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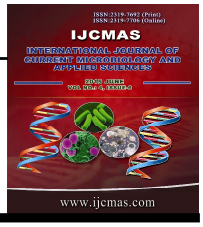

Original Research Article

Effect of plant extracts on planktonic growth and biofilm of *Staphylococcus aureus* and *Candida albicans*

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Keywords

Medicinal plants, Antimicrobial, Biofilm, Synergism

A B S T R A C T

The therapeutic use of plant derivatives has increased in recent years, providing major advances in chemical and pharmacological aspects. In this study, aqueous and hydroalcoholic extracts of *Eugenia uniflora, Piper diospyrifolium, Piper hispidum, Psidium guajava, Rosmarinus officinalis, Senna spectabilis* and *Tetradenia riparia* were investigated for antimicrobial activity, employing the broth microdilution technique. The microorganisms tested were *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Candida parapsilosis* and *Candida tropicalis*. All species showed some antimicrobial activity against bacteria and/or yeast species. *S. aureus, B. subtilis* and *Candida* species were most susceptible to the extracts. Five species were active against biofilm cells of *C. albicans* and *S. aureus*, and six species acted synergistically with fluconazole and/or nystatin against *C. albicans*, with FIC indexes less than 0.5. This study showed that these plant species could be useful sources for new antimicrobials.

Introduction

Medicinal plants are important in global health. Plants are used to treat various ailments including fever, gastrointestinal disorders, acne, and bacterial infections, and are used in various forms such as teas, decoctions, herbal powders and other formulations. The use of plants is prevalent as an alternative treatment for health problems, primarily in South American and African countries (Shale, 1999; Balunas, Kinghorn, 2005; Halberstein, 2005).

Many species have been investigated for their antimicrobial properties; and most studies have been conducted as screening in search of plants with antimicrobial activity, which allows for the selection of plant extracts with useful properties to be used in new chemical and pharmacological studies (Martinez et al., 1997; Machado et al., 2003). Brasileiro et al. (2006) assessed the antimicrobial activity of 33 Brazilian medicinal plants; although none of the

extracts was effective against Gram-negative bacteria, 13 extracts showed good activity against *Staphylococcus aureus*. Microbial resistance has been a major challenge for public health, especially when associated with biofilm-forming cells that are more resistant to drug treatment. In addition, the great concern caused by the undesirable effects of antimicrobials has contributed to a growing search for plant-derived therapeutic alternatives (Spellberg et al., 2004; Alviano et al., 2009). The high incidence of infections, particularly in immune-compromised individuals, increases the importance of and the search for new alternative therapeutic compounds. Natural products have great potential in research on and treatment of infectious diseases. Various classes of substances have been isolated from species of the genus *Piper*, such as alkaloids, neolignans, terpenes, lignans, which have demonstrated bioactivity (Nakamura et al., 2006). According to Nascimento et al. (2000), the use of plants to treat infectious diseases, has been extensively applied, and information from the literature and the results show the great potential of plants for therapeutic treatments, despite the fact that they do not have been thoroughly studied, and more studies need to be conducted to search for new components. The antimicrobial activity of some plants has been attributed to oxygenated terpenes, tannins, aldehydes and cardiac glycosides (Bertini, 2005). In this study, we evaluated antimicrobial effects of extracts from different plant species (Table 1) against planktonic and biofilm bacterial and fungal cells. Combinations of extracts and antimicrobial drugs were also evaluated.

Material and Methods

Plant material

Plant material was collected in April 2012, in the morning. Leaves from *Eugenia uniflora*, *Piper hispidum*, *Psidium guajava*, and *Rosmarinus officinalis*, and leaves, flowers and twigs from *Senna spectabilis* were collected in the Garden of Medicinal Plants, State University of Maringá (UEM), Maringá, Paraná. Leaves from *Piper diospyrifolium* were collected at the Horto Florestal Dr. Luiz Teixeira Mendes in Maringá. Specimens of the plant material are deposited at the UEM Herbarium. *Tetradenia riparia* was collected in the Medicinal Garden of the Universidade Paranaense (UNIPAR), Umuarama, Paraná, and a specimen is deposited at the UNIPAR Herbarium.

Preparation of extracts

Plant material was dried in a circulating-air oven at 40°C and then ground. Subsequently it was soaked in 90/10% (v/v) ethanol-water for 48 h at 25°C, protected from light. Extracts were obtained by vacuum evaporation, resulting in two residues, referred to as the aqueous extract which was lyophilized, and the hydroalcoholic extract, which was taken with ethyl acetate and left at room temperature until complete evaporation of the solvent. The extracts were stored at - 10°C.

