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Antimicrobial Action of Hydroalcoholic Extract from *Cymbopogonous citrus* Staupf (capim-limão), *Rosmarinus officinalis* L (alecrim) and *Lychnophora ericoides* (arnica)

Ação Antimicrobiana de Extrato Hidroalcóolico de Folhas de C*ymbopagon citratus* Staupf (capim-limão), R*osmarinus officinalis* L (alecrim) e L*ychnophora ericoides* (arnica)

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Abstract

Infectious diseases are always a concern, since the effectiveness of several antimicrobials in certain situations has decreased due to the microbial resistance developed over the years. Research in the area of antimicrobial drug development has been intensified in recent decades. The objective of this work was to verify in vitro the antimicrobial activity of hydroalcoholic extracts of lemongrass, rosemary and arnica. Bacteria used in the test were *S. aureus, K. pneumoniae* and *E.coli*. The extracts were obtained from the plants dry leaves in maceration with 70% cereal alcohol for seven days. The total soluble solids concentration of each extract was obtained by gravimetry. The extracts antimicrobial activity was obtained by the pour-plate technique, in which the colonies were counted. Under the conditions in which the tests were performed in the stage of microbial growth was observed with lemongrass and rosemary extracts, and the stage rate was zero. However, *S. aureus* was sensitive to the arnica hydroalcoholic extract in the concentration of 4.00 mg / mL with inhibition rate of 100%, reducing as the extract concentration was lower. At different concentrations, the arnica hydroalcoholic extract had a low inhibitory capacity for *K. pneumoniae* and *E. coli*. The plants antimicrobial activity varies greatly in relation to the botanical characteristics (cultivation, soil, harvest) and the extraction method (solvents of different polarities and techniques), for that reason, there are many varied results in several studies. Therefore, many studies are needed to confirm the antimicrobial efficacy of plant strata.

Keywords: Anti-Infective Agents. Phytochemicals. Microbial Sensitivity Tests.

Resumo

As doenças infecciosas são sempre preocupantes, pois a eficácia de vários antimicrobianos em certas situações, diminuiu em função da resistência microbiana, desenvolvida ao longo dos anos. As pesquisas na área de desenvolvimento de fármacos antimicrobianos, intensificaram-se muito nas últimas décadas. O objetivo desse trabalho foi verificar in vitro a atividade antimicrobiana de extratos hidroalcoólicos de capim-limão, alecrim e arnica. As bactérias utilizadas no teste foram S. aureus, K. pneumoniae e E.coli. Os extratos foram obtidos das folhas secas das plantas, em maceração com álcool cereal a 70%, durante sete dias. A concentração de sólidos solúveis totais de cada extrato foi obtida por gravimetria. A atividade antimicrobiana dos extratos foi obtida pela técnica pour-plate, em que se procedeu a contagem de colônias. Nas condições em que os testes foram realizados não foram observados inibição do crescimento microbiano com os extratos de capim-limão e alecrim, sendo a taxa de inibição zero. Entretanto, o S. aureus mostrou-se sensível ao extrato hidroalcóolico de arnica na concentração de 4,00mg/mL com taxa de inibição de 100%, reduzindo conforme a concentração do extrato era menor. Nas diferentes concentrações, o extrato hidroalcoolico de arnica, apresentou uma baixa capacidade inibitória para a K. pneumoniae e E.coli. A atividade antimicrobiana das plantas varia muito em relação as características botânicas (cultivo, solo, colheita) e ao método de extração (solventes de polaridades diferentes e técnicas), por essa razão, há resultados bem variados nas diversas pesquisas. Assim sendo, muitos estudos são necessários para confirmar a eficácia antimicrobiana dos extratos vegetais.

Palavras-chave*: Anti-Infecciosos. Compostos Fitoquímicos. Testes de Sensibilidade Microbiana.*

1 Introduction

The use of medicinal plants with the aim is an ancient practice and in recent decades has been widely exploited, mainly in what refers to the antimicrobial, anti-inflammatory, antioxidant and antitumor activity. The plants active components are synthesized during the secondary metabolism and for this reason the variability of phytochemicals (flavonoids, alkaloids, tannins, terpenes, plant steroids) present in the plant configure them with many medicinal properties[1,2].

The *Poaceae* family, of grasses, the species *Cymbopogon citratus* Stapf in Brazil is known as lemongrass, lemongrass, lemon balm, capim cidreira, capim-cidró. The cultivation is easy with preference for the South and Southeast regions. The consumption is in the form of teas or essential oil. The main indications of this plant are as calmative, carminative, diuretics, antimicrobial, antitumor, insect repellent and insecticide and source of vitamin A[3].

Rosmarinus officinalis L, is known usually as rosemary, *rosmarinho, flor do olimpo, rosa marinha* and belongs to the family *Labiatae*, with distribution throughout Brazil. It has

anti-hypertensive, digestive, healing, antiseptic, antimicrobial, sedative, stimulant of memory, antioxidant properties[4-6].

