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Research Paper

Evaluation of the antibacterial potential of *Petroselinum crispum* and *Rosmarinus officinalis* against bacteria that cause urinary tract infections

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Abstract

In this study we evaluated the antibacterial activity of the crude hydroalcoholic extracts, fractions, and compounds of two plant species, namely *Rosmarinus officinalis* and *Petroselinum crispum*, against the bacteria that cause urinary tract infection. The microdilution method was used for determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The crude hydroalcoholic extract of *R. officinalis* displayed *in vitro* activity against Gram-positive bacteria, with satisfactory MBC for the clinical isolate *S. saprophyticus.* The fractions and the pure compound rosmarinic acid did not furnish promising results for Gram-negative bacteria, whereas fractions 2, 3, and 4 gave encouraging results for Gram-positive bacteria and acted as bactericide against *S. epidermidis* as well as *E. faecalis* (ATCC 29212) and its clinical isolate. R. officinalis led to promising results in the case of Gram-positive bacteria, resulting in a considerable interest in the development of reliable alternatives for the treatment of urinary infections.

Key words: antibacterial activity, bioassay, *Rosmarinus officinalis*, *Petroselinum crispum*, Urinary Tract Infections (UTI).

Introduction

Urinary tract infections (UTIs) consist of the microbial invasion of any tissue of the urinary tract from the urethra to the kidneys. Infections of the prostate and epididymis are also often included in this definition (Ronald *et al.*, 2001; Farrel *et al.*, 2003).

The UTI etiology may vary according to sex, age, previous use of antibiotics, and contamination within or outside the hospital, not to mention that they differ from one environment to another. The microorganisms that mainly account for UTI are the Gram-negative enteric bacteria, especially *Escherichia coli*, which is the most frequently studied microorganism, followed by other Gram-negative bacteria such as *Klebsiella* sp, *Enterobacter* sp, *Acinetobacter* sp, *Proteus* sp, and *Pseudomonas* sp (Foxman, 2010; George and Manges, 2010). In addition, *Staphylococcus saprophyticus*, a Gram-positive bacterium, has been shown to be the second most frequent cause of UTI. The latter microorganism has also been demonstrated to be a saprophyte of the commensal flora of the urinary tract, mucosa, and skin, like *S. epidermidis* (Faro and Fenner, 1998; Mehnert-Kay, 2005).

The use of medicinal plants has become increasingly widespread and has been enriched by the vast biodiversity and the mixing of indigenous, African, and European cultures (Oliveira *et al.*, 2003). The antimicrobial properties of plant extracts and isolated compounds have been investigated by a number of researchers worldwide (More *et al.*, 2008; Porto *et al.*, 2009; Al-Bakri *et al.*, 2010). In Brazil, the consumption of herbal medicines is growing at a rate of 20% a year, following the re-evaluation of the global use of medicinal plants for the treatment of several diseases (Cartaxo *et al.*, 2010).

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Parsley (*Petroselinum crispum*) is a member of the Umbelliferae family, and it has been employed in the food, pharmaceutical, perfume, and cosmetics industries (López *et al.*, 1999). It is popularly known as cilantro, salsa, and salsa-pea. In traditional medicine, it is considered to be a diuretic, uterine stimulant, sedative, emollient, and anti-parasitic agent, and it is commonly employed for the treatment of chronic bronchitis, bronchial asthma, and dyspepsia. Its leaves and stems are indicated in the cases of menstrual problems, cystitis, edema, kidney stones, prostatitis, cramps, indigestion, anorexia, arthritis, and rheumatism (Yarnell, 2002; Yanardag *et al.*, 2003; Wright *et al.*, 2007). The constituents of parsley, which include ascorbic acid, carotenoids, flavonoids, coumarins, myristicin, apiole, various terpenoic compounds, phenyl propanoids, phthalides, furano coumarins and tocopherol, have been chemically investigated (Tunali *et al.*, 1999; Yanardag *et al.*, 2003).

