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Ruta graveolens, *Pelargonium graveolens* AND *Hibiscus cannabinus* AS NATURAL INHIBITORS OF GROWTH OF *Candida albicans*

Ruta graveolens, *Pelargonium graveolens* e *Hibiscus cannabinus* COMO INIBIDORES NATURAIS DO CRESCIMENTO DE *Candida albicans*

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ABSTRACT

Candidiasis is an infection caused by fungi of the genus *Candida*, *Candida albicans* being the species that most causes the disease in Brazil. The Fluconazole is the conventional medicine used as a treatment of these infections; however, it causes many adverse reactions in the patient and its continued use can induce the resistance of the pathogen. The medicinal plants can be very effective as an alternative treatment of diseases and have been used in folk medicine for years. In this study, it was evaluated the antifungal activity of extracts of *Ruta graveolens*, *Pelargonium graveolens* and *Hibiscus cannabinus* against the fungal development of *Candida albicans*, comparing the efficiency of these extracts to Fluconazole. The extracts were prepared in the concentration of 1g of the plant for each 5 mL of alcohol 70%. The data were obtained using the agar diffusion method. The minimum inhibitory concentration test (MIC) was performed, and showed that *R. graveolens* was able of inhibiting 100% of the pathogen when using 100 mg.ml-1 concentration. From the results obtained, it was possible to conclude that *R. graveolens* was the most effective extract when compared to Fluconazole, suggesting that this extract can be used as an alternative to conventional treatment to improve the efficiency of current treatments. The extracts of *P. graveolens* and *H. cannabinus* also presented antifungal activity but in smaller proportion than Fluconazole. The data from this study suggests that R. graveolens extract can be tested in future *in vivo* studies with the objective of proposing its use in alternative or simultaneous treatment of the synthetic drug used.
Keywords: Antifungal. Candidiasis. Fungal resistance. Medicinal plants.

RESUMO

A candidíase é uma infecção causada por fungos do gênero *Candida*, sendo *Candida albicans* a espécie que mais causa a doença no Brasil. Fluconazol é um antifúngico utilizado para o tratamento dessas infecções, porém, causa muitas reações adversas no paciente e o uso contínuo pode favorecer a resistência do patógeno. As plantas medicinais podem ser muito eficientes no tratamento alternativo de doenças e têm sido utilizadas na medicina popular há anos. Nesse estudo, foi avaliado *in vitro* a atividade antifúngica dos extratos de *Ruta graveolens*, *Pelargonium graveolens* e *Hibiscus cannabinus* contra o crescimento de *Candida albicans*, comparando a eficácia desses extratos vegetais com fluconazol. Os extratos foram preparados na concentração de 1g da planta para cada 5 mL de álcool 70%. Os dados foram obtidos através de difusão em ágar, e posteriormente foi determinada a concentração inibitória mínima (CIM) pela técnica de microdiluição em caldo. Os resultados mostraram que *R. graveolens* é eficaz na inibição de *C. albicans* com CIM de 100 $mg.mL^{-1}$, quando comparado ao tratado com fluconazol. Os extratos de *P. graveolens* e *H. cannabinus* foram capazes de inibir a propagação fúngica de *C. albicans*, porém de forma menos eficiente quando comparados aos medicamentos comumente usados no tratamento. Desse modo, os resultados sugerem que o extrato de *R. graveolens* possa ser testado em futuros estudos *in vivo*, a fim de propor sua utilização em tratamento alternativo ou simultâneo com fármacos já utilizados.
Palavras-chave: Antifúngico. Candidíase. Plantas medicinais. Resistência fúngica.

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INTRODUCTION

Candidiasis is a disease caused by fungi of the *Candida* genus. This genus comprises of 200 species, approximately 20 of which are pathogenic. It is a natural yeast in the human body and can colonize the skin and the gastrointestinal, vaginal, oral and urinary tracts (SILVA, 2011).

The presence of this fungus in the human microbiota is natural, however, when there is an imbalance in the host's immune system, *Candida* species can manifest itself aggressively. Mycoses caused by this genus can present in different clinical forms, where the lesion can be mild, acute or chronic, superficial or deep (BARROS, 2017).

In Brazil, fungal infections caused by the genus *Candida* (MAGALHÃES *et al.*, 2019) corresponds to 80%. About 30% of women have vaginal Candida colonization. Already in 80% of the healthy adult population there is *Candida* spp. in the gastrointestinal tract. Research carried out in Brazil indicates that 60% of clinical isolates include the *Candida albicans* species, making it the largest candidiasis agent in the country (BARBEDO, 2010).

