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Phytochemical profile and antioxidant and antimicrobial activities of hydroethanolic extracts of *Ficus pumila*

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Natural products, mainly of plant origin are widely used in popular medicine. This study aimed to evaluate the therapeutic potential by examining the hydroethanolic extracts of fresh and dried leaf, stem, root and fruit of *Ficus pumila* concerning their phytochemistry profile and antimicrobial, antioxidant and cytotoxic activities. The results show the presence of tannins and flavonoids in all extracts. The dried root extract exhibited the highest content of phenolic compounds (724.39 mg GA/g) and the dried leaf showed the highest content of flavonoids (15.30 mg quercetin/g). The extracts showed activity against *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Proteus mirabilis*. None of the extracts showed anti mycobacterial or antifungal activity. The highest antioxidant activity by scavenging of free radical DPPH was exhibited by the extract of fresh stem (EC_{50} = 12.81 µg/mL) and only the fresh leaf extract showed cytotoxicity with CC50 at 5 mg/mL.

Key words: *Ficus pumila*, antioxidant activity, antimicrobial activity, phenolics, flavonoids, cytotoxicity.

INTRODUCTION

During the last decades, the development of efficient drugs to combat microbial infections has revolutionized medical treatment, leading to drastic reduction in mortality from these diseases. Moreover, the widespread use of antibiotics unfortunately made the microorganisms develop resistance to antimicrobial agents, including drugs used to treat tuberculosis (Silveira et al., 2006).

Microbial resistance is considered a major global public health problem because it imposes restrictions for the treatment of infections. Thus, the increase of multi-resistant strains to antimicrobial drugs available in the market has led to search for new antimicrobial agents (Oplustil, 2012).

Natural products, especially of plant origin, are an excellent source for finding new antimicrobial molecules as the natural compounds to countain a much higher molecular diversity to those derived from synthetic products. For a long time, numerous plants have been used in the treatment of several diseases. Thus, research focused on the study and evaluation of plants can lead to

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discovery of new drugs, especially those with antimicrobial activity (Novais et al., 2003).

Ficus pumila, known as Ivy-girl or cat's nail, is an ornamental Moraceae family plant, native from Japan, China and Australia, which has been widely used in traditional Chinese medicine, as having analgesic and anti inflammatory activities. Although it has several biological activities, there are still few reports of its antioxidant and antimicrobial activities (Liao et al., 2012; Lorenzi and Hermes, 2001).

In this context, this study aimed to analyze the chemical composition, antimicrobial and antioxidant activities of hydroethanolic extracts of root, stem, leaves and dried and fresh fruits of *F. pumila* as a source of possible new agents to be used in the treatment of infections caused by Gram-positive and Gram-negative bacteria, mycobacteria and yeasts.

MATERIALS AND METHODS

Collection and identification of *F. pumila*

Sample collection of root, stem, leaf and fruit of *F. pumila* were performed at the Airport Garden District in Alfenas/MG (21º 27'50.70"S; 45º 56'32.28" W), to a rise of 908 m, in November 2012. The plant exsicata was deposited and identified at Federal University of Alfenas (MG, Brazil) receiving the registration number 2339.

Obtaining the extracts

Hydroethanolic fresh plant extracts were prepared and dried in a proportion of 20% (w/v) using ethanol 70% (v/v). Prior to extraction, the samples of root, stem, leaf and fruit of the plant were washed in running water and manually cut into smaller pieces with the help of a knife. For the preparation of the dried extracts, the plant samples were dried, crushed and subjected to the analysis of particle size.

The extracts were prepared from fresh and dried plant by maceration. After extraction, all extracts were filtered in filter paper. Then, they were concentrated by rotaevaporator apparatus using negative pressure of 500 mm Hg at a temperature of 60ºC, frozen and lyophilized (Silva et al., 2010).