Strains and growth conditions

The bacterial strains were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6623 and *Staphylococcus aureus* ATCC 25923. They were maintained in Mueller-Hinton Agar (MHA-Difco) at 4°C and cultured in Mueller-Hinton Broth (MHB-Difco) before each experiment.

The yeast strains were *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 28707, maintained on Sabouraud Dextrose Agar (SDA-Difco) at 4 °C and cultured in

Sabouraud Dextrose Broth (CSD) (Difco) at 37 °C before each experiment.

Microdilution MIC determination

Antibacterial and antifungal assays were performed by the microdilution method in sterile flat-bottom microplates, according to CLSI (2008, 2013). Each well contained appropriate test samples, culture medium, and approximately 10^5 cells/mL for bacteria and 10^3 cells/mL for yeasts. Serial two-fold dilutions of each extract were done in a microdilution plate (96 wells) containing 100 µl of culture medium. Next, the inoculum was added to each well. The microplates were incubated at 37 °C during 24 h for bacteria and 48 h for yeast. The MIC was defined as the lowest concentration which resulted in inhibition of visual growth. Minimal bactericidal and fungicidal concentrations (MBC and MFC) were determined by subculturing 10 µl of the culture from each negative well and from the positive control.

Checkerboard

Checkerboard tests were performed with *C. albicans*, by the broth microdilution reference procedure of the CLSI at a final inoculum of 0.5 x 10^3 to 2.5 x 10^3 cells/mL, using RPMI 1640 medium buffered with 0.165 M MOPS. The final concentrations ranged from 0.24 to 250 µg/mL for fluconazole and nystatin, and 15.60 to 1000 µg/mL for the extracts. Plates were incubated at 37 °C for 48 h. The fractional inhibitory concentration (FIC) index is defined as the sum of the MIC of each drug in combination, divided by the MIC of the drug used alone. An FIC index $\leq$ 0.5 is considered synergism; an FIC index > 4 is antagonism; and a result > 0.5 but $\leq$ 4 is indifferent (ODDS, 2003)].

Biofilm

C. albicans biofilm was formed on 96-well microtiter polystyrene plates. Assays were done twice in triplicate. 100 µl of a suspension containing 1 x 10^6 cells/mL in RPMI 1640 medium with L-glutamine without bicarbonate, buffered with 0.165 M MOPS was seeded in wells and incubated at 37°C for 48 h. Next, the well content was removed, the wells were washed with phosphate buffered saline (PBS), and dilutions of extracts ranging from 15.6 to 1000 µg/mL were added to each well. After incubation at 37 °C for 24 h, the wells were rinsed with PBS. For the MTT reduction assay, slight modifications of the method utilized by Schillaci et al. (2008) were made. 20 µl of MTT solution (5 mg/mL in PBS) was added to each well and the plates were incubated at 37 °C for 4 h. After staining, the MTT solution was removed from each well and 100 µl of DMSO was added to dissolve the MTT formazan product. 100 µl of DMSO was transferred to a new plate, and the optical density was measured at 570 nm using a microplate reader.

The *S. aureus* biofilm was formed in 96-well microtiter polystyrene plates. Assays were done twice in triplicate. 100 µl of a suspension containing 1 x 10^8 cells/mL TSB medium supplemented with 1% glucose were seeded in wells and incubated at 37 °C for 24 h. The remainder of the procedure was the same as described above for *C. albicans*.

Ergosterol assay

MICs of extracts against *C. albicans* were determined as described previously (SORTINO et al., 2007) in the absence and presence of ergosterol at the concentrations of 50, 100, 200 and 400 µg/mL in RPMI

medium. Amphotericin B was used as the control drug.

Results and Discussion

Antibacterial and antifungal effects

Aqueous and hydroalcoholic extracts from seven species of plants were evaluated for antimicrobial activity, using the technique of broth microdilution. Good activity was considered to be MICs ≤ 100 μg/mL, moderate activity a MIC of 100 to 500 μg/mL, and weak or inactive a MIC > 500 μg/mL (HOLETZ et al., 2002).

Several hydroalcoholic extracts from different plant species showed good activity against Gram-positive bacteria and yeasts (Table 2). The antifungal activity of *P. hispidum* may be due to the presence of amides (NAVICKIENE, 2000). In recent years, several studies have reported antifungal activity of *Piper* species, particularly against *C. albicans*, *Saccharomyces cerevisiae* and *Trichophyton mentagrophytes*, which led to the isolation of active compounds such as pyrrolidine and piperidine amides from *P. hispidum* and benzoic acid from *P. dilatum* (PESSINI, 2005).

