 28g of MGFE. We used the ethanol extract to present more material.

The isolation and purification of the chemical constituents of the barks of the ethanolic extract were performed by gas chromatography, using as bonded phase silica gel of Merck and Vetec ( $\mu\text{m}$  63-200). The length and diameter of the column varied according to the amounts of the samples and of silica to be used. For the Thin Layer Chromatography (TLC) were used chromatoplates of silica gel 60 ( $\mu\text{m}$  63-200). Above polyester T - 6145, Sigma Chemical CO (with fluorescence label of 250 nm).

The solvent used in the chromatographic elutions were: hexane, ethyl acetate and methanol, pure or combined in increasing degree of polarity. The disclosure of the chromatographed substances in CCD was made by exposition of the analytic chromatoplates to ultraviolet light (UV), reveled in the wavelength of 254 nm and by pulverization in an universal developed (mix of ethanol: acetic acid: sulphuric acid - 80:10:10), followed of warming in an incubator at 100°C, by approximately five minutes.

The mass spectra were obtained by electron impact (70 Ev) in a GC/MS Hewlett - Packard 5971 device using capillary column (30 m x 0,25 mm) dimethylpolysiloxane BD-1, having He as drag gas and the temperatures of 250°C in the injector, 200°C in the detector and in the column varying 1°/min between 35-180°C and 10°C/min in the range of 180-250°C.

The structure elucidation of the chemical constituents isolated from the bark of *M. guianensis* were Facundo et al. (2015).

After, the MGFE was used for the antifungal tests that were performed at the microbiology laboratory of Center of Teaching São Lucas, wherein the disk diffusion technique was used, in which disks of five mm of diameters of *C. albicans* culture (ATCC 10.231), were put in the center of Petri plaques containing Potato Dextrose Agar (PDA) medium, in the peripheral area of the plaques, four disks of filter paper were organized symmetrically, and were plunged in 1mg.mL<sup>-1</sup> of plant extract during 1 minute, resulting in 0,12mL of extract to each disk. For the negative control, disks plunged in distilled water were used, and for the positive control, the chemical Kasumin<sup>®</sup> and ethanol (solvant control) were used on the concentration of 1mg. mL<sup>-1</sup>, this concentration was based on the results of wells technique of the experiment is determined by the lowest inhibitory concentration (MIC) (LIMA et al., 2016).

After this process, the plaques were incubated at 25°C during five days. The evaluation consisted in measuring the diameter of the colonies (the mean of two steps that are diametrically opposed to each other) initiated after 24

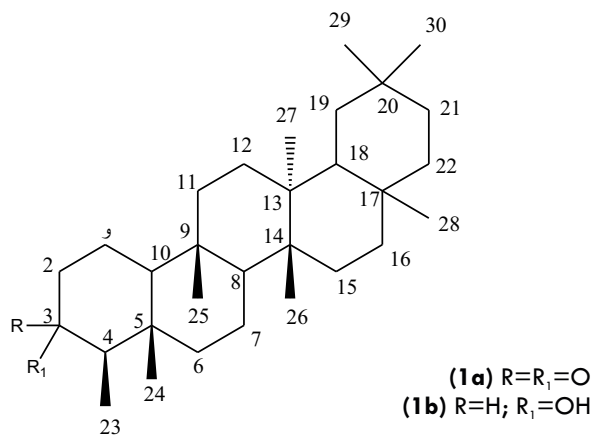
hours of incubation, lasting five days, that is, until the time the fungal colonies of the witness treatment reached the whole surface of the plaque. The statistical design was entirely randomized, wherein there was three repetitions per treatment. The data were subjected to analysis of variance (ANOVA) and the Tukey test, in which  $p < 0,05$  was considered significant. The analysis was done from the software Graphad Prism 5.0.

The emulsificant chemical Kasumin<sup>®</sup>, used in this work, was used for the comparison with our results, because it is a non-ionic surfactant, antifungal, antibacterial and systemic antibiotic, that has been used as a dispersant agent in the preparation of solutions, resulting in a more reliable procedure for the preparation of the inoculum. However, the surfactants might interact with organisms and drugs, affecting the *in vitro* activity of antimicrobial agents (NASCIMENTO et al., 2008).

### 3. Results and Discussion

The MGFE extract, which obtained the highest yield, was subjected to silica gel chromatography, and hexane, chlorophorm and acetone were used as solvents. After the evaporation of the solvents, the following yields of eluates were obtained: hexanic eluate (MGFEH, 9,1 g), chlorophormic eluate (MGFEC, 3,2 g) and acetone eluate (MGFEAC, 93,0 g). The MGFEAC eluate (45,0 g) was subjected to silica gel chromatography with hexane and chlorophorm in increasing polarity, thus yielding 45 subfractions.

