ORIGINAL ARTICLE

Determination of the antimicrobial activity in the hydroalcoholic extract of the plant *Plectranthus ornatus* Codd (Bilberry Chinese)

Determinação da Atividade Antimicrobiana do Extrato Hidroalcoólico da Planta Plectranthus ornatus *Codd (Boldo Chinês)*

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ABSTRACT

Plectranthus ornatus Codd is a plant from Mediterranean and Near East countries. It is indicated for liver illnesses and digestion problems in popular medicine. It is used in the treatment for the control of gastritis, dyspepsia, heartburn, gastric discomfort, and hangover. This study aims to evaluate the antimicrobial activity in extracts of P. ornatus using 15 standardized micro-organisms. Antimicrobial tests were performed on Mueller Hinton agar through the technique of wells. Broth microdilution tests were used for the determination of the minimum inhibitory concentration (MIC), and seeding on plates containing Mueller Hinton agar for the determination of the minimum microbicide concentration (MMC). The broth microdilution tests demonstrated that the extract inhibited the growth of Bacillus cereus, Streptococcus pyogenes, and Enterococcus faecalis at the concentrations of 20.31, 325, and 650 mg/mL, respectively. The microdilution tests showed growth inhibition of Saccharomyces cerevisiae at a concentration of 1,300 mg/mL. The extract did not show inhibitory activity on the other tested strains. As a result of increased multiple resistances to antibiotics, microbial research in the development of new drugs that are economically viable and offer an effective safety margin have won space in the scientific community.

Key words: Medicine, Traditional; Plectranthus; Peumus; Phytotherapy Products with Antimicrobial Action; Anti-Infective Agents; Microbial Sensitivity Tests.

RESUMO

A planta Plectranthus ornatus Codd é originária dos países do Mediterrâneo e Oriente Próximo. Na medicina popular é indicada para males do fígado e problemas de digestão. É utilizada no tratamento para o controle da gastrite, na dispepsia, azia, mal-estar gástrico e ressaca. Este trabalho objetiva a avaliação antimicrobiana do extrato de P. ornatus, utilizando-se 15 microrganismos padronizados. Os testes antimicrobianos foram realizados em ágar Mueller Hinton pela técnica de poços. Foram utilizados os testes de microdiluição em caldo, para a determinação da concentração inibitória mínima (CIM) e semeadura, em placas contendo ágar Mueller Hinton para a concentração microbicida mínima (CMM). Os testes de microdiluição em caldo demonstraram que o extrato nas concentrações de 20,31; 325 e 650 mg/mL inibiu o crescimento bacteriano de Bacillus cereus, Streptococcus pyogenes e Enterococcus faecalis, respectivamente. Para o fungo Saccharomyces cerevisiae os testes de microdiluição demonstraram inibição do crescimento na concentração de 1.300 mg/mL. Para as demais cepas testadas, o extrato não demonstrou atividade. Em decorrência da crescente resistência múltipla microbiana aos antibióticos, pesquisas para o desenvolvimento de novos medicamentos que sejam economicamente viáveis e com margem de segurança efetiva têm ganhado espaço na comunidade científica.

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Palavras-chave: Medicina Tradicional; Fitoterapia; Peumus; Plectranthus; Produtos com Ação Antimicrobiana; Anti-Infecciosos; Testes de Sensibilidade Microbiana.

INTRODUCTION

The emergence and spread of microorganisms that are resistant to the antimicrobials available in the market have been reported for decades, stimulating the search for new sources of substances with efficient antimicrobial activity, such as plants used in popular medicine.[1]

The treatment of diseases using plant extracts is the oldest method of natural medicine. The source of this knowledge is still remote, and it is believed that a large portion was acquired by observation of human and animal instincts. Thus, the man began to distinguish edible plants, or those that could treat diseases, from toxic plants. This knowledge has been passed down from generation to generation by communities that co-existed with herbs and depended on them to treat diseases. [2]

Brazil has a great potential for the development of studies and discovery of medicinal plants and drugs derived from them because about 20% of the 250 thousand medicinal species catalogued by the United Nations Educational Scientific and Cultural Organization (UNESCO) are native to Brazil, which facilitates the use of their curative potential for the treatment of diseases in the country.[3] The Amazon ecosystem has the greatest biodiversity on the planet, with numerous plant species with reported medicinal properties and others with still unknown therapeutic effects.[1,4]