Popularly known as rosemary, the *Rosmarinus officinalis* plant has been more often utilized due to religious reasons and it is commonly employed as a ritual ornament for deities and human beings. Its virtues were discovered in the Middle Ages, and since then it has been widely used for culinary and medicinal purposes worldwide (Leal *et al.*, 2003; Ozcan, 2003; González-Trujano *et al.*, 2007). Its main constituents are rosmarinic acid, carnosic acid, carnosol, ursolic acid, oleanolic acid, genkwanin, apigenin, and luteolin (Lamaison *et al.*, 1991; Cuvelier *et al.*, 1996; Frankel *et al.*, 1996).

Because there has been a considerable rise in the prevalence of infectious diseases caused by bacterial resistance, researchers have become more and more engaged in the search for new effective antimicrobials from plant sources. A survey of the literature, namely the works of Ojala *et al.* (2000), Oluwatuy *et al.* (2004), Bozin (2007), Fu *et al.* (2007), Weckesser *et al.* (2007), and Silva *et al.* (2008) did not retrieve any information on the use of *P. crispum* and *R. officinalis* for the treatment of bacterial UTI. Therefore, this study aimed to evaluate the antibacterial activity of the crude hydroalcoholic extracts, fractions, and compounds of two plant species, namely *Rosmarinus officinalis* and *Petroselinum crispum*, against the bacteria that cause UTI.

Material and Methods

Microorganisms

To determine the antibacterial activity of the crude extracts, fractions, and substances isolated from the plant species investigated here, the following strains from the American Type Culture Collection (ATCC) were employed: *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 29906), *Klebsiella pneumoniae* (ATCC 10031), *Enterobacter aerogenes* (ATCC 13048), *Pseudomonas aeruginosa* (ATCC 14502), *Staphylococcus saprophyticus* (ATCC 35552), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), and their clinical isolates. The clinical bacterial isolates and standard strain (ATCC) used in this study were maintained at -20 °C in a freezer in the Laboratory of Research in Applied Microbiology (LaPeMA) of the University of Franca.

Collection of crude extracts, fractions, and the pure compound rosmarinic acid

The aerial parts of the plant species *R. officinalis* were collected (3.5 kg) in the urban area of Patrocínio, located in the western region of the Brazilian state of Minas Gerais in May 2007. The geographical position of the town is latitude 18°17'00" S, longitude: 46°59'36" W; average height of 972 meters.

The aerial parts of the plant species *P. crispum* were collected (4 kg) from a traditional garden, located in the city of Guaxupé, located in southern region of the Brazilian state of Minas Gerais in October 2007. The geographical position of the town is latitude 21°18'20" S, longitude 46°42'41" W; average elevation of 830 meters.

Both plants were identified by Prof. Dr. Milton Groppo, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo (FFCLRP-USP), and a voucher specimen was deposited in the herbarium of the Institution, labeled as SPRF 11911th plant species *P. crispu*m and SPRF 11912th plant species *R. officinalis*.

The plants were dried and stabilized in circulating air oven at a temperature of about 40 °C, followed by grinding to a powder in a knife mill (Marconi, Piracicaba, São Paulo, Brazil). The powder resulting from each plant was subjected to exhaustive extraction by maceration with ethanol (Merck KgaA, Darmstadt, Germany)/water (96:4 v/v) at room temperature. A hydro solvent was employed, in order to obtain more polar substance(s). Three successive extractions were accomplished, with one-week interval between them. All the material resulting from the maceration process was filtered and concentrated under reduced pressure at 60 °C until complete elimination of the solvent, using a rotary evaporator (Marconi, Piracicaba, São Paulo, Brazil).

