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In vitro effects of *Eugenia pyriformis* Cambess., Myrtaceae: Antimicrobial activity and synergistic interactions with Vancomycin and Fluconazole

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The *Eugenia pyriformis* Cambess. species, Myrtaceae, also known by the popular name as *uvaia* was evaluated for its antimicrobial activity. Broth microdilution method was used to determine the Minimum Inhibitory Concentration (MIC) against selected pathogenic strains of bacteria, and fungi. Checkerboard method was used to evaluate the synergistic interactions of *E. pyriformis* with Vancomycin and Fluconazole. The leaf and stem crude extract showed for Gram-positive strains MIC values of 125 and 250 µg/ml and for leveduriform fungi MIC values ranging from 7.81 to 62.5 µg/ml. Ethyl acetate, hydroalcoholic fractions, and leaf acetonic extract showed MIC values between 62.5 and 125 µg/ml for Gram-positive strains. The ethyl acetate fraction and leaf acetonic extract showed MIC values ranging from 7.81 to 62.5 µg/ml for leveduriform fungi; the stem acetonic extract MIC value was 62.5 µg/ml against Gram-positive strains and MIC value of 7.81 µg/ml for leveduriform fungi. The combination of *E. pyriformis* with Vancomycin and Fluconazole showed synergistic activity for strains of *Enterococcus faecalis*, *Candida albicans*, *Candida krusei* and *Candida parapsilosis* with fractional inhibitory concentration indices (FICI) below of 0.5. The extracts and fractions of this medicinal plant were able to inhibit the growth of bacteria and fungi *in vitro*.

Key words: *Eugenia pyriformis* Cambess, antimicrobial activity, synergistic interaction.

INTRODUCTION

Medicinal plants produce a variety of compounds that show biological activities, which are employed for developing drugs, representing a source of great importance in research of new antimicrobial agents (Newman

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and Cragg, 2012).

These compounds derive from several secondary metabolic pathways, and include alkaloids, flavonoids, lignins, phenolic compounds and terpenoids (Saleem et al., 2009).

The extensive use of antimicrobials has led to growing resistance and the spread of many bacterial and fungal pathogens, which now constitutes a serious medical problem. The combination of antimicrobial therapy has become an alternative for the treatment of infectious diseases caused by multiresistant bacteria (Wolska et al., 2012). Essential oils, extracts and isolated compounds containing secondary metabolites are able to delay or inhibit bacteria, yeasts and leveduriform fungi growth (Tiwari et al., 2009). These compounds display antimicrobial activity when used alone, but there is also the possibility of using them in combination with conventional antimicrobials in order to improve their efficacy (Wolska et al., 2012).

The Myrtaceae family considered the most complex from the taxonomic point of view shows in its leaves a great amount of volatile constituents (Stieven et al., 2009). It is widely found in the Americas and Oceania, and in Brazil, it is represented by 23 genera and a thousand species distributed all over the country, mainly through the Atlantic Forest and *restinga*, with about a third of these species belonging to the *Eugenia* genus (Landrum and Kawasaki, 1997; Farias et al., 2009).

The species *Eugenia pyriformis* Cambess, representative of this family, is a common plant in the states of São Paulo, Paraná, Santa Catarina and Rio Grande do Sul, known by the popular name of *uvaia*, *uvaieira*, *uvaia-do-campo*, *uvalha* or *uvalha-do-campo* (Armstrong et al., 2012). The plant is grown in orchards and employed in popular medicine, its blooming occurs from November to January and edible fruits ripen becoming yellow in January and February, and they present high levels of antioxidant activity and phenolic compounds (Stefanello et al., 2009).

The *uvaia* is a plant that can be used in reforestation programs, showing easy cultivation and growing in gardens, its rich nutritional value fruits are used in industrial manufacturing of several products (Lorenzi et al., 2006) and its leaves act in treatment for gout (Schmeda-Hirschmann et al., 1987; Theoduloz et al., 1988). The fruit extract of *E. pyriformis* showed antimicrobial activity against *Enterococcus faecalis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Stieven et al., 2009).