Due to the prevalence of candidiasis in recent years, studies about antifungal drugs that aim to alleviate cases of the disease have increased. Currently, the most used drugs against candidiasis are triazoles, such as fluconazole and itraconazole (VIEIRA *et al.*, 2017). However, the indiscriminate use of drugs such as those can favor fungal resistance to the main therapeutic resources used for this infection (MAGALHÃES, 2019). This causes an increase in the rate of ineffectiveness of its treatment as well as the morbidity and mortality caused by candidiasis (VANDEPUTTE *et al*., 2005).

Thus, it is necessary to develop resources capable of controlling or preventing multi-resistance to antifungals, as has already occurred with bacterial multi-resistance to antibiotics (FERREIRA, 2019). Medicinal plants are effective alternatives to the treatment of diseases in folk medicine, in addition to providing the main raw material for drug synthesis (BARROS, 2017).

The use of medicinal plants in the treatment of diseases is an old habit that has been arousing interest in the scientific field, making the number of researches in the area high (CORRÊA, 2017). According to Oliveira (2016), essential oils and plant extracts contain compounds capable of inhibiting and preventing the development of some pathogens. The use of these products as a treatment for some diseases can increase immunity as well as reduce the side effects caused by conventional treatments.

Considering the antifungal potential of *Ruta graveolens*, *Pelargonium graveolens* and *Hibiscus cannabinus*, the present study aimed to evaluate in vitro the inhibitory potential of natural extracts of these plants against the growth of *C. albicans.*

MATERIAL AND METHODS

The tests were conducted at the Laboratory of Applied Microbiology of the State University of Minas Gerais – Ibirité Unit (LAMAP).

The yeast used belongs to the collection of microorganisms from LAMAP – UEMG Ibirité. The *C. albicans* strain was thawed and cultivated in Sabouraud Dextrose 2% (v/v) agar, pH 4.0 in a Petri dish. The plates with the culture were incubated in an oven at 37 °C for 24 hours. Subsequently, they were stored in a refrigerator and repeated every ten days to maintain the microorganism.

The natural extracts were obtained by maceration of the leaves, stems and flowers of: *Ruta graveolens*, *Pelargonium graveolens* and *Hibiscus cannabinus*, which formed three distinct groups respectively; Group A, Group G and Group H. For this process, the methods proposed by the Brazilian Pharmacopoeia (2010) were used with modifications, using the proportion of 1g of the plant for each 5 mL of alcohol 70% (v/v).

The plant materials were washed with running water and dried at room temperature, then cut and macerated with the aid of a pistil, by adding alcohol 70% (v/v) gradually. The extract together with the vegetable residue was stored in an amber screw-capped bottle at room temperature for 15 days, being manually shaken daily. After 15 days, the final product was obtained by filtration using

filter paper and funnel. The extracts obtained were placed in amber vials and stored in a refrigerator at 4 °C for later use.

Agar diffusion disk tests were performed six times in triplicate. The microorganism was inoculated in a Petri dish using the spreading technique and with the aid of a pipette and sterile tips, in the proportion of $1x10^6$ cells.mL^{-1} in a dish containing Mueller Hinton Agar, prepared from dehydrated compost according to the manufacturer's recommendations , with pH 7.2 (CARVALHO, 2016). Then, sterile filter paper discs of 0.6 mm in diameter were placed on the newly inoculated microorganism, soaked with the extracts of the tested groups, namely: Group A (Arruda), G (Geranium) and H (Hibiscus), prepared according to the methodology described above; both at a concentration of 0.2 g/ml. Tests were also performed using fluconazole (150 mg.mL^{-1}), which is a drug indicated for the treatment of candidiasis (CANNON *et al.*, 2007), as well as tests with 70% alcohol (v/v), the diluent used to prepare the extracts.

After these procedures, the plates were incubated in a mycological incubator at 37 °C for 12 hours. After this period, the inhibition zones were measured with the aid of a caliper. Data were evaluated by statistical analysis comparing the results with controls. Statistical analysis was performed using the Two-Way Anova tool by the Graph Pad Prism 5.0 program. Analysis of variance was performed and means were compared by Tukey's test ($P \leq 0.05$) of the data obtained.