R. officinalis was active against *S. aureus* with an MIC of 15.6 μg/mL, *B. subtilis* with an MIC of 62.5 μg/mL and *C. parapsilosis* with an MIC of 62.5 μg/mL. According to Hussain et al. (2010), essential oil from rosemary has good antimicrobial activity, with an inhibition zone comparable to the control ciprofloxacin; these authors attributed the activity to the major compound 1,8-cineol. Santoyo et al. (2005) attributed antimicrobial properties to the compounds α-pinene, camphor, verbenone and borneol. An important feature of essential oils is hydrophobicity, which facilitates penetration into the cell membrane of Gram-positive bacteria, causing increased cell death (DENYER, 1991).

In this study, different extracts from *S. spectabilis* were active against *B. subtilis*, *C. parapsilosis* and *C. tropicalis*, but leaves and branches were practically inactive against all microorganisms tested, stated that the chemical constituents of the same plant are different depending on which parts are used in the study. *T. riparia* was active against *C. albicans*, with an MIC of 62.5 μg/mL. Gazim et al. (2010) attributed the antimicrobial activity of *T. riparia* to oxygenated sesquiterpenes and oxygenated monoterpenes and diterpenes found in the essential oil obtained from its leaves.

Our aqueous extracts were less effective than hydroalcoholic extracts, but some extracts showed good activity. *E. uniflora* was effective against *S. aureus* with an MIC of 31.2 μg/ml, and against *C.parapsilosis* and *C. tropicalis*, with an MIC of 15.6 μg/mL. *P. guajava* inhibited *C. parapsilosis*, with an MIC of 15.6 μg/mL. *R. officinalis* was moderately active against *C. parapsilosis* with an MIC of 125 μg/mL. Flowers of *S. spectabilis* were active against *C. parapsilosis* and C. *tropicalis*, with an MIC of 125 μg/mL. Not all extracts that show good inhibition of certain pathogens have the ability to kill microorganisms. After subculturing 10 μl of the culture from each negative well, it was possible to determine the Minimal Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MFC). *P. diospyrifolium* had an MBC at 62.5 μg/mL against *B.subtilis*. *P. hispidum* showed good bactericidal activity against *S. aureus* and *B. subtilis*, with an MBC of 62.5 μg/mL. The species with the best fungicidal activity was *R. officinalis*, with MFC values of 62.5 and 250 μg/mL for *C. parapsilosis* and *C. tropicalis*, respectively.

Table.1 Plant material used to testing antimicrobial activity

Specie (Family) (Herbarium number according to colector)	Used parts	Popular use
Eugenia uniflora (*Mirtaceae* (8419)	Leaves	hypertension, rheumatism, diarrhea
Psidium guajava (*Mirtaceae*)(8423)	Leaves	colic, colitis, diarrhea, stomach pain, wounds and ulcers
Piper diospyrifolium (*Piperaceae*)(15095)	Leaves	gynecological diseases, intestinal disorders
Piper hispidum (*Piperaceae*)(9137)	Leaves, roots and fruits	astringent, diuretic, hemorrhoid, rheumatism, dysenteria
Rosmarinus officinalis (*Labiateae*)(10480)	Leaves	antispasmodic in renal colic, disminorréia of respiratory disorders
Senna spectabilis (*Leguminosae*)	Leaves	colds and flu, laxative, antimicrobial
Tetradenia riparia (*Lamiaceae*) (2502)	Leaves	malaria, angina, skin disease, gastroenteritis, gonorrhea, diarrhea, headaches, bronchitis, coughs, ulcers, kidney disease

Table.2 Minimal Inhibitory Concentrations (µg/mL) of hydroalcoholic extracts against bacteria and yeasts

Plant	*S. aureus*	*B. subtilis*	*E.coli*	*P. aeruginosa*	*C. albicans*	*C. parapsilosis*	*C. tropicalis*
E. uniflora	> 1000	> 1000	> 1000	> 1000	125	31.2	31.2
P. guajava	62.5	62.5	> 1000	> 1000	125	15.6	> 1000
P. diospyrifolium	62.5	7.8	> 1000	> 1000	250	> 1000	> 1000
P. hispidum	62.5	7.8	> 1000	> 1000	62.5	15.6	15.6
R. officinalis	15.6	62.5	> 1000	> 1000	250	62.5	125
S spectabilis (flowers twigs)	> 1000	62.5	> 1000	> 1000	> 1000	15.6	> 1000
S spectabilis (flowers)	> 1000	1000	> 1000	> 1000	250	62.5	15.6
S spectabilis (leaves)	> 1000	> 1000	> 1000	> 1000	250	250	500
S spectabilis (twigs+leaves)	> 1000	> 1000	> 1000	> 1000	> 1000	125	> 1000
T. riparia	> 1000	> 1000	> 1000	> 1000	62.5	125	> 1000