The evaluation of the phytochemical profile of extracts

Phytochemical screening tests based on colorimetry and precipitation for detection of anthraquinones, flavonoids, tannins, saponins and alkaloids were performed (Costa, 1994). The total phenolic content was measured from an aliquot of 0.5 mL each extract at 0.1 mg/mL and mixed with 2.5 mL of Folin-Ciocalteau reagent (diluted 1:10 in distilled water) plus 2.0 mL of Na_2CO_3 4% (w/v) in distilled water.

After 2 h of incubation and being protected from light at room temperature, the absorbance was measured at 750 nm in a spectrophotometer. The results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GA/g) and they were calculated by using a calibration curve ranging from 5 to 100 µg/mL of gallic acid (Singleton et al., 1999).

The content of flavonoids was measured in an aliquot of 0.5 mL of each extract (at a concentration of 1.5 mg/mL) and mixed with 1.5 mL of ethanol, 0.1 mL of aluminum chloride ($AlCl_3.6H_2O$) 10% (w/v), 0.1 mL 1 M potassium acetate plus 2.8 mL of distilled water, and 5 mL of final volume. After 30 min, the absorbance of the mixture was measured at 425 nm. The total flavonoid standard curve was made using a quercetinstandard solution. The amount of flavonoids was expressed as milligrams of quercetin equivalents per gram of sample (mg quercetin/g), and the values were shown as mean of triplicate determinations (Kalia et al., 2008).

The microbial strains used

For microbiological evaluation. the following microorganisms were used: a) Gram positive bacteria: *B. cereus (ATCC 11778)*, *B. subtilis (*ATCC 6633), *M. luteus (*ATCC *9341)*, *E. faecalis* (ATCC51299), *S. aureus (*ATCC *6538)*;b) Gram negative bacteria: *Escherichia coli (*ATCC8739*)*, *Enterobacter aerogenes* (LMI-UNIFAL), *Proteus mirabilis (*ATCC*25922)*, *Pseudomonas aeruginosa (*ATCC *9027)*, *Salmonella typhimurium (*ATCC *14028)*,*Serratia marcescens (LMI-UNIFAL)*; c) Mycobacteria: *Mycobacterium bovis* (BCG) ATCC 27289, *Mycobacterium tuberculosis* (H37) ATCC 27294 and d) Yeast: *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisae* (ATCC 2601). The strains were maintained at 4°C in BHI Agar. Before testing, they were inoculated in BHI agar and incubated for 24 h at 37°C.

Evaluation of antimicrobial activity of the extracts

The antimicrobial activity was evaluated by agar diffusion according to the methodology proposed in document M7-A6 (CLSI, 2003) for bacteria, M24-A2 (CLSI, 2008b) for mycobacteria and M44-A2 (CLSI, 2008a, 2009) for fungi.

For bacteria, Mueller Hinton agar and for yeasts Mueller Hinton agar with 2% glucose were used. The lyophilized extracts were dissolved in DMSO at a final concentration of 50 mg / mL and placed in wells punched into the agar in a volume of 40 microliters.

Then, the medium was inoculated with microorganism's suspensions in saline solution with turbidity corresponding to the 0.5 tube on MacFarland scale. The plates were incubated at 37°C for 24 h.

The activity against *Mycobacterium* was determined by diffusion in agar Middlebrook 7H10 medium added to Middlebrook OADC Enrichment®. The plant extracts at 50 mg/mL concentration, in a volume of 10 µL, was placed on disks of filter paper syrup type at 10 mm diameter and dried at 37°C. The agar Middlebrook 7H10 medium was inoculated with a suspension of *Mycobacterium* with turbidity corresponding to the 2.0 tube of MacFarland scale. The cultures were incubated at 37°C for 28 days.

The minimum inhibitory concentration (MIC) was taken for fungi and bacteria by broth microdilution according to the methodology proposed in document M27-A3. The minimum microbicidal concentration (MMC) was performed using nutrient agar (CLSI, 2008). All experiments were performed in triplicates.