Lychnophora ericoides L, family *Compositae* (*Asteraceae*), known as arnica, candeia, pau de candeia, veludinho, has preference for high altitudes and regions due to the benefits and high consumption, this plant is in a group of plants into extinction. Among the medicinal properties, it stands out healing, anti-inflammatory, in mechanical trauma accompanied by pain and hematomas[7].

Microbial resistance is a worldwide problem, since several antimicrobial drugs lose their effectiveness and on account of this, the infectious state is aggravated. Therefore, the search for alternatives that can contribute to the reduction of the resistant micro-organisms proliferation becomes a constant challenge. Therefore, the objective of this work was to verify in vitro the antimicrobial activity of hydroalcoholic extracts of lemongrass, rosemary and arnica.

2 Material and Methods

The research was conducted in the Food Analysis Laboratories and Microbiology of Filadélfia University Center - Londrina/PR.

2.1 Pathogenic Bacteria

The pathogenic micro-organisms (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus*) were obtained from the bacteria bank of the Microbiology Laboratory of the Filadélfia University Center - Londrina/PR. These micro-organisms had already been identified biochemically by tests of catalase, cytochrome oxidase, citrate, urease, indole, hydrogen sulfide gas, fermentation of carbohydrates, methyl red, Voges-Proskauer, nitrates reduction and motility.

For the inhibition tests, these bacteria were recovered in nutrient broth and incubated at 35 ºC for 18 hours. Then, each culture was diluted in sterile peptone water (0.1%) to obtain 10^6 CFU/m. For this purpose, the standard test of turbidity of 0.5 scale of Mac Farland was used.

2.2 Obtaining the extracts

The techniques for obtaining the plants extracts are very variable and, for this reason, the comparison of results among the several papers becomes complex[8]. In this study, the maceration process of dried leaves in hydroalcoholic solution at 70% was adopted, because it is a technique widely used in pharmaceutical formulations.

For the preparation of the hydroalcoholic extracts (EHA) of *C. citratus* S, *R. officinalis* L and *L. ericoides* L 20 g of dry leaf in 200 mL of cereal alcohol were used (ALKOFLORA) at 70%. The extracts were placed in amber flasks and kept under the light and at room temperature for 15 days, with manual agitation, twice a day. After this time, the extracts were filtered in filter paper, Whatman number 01 and the pH was determined in potentiometer (TECNAL). These extracts were kept in Amber bottle until the time of the tests.

2.3 Determination of Total Soluble Solids (TSS)

The SST determination was performed by gravimetric method (ABNT/NBR 10664, 1989), modified. For the determination of the SST concentration, aliquots of 1 mL of each extract were placed in weighing bottle, previously calibrated, and then sent to the greenhouse (QUIMIS) for 30 minutes at 85ºC. After this time, they were sent to the desiccator for cooling and subsequent weighing. This procedure was repeated until a constant weight. All analyzes were performed in triplicate.

2.4 Minimum Inhibition Concentration (MIC)

For the IMC determination, it was used as a means of cultivating the Standard Agar (AP).[9] The AP composition favors the microorganisms growth in study, and for that reason it was adopted in this experiment. In sterile Petri plates (60x15 mm), plants hydroalcoholic extracts were placed, separately, in different volumes, with the aim of obtaining different concentrations. Then they were sent to oven at 45 °C overnight, for the alcohol evaporation. When the plates were dried (to ensure the alcohol evaporation), they were removed from the oven and 0.5 mL of bacterial suspension of each micro-organism was added (diluted to 10^{-6}). Then AP was enough to reach a final volume of 5 mL. The content was homogenized performing descriptive movement of the number eight and left to rest for solidification. After the medium solidification, the plates were incubated at 35ºC ± 2°, in a reversed was, during 24 to 48 hours. After this period, the IMC was determined as the lowest concentration of SST (mg/mL) of the extract in which there was no bacterial growth.

In parallel, two controls were carried out. For the first control 1mL of each extract for plates were transferred, separately, and then placed in the oven at 45 °C for 1 hour. Soon after the alcohol evaporation, when the plates were already dried, they were withdrawn from the oven and added to 4.0 mL of Standard Agar. Whereas in the second control 0.5ml of bacterial suspension of each micro-organism (diluted to 10^{-6}) was added to the Petri dishes, then added to 4.5 mL of the Standard Agar completing a final volume of 5 mL, both controls were incubated at 35°C, in a reversed way, during 24 to 48 hours.

The controls were conducted to test the tests effectiveness and therefore prove that there was no contamination during the whole research procedure.

