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### Antifungal and antibacterial effects of *Ruta- graveolens* extracts

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#### ABSTRACT

Indiscriminate and excessive uses of antibiotics may lead to the emergence of antibiotic-resistant microorganisms and side effects in patients. Therefore, researchers recently have focused on the use of natural resources, especially medicinal plants. *Ruta graveolens* is a kind of medicinal plant which contains antifungal and antibacterial components and is used in traditional medicine in Iran and other nations. The main goal of this research was the investigation of antimicrobial effects of aquatic and alcoholic extracts of this plant on Gram-positive and Gram-negative bacteria and four *Candida albicans* strains. For this aim, antibacterial and antifungal activities of *Ruta graveolens* extracts on *Staphylococcus aureus* (PTCC 1431), *Bacillus subtilis* (PTCC 1720), *Escherichia coli* (PTCC 1763) and *Pseudomonas aeruginosa* (PTCC 1599) and four *Candida albicans* strains isolated from denture stomatitis were evaluated by measurement of minimum inhibitory concentration (MIC) and minimum bactericidal and fungicidal concentration (MBC, MFC), disc diffusion and well diffusion methods. The results indicated that aquatic, methanolic and ethanolic extracts with different MIC and MBC have been effective on growth of selected bacteria and *Candida albicans*.

#### 1. Introduction

Although applications of antibiotics and antifungal drugs are useful for treatment of different bacterial and fungal infections, indiscriminate and excessive uses of them have increased the emergence of antibiotic-resistant microorganisms and side effects in patients (Azizi et al., 2012, Hashemi Karouei et al., 2012). Therefore, 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 medicinal 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 and fungi became inevitable (Azizi et al., 2012, Preethi et al., 2008). The applications of plants were common among Iranians and Iranian's traditional medicine since long time ago. *Ruta graveolens* is a medicinal plant that has been used for traditional treatment in Iran and other nations. *Ruta* has been reported as a medicine with significant treatment effects (Azizi et al., 2012, Standards NCCLS M7-A5). It has a significant treatment effect for several diseases. 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,

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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 (Azizi et al., 2012, Ivanova et al., 2005). However, there are not any studies about the valuable therapeutic effect of *Ruta graveolens* in traditional treatments. Although this plant has been widely used, the exact mechanism and its effective components have been unknown. According to the documents that remained from ancestors, this plant has a significant therapeutic value especially in some refractory disease such as cancer and Alzheimer.

Therefore, investigation about this plant has valuable results for treatment of different disease. *Ruta graveolens* has anti-bacterial and anti-cytotoxic effects against different species of *Staphylococcus* such as *Staphylococcus aureus* and *Staphylococcus epidermis*, *Listeria monocytogenes* and *Bacillus subtilis* (Ivanova et al., 2005, Standards NCCLS M7-A5, Jalali Moghadam et al., 2012). Moreover, the aquatic extracts of this plant with the aquatic extracts of *Viola* have a growth suppressor effect on *Trichomonas vaginalis*. The phenolic, alkaloid and terpenoid components of *Ruta graveolens* have 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. (Owlia et al., 2004). Therefore, it seems that *Ruta graveolens* can be used against infections on animals, plants and human. Nevertheless, *Ruta graveolens* is an endemic plant in the north of Iran and it has been widely used in traditional medicine for treatment of infectious and inflammatory diseases. However, there are not any clinical studies about it till now. The aim of this study is to investigate the antimicrobial effect of aquatic and alcoholic extract of *Ruta graveolens* on gram-positive and gram-negative bacteria and four *Candida albicans* isolated from Denture Stomatitis. The focus of this study is antimicrobial activities on particular gram-negative bacteria such as *P. aeruginosa* and *E. coli* and *Candida albicans* which have a significant influence on different disease such as urinary tract infection and denture Stomatitis.

## 2. Materials and Methods

### 2.1. Collection and Preparation of plant

*Ruta graveolens* has been collected from the mountainous area of Tonekabon in North Iran and prepared powder from it after washing and drying.

### 2.2. Extraction

The aquatic, ethanolic and methanolic soluble were prepared from the powder with Soxhlet method. After extraction, the alcoholic solvents with a rotary evaporator and aquatic solvent in 45°C were removed and yielding the pure extracts was prepared and then dilution 10<sup>-1</sup> with DMSO prepared from each extract (Ivanova et al., 2005, Standards NCCLS M7-A5, Jalali Moghadam et al., 2012).

