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Vol. 9(2), pp. 42-47, 10 January, 2015
DOI: 10.5897/JMPR2014.5667
Article Number: 92392F550188
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Antifungal activity of the aqueous extract of *Stachytarpheta cayennensis,* (Rich.) Vahl. (Verbenaceae), on oral candida species

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Received 30 October, 2014; Accepted 28 December, 2014

The objective of this study was to evaluate the antifungal activity of the aqueous extract of the leaves of the "verbena" (*gervão roxo*) *Stachytarpheta cayennensis,* (Rich.) Vahl. (Verbenaceae), by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) on different species and strains of the genus Candida. In this study, the species *Candida albicans, Candida tropicalis, Candida glabrata, Candida stellatoidea, Candida dubliniensis* and *Candida krusei* were included. Four strains of *C. albicans* and three of *C. tropicalis* were used, while for the other species only one strain was tested. These yeasts were used in this study because of their importance and frequency in the oral cavity. The yeasts were subjected to treatments with concentrations of the aqueous extract ranging from 0.09 to 25 mg mL^{-1}. The results indicated, however, that concentrations of less than 12.5 mg mL^{-1} were insufficient to show a fungistatic or fungicidal effect. The concentration of 12.5 mg mL^{-1} showed a fungistatic effect on most tested strains and species, *C. albicans* CαFB-12 and ATCC-44858; *C. tropicalis* CTFB-22 and CTFB29. The fungicidal effect (MFC) was observed only on the species *C. krusei* for the concentration of 12.5 mg mL^{-1}. Based on the employed methodology, it is concluded that the aqueous extract of *S. cayennensis* showed an antifungal, mainly fungistatic, effect on oral *Candida* species.

Key words: Yeasts, candidiasis, metabolites, *Stachytarpheta cayennensis.*

INTRODUCTION

Using plants for the treatment and cure of diseases is as old as the human species itself, with popular knowledge making a great contribution to the dissemination of the therapeutic virtues of these plants. This knowledge has

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often represented a therapeutic resource for many communities and ethnic groups (Maciel et al., 2002) who do not have access to other forms of treatments offered by medicine, or who prefer it in relation to traditional medicine because of cultural issues.

This way, users of medicinal plants from various parts of the world have maintained the practice of phytotherapy and validated the therapeutic information that has been accumulated over centuries. Indirectly, popular medicine awakens the interest of researchers in multidisciplinary studies that enrich the inexhaustible knowledge regarding the therapeutic use of medicinal plants. It is estimated that in continents such as Africa, up to 80% of the population uses medication from plant origins. In Germany and France, this is 75%, in Canada 70%, and in the US 42% (Gregorio, 2006).

The genus *Stachytarpheta* (Verbenaceae) has 133 species. It is distributed throughout Brazil. The species of this genus are generally shrubs, branching subshrubs or, in rare cases, herbs that range from 0.5 to 1.5 m in height, although certain species may reach up to 4 m (Salimena-Pires and Giulietti, 1998).

Its flowers are arranged in a spiral along the axis of the inflorescence in a very compact way, reaching up to 60 cm in length. Its corrolas are quite striking and easily located at a distance in the field. Usually they are blue, but they can have several colors depending on the species, such as red, violet, orange, white or black (Costa, 1960).

Stachytarpheta cayennensis (L.C. Rich) Vahl (Figure 1) is an erect, perennial, branching, somewhat angular, fibrous subshrub that is very resistant to traction. It usually has opposite, ovate leaves with a distinct petiole and serrated and indented edges, an acute or subacute apex, a slightly wrinkled appearance, green color, terminal inflorescence with linear stalks, sessile flower with a gamosepalous calyx, pilose on the dorsum, a corolla with five petals welded at the base, of a lilac coloring, with an androecium with two fertile stamens and two staminodes.

S. cayennensis (Rich.) Vahl, popularly known as verbena, belongs to the family Verbenaceae (Pio Correa, 1984). This species is found in the tropical and subtropical Americas, from Mexico to Brazil (Lopes, 1977; Troncoso, 1979), and it has been used in traditional medicine as an anti-inflammatory, analgesic, antipyretic, hepatoprotective and laxative agent, and in the treatment of gastric disorders (Mathias and Emily, 1993; Mesia-Vela et al., 2004). Crushed leaves and roots have also been applied in the treatment of skin lesions (Caribe and Campos, 1991), including ulcerated lesions caused by *Leishmania* species (Moreira et al., 1998, 2002). Some of the effects suggested by the population have already been demonstrated experimentally, such as the anti-inflammatory, analgesic, gastroprotective, antibacterial and antifungal activity (Schapoval et al., 1998; Mesia-Vela et al., 2004; Duarte et al., 2004; Falcão et al., 2005; Okoye et al., 2010; Oliveira et al., 2011; Trabulsi Filho et al., 2013; Neiva et al., 2014).