To determine the microbial inhibition rate, the following expression [10] was used:

% inhibition = (C - T)/C x 100, where

C: CFU/mL of the microorganism control without adding the hydroalcoholic extract

T: CFU/mL of the tested microorganism in the presence of hydroalcoholic extract

3 Results and Discussion

3.1 Determination of pH

The hydroalcoholic extracts obtained exhibited pH 6.20, being compatible with the micro-organisms growth in the study. pH was not an interfering factor in the tests, because the tested bacteria have an optimum pH for growth in the range from 6.0 to 8.0.[11]

3.2 Determination of Total Soluble Solids (TSS)

The values of the TSS concentration in hydroalcoholic extracts were obtained by gravimetry at 85 ºC. In 60 minutes 7.87mg/mL were obtained of TSS of *C.citrus* , while it took 90 minutes to obtain 19.97mg/mL of SST *R officinalis* and 2 mg/mL of SST of *L. Ericoides*. The concentrations used of each EHA in tests were obtained using different volumes (Table 1)

Table 1 - TSS concentration, obtained in different aliquots of hydroalcoholic extracts (EHA)

EHA Volume (mL)	TSS concentrations (mg/mL)			
	2.0	**1.5**	**1.0**	**0.5**
C. citrus	15.74	11.81	7.81	3.91
R. officinalis	39.94	29.96	19.97	9.98
L erioides	4.00	3.00	2.00	1.00

Source: Research Data.

3.3 Determination of the Minimal Inhibitory Concentration (MIC)

The indiscriminate use of antibiotics has facilitated the emergence of microbial resistance and, therefore, the importance of developing new products that are capable of inhibiting the micro-organisms growth. In this sense, for decades, the active components of plants that have antimicrobial activity have been studied[12].

MIC was determined as the lowest concentration of the extract in which there was no bacterial growth. In the conditions in which the tests were performed, it was found that the EHA of *C. citrus and R. Officinalis*, in different concentrations, did not inhibit the bacteria growth in the study. These results corroborate the work information of Schuck et al.[12], in which the aqueous extracts of infused and in decoction of fresh leaves of *C*. *citratus* were also negative for *S. aureus* and *E. coli*.

However, Porte and Godoy[14] found for essential oils, high sensitivity of Gram-positive bacteria to the essential oils of rosemary, including *S. aureus*, *Micrococcus* sp. and *Sarcina* sp., as well as the yeast *Saccharomyces cerevisiae* and no or very little effect was verified against Gram-negative bacteria *Pseudomonas fluorences*, *E coli* and *Serratia marcescens.*

The antibacterial activity of *C. citratus* was described by several authors, but only observed on the essential oil, which contains geraniol and citral.[15-17]

Despite the EHA of *R officinalis* L tested in this experiment having not provided satisfactory results of bacterial inhibition, other authors such as Hammer *et al.*[18] found values of MIC of 9 mg/mL of *M. officinalis* L essential oil on strains of *E. coli*.

The low antibacterial activity of alcoholic extract of *R. Officinalis* was observed when tested against *S. aureus*, while gram-negative bacteria were resistant to this same type of extract[19].

The chloroform extract of *R officinalis* showed higher antimicrobial efficacy for *S. aureus* when compared with the ethanolic extract[20]. Furthermore, *S aureus* showed to be more sensitive than *E.coli*. The essential oils of *R. officinalis* also showed similar activity, i.e., were more effective in Gram-positive bacteria than for the gram-negative [21].

In this experiment, when tested in *S. aureus*, EHA of *L. ericoides* L in the concentration of 4.00mg/mL, showed efficacy of 100% of microbial inhibition, which was not observed in other micro-organisms, in different concentrations of this extract (Table 2). In observation to the growth in Petri dish, it was found that the extent to which increased the extract concentration , the size of the colonies of *K.* Pneumoniae decreased. *E. coli* was the one that proved to be more resistant to the inhibitory effect of EHA of *L.ericoides* L.

Table 2 - Inhibition Rate (%) of hydroalcoholic extracts of dried leaves after 48 hours of incubation (32 ºC (±2)*

	Concentration of hydroalcoholic Extract of *l. Ericoides L* (mg/mL)			
Microorganisms	**4.00**	**3.00**	**2.00**	**1.00**
S. aureus	100	77	36	34
K. pneumoniae	72	6	(-)	(-)
E. coli	47	44	26	14

* All tests were performed in duplicate and compared with a control group in which the number of colonies formed without the presence of the hydroalcoholic extract was observed

(-) Microorganism resistant to the tested extract concentrations

Brasileiro et al.[22] in their studies, indicated IMC of the ethanolic extract of arnica against *S aureus* of 0.5 mg/mL, different from what was found in this experiment with hydroalcoholic extract.

4 Conclusion

The concentration of plants active constituents varies depending on season, soil and climate, time of collection, storage condition, parts to be tested, techniques of extraction and solvents used.

In the conditions in which this experiment was conducted, IMC was not detected the IMC of hydroalcoholic extracts of dried leaves of lemon grass and rosemary in the bacteria *Staphylococcus aureus, K pneumoniae* and *E. coli*. However, *S. aureus* showed sensitive to arnica hydroalcoholic extract. Somehow, in this experiment gram-positive bacteria showed to be more sensitive than the negative ones, and this type of result was also observed in other studies.

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