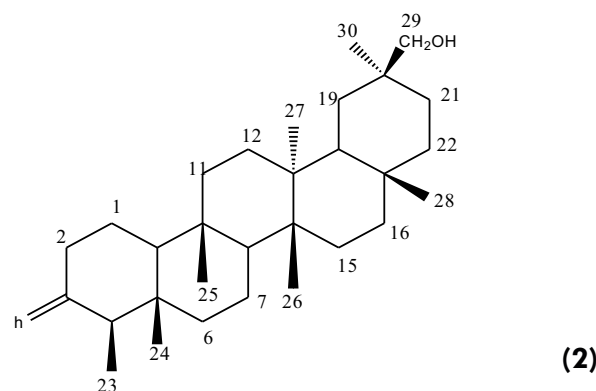
Repeated chromatographic procedures led to the isolation of four substances which were called **MGFEAC-1**, **MGFEAC-2**, **MGFEAC-3** and **MGFEAC-4**. The substance named **MGFEAC-1** appeared as a white amorphous solid, soluble in chlorophorm, and when it was treated with acetic anidride and concentrated sulphuric acid (Liebermann-Buchard test), it displayed a red coloration, so confirming the triterpenic nature of this compound. All of the spectroscopic data of RMN-1H and RMN-13C, uni and bidimensional, M.E. and I.R., revealed that MGFEAC-1 is a mixture of two triterpenes of the friedelane class, which were already isolated from other *Maytenus* species and named **MGFEAC-1a** friedelina and **MGFEAC-1b** friedelanol (Figure 1) (FACUNDO et al., 2015).



**Figure 1.** Structure of the triterpenes friedelina (1a) and friedelanol (1b). / **Figura 1.** Estrutura do friedelina triterpenos (1a) e friedelanol (1b).

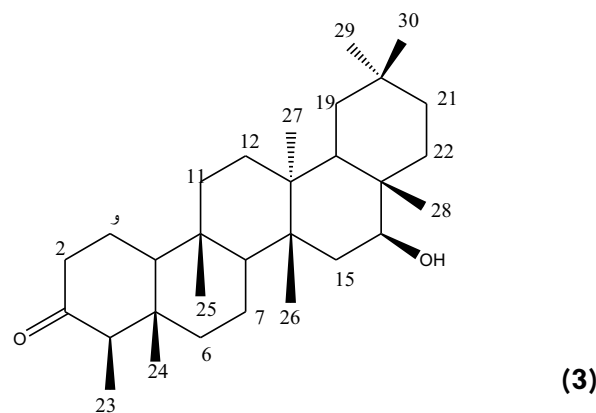
Phytochemical studies performed in plants of the genus *Maytenus*, revealed these two triterpenes can be considered chemosystematic markers of the genus (NOSSACK et al., 2000).

The compound MGFEAC-2 appeared as amorphous white solid, soluble in chlorophorm, melting point of 268-269 °C, thin Layer Chromathography revealed only one spot and presented positive test for triterpenes when it was treated with Liebermann-Burchard reagent. The spectroscopic data of RMN-1H and RMN-13C, uni and bidimensional, M.E. and I.R., demonstrated the substance is a pentacyclic triterpene of the friedelane class. It is about the triterpene 29- hidroxyfriedelan-3-one (2) (Figure 2), already isolated in other plants of the genus *Maytenus* showing signs assigned to the carbonyl group of friedelanol class (BAY-HURTADO, 2014).



**Figure 2.** Structure of the triterpene 29- hidroxyfriedelan-3-one. / **Figura 1.** Estrutura do triterpeno 29- hidroxifriedelan-3-ona.

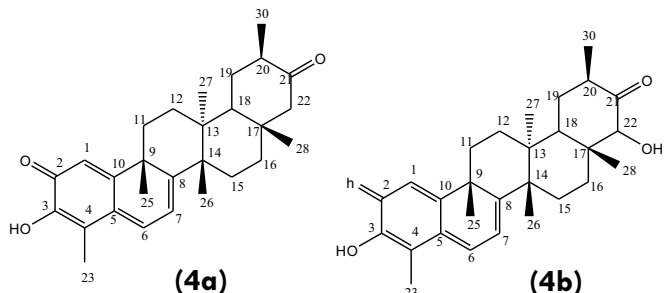
The compound named **MGFEAC-3** appeared as amorphous white solid, soluble in chlorophorm, melting point of 278-280 °C, presented only one spot when analyzed by thin layer chromatography and presented positive test for triterpenes when it was treated with Liebermann-Burchard reagent. The spectroscopic data of RMN-1H and RMN-13C, uni and bidimensional, M.E. and I.R., demonstrated the compound is the triterpene 16-hidroxyfriedelan-3-one (3) (Figure 3).