The dried plant extracts were placed in an amber bottle covered with a lid and were stored in the refrigerator until the tests were conducted. Fractionation of the extract of *R. officinalis* (12.0 g) was performed by vacuum liquid chromatography (VCL-silica gel 60, Merck) using *n*-hexane, ethyl acetate, ethanol (all purchased from Merck), or mixtures of these solvents in increasing polarity gradient as eluent, which furnished seven fractions. A volume of two liters of solvent or solvent mixtures was necessary for the collection of each fraction. Fractions 1, 2, 3, 4, 5, 6, and 7 were eluted with *n*-hexane, hexane/ethyl acetate (75:25 v/v), hexane/ethyl acetate (50:50 v/v), ethyl acetate, ethyl acetate/ ethanol (75:25 v/v), ethyl acetate/ ethanol (50:50 v/v), and ethanol, respectively, which yielded masses of 0.4, 2.4, 4.4, 1.8, 0.6, 0.7, and 0.5 g, respectively.

A yellowish solid substance, namely rosmarinic acid (designated RA), was isolated from the extraction. To this end, 200 g powdered leaves of the plant that had been submitted to extraction by maceration (room temperature) for seven days using water/acetic acid (85:15 v/v) were employed. The product of maceration was filtered, and the pH was adjusted to 10 by addition of a calcium hydroxide (Merck) solution. This gave a precipitate (27 g) that was identified by comparison with an authentic RA sample. The final identification was carried out by ^{1}H and ^{13}C NMR. The data were compared to those published for rosmarinic acid (Kuhnt *et al.*, 1995), which confirmed that RA was actually rosmarinic acid.

Biological assays

To evaluate the antibacterial activity of the crude extracts, fractions, and the isolated substance, the Minimum Inhibitory Concentration (MIC) was determined by the microdilution technique performed in 96-well microtiter plates (Techno Plastic Products, Trasadingen, Switzerland), as recommended by the CLSI (2006). For dilution of the extracts, fractions, and isolated compound, one milligram of the samples was dissolved in 125 μL dimethyl sulfoxide (DMSO). Controls were monitored in culture. Sterility was controlled by means of the Mueller-Hinton broth (Difco, Sparks, MD, USA). The negative control was the solvent dimethyl sulfoxide (DMSO), used at concentrations ranging from 1 to 5%. In the case of the positive control, the antibiotics gentamicin and penicillin (Merck Sharp & Dohme, São Paulo, Brazil) were employed for Gram-negative and Gram-positive bacteria, respectively, at concentrations ranging from 0.0115 g/mL to 5.9 μg/mL.

The use of the controls allowed for analysis validation. The crude extracts, fractions, and pure compound were evaluated at concentrations between 20 μg/mL and 400 μg/mL. To this end, microplates were capped and incubated at 37 °C, for 24 hours. Following this incubation period, 15 μL of a resazurin (Sigma-Aldrich, New York, USA) solution at 0.02% in sterile water was added to each well, for analysis of the results. This system facilitates microbial growth detection; the blue color indicates the absence of microbial growth, while the red color indicates growth of viable cells. For determination of the Minimal Bactericidal Concentration (MBC), before the addition of resazurin an aliquot of the inoculum was aseptically removed from each well presenting no apparent growth, and then plated onto agar Mueller-Hinton (Difco) supplemented with sheep blood (5%); the plates were incubated as previously described.

Results and Discusssion

The crude extract obtained from the leaves and stems of the plant species *P. crispum* furnished MIC and MBC > 400 μg/mL for all the tested bacterial strains, except for *P. aeruginosa* (ATCC 14502), which yielded MIC and MBC of 350 μg/mL and > 400 μg/mL, respectively, which resulted in a bacteriostatic effect. Therefore, the plant extract obtained from *P. crispum* did not furnish promising results against the selected bacteria. Rios and Recio (2005) described that the crude extract of a plant can only be considered promising when MIC < 100 μg/mL is achieved (Table 1).

The hydroalcoholic extract from *R. officinalis* leaves led to satisfactory results according to the criteria of Rios and Recio (2005) for the Gram-positive bacteria *S. saprophyticus* (ATCC 35552), *S. epidermidis* (ATCC 12228), *E. faecalis* (ATCC 29212), and their clinical isolates, for which the MIC values ranged from 70 to 150 μg/mL. Analysis of the MBC results revealed bactericidal activity against *S. saprophyticus* and bacteriostatic effect against the other tested Gram-positive bacteria. For Gram-negative bacteria, though, results were not so promising. MIC and MBC were > 400 μg/mL, with the exception of the bacterium *P. aeruginosa* (ATCC 14502), for which there was a bacteriostatic effect (Table 1).