### 2.3. Bacteria and fungi

In this study, the lyophilized *Staphylococcus aureus* (PTCC 1431), *Bacillus subtilis* (PTCC 1720), *Escherichia coli* (PTCC 1763) and *Pseudomonas aeruginosa* (PTCC 1599) were obtained from Persian Type Culture Collection (PTCC) of scientific and industrial center of Shahryar in Iran and four *Candida albicans* (*Candida albicans* 1, *Candida albicans* 2, *Candida albicans* 3 and *Candida albicans* 4) isolated from Denture Stomatitis in dental clinic. Then a suspension adjusted to the 0.5 McFarland standards of each bacteria and fungi was prepared.

### 2.4. Disc diffusion method (Kirby Bauer test)

Disc diffusion method for antimicrobial susceptibility testing was carried out to assess the presence of antibacterial and antifungal activities of the plant extracts. In this method first inoculated 10 microliter of bacteria and fungi suspension adjusted to 0.5 McFarland standards to Muller Hinton agar for bacteria and subouraud dextrose agar for fungi and has been cultured with sterile swab. In other step the discs contain 30, 40, 50 and 60 microliter of plant extracts (from the dilution of 1/10) was placed on the Mueller-Hinton agar and subouraud dextrose agar surface. After incubation at 37°C for 24 hours, they examined for inhibition zone. The experiments were repeated three times for each sample and determined the average of the inhibition zone diameter (Standards NCCLS M7-A5; Ivanova et al., 2005, Jalali Moghadam et al., 2007).

### 2.5. Well Diffusion Method

Agar well diffusion method for antimicrobial susceptibility testing was carried out to assess the presence of antibacterial activities of plant extract. After preparation of 4 wells on the Muller Hinton agar plate in sterilized situation, the extracts with different amount of 70, 80, 90 and 100 microliter were loaded to each well. In the next step, 10 microliter of bacteria and fungi suspension adjusted to 0.5 McFarland standards to Muller Hinton agar for bacteria and subouraud dextrose agar for fungi and has been cultured with sterile swab. After incubation of plates at 37°C for 24 hours, they examined for inhibition zone. The experiments were repeated three times for each sample and determined the average of the inhibition zone diameter (Standards NCCLS M7-A5; Ivanova et al., 2005).

### 2.6. Minimum Inhibition Concentration Determination (MIC)

*Ruta graveolens* extracts MICs were performed using a broth macrodilution method as recommended by NCCLS (M7-A5 and M27 – A2). Ten microliter of bacterial and fungal suspensions (with 0.5 McFarland standards) was added to the 11 tubes consisting Muller Hinton broth and subouraud dextrose agar with dilution of 1/2. Then, MIC was determined after incubation of samples at 37°C for 24 hours. Subsequently, 10µl of each tube (before incubation for MIC) were cultured in Muller Hinton agar and subouraud dextrose agar and incubated similarly for detection of extracts MBC and MFC. The experiments were repeated three times for each samples and determined the average of MIC and MBC of extractions [Standards NCCLS M7-A5]

## 3. Results

This study showed the inhibition effect of the aquatic and alcoholic *Ruta graveolens* extracts on *Staphylococcus aureus* and *Bacillus subtilis* in both well diffusion and disc diffusion methods. 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). The most important result of this study was the effect of different amount of

aquatic extracts on all bacteria samples that were used in this project. Although, 30 and 40 microliter 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 inhibition effect of different amounts of aquatic extract of *Ruta graveolens* with disc and well diffusion methods on *Pseudomonas aeruginosa* was one of important results of this study (Figure 4). Ethanolic and methanolic *Ruta graveolens* extracts had inhibition effect neither disc diffusion nor well diffusion methods on *E.coli* growth (Table 1 and 2).

In the disc diffusion method *Candida albicans* 1 and *Candida albicans* 3 grown in around of discs contain different amounts of ethanolic, methanolic and aquatic *Ruta graveolens* extracts but *Candida albicans* 2 and *Candida albicans* 4 not grown and produced different diameters of growth inhibition zone. In the well method with increasing extracts amount compare with disc diffusion increased antifungal effects of them. Antifungal effects of *Ruta graveolens* ethanolic extract on *Candida albicans* 1, *Candida albicans* 2 and *Candida albicans* 3 was more than *Ruta graveolens* methanolic and aquatic extracts (Table 1 and 2). Moreover, the MIC and MBC of methanolic extracts on *Staphylococcus aureus* were recorded to be 1662µg/ml and 126×10<sup>2</sup> µg/ml, respectively. However, the MIC and MBC of aquatic and methanolic extracts on *Staphylococcus aureus* and *Bacillus subtilis* are equal. The MIC and MBC are 6259 µg/ml and 125×10<sup>2</sup> µg/ml, respectively. For methanolic extract on *E.coli* the MIC was 125×10<sup>2</sup> µg/ml and MBC was 5×10<sup>4</sup> µg/ml. However, MIC and MBC of aquatic and methanolic extracts on *Pseudomonas aeruginosa* and *E.coli* are the same and equal to 25×10<sup>3</sup> µg/ml and 5×10<sup>4</sup> µg/ml respectively (Table3).