**Figure 3.** Structure of the triterpene 16β-hidroxyfriedelan-3-one. / **Figura 3.** Estrutura do triterpeno 16β-hidroxifriedelan-3-ona

The compound **MGFEAC-4** appeared as yellow crystals, soluble in chlorophorm, unknown melting point, analysis in thin layer chromatography revealed only one stain and presented positive test for triterpenes when treated with

Liebermann-Buchard reagent. Despite this compound has showed only one stain in TLC, the spectral data demonstrated it is a mixture of two quinonamethide triterpenes, named tingenine (**4a**) and tingenone (**4b**) (Figure 4) (FACUNDO et al., 2015), isolated from other plants of the genus *Maytenus*. Quinonamethide triterpenes are secondary metabolites whose occurrence is limited to species of the family Celastraceae (CORSINO et al., 2000).



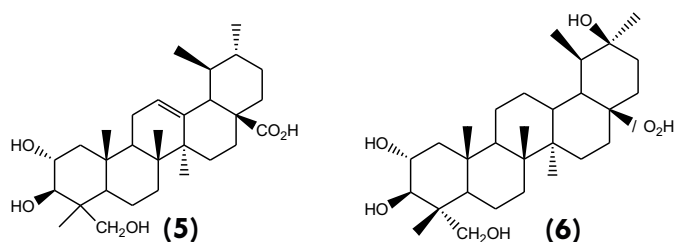
**Figure 4.** Structure of the following quinonamethide triterpenes tingenine (4a) e tingenone (4b). / **Figura 4.** Estrutura da sequência de “quinona-methide” triterpenos tingenina (4a) e tingenona (4b).

Phytochemical assays, coupled with gas chromatography linked to mass spectrometer of the extract MGFE, revealed the presence of other quinonamethide triterpenes, besides tingenine (4a) and tingenone (4b). In following steps to this research, new chromatographic procedures are being performed in order to lead to the isolation of other quinonamethide triterpenes of this extract. We also observed in these assays, the presence of substances belonging to the flavonoid class.

Pentacyclic triterpenes are of great importance due to various biological activities they presents, serving as candidates or prototypes of new drugs (ALVARENGA; FERRO, 2006). Studies with friedeline (1a), (Figure 4) indicated the antiproliferative activity, proapoptotic (MARTUCCIello et al., 2010), anti-inflammatory, analgesic and antipyretic (ANTONISAMY et al., 2011).

According to studies carried out in Germany, pentacyclic triterpenes are responsible by the activity observed in the treatment of actinic keratosis, a wound on the skin caused by the sun which is characterized by the presence of reddish or slightly brownish areas with a harsh surface, indicates the pentacyclic triterpenes are the substances responsible by the activity observed (HUYKE et al., 2006).

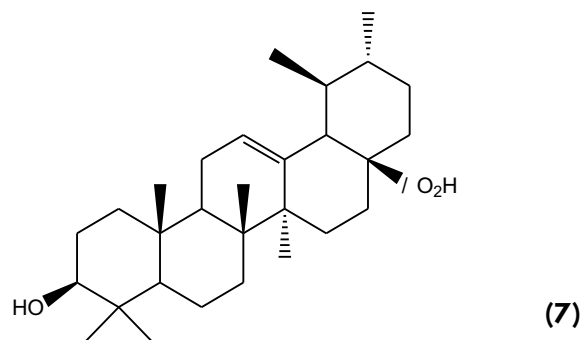
Asiatic (**5**) and madasiatic acid (**6**) (Figure 5) are components of a magistral preparation knowed as Madécassol<sup>®</sup>, which can be used topically and internally, used as wound healer in burns and in the treatment of chronic venous insufficiency (JAMES et al., 2009).



**Figure 5.** Structure of the madasiatic acid (5) and asiatic acid (6). / **Figura 5.** Estrutura do ácido asiático louco (5) e ácido asiático (6).

Another triterpene that has received attention is the

ursolic acid (**7**) (Figure 6). A study performed at University of Iowa, United States, found the ursolic acid reduces muscle atrophy, fats, glycemia, cholesterol and triglycerides, besides promoting muscle growth.



**Figure 6.** Structure of Ursolic Acid (7). / **Figura 6.** Estrutura do ácido ursólico (7).

Quinonamethide triterpenes are compounds that, until now, had only been isolated from species of the family Celastraceae (CORSINO et al., 2000). Tingenone (4b) (Figure 4) is a quinonamethide triterpene, isolated from various plants of this family, including *Maytenus acanthophylla* (OLIVEIRA et al., 2006). Recent studies carried out by Silva and coworkers (2013), indicated the tingenone possesses great activity against *Microcystis novacekii*, a species of cyanobacterium that possesses a great capacity to form blooms and produce toxins, denominated microcystins, which are involved in environmental accidents, and are responsible by the majority of cases of animal and human intoxication.