Antioxidant activity of extracts

To determine the antioxidant activity of different concentrations of the extracts (from 400 to 1.56 µg/mL, in serial dilution ratio 2) in an ethanolic solution (2 mL), we admixed 0.5 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.5 mM, diluted in ethanol). After incubation for 30 min in the dark and at room temperature, the absorbance was measured at 517 nm. The blank consisted of all reagents except for the extracts. Ascorbic acid, BHT and quercetin were used as a positive control. The scavenging property was calculated as a percentage of DPPH scavenged radicals, using the following equation: scavenging DPPH radical (%) = [(absorbance of blank - absorbance of sample)/ (absorbance blank)] x 100, and also led the

Table 1. Content of total phenolics and flavonoids in the extracts of *F. pumila.*

Extract	Phenolic compounds * (mg GA/g) **	Flavonoids compounds * (mg quercetin/g) ***
Fresh leaf	153.21 ± 8.4[b]	9.38 ± 0.1[b]
Dry leaf	154.29 ± 3.5[b]	15.30 ± 0.6[a]
Fresh stem	84.8 ± 3.3[a]	2.56 ± 0.2[d]
Dry stem	492.57 ± 7.9[f]	1.29 ± 0.1[e]
Fresh root	370.24 ± 11.7[d]	2.71 ± 0.1[d]
Dry root	724.39 ± 27.7[g]	1.63 ± 0.1[e]
Fresh fruit	298.08 ± 6.6[c]	5.70 ± 0.4[c]
Dry fruit	407.26 ± 19.1[e]	3.05 ± 0.1[d]

*Results expressed as mean ± standard deviation (n = 3). Means with different letters are statistically different in the same column or compound (Scott -Knott $p < 0.05$). ** Milligrams of gallic acid (GA) per gram of sample. *** Milligrams of quercetin per gram of sample.

EC_{50} of each extract. Values are presented as an average of triplicate independent experiments (Yen et al., 2005).

Evaluation of the cytotoxic activity of extracts on cell culture

Cytotoxicity was assessed by 3 - (4,5-dimethylthiazol-2YL) -2,5-diphenyltetrazolium bromide (MTT) method. In this test, 1 x 10^4 BHK-21 cells (newborn hamster's kidney) per well were seeded in 96-well plates containing the medium Eagle's Minimum Essential (MEM) (plus 10% fetal bovine serum and antibiotics). 0.1 mL of MEM containing 1% fetal bovine serum with decreasing dilutions of the extracts (5 to 0.039 mg/mL) was added to the cultures for 24 h. After incubation, 10 µL of MTT at a concentration of 5 mg/mL was added and incubated for 4 h at room temperature for the MTT incorporation and for the formation of formazan crystals. Spectrophotometric analysis was performed on a microplate reader at 570 nm. The percentage of cytotoxicity was calculated by using the formula [(AB)/ AX100], where A and B are values of the optical densities of the treated and controlled cells, respectively (Araújo et al., 2008). All experiments were performed in triplicates.

Statistical evaluation

The statistical analysis was performed using the SISVAR 5.3 software. Analysis of variance (ANOVA) and also the Scott-Knott test was applied to observe significant differences between average values ($p < 0.05$).

RESULTS

Concerning the phytochemical screening, all extracts showed the presence of tannins and flavonoids, but no anthraquinones, alkaloids or saponins. The highest content of total phenolic compounds was presented by the dried root (724.39 mg GA/g) and the lowest on the fresh stem (84.8 mg GA/g). The highest content of flavonoids was observed on the dried leaf (15.30 mg quercetin/g) and the lowest in the dried stem (1.29 mg quercetin/g) (Table 1).

For antimicrobial activity, it was possible to observe that only *B. subtilis*, *B. cereus*, *M. luteus*, *E. faecalis*, *S. aureus* and *P. mirabilis* were sensitive to the extracts of *F. pumila*. *M. luteus* was the most sensitive bacteria except for the dried leaf extract (halos from 10-16 mm). *B. cereus* was sensitive to all extracts, but showed smaller halos from 6-14 mm. No inhibition was observed for *E. aerogenes*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. marcescens*, *M. bovis*, *M. tuberculosis*, *C. albicans* and *S. cerevisiae*. The best antimicrobial activity was exhibited on the extract of dried root (Table 2).

