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Survey of the Antibacterial Effect of Aquatic and Alcoholic Extracts of *Ruta-graveolens*

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Abstract: Indiscriminate and excessive uses of antibiotics may promote the emergence ofantibiotic-resistant microorganisms and side effects in patients. Therefore the researchers recently have focused on the use of natural resources, especially medicinal plants. Rutagraveolensis a kind of medical plants which contains antifungal and antibacterial components and is used in traditional medicine in Iran and other nations. The main goal of this research was investigation of antimicrobial effect of aquatic and alcoholic extracts of this plant on Gram-positive and Gram-negative bacteria. For this aim antibacterial activities of Rutagraveolens extracts on Staphylococcus aureus (PTCC 1431), Bacillus subtilis (PTCC 1720), Escherichia coli (PTCC 1763) and Pseudomonasaeruginosa (PTCC 1599) were evaluated by measurement of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), disc and well diffusion methods. The results indicated that aquatic, methanolic and ethanolic extracts with different MIC and MBC are effective on the growth of selected bacteria.

Key words: Antibacterial effect · Rutagraveolens · MIC · MBC

INTRODUCTION

Although applications of antibiotics are useful for treatment of different bacterial infections, indiscriminate and excessive uses of them have increased the emergence ofantibiotic-resistant microorganisms and side effects in patients [1, 2]. Therefore, the researchers recently have focused on the use of natural resources, especially medicinal plants mainly because of necessity for new antimicrobial treatment. Although different extracts of traditional medical plants have been investigated and some of them have been confirmed as antibacterial medicines, the recognition of new materials that are active against pathogenic resistant bacteria became inevitable [1-3]. The applications of plants were common among Iranians and Iranian's traditional medicine since long time ago.Rutagraveolensis a plant medicine that has been used for traditional treatment in Iran and other nations.Ruta has been reported as a medicine with significant treatment effects[1-5]. Nowadays, it has been used by different nations of the world because of its interesting and unique treatment effects. Its applications as an anti-inflammatory, anti-cancer, anti-arrhythmic, anti-blood pressure, anti-microbial, anti-fungi, anti-parasites, reducer of nervous system activity, contraception and abortion of fetuses have been confirmed in numerous clinical and laboratory studies[1-7]. However, there are few studies about the valuable therapeutic effect of Rutagraveolens in traditional treatments. Although this plant has been widely used, the exact mechanism and its effective component(s) are unknown. According to the documents that remained from ancestors, this plant has a significant therapeutic value especially in some refractory diseases such as cancer and Alzheimer. Therefore, investigation about this plant has valuable results for treatment of different diseases. Rutagraveolens has anti-bacterial and anti-cytotoxic effects against different specise of *Staphylococcus* such as *Staphylococcus aureus* and *Staphylococcus epidermis*, *Listeria monocytogenes* and *Bacillus subtilis*[4, 5, 7, 8]. Moreover, the aquatic extracts of this plant with the aquatic extracts of Viola have a growth suppressor effect on

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Trichomonasvaginalis. The phenolic, alkaloid and terpenoid components of Rutagraveolenshave antimicrobial effects. It has been reported that antimicrobial activity of the extract of this plant against Gram-negative bacteria such as Pseudomonas aeruginosa and Salmonella, is same as the antimicrobial activity of gentamicin. [9].Therefore, it seems that Rutagraveolenscan be used against infections in animals, plants and humans. The aim of this study was to investigate the antimicrobial effect of aquatic and alcoholic extracts of Rutagraveolenson Gram-positive and Gram-negative bacteria. The focus of this study was of antimicrobial activities on particular Gram-negative bacteria such as Pseudomonasaeruginosa and E.coli which have a significant influence on different diseases such as urinary tract infections.

MATERIALS AND METHODS

Preparation of Plant Extracts: Rutagraveolenshas been wascollected from the mountainous area of Tonekabon in North-Iran and prepared as a powder after washing and drying,The aquatic, ethanolic and methanolic solubleextracts were prepared from the powder with soxhlet method. After extraction, the alcoholic solvents with arotary evaporatorand aquatic solvent in $45^{°C}$ were removed and then dilution 10^{-1} with DMSO was prepared from each extract [2, 4-8, 10].

Bacteria: In this study, the lyophilized Staphylococcus aureus (PTCC 1431), *Bacillus subtilis (*PTCC 1720), Escherichia coli (PTCC 1763) and *Pseudomonas* aeruginosa (PTCC 1599) were obtainedfrom Persian Type Culture Collection (PTCC) ofscientific and industrial center of Shahryar in Iran. Then a suspension adjusted to 0.5 McFarland standards of each bacterium was prepared.

Disc Diffusion Method: Disc diffusion method for antimicrobial susceptibility testing was carried out to assess the presence of antibacterial activities of the plant extracts. In this method first 10 micro-liter of bacterial suspension adjusted to 0.5 McFarland standards were inoculated onto Muller Hinton agar with using a sterile swab. In other step the discs containing30, 40, 50 and 60 micro-liter of plan extracts (from the dilution of 1/10) was placed on the Mueller-Hinton agar surface. After incubation at 37°C for 24 hours, they were examined for the inhibition zone. The experimentwas repeated three times for each sample and the average of the inhibition zone diameter was determined [2, 4-8, 9-11].

Agar Well Diffusion Method: 10 micro-liter of each bacterial culture which has been adjusted to 0.5 McFarland standards was used to make lawn Muller Hinton agar plates evenly using a sterile swab. 4 wells on the Muller Hinton agar plate in sterilized situation were made and the extracts with different amounts of 70, 80, 90 and 100 micro-liter were loaded to each well. After incubation of plates at 37°C for 24 hours, they were examined for the inhibition zone. The experiment was repeated three times for each sample and the average of the inhibition zone diameters was determined [2, 5, 7, 8, 11].