The triterpenes found in this work may be originated from saponins, since the saponins possess both lipophilic (triterpenes or steroids) and hydrophilic components in its structure, a property that is related with the reduction of the surface tension of the water and its action as detergent and emulsificant. Triterpenes have shown great potential in some biological activities: are anti-inflammatory, antibacterial, antifungal, antiviral, analgesic, cardiovascular, antitumor (IKEDA, 2008).

An important factor to be taken into account when research involving medicinal plants is carried out, and which is usually also neglected, are the environmental conditions established at the time of the collection of the plant, like seasonality, weather, type of soil and air temperature. According to Freitas et al. (2004), the production of secondary metabolites by the plant organism works according to the interaction of the plant with its environment in response to chemical and biological factors. It may explain divergent results of extracts of the same species, when specimens of this species are collected in different locations and seasons.

In respect to the antifungal potential, we verified the ethanolic extract of the bark of *M. guianensis* presented inhibition on *C. albicans*, in which in the end of 120 hours, the growth mean of the fungi colonies in which the plant extract was used was 1.52 mm; in the negative control, using the sterile distilled water, the mean was 2.9 mm; using ethanol, the mean was 2.11 mm, whilst in the positive control, using the chemical, the mean inhibition was 2.87 mm (Table 1).



**Table 1.** Mean inhibition of the growth (mm) of the fungus *C. albicans* subjected to exposition to the plant extract of the bark of *M. guianensis* in vitro during 120 hours. / **Table 1.** Média de inibição do crescimento (mm) do fungo *C. albicans* submetido a exposição ao extracto vegetal de casca de *M. guianensis* in vitro durante 120 horas.

Treatments	Hours					Means
	24	48	72	96	120	
Plant Extract	1,36	1,53	1,53	1,6	1,6	1,52±0,59
Positive control	2,66	2,83	2,83	3,03	3,03	2,87±0,29
Negative control	1,5	2,4	2,7	3,3	4,6	2,9±0,25

Statistically significant relative to distilled water \*  $p < 0,05$ ; statistically significant relative to the chemical #  $p < 0,05$ .

According to the statistic analysis above, one can be seen that the results using the plant extract were significant in comparison to distilled water, noting a satisfactory result, however it was not satisfactory as the result of that of the chemical, and because of it, a difference relative to the chemical can also be observed, but the results are encouraging since the extract tested was the crude extract, being indicated further studies with secondary metabolites, for a better evaluation against *C. albicans*. Another important point is that the extract used showed better results than that of the ethanol, since the ethanol did not have significance relative to distilled water.

Similar results were found by Annan et al. (2009) using friedeline, also isolated in the present work, from the methanolic extract of *Paullinia pinnata* L. roots against *S. aureus* and MRSA using the MIC on the concentration of 256 µg/mL relative to positive control using tetracycline (MIC= 128 µg/mL). However, other authors suggest that biological assays aiming toxicity at different concentrations must be carried out.

Plants of this family were widely investigated as a source of secondary metabolites and it was found they may be powerful relative to its insecticide and antifungal activities (SANTOS et al., 2014).

The antimicrobial activities were described for pentacyclic triterpenes, such as oleananes, ursanes e friedelane lupanes. It is speculated the mechanism of action of triterpenes is due to membrane lysis of cellular microorganisms. For this purpose, the hexanic extract and three oxofriedelane-triterpenes (1), 3-oxo-12 $\alpha$ -hydroxyfriedelane (3), 3,16-dioxofriedelane (5) e 3-oxo-12 $\alpha$ , 29-dihydroxyfriedelane (6) of *M. gonoclada* were tested against the pattern of two lineages of *Escherichia coli* bacterium, *Citrobacter freundii*, *Bacillus cereus* and against the yeast *Candida albicans*, using the disk-diffusion test (AWANCHIRI et al., 2009).

Many of the secondary metabolites present in the family Celastraceae, presents pronounced biological activities, in which are included antifungal activities (MARQUES et al., 2007).

The results similar were of Lima et al. (2016) together with the data described in the literature, clearly show that the *M. guianensis* species is a rich source of triterpenes from the classes of friedelan and quinone-methide, and has various biological activities, provides the first reports of results that demonstrate the effectiveness of substances, obtained from *M. guianensis*, on four ATCC bacteria, evaluated using the minimum inhibitory concentration test,

ranging, in most, from 250 to 1.95 µg/mL concentrations.

#### 4. Conclusion

The results of this study showed that *Maytenus guianensis* is a rich source of triterpenes of the friedelane and quinonamethide classes. According to data on literature cited along this manuscript, the triterpenes belonging to these classes has shown several biological activities. Furthermore, it was found the ethanolic extract inhibited the growth of *C. albicans*, relative to the chemical. However, other concentrations and methodologies using new isolated compounds from *M. guianensis* must be tested in order to improve the comprehension of the fungus-plant relationship.

#### 5. Acknowledgments

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