In assessing the minimum inhibitory concentration (MIC), all microorganisms tested were sensitive to the extracts of *F. pumila*. The results show minimal difference between the MIC prepared with the dried leaves and cooked with fresh plant extracts. The best result for the MIC was for the extract of fresh stem (Table 3).

Regarding the evaluation of minimal microbicidal concentration (MMC) test, the extracts prepared with dried plant exhibited better results, and the most efficient was the dried stem extract (Table 4).

For the DPPH free radical scavenging activity, the highest antioxidant potential was shown on the extract of dried stem, which had the lowest concentration capable of sequestering 50% of DPPH radicals, with EC_{50} of 12.81 µg/mL (Table 5). Correlation between phenolic compounds and antioxidant activity was positive (r^2= 0.32), varying from weak to moderate.

Only the fresh leaf extract showed cytotoxic activity with CC_{50} at 5 mg/mL.

DISCUSSION

Ficus species generally exhibit flavonoids and tannins in their chemical composition (Sirisha et al., 2010). Several studies have reported the presence of flavonoids in extracts of leaf and stem of *F. pumila*, and the presence of tannins in the methanol extract of the leaves (Pistelli et al., 2000; Leong et al., 2008; Kaur, 2012). Thus, the results obtained in this study confirm data previously

Table 2.Antimicrobial activity of extracts of *F. pumila*. Agar diffusion test. Inhibition zones in mm.

Microorganism	Extract							
	Fresh leaf	Dry leaf	Fresh stem	Dry stem	Fresh root	Dry root	Fresh fruit	Dry fruit
Bacillus subtilis	11	10	0	9	0	10	0	0
Bacillus cereus	6	9	11	12	11	14	7	9
Micrococcus luteus	0	11	14	15	14	16	10	11
Enterococcus faecalis	8	9	8	9	9	10	0	9
Staphylococcus aureus	0	7	11	12	11	12	7	13
Escherichia coli	0	0	0	0	0	0	0	0
Enterobacter aerogenes	0	0	0	0	0	0	0	0
Serratia marcescens	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0
Proteus mirabilis	0	8	10	11	10	11	7	9
Salmonella typhimurium	0	0	0	0	0	0	0	0
Candida albicans	0	0	0	0	0	0	0	0
Saccharomyces cerevisae	0	0	0	0	0	0	0	0
Mycobacterium tuberculosis	0	0	0	0	0	0	0	0
Mycobacterium bovis	0	0	0	0	0	0	0	0

Table 3. Minimal inhibitory concentration (mg/mL) extracts of *F. pumila*.

Microorganism	Extract							
	Fresh leaf	Dry leaf	Fresh steam	Dry steam	Fresh root	Dry root	Fresh fruit	Dry fruit
Bacillus subtilis	3.12	12.5	6.25	6.25	12.5	6.25	6.25	12.5
Bacillus cereus	6.25	6.25	0.39	0.39	0.78	0.78	0.78	0.78
Micrococcus luteus	6.25	6.25	3.12	6.25	0.78	6.25	0.39	3.12
Enterococcus faecalis	6.25	3.12	6.25	3.12	12.5	6.25	6.25	12.5
Staphylococcus aureus	6.25	12.5	6.25	12.5	12.5	12.5	12.5	12.5
Escherichia coli	6.25	3.12	3.12	6.25	12.5	6.25	12.5	6.25
Enterobacter aerogenes	6.25	6.25	3.12	6.25	6.25	6.25	6.25	12.5
Serratia marcescens	6.25	6.25	6.25	3.12	6.25	6.25	6.25	6.25
Pseudomonas aeruginosa	6.25	6.25	6.25	6.25	6.25	6.25	12.5	6.25
Proteus mirabilis	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
Salmonella typhimurium	6.25	6.25	6.25	6.25	6.25	12.5	6.25	12.5
Candida albicans	3.12	3.12	3.12	6.25	3.12	6.25	3.12	6.25
Saccharomyces cerevisae	6.25	6.25	12.5	6.25	6.25	6.25	6.25	12.5

reported in the literature on the chemical composition of *F. pumila* (Table 1). Regarding the content of flavonoids and phenolic compounds, it is believed that phenolic compounds such as tannins and flavonoids from plants may be related to antioxidant and antimicrobial activities (Einbond et al., 2004; Banerjee et al., 2005; Choi et al., 2006).