Minimum Inhibition Concentration Determination (MIC): MICs of Rutagraveolensextracts were performed using a broth macrodilution test as recommended by NCCLS [11]. 10 micro-liter bacterial suspensions (with 0.5 McFarland standards) were added to the 11 tubes containing Muller Hinton broth and were serially diluted. Then, MIC was determined after incubation of samples at 37°C for 24 hours. Subsequently, 10 micro-liter of each tube (before incubation for MIC) were cultured onto Muller Hinton agar and incubated similarly for detection of MBC. The experiments were repeated three times for each sample and the average of MIC and MBC of extractions was determined [2, 4, 5, 8, 11].

RESULTS

The inhibition effect of ethanolic extracts with higher inhibition zone diameter was more than methanolic and aquatic extracts. The results exhibited that the rate of inhibition effect increases by raising the amount of extracts (Table 1 and 2). Although, 30 and 40 micro-liter aquatic extract with disc diffusion method did not have any effect on the growth of E.coli, increasing the rate of extracts in disc and well diffusion methods cause the inhibition effects (Table 2). The MIC and MBC of methanolic extracts on Staphylococcus aureus were recorded to be 16×10^{2}µg/ml and 13×10^{3}µg/ml, respectively. For methanolic extract on E.coli the MIC was 13×10^{3} µg/ml and MBC was 5×10^{4} µg/ml. However, MIC and MBC of aquatic and methanolic extracts on Pseudomonasaeruginosa and E.coli were the same and equal to 25×10^{3} µg/ml and 5×10^{4} µg/ml respectively (Table 3).

Table 1: Diameter of growth inhibition zone of Rutagraveolens extracts on bacteria by well diffusion method (mm)

Extract	Aquatic				Ethanolic				Methanolic			
Amount of extract (µl)	70	800	90	100	70	80	90	100	70	80	90	100
B. subtilis	13	14	15	16	23	24	25	26	20	21	21	22
Staph. aureus	15	16	16	17	20	20	20	22	19	20	20	22
E. coli	11	12	11	15	0	0	0	0	0	0	0	0
Ps.aeruginosa	11	11	12	12	0	0	0	0	20	20	23	20

Table 2: Diameter of growth inhibition zone of Rutagraveolens extracts on bacteria by disc diffusion method (mm)

Extract	Aquatic				Ethanolic				Methanolic			
Amount of extract (µl)	30	40	50	60	30	40	50	60	30	40	50	60
B. subtilis	10	11	10	12	14	23	24	26	18	20	21	21
Stap. aureus	12	12	12	15	13	17	19	21	12	14	17	19
E. coli	0	0	7.5	7.5	0	0	0	0	0	0	0	0
Ps.aeruginosa	8	8	8	9	0	0	0	0	0	0	0	0

Table 3: The MIC and MBC of different extracts of Rutagraveolens on different bacteria (µg/ml)

Extract	Aquatic		Ethanolic		Methanolic	
Methods	MBC	MIC	MBC	MIC	MBC	MIC
B. subtilis	13×10^3	63×10^2	63×10^2	31×10^2	13×10^3	31×10^2
Staph. aureus	13×10^3	63×10^2	13×10^3	31×10^2	13×10^3	16×10^2
E. coli	5×10^4	25×10^3	5×10^4	13×10^3	5×10^4	25×10^3
Ps.aeruginosa	5×10^4	25×10^3	5×10^4	25×10^3	5×10^4	25×10^3

DISCUSSION

Rutagraveolens has a significant treatment value. This plant with a lot and high variation of chemical components is a kind of natural fungicidal and bactericidal plant and has a growth inhibition effect on several bacteria and fungi [4, 5, 7].The glycosides, alkaloids, quinolene, comarine, lignins and flavonoids are the most important components of this plant. The antimicrobial effect of these components on different kinds of fungi and bacteria has been proved [9, 12].The applications of antibiotics are common for deletion of microbes from environment for example during infectious diseases but resistance of bacteria to chemical components is a great problem. Method of extraction, kind of solvents (solubility of extract) and amount of extracts particularlyphytochemicals, kind and number of bacteria are effective in diameter inhibition zone [4, 5, 11]. The chemical structures and different metabolisms of Gram-positive and Gram-negative bacteria cause different response to the condition and stresses of environments like antibiotics. So, the gram-negatives are more resistant to antibiotics than gram-positives. On the other hand they can get resistant to antibiotics faster than gram-positives. Thereby, recognition and identification of proper chemical components with antibacterial effects on Gram-negative bacteria are so important. [4, 5, 8, 11]. In this research, growth inhibitions of gram-positive and gram-negative bacteria were observed according to the MIC and MBC methods. Another significant finding of this work is the growth inhibition of Gram-negative bacteria specially Pseudomonasaeruginosa with different extracts. The special cell wall of Pseudomonasaeruginosa prevents the entrance of chemical materials and causestheir resistance[4-6, 8, 9, 11]. The effect of the plant on Pseudomonasaeruginosa in this study confirms the research by Alzorekyand Nakahara[12]. They announced that the antimicrobial effect of this plant on Pseudomonasaeruginosa is equal with gentamicin. In another study by Ouliaand Saderi[9].The inhibition effect of this extract on Pseudomonasaeruginosa was confirmed [9]. Since the sensitivity of Gram-positive bacteria is more than Gram-negative bacteria, in all methods Staph. aureus and B. subtilis had a different amount of sensitivity on the different extracts. The results of thestudy of Ivanovaand *et al* on antimicrobial effect of hydroalcoholic and aquatic extract of seeds and stems of what confirm the results of this study [8].

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