Given the importance of Myrtaceae family and due to scarce studies conducted so far, this plant represents a great potential of exploration and a promising field for development of antibacterial and antifungal agents for treatment of human and animal infections. This study aims at evaluating the *in vitro* antimicrobial activity and potential synergistic of the extracts and fractions of *E. pyriformis* Cambess.

MATERIALS AND METHODS

Plant and preparation

Aerial parts of *E. pyriformis* Cambess were collected in *campo limpo* and in *borda de capão* at Curitiba's Jardim Botânico, under the coordinates 25° 26' S; 49° 14' W, at an altitude of 930 m, in June. The plant identification was performed by the botanist Gert Hatschbach, at Botanical Garden of Curitiba (MBM) herbariums, under number 204990.

The crude ethanolic extract was prepared with 96° GL ethanol, in continuous reflux for 6 h, at 50°C in modified Soxhlet device. Fractions were obtained through the liquid-liquid partitioning method. In the technique, solvents of analytical standard PA were used in increasing order of polarity (hexane, chloroform and ethyl acetate), being the fraction remaining to the hydroalcoholic. The crude acetonic extract was obtained from leaves and stem extracted with acetone at 30°C during a period of 6 h in modified Soxhlet device.

The screening phytochemical was performed in thin layer chromatography (TLC) silica gel 60 F254 (Merck) analysis of the crude ethanolic extract, fractions and crude acetonic extract in mobile phase and reveals specific to indicate the presence of sterols/triterpenoids (vanillin-sulfuric acid 1%), tannins (ferric chloride 1%) and phenolic compounds (Neu-reagent). The following solvents moistures were used toluene/ethyl acetate (97:3) for steroids/triterpenes, ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:27) for tannins and phenolic compounds.

For the microbiological analysis, the extracts and fractions were prepared in 10% ethanol and 2% dimethyl sulfoxide (DMSO), and filtered through 0.22 µm Millipore membrane (TPP, Trasadingen, Switzerland) in order to assure its sterility.

Antibacterial activity

The antibacterial activity tests were performed with the following strains: *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853.

The Minimum Inhibitory Concentration (MIC) values were determined through broth microdilution method (Clinical and Laboratory Standards Institute [CLSI], 2008a). Bacterial suspensions were prepared in saline solution at concentration of 1.0×10^8 CFU/ml, corresponding to 0.5 McFarland tube and they were subsequently inoculated in a 5 µl volume into the wells, thus remaining a final concentration of 10^4 CFU/ml.

The inhibitory activity negative control of the diluents, ethanol and DMSO was prepared by adding 100 µl of 10% ethanol and 2% DMSO solution in 100 µl of Mueller-Hinton broth (MHB) and 5 µl of the bacterial inocula. For the sterility control, 100 µl of MHB and 100 µl of the extract or fraction were used. The bacterial viability or positive control was prepared with 100 µl of MHB and 5 µl of the bacterial inocula.

Microplates were incubated in bacteriological incubator at 35°C for 16 to 20 h. After this time interval, 20 µl of aqueous solution of 0.5% Triphenyltetrazolium Chloride (TTC – Merck, Darmstadt, Germany) were added, and the microplates were incubated again for 3 h at 35°C. The results reading were subsequently performed, where the red coloration formation in the wells was interpreted as absence of antimicrobial activity for the studied substance.

For the results analysis, the MIC values obtained were classified as having good inhibitory potential (up to 100 µg/ml); moderate inhibitory activity (between 100 and 500 µg/ml); weak inhibitory activity (between 500 and 1000 µg/ml), and absence of inhibitory activity (higher than 1000 µg/ml) (Ayres et al., 2008).

Table 1. Antimicrobial activity of stem and leaves of *E. pyriformis* Cambess.