In the agar diffusion test conducted to evaluate the antimicrobial activity of the extracts, the dried root extract showed better results for the spectrum of action and inhibition zone diameter. However, in determining the MIC and the MMC, the stem extract was more effective. Furthermore, the *E. coli*, *E. aerogenes*, *S. marcescens*, *P. aeruginosa* and *S. typhimurium* bacteria, as well as the yeast *C. albicans* and *S. cerevisiae* showed no inhibition zone in the agar diffusion test, but were sensitive to extracts of *F. pumila* during the broth microdilution test (MIC) (Table 2). This difference in results may possibly be related to the physical characteristics of the culture method, to the solubility of the compounds of each process and the sensitivity of the method.

In the literature, there are few reports on the antimicrobial activity of *F. pumila*. However, several studies have confirmed the antimicrobial activity of different species of the *Ficus* genus. Studies have described the activity of the methanol extract of fresh

Table 4. Minimal microbicidal concentration (mg/mL) of the extracts of *F. pumila*.

Microorganism	Extract							
	Fresh leaf	Dry leaf	Fresh steam	Dry steam	Fresh root	Dry root	Fresh fruit	Dry fruit
Bacillus subtilis	ND	12.5	ND	12.5	ND	ND	ND	ND
Bacillus cereus	ND	ND	6.25	ND	ND	ND	ND	25
Micrococcus luteus	25	25	12.5	25	ND	12.5	25	25
Enterococcus faecalis	25	12.5	25	12.5	25	12.5	25	12.5
Staphylococcus aureus	12.5	12.5	6.25	12.5	ND	12.5	12.5	12.5
Escherichia coli	25	25	25	12.5	ND	12.5	25	12.5
Enterobacter aerogenes	ND	12.5	ND	6.25	12.5	12.5	12.5	12.5
Serratia marcescens	12.5	ND	ND	25	ND	25	25	25
Pseudomonas aeruginosa	25	25	25	25	ND	25	25	25
Proteus mirabilis	25	12.5	25	12.5	ND	12.5	ND	12.5
Salmonella typhimurium	ND	ND	25	ND	ND	25	25	25
Candida albicans	6.25	6.25	12.5	12.5	6.25	12.5	6.25	6.25
Saccharomyces cerevisae	25	12.5	25	12.5	25	12.5	25	12.5

ND, Not detected at the concentrations used in the test.

Table 5. Antioxidant activity of extracts of *Ficus pumila*.

Extract	% DPPH radicals scavengeing (100 µg/mL)*	EC_{50} (µg/mL)
Fresh leaf	61.85 ± 1.1^{a}	58.56 ± 0.5^{i}
Dry leaf	83.18 ± 0.6^{e}	25.28 ± 0.1^{e}
Fresh stem	82.93 ± 0.4^{e}	12.81 ± 0.2^{c}
Dry stem	81.85 ± 0.4^{d}	25.79 ± 0.3^{f}
Fresh root	83.73 ± 0.4^{e}	25.03 ± 0.1^{d}
Dry root	81.73 ± 0.5^{d}	13.20 ± 0.2^{c}
Fresh fruit	77.13 ± 0.4^{c}	36.60 ± 0.3^{h}
Dry fruit	87.59 ± 0.7^{f}	28.50 ± 0.2^{g}
Quercetin	81.44 ± 0.2^{d}	4.57± 0.1^{a}
Ascorbic acid	90.43 ± 0.3^{g}	6.49 ± 0.1^{b}
BHT**	63.70 ± 0.1^{b}	70.11 ± 0.5^{j}