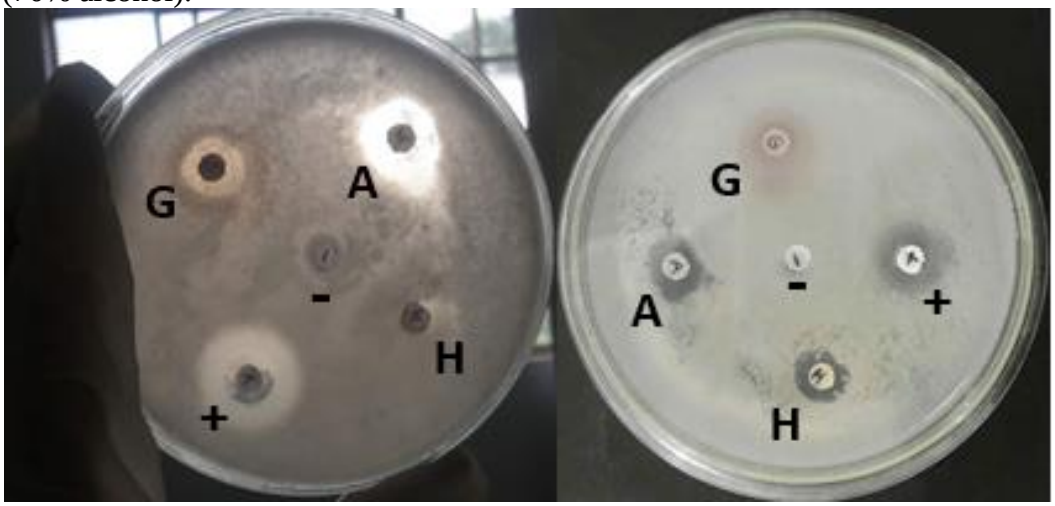
The minimum inhibitory concentration (MIC) was performed in duplicate using the methodology described by Carvalho (2016) with changes. The 96-well microplate microdilution method was used. The tests were performed in duplicate for each extract: Arruda (A), Geranium (G) and Hibiscus (H).

For each extract, a serial dilution of the extracts was carried out in Mueller Hinton broth (Laborclin, 2019) obtaining the following extract concentrations: 200 mg.mL^{-1}, 100 mg.mL^{-1}, 50 mg.mL^{-1}, 25 mg.mL^{-1}, 12,5 mg.mL^{-1}, 6,25 mg.mL^{-1}, 3,125 mg.mL^{-1} and 1,5625 mg.mL^{-1} (Figure 1). After dilution, 100 µL of suspension of 1x106 cells.mL-1 of *C. albicans* were added in each of these dilutions. Fluconazole was also used at a concentration of 1µg/mL and 100 µL of alcohol 70% (v/v) per well. For data analysis, the Excel program was used.

RESULTS AND DISCUSSION

The extracts of *Ruta graveolens*, *Pelargonium graveolens* and *Hibiscus cannabinus* were effective in inhibiting the fungal growth of *C. albicans*, and all three showed halos of inhibition in the agar diffusion test (Figure 1).

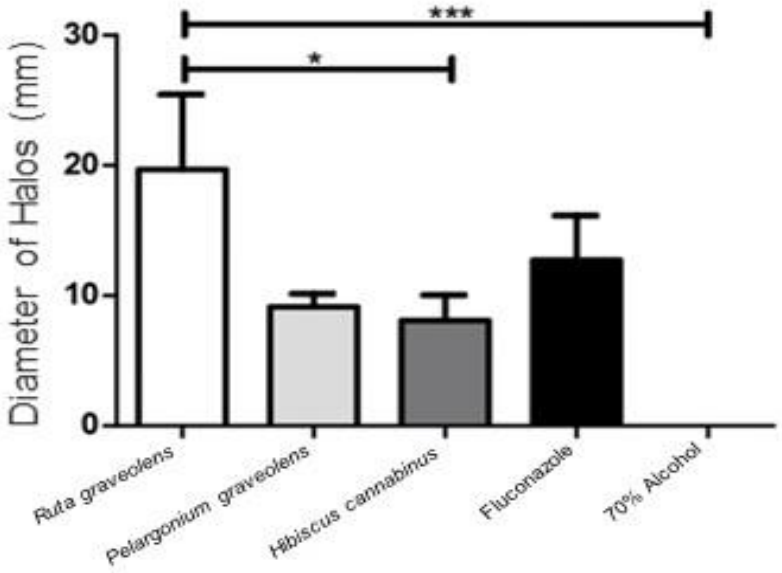
Figure 1 - Agar Diffusion Test. Inhibition halos of extracts of *Ruta graveolens* (arruda) (**A**), *Pelargonium graveolens* (geranium) (**G**) and *Hibiscus cannabinus* (hibiscus) (**H**) Fluconazole (**I**) Diluent (70% alcohol).



Source: The authors.

It is possible to observe that the extract of *R. graveolens* (arruda) showed greater capacity to inhibit the growth of *C. albicans*, with an inhibition pattern similar to that shown by fluconazole (Figure 2).