Microorganism	Stem extracts and fractions (µg/ml)						Leaf extracts and fractions (µg/ml)					
	RE	HEF	CF	EAF	HF	AE	RE	HEF	CF	EAF	HF	AE
Gram-positive												
E. faecalis ATCC 29212	250	500	1000	1000	-	62.5	125	-	250	62.5	62.5	62.5
S. aureus ATCC 25923	250	-	-	-	-	62.5	125	-	250	62.5	62.5	125
Gram-negative												
E. coli ATCC 25922	1000	-	-	-	-	250	1000	-	500	250	500	250
K. pneumoniae ATCC 700603	1000	-	-	-	-	-	1000	-	-	250	-	1000
P. aeruginosa ATCC 27853	1000	-	-	1000	-	1000	1000	-	500	250	1000	500
Leveduriform fungi												
C. albicans ATCC 40175	31.25	-	-	-	-	7.81	31.25	-	-	62.5	-	7.81
C. krusei ATCC 40174	31.25	-	-	-	-	7.81	7.81	-	-	31.25	-	7.81
C. parapsilosis ATCC 40038	62.5	-	-	-	-	7.81	15.62	-	-	31.25	-	7.81

RE: Crude Extract, HEF: Hexane Fraction, CF: Chloroform Fraction, EAF: Ethyl Acetate Fraction, HF: Hydroalcoholic Fraction, AE: Acetonic Extract, (-) no effect on inhibition of growth with the concentrations tested.

Antifungal activity

The tests were performed with the *Candida albicans* ATCC 40175, *Candida krusei* ATCC 40147 and *Candida parapsilosis* ATCC 40038 strains.

Serial dilutions of the extracts and fractions in a concentration range from 1000 to 7.81 µg/ml were prepared with liquid medium RPMI 1640 (Gibco/Invitrogem, New York, USA) in 96-well, U-shaped bottom sterile microplates (CLSI, 2008b). The distinct fungal suspensions were prepared in saline solution at initial concentration of 1.0×10^8 CFU/ml. These suspensions were diluted in liquid medium until a 1.0 to 5.0×10^3 CFU/ml final concentration was reached and subsequently inoculated 100 µl into the wells. The microplates were incubated for 48 h at 35°C. After this period, 20 µl of 0.5% TTC were added and the plates were incubated again for 3 h at 35°C. The results reading and analysis were performed according to the same methodology as the antibacterial activity.

Synergistic activity

The analyses of synergism were determined through Checkerboard method using extracts and fractions of *E. pyriformis* that showed MIC values below 100 µg/ml in combination with the antimicrobials Vancomycin and Fluconazole.

The antimicrobial in the combination was serially diluted along the ordinate of the microplate, while the extracts and fractions were diluted along the abscissa. The concentrations were prepared corresponding to MIC/8, MIC/4, MIC/2, MIC, MICX2 and MICX4. The combination for each reference strain was tested in duplicate. The first antagonistic, additive or synergistic effect of the extracts and fractions in combination with the antimicrobial was determined with calculation of fractional inhibitory concentration indices (FICI). FICI was calculated as $FIC_A + FIC_B$, where $FIC_A = MIC_A$ of the combination/MIC_A alone and $FIC_B = MIC_B$ of the combination/MIC_B alone. The results were interpreted as synergism (FICI < 0.5), addition (0.5 < FICI > 4) or antagonism (FICI > 4) (Chung et al., 2011).

The second method involved plotting the data as isobolograms (Hemaiswarya and Doble, 2010). The graph is represented with the ratio to the FIC of the *E. pyriformis* on the x-axis and the ratio of the FIC of the antimicrobial on the y-axis. A straight line that connects the ratio 0.5 in the ordinate and 0.5 in the abscissa indicates the line synergism. A straight line that connects the ratio 4.0 in the ordinate and 4.0 in the abscissa indicates the line additivity, the location of the FIC of the combination considerably above the line indicates antagonism.