Bilberries belong to the group of plant species with cholagogue properties. The Bilberry of Chile (*Peumus boldus* Molina) is known as the true bilberry. [5] Brandão et al. registered in the first Brazilian Pharmacopeia of 1929, that the plant popularly known as bilberry was the Bilberry of Chile. The differentiation and correct species identification is important because true and false bilberries (*Plectranthus barbatus* Ard.) contain compounds that cause side effects.[6]

The Lamiaceae family is mainly originated from Mediterranean and Eastern countries and consists of approximately 200 genera and 3,200 species, most notably the genus *Plectranthus* with various species with therapeutic use.[7] *Plectranthus* species are rich in diterpenes and are used in popular medicine in many parts of the world.[8,9]

Plectranthus ornatus Codd is popularly known as Chinese bilberry, gambá bilberry, small bilberry, or creeping bilberry[5]. It is indicated for liver and digestion problems in popular medicine; it can be used in the treatment of gastritis, dyspepsia, heartburn, and gastric discomfort; its bitter taste stimulates digestion and appetite.[5,10]

The leaves contain substances with analgesic activity, showing no side effects. Mild sedative activity have been observed, which can be associated with the analgesic action, and bactericide and fungicide activity not yet specified in the literature.[5]

MATERIAL AND METHODS

Collection of specimens: leaves of *Plectranthus ornatos* were collected in the municipality of Alfenas, Minas Gerais State, Brazil, at the edge of Highway MG-179, Km 0, in September of 2011, and identified in the Botanical Laboratory of the Federal University of Alfenas (UNIFAL). The specimen is stored in the Herbarium of the José do Rosário Vellano University (UNIFENAS), Alfenas, under number 320.

Obtaining the extract: *Plectranthus ornatos* fresh leaves extract was obtained using 70% ethanol (400 g of leaves in 1,600 mL of 70% alcohol) according to technique already described.[11,12] This mixture was macerated in a volumetric flask (2,000 mL), stored at room temperature in the dark for 15 days, and filtered and kept at 4° C in a sterile amber bottle. It was subsequently concentrated in a rotational evaporator and lyophilized. The extract was resuspended in sterile distilled water and filtered in 0.22 µm Millipore® filter at the time of use.

Antimicrobial activity: antimicrobial activity tests were performed with standardized bacteria and fungi species (ATCC and NEWPROV): 1. Bacteria: *Bacillus cereus* (ATCC 11778), *Bacillus stearothermophilus* (ATCC 7953), *Bacillus subtilis* (ATCC 6633), *Enterobacter aerogenes* (ATCC 13048), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Proteus mirabilis* (ATCC 25933), *Pseudomonas aeruginosa* (ATCC 25619), *Shigella flexineri* (NEWPROV 0122), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (NEWPROV 25923), and *Streptococcus pyogenes* (NEWPROV 19615); and, 2. Fungi: *Candida albicans* (ATCC 1023-1) and *Saccharomyces cerevisiae* (ATCC 2601). Bacteria were kept on BHI agar and fungi in Sabouraud agar, at 4 *°C until testing.*

Evaluation of sensitivity profiles: the antimicrobial activity of the *Plectranthus ornatos* extract was evalu-

ated through the agar diffusion test, minimum inhibitory concentration (MIC), and minimum microbicide concentration (MMC) according to the standards of the National Committee for Clinical Laboratory Standards (NCCLS, 2002).[13-15] The following concentrations were tested 1,300; 650; 325; 162.5; 81.25; 40.62; 20.31; 10.15; 5.07; and 2.53 mg/mL. Sensitivity profile determinations were conducted using the microdilution methodology in RPMI 1640 media for yeasts according to the M27A3 protocol (CLSI, 2008)[13], and microdilution in Mueller-Hinton broth for bacteria according to the M7A6 protocol (CLSI, 2003)[14]. Tests were performed in duplicate.

MIC was determined in extracts that showed inhibitory activity on the agar diffusion test (NCCLS, 2002)[15] in microplates with 150 *µL* of Mueller-Hinton broth 2X concentrate were placed into all wells. The first well received the extract concentration of 1,300 mg/mL, and serial decreasing dilutions were prepared from this well on. Ten *µL* of microbial suspension, at turbidity equivalent to 0.5 in the MacFarland scale, were added to the wells.