*Results expressed as mean ± standard deviation (n = 3). Means with different letters are statistically different (Scott -Knott $p < 0.05$).
**BHT: Butylated hydroxytoluene.

leaves of *F. pumila* against *B. subtilis* and methanolic extracts of fruit and stem of *Ficus microcarpa* against Gram-positive and Gram-negative bacteria (Ragasa et al., 1999; Sirisha et al., 2010). The activities of the methanol bark extract of *Ficus glomerata* against *B. subtilis* and ethanol root extracts of *Ficu sracemosa* and *Ficus benghalensis* against *S. aureus* and of the ethanolic root extract of *Ficus racemosa* against *E. coli*, *E. cloacae*, *P. aeruginosa*, *B. subtilis* and *C. albicans* has been mentioned (Murti and Kumar, 2011; Goyal, 2012; Jagtap et al., 2012) .

The antimicrobial activity of different speciesfrom *Ficus sp.*, can be related to the presence of tannins and flavonoids. It is believed that flavonoids are capable of complexing with the bacterial cell wall, causing the death of the microorganism and the tannins are able to inactivate enzymes, transport proteins and microbial adherence (Goyal, 2012).

The antioxidant activity of ethanol extracts of leaf, stem, fruit and root of *F. pumila* was determined by the DPPH method. This technique allows the evaluationof antioxidant activity through the ability to scavenge free radicals in a given period of time. In this case, the antioxidant activity is determined by 50% reduction on the initial concentration of DPPH, or the EC_{50} value. It is considered that the lower the EC_{50} value, the higher the antioxidant activity of the extract. Thus, according to the EC_{50} values, the highest antioxidant activity was exhibited by the extract of fresh stem (Table 3).

The antioxidant activity of plants of the genus *Ficus* is

due to the presence of flavonoids and phenolic compounds in its composition. Studies reported the antioxidant activity of methanol extracts of the stem, fruit and leaves of *F. microcarpa* and aqueous extract of dried stem of *F. glomerata*(Sirisha et al., 2010). A high antioxidant activity of hydroethanolic leaf extract of *F. pumila* was also described which occurred in the presence of phenolic compounds and flavonoids (Leong et al., 2008).

Ficus species are rich in flavonoids and phenolic compounds that produce a strong antioxidant property of these plants, aiding in the prevention and treatment of many diseases (Sirisha et al., 2010). Phenolic compounds act as radical scavengers and sometimes as metal chelators, being probably responsible for the antioxidant activity of *F. pumila* (Moreno and Neuza, 2010).

The positive correlation between the phenolic content and the antioxidant activity indicates that phenolic compounds have an important role in the antioxidant activity presented by the ethanolic extracts of *F. pumila*.

The cytotoxicity evaluation allows determination of the concentration of drug to be used and provides important information about the possible cell damage (Araújo et al., 2008). In this study, the cytotoxic activity of the extracts of *F. pumila* was evaluated in BHK 21 cells. These mammalian cells are derived from baby hamster kidney and were used to determine the cytotoxic activity to various biological compounds (Carbonell et al. 2003; Joshi and Chauhan, 2013). According to the results, only the fresh leaf extracts were cytotoxic for the culture of BHK-21 cells at 5 mg/mL concentration. This cytotoxicity may be related to the high content of flavonoids found in the leaves of *F. pumila* (Xie et al 2009).

There are few studies on the cytotoxicity of *F. pumila*. Ramcharani et al. (2010) have shown cytotoxic activity of methanol leaf and stem extracts of *F. pumila* to the human leukemia cells (MT-4). This activity was attributed to the presence of phenolic compounds. It is believed that these compounds may trigger intracellular signaling pathways that induce death of leukemic cells. The difference in result may be related to the cell type used in the evaluation, since the same methodology was used to assess cell viability.

The results of this study show for the first time that the hydroethanolic extracts of *F. pumila* have active compounds with antioxidant and antimicrobial activities and should be taken into consideration for the development of new drugs.

Conflict of Interest

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

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