RESULTS AND DISCUSSION

The antimicrobial activity *in vitro* of extracts and fractions of stem and leaves of *E. pyriformis* Cambess was determined in this study. The values obtained in the microbiological assays are presented in Table 1.

According to this established profile, the leaf hydroalcoholic and ethyl acetate fractions showed pronounced inhibitory activity for *E. faecalis* and *S. aureus* (MIC=62.5 µg/ml), and the results were considered good in the scale established. Similar results were obtained to stem acetonic extract (*E. faecalis* and *S. aureus*) and leaf acetonic extract (*E. faecalis*) showed good inhibitory potential (MIC= 62.5 µg/ml).

The phytochemical screening showed the presence of sterols/triterpenes in stem and leaf acetonic extract, hexane and chloroform fraction; tannins and phenolic compounds in the acetonic extract, chloroform, ethyl acetate and hydroalcoholic fractions. The reagent Neu showed yellow bands which are characteristic of flavonoid compounds (Riffault et al., 2014). Chavasco et al. (2014) reported that leaf extracts of *E. pyriformis* showed the presence of alkaloid, flavonoid, tannin, saponin and stem extracts showed tannin and saponin in their composition.

The observed antibacterial activity is attributed to the presence of different bioactive compounds which have an impact on growth and metabolism of microorganisms. The phenols and flavonoids significantly contribute to the

Table 2. FIC indices of *E. pyriformis* Cambess with Vancomycin and Fluconazole against strains of Gram-positive and leveduriform fungi.

Compound		Gram-positive									
		E. faecalis ATCC 29212					*S. aureus* ATCC 25923				
A	B	$^{*}MIC_A$	$^{*}MIC_B$	$^{**}FIC_A$	$^{**}FIC_B$	$^{\delta}FICI$	$^{*}MIC_A$	$^{*}MIC_B$	$^{**}FIC_A$	$^{**}FIC_B$	$^{\delta}FICI$
AES	VAN	62.5	1	>8	>8	>8	62.5	2	0.12	2.00	2.12
AEL	VAN	62.5	1	8	0.12	>8					
EAFL	VAN	62.5	1	8	0.12	>8	62.5	2	1.00	0.25	1.25
HFL	VAN	62.5	1	0.25	0.12	0.37	62.5	2	0.12	2.00	2.12

Compound		Leveduriform fungi														
		C. albicans ATCC 40175					*C. krusei* ATCC 40174					*C. parapsilosis* ATCC 40038				
A	B	$^{*}MIC_A$	$^{*}MIC_B$	$^{**}FIC_A$	$^{**}FIC_B$	$^{\delta}FICI$	$^{*}MIC_A$	$^{*}MIC_B$	$^{**}FIC_A$	$^{**}FIC_B$	$^{\delta}FICI$	$^{*}MIC_A$	$^{*}MIC_B$	$^{**}FIC_A$	$^{**}FIC_B$	$^{\delta}FICI$
RES	FLU	31.25	0.50	0.50	2.00	2.50	31.25	64	0.50	0.12	0.62	62.50	4	0.12	1.00	1.12
REL	FLU	31.25	0.50	0.50	0.50	1.00	7.81	64	0.25	0.25	0.50	15.62	4	0.25	0.12	0.37
AES	FLU	7.81	0.50	1.00	0.25	1.25	7.81	64	0.50	0.12	0.62	7.81	4	0.12	1.00	1.12
AEL	FLU	7.81	0.50	0.50	0.12	0.62	7.81	64	0.12	0.12	0.24	7.81	4	0.50	0.12	0.62
EAFL	FLU	62.5	0.50	0.25	0.12	0.37	31.25	64	0.12	0.12	0.24	31.25	4	0.25	0.12	0.37

AES: Acetonic Extract Stem, AEL: Acetonic Extract Leaf, EAFL: Ethyl Acetate Fraction Leaf, HFL: Hydroalcoholic Fraction Leaf, RES: Crude Extract Stem, REL: Crude Extract Leaf, VAN: Vancomycin, FLU: Fluconazole. *Minimum inhibitory concentration for combination of two compounds expressed in µg/ml. **Fractional inhibitory concentration of individual compounds. δFractional inhibitory concentration of two compounds in the combination.

to the antibacterial activity, can form complexes with cell wall and also disrupt bacterial envelopes (Kurek et al., 2011).