Its chemical composition includes alkaloids, glycosides (verbenalin and verbenin), tannins, saponins, flavonoids, steroids, quinones, phenolic compounds and gluconic acid (Mathias and Emily, 1993).

The objective of this study was to evaluate the antifungal effect of the aqueous extract of the leaves of the *S. cayennensis* (Rich.) Vahl. (Verbenaceae), collected in the Southwest region of Paraná - Brazil, by determining its MIC and MFC on different species and strains of the genus *Candida.*

MATERIALS AND METHODS

Collection and preparation of the raw material

The aerial parts of the plant (*gervão roxo*) [*S. cayennensis* (Rich.) Vahl.], belonging to family Verbenaceae, were collected in the municipality of Francisco Beltrão - Paraná - Brazil, during its flowering period (Spring). The entire collection was performed on the same day, in the month of November, 2013. The plants were exsiccated in the Laboratory of Chemistry and Biochemistry, Universidade Paranaense - UNIPAR - Francisco Beltrão Campus - Paraná - Brazil. One voucher specimen was deposited in the herbarium of UNIPAR under the number 12,643.

The plants were then stored in a dehumidification chamber at a temperature of 24°C during 45 days for drying. After this period, the leaves were separated and crushed, obtaining the plant biomass (powder) for the preparation of the aqueous extract.

The preparation of the Aqueous Extract of *S. cayennensis* (Rich.) Vahl. was carried out in the Laboratory of Chemistry and Phytochemistry, União de Ensino do Sudoeste do Paraná - Unisep - Dois Vizinhos - Paraná - Brazil. The isolation and growth of the yeasts, in addition to the microbiological tests, were performed at the Laboratory of Microbiology, Universidade Paranaense - Unipar - Francisco Beltrão Campus - Paraná - Brazil.

Preparation of the aqueous extract

Twenty grams of dry powder of the leaves of *S. cayennensis* (Rich.) Vahl. were used for 1000 ml of distilled water, using 5 stages of preparation to obtain the sterile aqueous extract: (1) 20 g of dry vegetable matter (powder) were suspended in 300 ml of distilled water at 70°C, remaining in infusion for 24 h, duly closed and protected from light; 2) After this period, the suspension was filtered, and 350 ml of distilled water was again added to the filtration plant residue at a temperature of 70°C, with this infusion being maintained for more than 24 h in similar conditions as the first step; 3) The extract obtained in this second step was filtered and 350 ml of distilled water at 70°C was added to the resulting residue. This infusion was maintained over 24 h, completing the extraction process; 4) After the last filtering, three crude aqueous extracts were obtained, which were mixed to produce one single extract; 5) The final extract was subjected to vacuum filtration, with the volume of extract to be used in the experiments being previously submitted to microbiological filtration with a Millipore® membrane of 0.22 μm. This solution was put into sterile glass tubes and kept at 4°C and protected from light.

Isolation of microorganisms

Pure collection strains (ATCC-44858) and three different strains of clinical isolates of *Candida albicans*, *Candida tropicalis* and

Figure 1. Botanical characteristics of verbena - *Stachytarpheta cayennensis* (L. C. Rich) Vahl.

Candida krusei, obtained from the Microbiology Laboratory, Universidade Paranaense - Unipar - Francisco Beltrão Campus - Paraná - Brazil, were used, which were collected from children between four and eight years of age. Strains of *C. albicans* (4 strains), *C. tropicalis* (3), *Candida glabrata* (1), *Candida stellatoidea* (1), *Candida dubliniensis* (1) and *C. krusei* (1) were used, totaling 11 fungal samples.

Preparation of the inoculum

The different yeast strains were activated in a Sabouraud dextrose agar medium, being planted and incubated for 24 h at 36°C. The cultures were suspended in 5 ml of sterile saline solution 0.85% (0.145 mol L^{-1}; 8.5 g L^{-1} NaCl). The resulting suspension was homogenized in a tube shaker for 15 s and the cell density was adjusted visually to the turbidity equivalent to 1.0 on the McFarland scale.

MIC determination

The antifungal activity tests were performed with the broth microdilution technique (BM), in accordance with the reference document M27A2 (NCCLS/CLSI, 2005). This methodology was used to test the yeasts in the aqueous extract of the leaves of *S. cayennensis,* (Rich.) Vahl., with the technique being adapted for this. The concentrations of the aqueous extract tested regarding the different species and strains of *Candida* varied from 0.09 to 25 mg mL^{-1}. A synthetic RPMI 1640 medium (with glutamine, without bicarbonate and with red phenol indicator) was used, buffered in 3-(N-morpholino) propanesulfonic acid (MOPSO), in accordance with the standards of the Clinical and Laboratory Standards Institute (CLSI) (NCCLS/CLSI, 2005).