MMC was evaluated at the extract concentrations that showed inhibition in bacterial growth. Confirmation of bactericidal/bacteriostatic and/or fungistatic/fungicidal dilutions was performed by plating the specific dilution, and the immediate up and down concentrations, in Mueller-Hinton agar media plates; presence or absence of microbial growth were analyzed.

Statistics - descriptive statistics was adopted in the analysis of the antimicrobial activity data.

RESULT

Halos between 18 and 22 mm were observed in the culture of microorganisms demonstrating growth inhibition and the antimicrobial activity of the *P. ornatus* extract in the gram-positive bacteria *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, and *Streptococcus pyogenes* NEWPROV 19615, and the fungi *Saccharomyces cerevisiae* ATCC 2601. There was no formation of halos in the culture of the following organisms: *Bacillus subtilis* ATCC 6633, *Bacillus sterothermophilus* ATCC 7953, *Candida albicans* NEWPROV 0031, *Enterobacter aerogenes* ATCC 13046, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 25933, *Pseudomonas aeruginosa* ATCC 25923 25619 NEWPROV, *Staphylococcus aureus*, *Streptococcus pyogenes* NEWPROV 19615, *Shigella flexineri* NEW PROV 0122, and *Salmonella typhimurium* NEWPROV 14 028 (Table 1).

DISCUSSION

The MIC and MMC results from the evaluation of the *P. ornatus* extract indicate positive antimicrobial activity in *Bacillus cereus* (ATCC 11778) and *Enterococcus faecalis* (ATCC 29212) at the 20.31 mg/mL concentration, and *Streptococcus pyogenes* (NEWPROV 19615) at the 325 mg/mL concentration. The MIC and MMC of 1,300 mg/µl were observed in *Saccharomyces cerevisiae* (ATCC 2601) (Table 1).

Table 1 - Inhibition halos, Minimum Inhibitory Concentration and Minimum Microbicide Concentration of the hydroalcoholic *P. ornatos extract*

Microrganismos	Halos de Inibição (mm)	CIM* (mg/mL)	CMM** (mg/mL)
Bacillus cereus (ATCC 11778)	18	20.31	1300.0
Bacillus subtilis (ATCC 6633)	–	–	–
Bacillus stearothermophilus (ATCC 7953)	–	–	–
Candida albicans (ATCC 10231)	–	–	–
Enterobacter aerogenes (ATCC 13046)	–	–	–
Enterococcus faecalis (ATCC 29212)	22	20.31	1300.0
Escherichia coli (ATCC 25922)	–	–	–
Klebsiella pneumoniae (ATCC 13883)	–	–	–
Proteus mirabilis (ATCC 25933)	–	–	–
Pseudomonas aeruginosa (ATCC 25619)	–	–	–
Saccharomyces cerevisiae (ATCC 2601)	29	1300.0	–
Shigella flexineri (NEWPROV 0122)	–	–	–
Salmonella typhimurium (NEWPROV 14028)	–	–	–
Staphylococcus aureus (ATCC 6538)	–	–	–
Streptococcus pyogenes (NEWPROV 19615)	22	325.0	1300.0

Source: Laboratory of Biology and Physiology of Microorganisms, 2013.
Note: * Minimum Inhibitory Concentration (MIC).
**Minimum Microbicide Concentration (MMC).

Fernandes et al. verified that the antimicrobial activity of extracts and vegetable oils result from secondary metabolism products such as terpenoids and phenolic compounds, as flavonoids and saponins, that in their pure form also exhibit antimicrobial activity. The different results in the antimicrobial activity described in plants could be related to different amounts of each active ingredient in the studied extracts, use of different techniques and procedures, and different plant harvesting seasons.[16]

Brasileiro et al. evaluated the antimicrobial and cytotoxic activity of *P. ornatus* extracts in other 31 plant species, using the agar diffusion method and Artemia lethality test, and observed that the *P. ornatus* extract showed toxicity to Artemia salina larvae (Ld50 < 1000 ppm) and did not show growth inhibition in *Sthapylococcus aureus* and *Escherichia coli*, confirming the results obtained in our study.[4]

Nogueira et al. pointed out that strains of *S. aureus* were susceptible to *Plectranthus amboinicus* extract when testing 10 isolates of *S. aureus*, eight of *Pseudomonas aeruginosa*, four of *Candida albicans*, and four of *Candida krusei* isolates from acute external otitis. *Pseudomonas aeruginosa* was resistant to this tested extract.[17]

Lopes and Almeida[18] also evaluated the antibacterial activity of the fruit extract from *Morinda citrifolia* L. on strains of *S. aureus* and *E. coli* demonstrating that it did not inhibit growth.