Table.3 FIC index for combination of extracts and antifungal agents

Plant species	Antifungal agent	FIC index
E. uniflora	Fluconazole	0.31
	Nystatin	0.49
P. diospyrifolium	Nystatin	0.31
P. hispidum	Fluconazole	0.37
	Nystatin	0.12
R. officinalis	Nystatin	0.24
S. spectabilis (flowers)	Nystatin	0.37
T. riparia	Nystatin	0.24

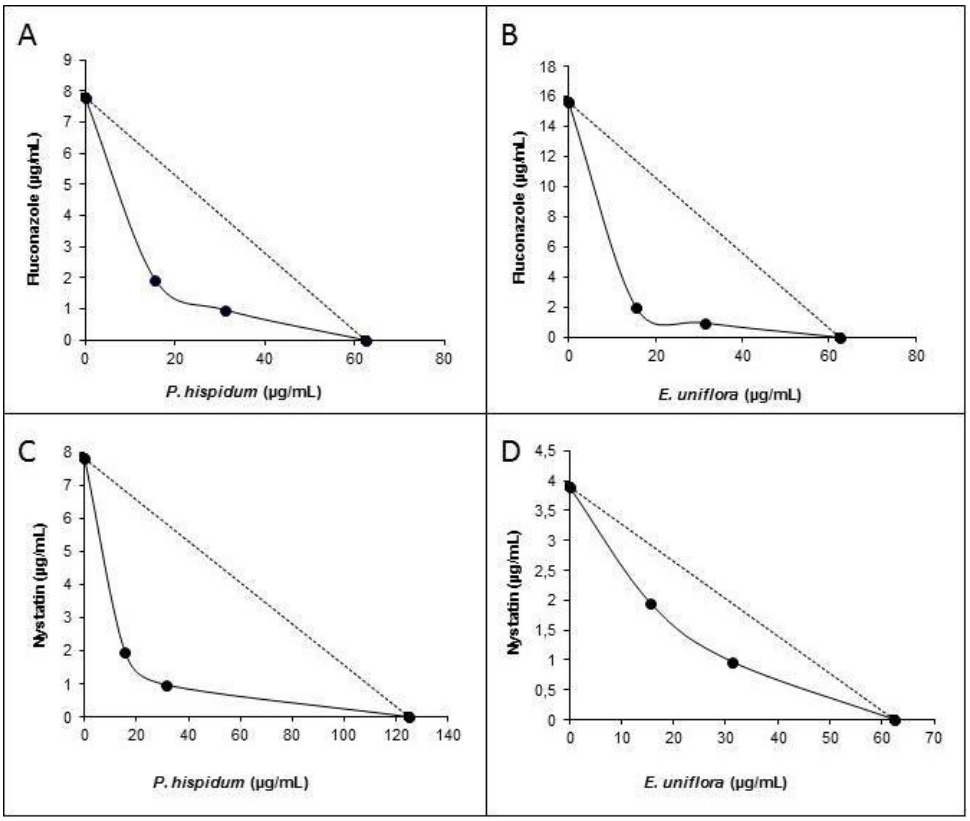
Table.4 Inhibitory concentrations to planktonic and biofilm cells of *Candida albicans* (µg/mL)

Plants	Planktonic cells	Biofilm 48h
E. uniflora	125	1000
P. guajava	125	>1000
P. diospyrifolium	250	500
P. hispidum	62,5	125
T. riparia	62,5	62,5
Fluconazole	7,8	>1000
Nystatin	3,9	7,8

Table.5 Inhibitory concentrations to planktonic and biofilm cells of *Staphylococcus aureus* (µg/mL)

Plants	Planktonic cells	Biofilm
P. diospyrifolium	62,5	250
P. hispidum	62,5	250
P. guajava	62,5	500
R. officinalis	15,6	125
Penicilin	0,02	>50
Vancomicin	-	>50

Figure.1 Isobolograms for combinations of extracts and antifungal agents. A and C) Synergism between *P. hispidum* and Fluconazole and Nystatin. B and D) Synergism between *E. uniflora* and Fluconazole and Nystatin. Concave curves indicate synergism