Positive controls, which were characterized by 100 μl of the RPMI culture medium and 100 μl of inoculum solution, and negative controls, consisting only of 200 μl of the liquid RPMI culture medium, were used for all tests. The dishes were incubated at 36°C

Table 1. Effect of the aqueous extract of *Stachytarpheta cayennensis,* (Rich.) Vahl., on the different species and strains of the genus *Candida*.

Fungus		MIC	MFC
Species	**Strain**	**(mg mL^{-1})**	**(mg mL^{-1})**
Candida albicans	CαFB-01	R	R
C. albicans	CαFB-01	R	R
C. albicans	CαFB-01	12.50	R
C. albicans	ATCC-44858	12.50	R
C. tropicalis	CTFB-03	R	R
C. tropicalis	CTFB-22	25.00	R
C. tropicalis	CTFB-29	12.50	R
C. glabrata	CGFB-2x	12.50	R
C. stellatoideia	CSFB-1Ɛ	12.50	R
C. dubliniensis	CDFB-4β	12.50	R
C. krusei	CKFB-0α	12.50	12.50

MIC: Minimum inhibitory concentration; MFC: minimum fungicidal concentration; R: Did not have effect on the microorganisms tested - Resistant.

for 72 h, with readings taken every 24 h.

For the reading of the tests, the yeast growth that occurred in the dishes related to the different concentrations tested, was compared with its growth in the positive control dish. The lowest concentration capable of producing inhibition of yeast growth in relation to the positive control dish was identified as the minimum inhibitory concentration (MIC) of the plant extract for this sample. All dilutions of the plant extract and control groups were tested in triplicate.

MFC determination

The MFC was determined based on the results obtained for MIC. An aliquot of 50 μl of the dishes that showed inhibition was placed on the surface of a dish containing Sabouraud dextrose agar and incubated at 36°C for 24 h. MFC was considered to be the lowest concentration of the crude aqueous extract of *S. cayennensis,* (Rich.) Vahl., that did not demonstrate any fungal growth on the surface of the culture medium after incubation. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Table 1 shows the effect of the S. *cayennensis*, (Rich.) Vahl., extract on the different species and strains of the genus *Candida*. By analyzing the obtained results, one can see that the extract had a MIC of 25 mg mL^{-1} on the clinical isolate of *C. tropicalis,* and of 12.5 mg mL^{-1} on some isolates and on the ATCC-44858 strain of *C. albicans*, as well as on the isolates of *C. tropicalis*, C. *glabrata*, C. *stellatoidea*, *C. dubliniensis* and *C. Krusei*. The extract did not inhibit the growth of an isolate *of C. tropicalis* and two of *C. albicans.* However, with respect to the fungicidal activity, the minimum fungicidal concentration (MFC) of the extract was 12.5 mg mL^{-1} for the isolate of *C. krusei*. For all other strains and species, the extract did not have a MFC.

According to this study, concentrations of less than 12.5 mg mL^{-1} were insufficient for the aqueous extract of *S. cayennensis,* (Rich.) Vahl., to have a fungistatic or fungicidal effect *in vitro*. The concentration of 12.5 mg mL^{-1} , however, had a fungistatic effect on most tested strains and species (*C. albicans*, C. *tropicalis*, C. *glabrata*, C. *stellatoideia*, *C. dubliniensis* and *C. krusei),* with a more pronounced antifungal behavior being observed for the strains of *C. albicans* and *C. tropicalis*, and a fungicidal effect being observed only for the species *C. krusei.*

The species *S. cayennensis,* (Rich.) Vahl., evaluated in this study therefore shows satisfactory antimicrobial activity in the biological assays of antimicrobial activity on such pathogenic bacteria as *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (Onofre et al., 2014).

According to Vargas (2006), both the root and leaves of *S. cayennensis* contain several substances with therapeutic potential, such as essential oils, polyphenols, an antibiotic compound similar to penicillin, vitamins B and C, calcium, phosphorus and iron.

The pharmacological effects suggested by the population have already been demonstrated experimentally, such as the anti-inflammatory, analgesic, gastroprotective, antibacterial and antifungal activity (Costa et al., 1960; Schapoval et al., 1998; Mesia-Vela et al., 2004; Duarte et al., 2004; Falcão et al., 2005).