The stem and leaf acetonic extract, hydroalcoholic fractions and leaves ethyl acetate showed good inhibitory potential against Gram-positive microorganisms; however, MIC values above of 250 µg/ml were shown for Gram-negative. Low inhibitory activity presented in Gram-negative bacteria in relation to Gram-positive bacteria could be ascribed to their differences in cell membrane constituents and their arrangement. The resistance of Gram-negative bacteria towards antibacterial substances may be due the presence of outer membrane as a permeability barrier, difficult for compounds diffusion through its lipopolysaccharide membrane (Chew et al., 2011). The absence of this barrier in Gram-positive bacteria allows direct contact of substances with the cell membrane phospholipid layer, thus allowing the increase in ionic permeability and leakage of vital intracellular constituents, or even resulting in its enzymatic systems deficiency (Zarai et al., 2011).

The antifungal activity analysis (Table 1) showed good inhibitory potential for crude extracts, with MIC values ranging from 7.81 and 62.5 µg/ml. These values were the lowest MICs found in relation to all the tested microorganisms. Results suggest an antifungal activity efficiency of *E. pyriformis* on leveduriform fungi, which despite being eukaryotic organisms, with more complex structural organization in comparison with bacteria (Teke et al., 2011), showed more significant MIC values. The leaves and stem acetonic extract also showed good inhibitory potential on leveduriform fungi (MIC=7.81 µg/ml), and the ethyl acetate fraction showed good inhibitory potential only for the leaf (MIC= 31.25-62.5 µg/ml). The other tested fractions did not show any inhibitory activity.

The results of the analysis of synergism of extracts and fractions from *E. pyriformis* with Vancomycin and Fluconazole were determined against Gram-positive and fungi leveduriform as depicted on Table 2.

The combination of leaf hydroalcoholic fraction and Vancomycin exhibited synergism against *E. faecalis* with FICI of 0.37 while the combination of Fluconazole with either leaf crude extract or leaf acetonic extract of *E. pyriformis* showed enhanced