The encouraging results obtained with the crude extract of *R. officinalis* motivated its fractionation by vacuum liquid chromatography, which enabled assessment of the bactericidal activity of these fractions. Fractionation of the plant *R. officinalis* gave rise to 7 fractions, designated fractions 1 to 7. In the present study, the compound rosmarinic acid was also isolated from *R. officinalis* leaves (Figure 1).

Fractions 1-7 afforded MIC values ranging between 200 and 400 μg/mL in the case of Gram-negative bacteria, while MBC was equal to or larger than 400 μg/mL for all the tested bacteria. As for Gram-positive bacteria, fraction 4 followed by fractions 2 and 3 gave the best results, with MIC values lying between 30 and 400 μg/mL (*S. saprophyticus, S. epidermidis* and *E. faecalis*). The remaining fractions and the isolated compound rosmarinic acid yielded MIC values ranging from 70 to 400 μg/mL. The MBC values revealed the bactericidal effects of fraction 3 against the Gram-positive bacterium *E. faecalis* (ATCC 29212) and its clinical isolate, and of fractions 2 and 3 against the Gram-positive bacterium *S. epidermidis* (ATCC 12228), which represents an improvement compared with the crude extract. It is considered that a bacteriostatic effect occurs when the MIC/MBC ratio is lower than 1 and a bactericidal effect is said to take place when the MIC/MBC ratio is equal to or greater than 1 (Table 1).

Many studies have reported that antibacterial activity investigation is based on folk medicine; that is, the selection of plants to be tested is based on the knowledge of various populations about the curative power of the plants. Some researchers, more specifically Ojala *et al.* (2000), Oluwatuy *et al.* (2004), Bozin *et al.* (2007), Fu *et al.* (2007), Weckesser *et al.* (2007), and Silva *et al.* (2008), have described the antibacterial activity of spices.

Table 1 - Results of Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) for the plants *P. crispum*, ethanol extract, and *R. officinalis*, ethanol extract, fractions, and pure compound.

Bacteria/ATCC		*Petroselinum crispum* (Parsley)		*Rosmarinus officinalis* (Rosemary)																		
		Extract		Extract		Fraction 1		Fraction 2		Fraction 3		Fraction 4		Fraction 5		Fraction 6		Fraction 7		RA		Drugs
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	
		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)
Gram negative	*E. coli/25922*	—*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.74[#]
	E. coli (Clinical isolate)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.74[#]
	P. mirabilis/29906	—	—	—	—	—	—	—	—	200	—	300	—	—	—	—	—	—	—	400	—	2.95[#]
	P. mirabilis (Clinical isolate)	—	—	—	—	—	—	200	400	—	—	—	—	—	—	—	—	—	—	400	—	2.95[#]
	K. pneumoniae/10031	—	—	—	—	—	—	—	—	400	400	—	—	—	—	—	—	—	—	—	—	0.39[#]
	K. pneumoniae (Clinical isolate)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.39[#]
	E. aerogenes/13048	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.74[#]
	E. aerogenes (Clinical isolate)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.74[#]
	P. aeruginosa/14502	350	—	350	—	—	—	400	—	300	—	400	—	400	—	400	—	400	—	400	—	5.90[#]
	P. aeruginosa (Clinical isolate)	—	—	—	—	400	—	—	—	300	—	—	—	—	—	—	—	—	—	—	—	5.90[#]
Gram positive	*S. saprophyticus/35552*	—	—	150	—	400	—	400	—	300	—	100	300	70	80	—	—	—	—	400	—	0.15[##]
	S. saprophyticus (Clinical isolate)	—	—	130	130	400	—	200	400	50	70	40	80	200	400	—	—	—	—	400	—	0.15[##]
	S. epidermidis/12228	—	—	120	—	200	300	80	80	50	50	—	—	—	—	—	—	—	—	—	—	5.90[##]
	S. epidermidis (Clinical isolate)	—	—	70	—	300	—	200	300	90	300	60	80	300	400	—	—	—	—	400	—	5.90[##]
	E. faecalis/29212	—	—	90	120	—	—	—	—	200	200	30	60	90	200	—	—	—	—	—	—	2.95[##]
	E. faecalis (Clinical isolate)	—	—	100	300	—	—	400	—	300	300	40	100	100	300	—	—	—	—	—	—	5.90[##]