Studies conducted by Robinson et al. (1990) and Vargas (2006) indicate that the triterpenoids obtained from the *S. cayennesis* have anti-inflammatory, antimicrobial, antiviral and analgesic effects. An *in vivo* experiment in rats has confirmed the analgesic and anti-inflammatory activity. The isolation in the extract of *S. cayennensis* of a glycosylated phenylpropanoid with anti-histaminic activity and of an iridoid, ipolamiide, with anti-histaminic and anti-bradykinin effect, contributed to

confirming the aforementioned effects.

Studies with the aqueous extract of *S. cayennensis* have demonstrated its antiulcerogenic effect and its inhibition of the secretion of gastric acid. This last effect is a consequence of the inhibition of the activity of the kinase protein dependent of AMPc (PKA). The anti-ulcer effect seems to involve the activation of defense mechanisms of the gastric mucosa, which is independent of the effect that inhibits the secretion of gastric acid (Vella, 1999; Penido et al., 2006).

In studies similar to this one that used the aqueous extract of *S. cayennensis*, the activity against strains of *Staphylococcus aureus*, *Micrococcus luteus, Bacillus subtilis, E. coli, P. aeruginosa* and *Salmonella typhimurium* has been observed (Duarte et al., 2004; Onofre et al., 2014).

In other studies with the aqueous extract of the leaves of *S. cayennensis* that sought to establish the intestinal transport of water and the effect on gastrointestinal propulsion in mice, a reduction in intestinal transit in relation to the control group was shown. This same work showed that the extracts of the leaves of *S. cayenensis* have a potential anti-diarrheal effect in infections by enteropathogens, highlighting the weak-to-moderate antibacterial activity *in vitro* on Gram-positive and Gram-negative bacteria (Rosas, 2004).

The bioautography assays revealed antimicrobial substances in the hexane fraction of the hydroalcoholic extract. In other studies, Vargas (2006), Okoye et al. (2010) and Okoye et al. (2014) observed that extract of the leaves of S. *cayennensis* proved to be effective in the treatment of *C. albicans* and other micro-organisms in oral cavity infections, inhibiting microbial growth.

The results of this study demonstrate the different effect of the extract of *S. cayenensis* on different species and strains of *Candida,* indicating that the aqueous extract of this plant has a considerable effect on the growth of these microorganisms. Studies in this sense were also carried out by Okoye et al. (2014). Their results showed that the components of the leaf extracts of *S. cayennensis* have immunomodulatory and antifungal activity.

The occurrence of candidiasis in Brazil and the clinical importance in immunocompromised patients justify this study with different strains of *Candida*. In this study, the antifungal effect of the crude aqueous extract of the leaves of *S. cayenensis* on one ATCC-44858 strain of *C. albicans*, and on three isolates of this species from the oral cavity, and on 7 strains of isolates of non-albicans *Candida* species, were evaluated.

The different behavior obtained for strains of the same species could be explained by the existence of genetic variability between the different strains, as can be seen in the results for the species *C. albicans* and *C. tropicalis*. In any research involving medicinal plants, and before attempting to extrapolate the results obtained, it is important to consider the environmental factors at the time of the collection of the plant, such as seasonality, climate, type of soil, and air temperature.

According to Freitas et al. (2004), the production of secondary metabolites by the plant occurs as a function of the plant-environment interaction in response to chemical and biological factors. This fact may explain the divergent results of extracts of the same species, but which were collected in different locations and time periods. The aqueous extract of *S. cayennensis* tested in this study was obtained from plants harvested in a single season (Spring), in a single month (November 2013) and in the same place of the municipality Francisco Beltrão - Paraná - Brazil.

The fungicidal effect of the aqueous extract of *S. cayennensis,* on the other hand, was only observed in one of the tested species. The concentration of 12.5 mg mL^{-1} was sufficient to inhibit the species *C. krusei*. However, even at the highest concentrations tested, no effect was observed on the other species, indicating the need for other tests involving concentrations exceeding 25 mg mL^{-1} of the aqueous extract of *S. cayennensis.*

Conclusion

The aqueous extract of the verbena (*gervão roxo*), *S. cayennensis* has an *in vitro* fungistatic effect on oral *Candida* species isolated from children between 4 and 8 years of age. The results allow the conclusion to be drawn that concentrations of less than 12.5 mg mL^{-1} were insufficient to show a fungistatic or fungicidal effect. The concentration of 12.5 mg mL^{-1} showed a fungistatic effect on the strains of *C. albicans* CαFB-12 and ATCC-44858; on *C. tropicalis,* strains CTFB-22 and CTFB29. The fungicidal effect was observed only on the species *C. krusei* (MFC = 12.5 mg mL^{-1}).

Conflicts of interest

The authors declare there are no ethical, publishing-related or financial conflicts of interest regarding the data of this study.

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