## 4. Discussion

In *Ruta graveolens* has a significant treatment value. This plant with a lot and high variation of chemical component is a kind of natural fungicidal and bactericidal and has a growth inhibition effect on several bacteria and fungi (Preethi et al., 2008; Azizi et al., 2012). The glycosides, alkaloids, quinolene, comarine,

lignins and flavonoids are the most important components of this plant. The antimicrobial effect of these components on different kind of fungi and bacteria has been proved (Standards NCCLS M7-A5; Ivanova et al., 2005). 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), amount of extracts particular phytochemical, kind and number of bacteria are effective in diameter inhibition zone. 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. For example in the soxhlet method oily components extracts more than cold method (Azizi et al., 2012; Hashemi Karouei et al., 2012).

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 *Pseudomonas aeruginosa* with different extracts. The special cell wall of *Pseudomonas aeruginosa* prevents the entree of chemical materials and casus their resistant effect of the plant on *Pseudomonas aeruginosa* in this study confirms the research by Alzoreky et al. They announced that the antimicrobial effect of this plant on *Pseudomonas aeruginosa* is equal with gentamicin (Ivanova et al., 2005; Alzoreky et al., 2003). In another study by Oulia et al. the inhibition effect of this extract on *Pseudomonas aeruginosa* were confirmed (Oulia et al., 2005). 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 the study of Sadari and et al on antimicrobial effect of hydroalcoholic and aquatic extract of seeds and stems confirm the results of this study (Ivanova et al., 2005).

**Table 1.** Diameter of growth inhibition zone of *Ruta graveolens* extracts on bacteria by well diffusion method (mm)

Extract	Aquatic				Ethanolic				Methanolic			
	70	80	90	100	70	80	90	100	70	80	90	100
Amount of extract $\mu$ ( $\mu$ l)	70	80	90	100	70	80	90	100	70	80	90	100
<i>B. subtilis</i>	13	14	15	16	23	24	25	26	20	21	21	22
<i>Staph. aureus</i>	15	16	16	17	20	20	20	22	19	20	20	22
<i>E. coli</i>	11	12	11	15	0	0	20	0	0	0	0	0
<i>Ps. aeruginosa</i>	11	11	12	12	0	0	0	0	20	20	23	20

**Table 2.** Diameter of growth inhibition zone of *Ruta graveolens* extracts on bacteria by disc diffusion method (mm)

Extract	Aquatic				Ethanolic				Methanolic			
	30	40	50	60	30	40	50	60	30	40	50	60
Amount of extract ( $\mu$ l)	30	40	50	60	30	40	50	60	30	40	50	60
<i>B. subtilis</i>	10	11	10	12	14	23	24	26	18	20	21	21
<i>Staph. aureus</i>	12	12	12	15	13	17	19	21	12	14	17	19
<i>E. coli</i>	0	0	7.5	7.5	0	0	0	0	0	0	0	0
<i>Ps. aeruginosa</i>	8	8	8	9	0	0	0	0	0	0	0	0

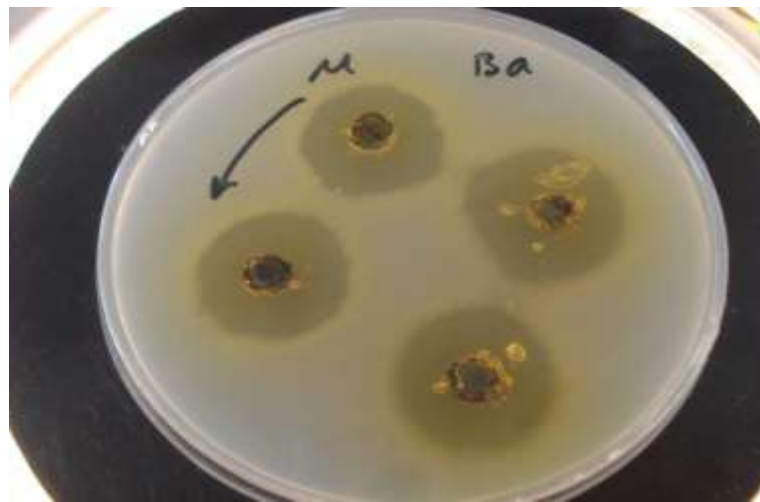
**Table 3.** The MIC and MBC of different extracts of *Ruta graveolens* on different bacteria ( $\mu$ g/ml)