[#]Gram-negative Control (Gentamicin) / [##]Gram-positive Control (Penicillin) / — > 400 µg/mL / RA = Rosmarinic acid.

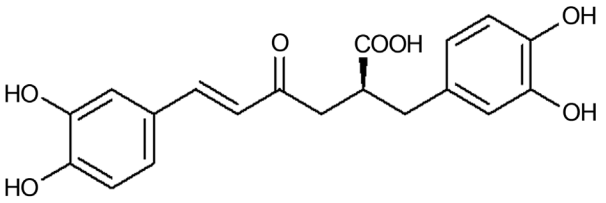

Figure 1 - Chemical structure of rosmarinic acid.

On the basis of MIC determination, Ojala *et al.* (2000) reported the antimicrobial activity of the methanol extract of the plant species *P. crispum*, containing coumarins, against the clinical isolates *Bacillus subtilis*, *P. aeruginosa*, *S. epidermidis*, *S. aureus*, and *Saccharomyces cerevisiae*. According to these authors, a modest antimicrobial activity, with MIC ranging from 200 μg/mL to 350 μg/mL, was achieved. Using the hydroalcoholic of this same plant, our group obtained the same MIC values against the standard strain of *P. aeruginosa*.

Oluwatuy *et al.* (2004) demonstrated the antibacterial activity of the methanol extract of *R. officinalis* against the clinical isolate of *S. aureus*, with MIC of 60 μg/mL. Bozin *et al.* (2007) showed that the Gram-positive *S. aureus* (ATCC 6538) and *S. epidermidis* (ATCC 12228) were sensitive to the essential oil from the plant species *R. officinalis*, with MIC values equal to 60 μg/mL and 150 μg/mL, respectively. According to Fu *et al.* (2007), combinations of two essential oils from *R. officinalis* displayed antibacterial activity against *S. epidermidis* (ATCC 12228), *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633), *E. coli* (ATCC 8739), *P. vulgaris* (ATCC 49132), and *P. aeruginosa* (ATCC 27853), with MIC ranging from 70 μg/mL to 150 μg/mL.

Silva *et al.* (2008) investigated the action of the hydroalcoholic extract of *R. officinalis* on *Streptococcus mitis* (ATCC 9811), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus mutans* (ATCC 25175), and *Streptococcus sobrinus* (ATCC 27609), the predominant bacterial species in the supragingival biofilm, and *Lactobacillus casei* (ATCC 7469), obtained by the MIC technique. The MIC values lay between 40 and 120 μg/mL, except for *S. mitis* (ATCC 9811), which yielded MIC greater than 400 μg/mL. According to these authors, the rosemary extract was effective against the tested strains, whereas in our study the selected bacterial strains did not give evidence of such promising results in the case of the hydroalcoholic extract. Nevertheless, good results were verified in the case of Gram-positive bacteria belonging to the species *S. epidermidis* and *E. faecalis*.

Thus, the results of these studies suggest that different organisms react differently to the same extract, as well as fractions and pure compounds. The plant *P. crispum* did not lead to satisfactory results for the bacteria tested herein, while the plant *R. officinalis* furnished promising results, particularly for Gram-positive bacteria. Taken together and compared with published data (Rios and Recio, 2005), these findings justify the considerable interest in the development of credible alternatives for the treatment of urinary tract infections.

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