Pinho et al.[19] carried out a study on antimicrobial activity of plant extracts using extracts of aroeira, barbatimão, and erva baleeira in diffusion agar assays on strains of *S. aureus* and *E. coli*; the studied extracts inhibited the growth of *S. aureus* but not of *E. coli*.

Alcoholic extracts of propolis, at concentrations of 11 and 20% inhibit the growth of *Bacillus cereus*, *Bacillus subtilis*, *Klebisiela pneumoniae*, *Streptococcus pyogenes*, *Enterobacter aerogenes*, *Micrococcus luteus*, *Candida albicans*, and *Saccharomyces cerevisae*.[20]

Silva et al. investigated antioxidant and antimicrobial activity of *Mimosa caesalpiniifolia* Benth extract in strains of *Bacillus cereus*, *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* using the agar diffusion technique. These authors found that the extract exhibited growth inhibitory activity in all tested microorganisms.[21]

Lopes et al.[22] analyzed the antimicrobial activity of insulin dry extract and copaíba oil in bacterial and fungal strains (*Salmonella typhimurium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Candida albicans*, and *Cryptococcus neoformans*) and verified that copaíba oil inhibited the growth of *S. aureus*, *E. coli*, *B. subtilis*, and *E. faecalis*; the other tested microorganisms were resistant. The MIC for the sensitive microorganisms ranged from 6.05 to 30.25 mg/mL, with MMC of 18.15 mg/mL for *E. faecalis*. The insulin extract did not show antimicrobial activity.[22-28]

Oregan, thyme, lippia, ginger, sage, rosemary, and basil extracts were studied by Pozzo et al.[29] Antimicrobial activity tests on 32 strains of *S. aureus* isolated from bovine mastitis showed inhibitory activity in all tested microorganisms.[29]

The evaluation of antimicrobial and antioxidant activity in the plant Ziziphus joazeiro, known as juazeiro or laranjeira do vaqueiro, using the agar diffusion technique and MIC, showed that the extract from leaves and bark have antioxidant activity and antimicrobial in 70% of the examined strains including *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*. The extract from leaves showed MIC between 0.25 and 0.5 mg/mL against *Micrococcus luteus*, and between 0.125-0.250 mg/mL against *Mycobacterium smegmatis*, whereas the bark extract presented MIC between 0.5 and 1.0 mg/mL against *M. smegmatis*.[24]

Phytochemical studies allowed the isolation of diterpenes from the genus *Plectranthus* sp. with ornantina A, barbatusina, labdane, and forskolin being the active principles; alkylphenols and flavonoids also were identified.[25,26] These active ingredients could be associated with the antimicrobial activity observed in the results described in this study.

Due to the wide variety in plant chemical composition, some studies detected flavonoids with several pharmacological actions, especially anti-inflammatory, cicatrizant, antitumor, antimicrobial, and antifungal mainly.[1,4,21,26-29] In this study, the antifungal activity of the evaluated product was identified at various concentrations resulting in growth inhibition of the fungus *Saccharomyces cerevisae* in presence of the *P. ornatus* extract. However, assuming the interference of several factors, there was no inhibition of *Candida albicans*.

Flavonoids and tannins have the ability to inactivate enzymes and form complexes with extracellular and soluble proteins and with bacteria cell wall, setting up the likely mechanisms of antimicrobial activity. The total rupture of microbial membranes can be caused by flavonoids with lipophilic features.[1,4,21]

Therefore, it is possible that these compounds are present in the *P. ornatos* extract and are responsible for its antimicrobial activity.

In *in vitro* antimicrobial activity studies using crude extracts of plants, the antimicrobial potential is often not related to just one single active ingredient. Therefore, the process of isolation of active substances in extracts can hinder its use as a phytotherapic product because its action is linked to the association of several active ingredients and not only one. [30]

CONCLUSION

The hydroalcoholic extract from *P. ornatus* leaves presented MIC from 20.31 mg/mL to 1,300 mg/mL, varying with the tested microorganism species. MMC was observed in three out of the four bacterial strains that showed inhibition halos. This study reaffirms the importance of ethno-pharmacological investigations in the selection of plants with therapeutic indication.

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