Extract	Aquatic		Ethanolic		Methanolic	
	MBC	MIC	MBC	MIC	MBC	MIC
<i>B. subtilis</i>	$125 \times 10^2$	6250	6250	3125	$125 \times 10^2$	3125
<i>Staph. aureus</i>	$125 \times 10^2$	6250	$125 \times 10^2$	3125	$125 \times 10^2$	1562.5
<i>E. coli</i>	$5 \times 10^4$	$25 \times 10^3$	$5 \times 10^4$	$125 \times 10^2$	$5 \times 10^4$	$25 \times 10^3$
<i>Ps. aeruginosa</i>	$5 \times 10^4$	$25 \times 10^3$	$5 \times 10^4$	$25 \times 10^3$	$5 \times 10^4$	$25 \times 10^3$

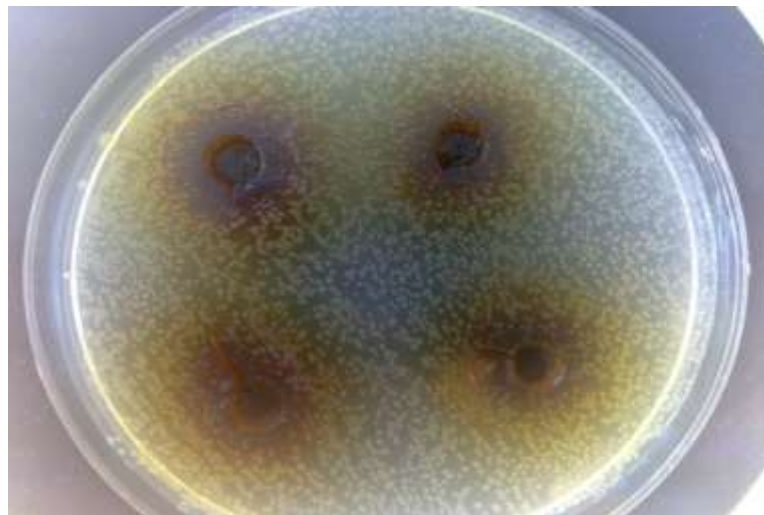




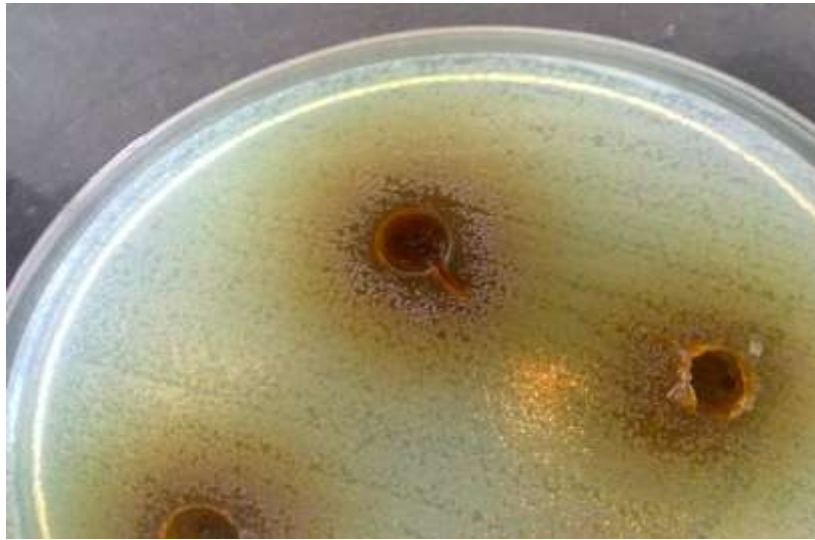
**Figure 1.** Inhibition zone diameter of *Bacillus subtilis* with different amount of ethanolic extracts in disc method.



**Figure 2.** Inhibition zone diameter of *Bacillus subtilis* with different amount of ethanolic extracts in well method.



**Figure 3.** Inhibition zone diameter of *E. coli* with different amount of aquatic extracts in well method.



**Figure 4.** Inhibition zone diameter of *ps. aeruginosa* with different amount of aquatic extracts in disc method.

## Refereces

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