Checkerboard

Synergism between hydroalcoholic extracts and fluconazole or nystatin can be checked by the FIC index (Table 3) and isobolograms (Figure 1). Six extracts showed synergism with fluconazole or nystatin, and *E. uniflora* and *P. hispidum* acted synergistically with both antifungals fluconazole and nystatin against *C. albicans*. The fractional inhibitory concentration (FIC) index is defined as the sum of the MIC of each drug in combination, divided by the MIC of the drug used alone. An FIC index ≤ 0.5 is considered synergism; FIC index > 4 is antagonism; and a result > 0.5 but ≤ 4 is indifferent (ODDS, 2003).

In view of the lack of new classes of antifungal drugs with novel mechanisms of action, the increase of infections caused by opportunistic fungi and fungal resistance, combinations of drugs may be an effective therapeutic strategy. Therapeutic interactions are described as synergistic, indifferent and antagonistic. Several plants have been shown to act synergistically with antibiotics, with an advantage for treatment, including benefits such as a broader spectrum of efficacy, improved safety and tolerability, reduced toxicity, and reduced resistance to antifungal agents (ENDO 2010; CABRAL, 2013). Some proposed mechanisms for the synergistic antifungal effect are (i) inhibition at different stages of the same biosynthetic pathway; (ii) greater penetration of an antifungal agent because of the effect on the wall or other agent of the membrane; (iii) simultaneous inhibition in different fungal targets (JOHNSON et al. 2004).

Biofilm

Inhibition of biofilm cells was evaluated by the MTT reduction assay for *C. albicans* strains. Plant extracts tend to be more effective in planktonic cells than in sessile (biofilm) cells, according to the results shown in Table 4. The hydroalcoholic extract of *T. riparia* was effective in inhibiting biofilm at a concentration of 62.5 μg/mL, the same inhibitory concentration obtained for planktonic cells. *T. riparia* was more effective than fluconazole, but less effective than nystatin, with an MIC of 7.8 μg/mL for biofilm cells. *P. hispidum* was effective against biofilm cells at 125 μg/mL, only one dilution above the MIC for planktonic cells. *P. diospyrifolium* was moderately effective, with an MIC of 500 μg/mL. *E. uniflora* and *P. guajava* were inactive against the biofilm formed by *C. albicans*.

The inhibition of the *S. aureus* biofilm was also evaluated by reduction of MTT. Table 5 shows that hydroalcoholic extracts were active against biofilms at concentrations at least three times above the inhibitory concentrations on planktonic cells; this occurs because cells in biofilms are more resistant than planktonic cells.

Biofilm cells have a unique developmental characteristic compared to planktonic cells, making them more resistant to antibiotics, increasing their tolerance to about 10 to 100 times greater than the planktonic form (HARRISON, 2004; REIS, 2011). *R. officinalis* was more effective in inhibiting biofilm at 125 μg/ml, and *P. diospyrifolium* and *P. hispidum* were effective at 250 μg/ml. Quave et al. (2008) reported that 10 plants showed inhibition of biofilm formation against *S. aureus*, one of them, *R. officinalis*, at 8 μg/mL. The difference in antimicrobial activity in the same species might be due to factors related to culture, such as soil type, moisture or light and harvest time, which can affect the chemical composition of the plant (CORREA Jr, 1991).

Five species were active against biofilm cells of *C. albicans* and *S. aureus*, which is very interesting since the formation of biofilms by microrganisms and infections related to biofilms has stimulated a search for new anti-biofilm strategies. Biofilms often cause chronic infections with high tolerance to antimicrobials compared to planktonic cultures, and resist macrophages and other components of the immune system.

Ergosterol assay

We also evaluated the ability of hydroalcoholic extracts from *T. riparia* and *P. hispidum* to bind directly to ergosterol by the method of MIC determination, with exogenous ergosterol added to the medium. Neither of the two extracts significantly altered MIC values against *C. albicans*, indicating no connection to its mechanism of action with the ergosterol molecule.

This study contributes to the knowledge of antimicrobial properties of plants commonly found in Brazil. Seven different species of plants were tested, and showed some antimicrobial activity against bacteria and/or yeast species. Six of the seven species acted synergistically with the antifungal against *C. albicans*, and five were also effective against biofilm cells of *C. albicans* and *S. aureus*. These *in vitro* assays indicate that these plant species are potential candidates for the development of new strategies to treat fungal and bacterial infections.

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