Figure 2 - Average of inhibition halos represented in millimeters. Comparison between extracts and positive and negative controls, where ***: p < 0.001 between Arruda (*Ruta graveolens*) and 70% alcohol; and *: p < 0.01 between Arruda and Hibiscus (*Hibiscus cannabinus*).



Source: The authors.

Compared to the diluent (70% alcohol), the rue (p<0.001) showed a significant difference, indicating that the inhibition potential of the extract was due to the constituent properties of the plant and not to the solvent used for the production of the extract. Thus, this result is in accordance with data obtained in the research by Araujo *et al.* (2008), in which the antifungal potential of *R. graveolens* was evaluated against several opportunistic microorganisms responsible for causing diseases in human beings, including some *Candida* species such as *C. albicans* and *Candida tropicalis*. For these yeasts it was observed that the essential oil of rue had halos of 10 to 20 mm. This result was similar to that found in the present study.

When compared to the extracts of *Pelargonium graveolens* and *Hibiscus cannabinus*, the extract of *R. graveolens* had a greater capacity to inhibit the growth of *C. albicans*, and compared to H. cannabinus (p<0.01) it showed a significant difference, as it Hibiscus did not show statistical difference with 70% alcohol.

The volatile oil of *P. graveolens* and its antifungal potential was proven to be synergistic with amphotericin B against *Candida* sp. in a study by Rosato *et al.* (2008), in which the inhibition of all *Candida* species including *C. albicans* was demonstrated, as well as the fungistatic activity against *Aspergillus flavus* strains. This result is in line with those obtained in the present research, noting that the alcoholic extract of the plant was used in this study, which is more economical and easily accessible for popular use. It is noteworthy that no other studies were found using the alcoholic extract of *P. graveolens* against the fungal growth of *C. albicans*.

Although the extracts of *P. graveolens* and *H. cannabinus* did not show significant difference compared to fluconazole and *R. graveolens*, the two extracts showed antifungal activity against *C.*

albicans, which corroborates an important scientific result. It was observed that the three extracts had a MIC of 100 $mg.mL^{-1}$, which was the most effective concentration to inhibit 100% of fungal growth.

A study conducted in 2008 at the University Hospital Lauro Wanderley tested the antimicrobial activity of rue essential oil against *Candida* spp. isolated from patients with acute otitis externa, and it was concluded that the essential oil of *R. graveolens* with its concentration of 4% (2500 μg/ml) was able to inhibit the strains of *Candida* spp. (NOGUEIRA *et al.*, 2008). The results obtained by these authors are in accordance with the data obtained in the present study, taking into account that it was developed with the use of an alcoholic extract that has a lower initial concentration (200 $mg.mL^{-1}$) than the essential oil used by Nogueira *et al* (2008).

With respect to the extract of *Pelargonium graveolens*, a research done by Raut *et al.* (2014) on the medicinal properties of essential oils demonstrated the fungicidal activity of the essential oil of Geranium against *C. albicans* in concentrations of up to 0.15% (RAUT *et al.*, 2014). In this study, *P. graveolens* also demonstrated antifungal activity at all concentrations tested, but only the highest concentrations of 200 $mg.mL^{-1}$ and 10 $mg.mL^{-1}$ were able to inhibit 100% of the growth of *C. albicans*. It should be taken into account that the essential oil is more concentrated since no solvent is used for its extraction.

No studies were found that evaluated the inhibitory activity of *H. cannabinus* against the fungal growth of C. albicans or any species belonging to the genus *Candida*, making this research a pioneer in this regard.

CONCLUSION

The results indicate that possibly the analyzed extracts of *Ruta graveolens, Pelargonium graveolens* and *Hibiscus cannabinus* are capable of inhibiting the growth of *C. albicans*. However, *Ruta graveolens* had the best antifungal activity against *C. albicans* among all tested extracts.

It is suggested that *in vivo* tests be carried out to prove this extract as a potential treatment for infections caused by *C. albicans*, mainly from the study of its chemical constituents. Further research is needed with the production of ointments and lotions from the extract, as *R. graveolens* is not recommended as a medicine for systemic use.

The other tested extracts, *Pelargonium graveolens* and *Hibiscus cannabinus* showed lower antifungal activity when compared to the antifungal activity presented by *R. graveolens* and fluconazole, even so, it can be suggested that both extracts can be used as a complementary treatment to the usual antifungals. *In vivo* research is needed to prove therapeutic activities, determination of dosages and type of use (systemic or topical).

This research was the first to evaluate the potential of inhibition of *Hibiscus cannabinus* against the growth of *C. albicans*, being of great relevance to support future studies involving the antifungal activity exerted by this and other plants that are a source of new drugs against the causative fungi of human pathologies.

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