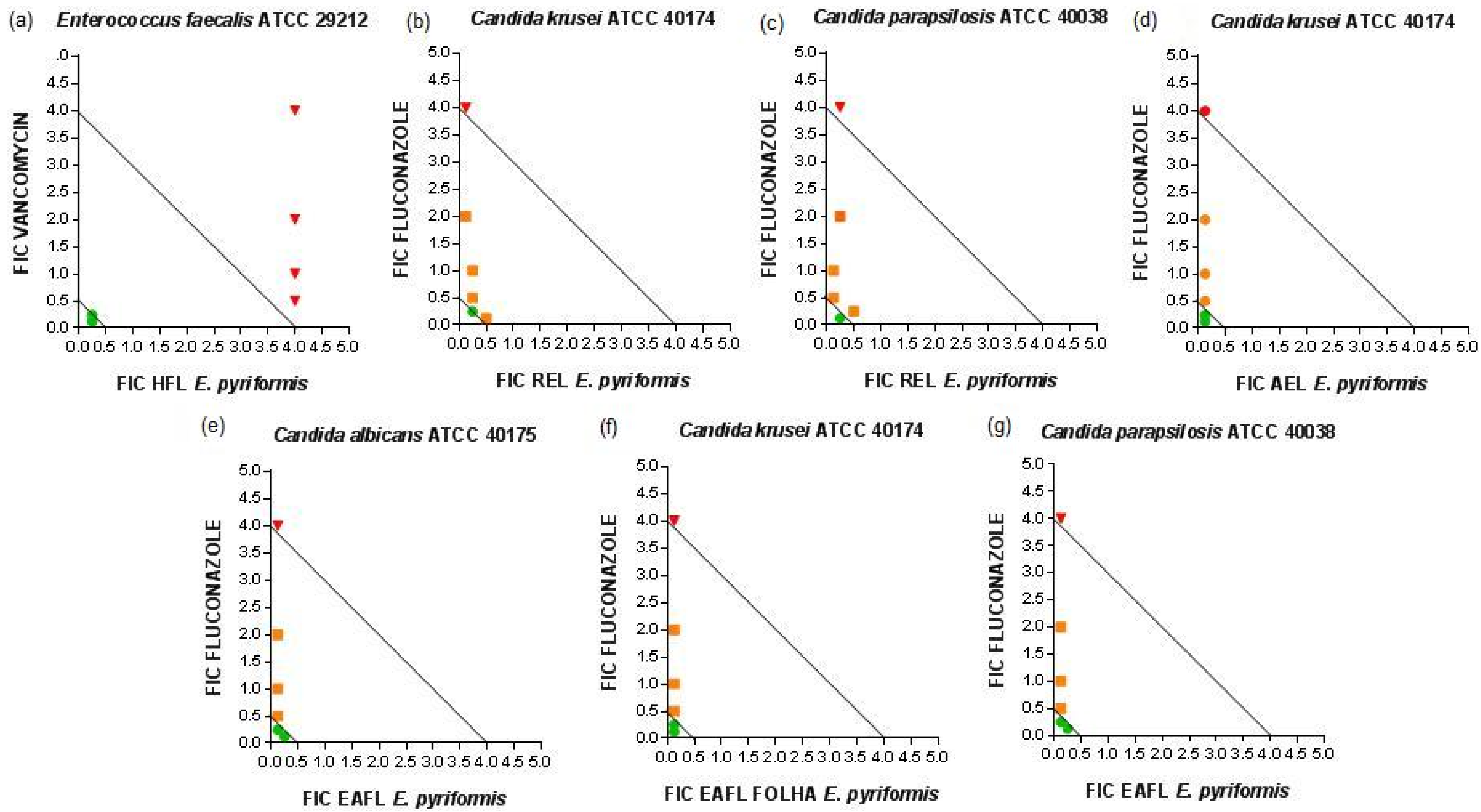

Figure 1. Representative isobolograms depicting the interaction of extracts and fractions of *E. pyriformis* with Vancomycin and Fluconazole. ( ) Synergistic, ( ) Additive, ( ) Antagonism, (a) HFL: Hydroalcoholic Fraction Leaf, (b) REL: Crude Extract Leaf, (c) REL: Crude Extract Leaf, (d) AEL: Acetonic Extract Leaf, (e) EAFL: Ethyl Acetate Fraction leaf, (f) EAFL: Ethyl Acetate Fraction leaf, (g) EAFL: Ethyl Acetate Fraction leaf. The lines connecting 0.5 on the abscissa and ordinate indicates synergism, 4.0 on the abscissa and ordinate indicates additivity and above the line indicates antagonisms.

enhanced efficacy against *C. krusei* and *C. parapsilosis* with FICI values ranging between 0.24 and 0.50. On the hand combination of Fluconazole with leaf ethyl acetate fraction showed enhanced efficacy against *C. albicans*, *C. krusei*, and *C. parapsilosis* with FICI values ranging from 0.24 to 0.37.

Representative isobolograms of the combination of extracts and fractions from *E. pyriformis* with Vancomycin and Fluconazole against all the microorganisms are as shown graphically in Figure 1. A synergistic interaction was observed for one combination with Vancomycin and six with Fluconazole for the microorganisms with the FIC below the line of synergism.

Conclusively, extracts and fractions obtained from *E. pyriformis* Cambess showed antimicrobial activity as exhibited by their ability to inhibit bacterial and fungal growth *in vitro*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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