

**ESSENTIAL OILS OF *CITRUS BERGAMIA* AS ANTIBACTERIAL AGENTS AGAINST SOME HUMAN PATHOGENIC BACTERIA.****Dr. Vijay Nigam\*, Dr. Sukhwant Singh, Bhagyashri Gupta, Aakansha Singh**

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**ABSTRACT**

Bacterial infections are a major problem in humans; as such, replacing the chemical drugs used against them with plant drugs is an important research. Here, the chemical compositions and antibacterial activity of the essential oils obtained from *Citrus bergamia* bung against ten human pathogenic bacteria were evaluated. Chemical compositions of essential oil were analyzed by gas chromatography- mass spectrometry (GC-MS) method. Antibacterial activity of essential oil was evaluated by well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the macrodilution method. The GC-MS spectrums showed 13 compounds, in which the highest chemical composition was related to

phenol (37.36%), thymol (22.65%) and p-cymen (19.29%) compounds. The essential oil of *Citrus bergamia* bung showed good activity against all tested bacteria, except for *Pseudomonas aeruginosa*, which is possibly due to the high levels of phenol in their compositions. The MIC and MBC values of the bacterial strains sensitive to the essential oil were in the ranges of 0.8 to 25 and 50 to 100 mg/ml, respectively. However, the essential oil of *Citrus bergamia* bung is a suitable plant drug against human pathogenic bacteria.

**Key words:** *Citrus bergamia*, antibacterial, essential oil.

**INTRODUCTION**

There is a popular and scientific interest to screen essential oils of plants used medicinally all over the world (Salehi et al., 2005). The use of medicinal plants as anti- bacterial and

anti-inflammatory drugs in folk medicine is a practice common in Iran, although in most cases, the active principles of the plants are unknown (Hajhashemi et al., 2002). In recent years, multiple drug resistance in human pathogenic microorganisms has been developed due to the indiscriminate use of commercial anti-microbial drugs commonly used in the treatment of infectious diseases. This situation forced scientists to search for new antimicrobial substances from various sources like medicinal plants (Clark, 1996; Cordell, 2000). Bergamot is a genus of the well-known medicinal plant of *Rutaceae* family and it comprises numerous species growing wild in the Mediterranean area (Bezi et al., 2005). Many members of the genus *Bergamot* have aromatic and medicinal characteristics (Sahin et al., 2003) and have been used also to treat various ailments such as cramps, muscle pains, nausea, indigestion, diarrhea and infectious diseases (Leung, 1996; Hajhashemi et al., 2000; Hajhashemi et al., 2002). More so, it has shown antispasmodic, anti-diarrhoeal, anti-oxidant, sedative as well as antimicrobial properties (Zargari, 1990). *Citrus bergamia* is an endemic plant and one of the most important of the twelve species of Iranian *Bergamot* species which is widely distributed in the southern region of Iran. There are some published investigations regarding the antimicrobial activity of various *Bergamot* species. Unfortunately, so far no reports are known which included the antimicrobial effect of the essential oil of *Citrus bergamia* on human pathogenic bacteria. Therefore, the aim of this work is to evaluate the chemical composition of *Bergamot* by using gas chromatography-mass spectrometry (GC-MS) analysis and to determine the antibacterial activity by the well diffusion method.

## MATERIALS AND METHODS

### Plant material

Apparently, healthy fruit of *Bergamot* were collected and identified during the flowering period, from the Mountain region in the southern tip of Italy.

### Isolation of essential oil

Air-dried plant material (100 g) was hydro distilled for 3 h using a Clevenger type apparatus. The essential oils were collected over water, separated and dried over anhydrous sodium sulphate, after which they were stored in sealed vials at 4 to 6°C prior to chemical analysis and antimicrobial screening.

**Gas chromatography mass spectrometry analysis**

GC-MS analysis of the oil was conducted using a Hewlett Packard 6890 instrument operating on EI mode and equipped with a 5MS- HP fused silica column (30 m×0.25 mm × 0.25 µm film thickness capillary columns). Helium (99.99%) was used as the carrier gas at the constant flow of 1 ml/min. The oven temperature was held at 60°C for 1 min, then programmed to 210°C at a rate of 6°C/min, and then held for 10 min. The injector and detector (FID) temperatures were kept at 250 and 280°C, respectively. The components of the oil were identified by comparison of their mass spectra with those obtained from authentic samples and/or the control. The agar plates were incubated for 24 h at 37°C and the diameter of the zone of inhibition surrounding the wells was measured. Assays were performed in triplicate and the data were shown as mean ± standard deviation (SD).

**Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

The MIC and MBC of the essential oil against the test microorganisms was determined by the broth macro dilution method. An eight-fold serial dilution of the essence in DMSO, including 100, 50, 25, 12.5, 6.25, 3.12, 1.6, 0.8 and 0.4 mg/ml was prepared in sterile test tubes. These dilutions were added to 1 ml Muller Hinton Agar medium containing  $1.5 \times 10^6$  CFU/ml bacteria. Two test tubes served as positive and negative control, respectively. The test tubes were incubated at 35°C for 24 h.

**Gas chromatography mass spectrometry analysis**

GC-MS analysis of the oil was conducted using a Hewlett Packard 6890 instrument operating on EI mode and equipped with a 5MS- HP fused silica column (30 m×0.25 mm × 0.25 µm film thickness capillary column). Helium (99.99%) was used as the carrier gas at the constant flow of 1 ml/min. The oven temperature was held at 60°C for 1 min, then programmed to 210°C at a rate of 6°C/min, and then held for 10 min. The injector and detector (FID) temperatures were kept at 250 and 280°C, respectively. The components of the oil were identified by comparison of their mass spectra with those obtained from authentic samples and/or the National Institute of Standards and Technology/ National Bureau of Standards (NIST/NBS) and Wiley mass spectral database. They were also confirmed by comparison of their retention indices (RI) (Van

den Dool and Kratz, 1963) and retention times (RT), either with those of authentic compounds or with published data (Adams,2001).

### Antibacterial activity determination

The microorganisms used in this study were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonasa aeruginosa* ATCC 27853, *Kellebsiella pneumoniae* ATCC27881, *Salmonella typhi* ATCC 10556, *Serratia marcesens* ATCC 35668, *Shigella dysenteriae* ATCC 15303, *Shigella flexneri* ATCC 15305, *Enterobacter aerogenes* ATCC 25933 and *Proteus vulgaris* ATCC 25942, provided from the Institute pasture of Iran. The antimicrobial activity of essential oils was tested by the agar well diffusion method on Muller Hinton Agar (MHA). Using a cork borer, five wells (6 mm in diameter) were made in the agar medium (one well was in the center and four were at the corner), and inoculums containing  $1.5 \times 10^6$  CFU/ml of the test bacteria were spread onto the surface of the medium with a sterile swab. In the case of essential oil, 10, 20, 30, 40 and 50  $\mu$ l of the essence was pipetted into the wells, whilst 50  $\mu$ l of dimethyl sulfoxide (DMSO) was served as a control. Gentamycine disk was used as a positive complete inhibition of the growth was observed was recorded as MIC. The MIC for each of the test bacteria was determined in triplicate assays and the data were shown as mean  $\pm$  SD. To determine MBC, broth was taken from each well and inoculated in MHA for 24 h at 37°C.

### RESULTS

The results obtained in the qualitative and quantitative analyses of essential oil are shown in Table 1.

**Table 1. Percentage composition of the essential oils isolated from *Citrus bergamia***

RI	<i>Citrus bergamia</i>	Compounds	#
988	0.76	$\beta$ -myrcene	1
1017	0.93	$\alpha$ -terpinene	2
1131	1.92	borneol	3
1268	22.65	thymol	4
1298	37.36	phenol	5
1567	2.02	caryophyllene oxide	6
1280	0.24	Carvacrole	7
1030	0.94	limonene	8
1010	19.29	p-cymene	9
1060	5.01	-terpinene	10

1076	4.92	L-linalool	11
1189	0.66	terpineol	12
1418	2.19	-Caryophyllene	13

From the essential oil of *Citrus bergamia*, 13 constituents were identified, of which phenol (37.36%), thymol (22.65%) and *p*-cymene (19.29%) were the major components. The results regarding the antibacterial activity of the essential oil from *Bergamot bakhtiarica* are indicated in Table 2. The essential oil of *Citrus bergamia* showed good activity against all test bacteria, except for *Ps. aeruginosa*, as compared to gentamycine antibiotic (as positive control). *E. coli*, *K. pneumonia* and *S. aureus* were the most susceptible. The control treatment (DMSO) did not show an inhibitory effect on any of the bacteria. However, the antibacterial activity of essential oil in all concentrations (from 10 to 50  $\mu$ l) was found for the tested bacteria. The MIC value of the essential oil of *Bergamot bakhtiarica* for all test bacteria is shown in Table 3. As shown in Table 3, the MIC value of the bacterial strains sensitive to the essential oil was in the range of 0.8 to 25 mg/ml. The lowest MIC was found for *Staphylococcus aureus*, *Shigella dysenteriae* and *Proteus vulgaris* (0.4 mg/ml), whereas the maximum MIC was found for *Ps. Aeruginosa* (25 mg/ml) and *K. pneumoniae* (12.5 mg/ml). The MBC value of the essential oil of *Citrus bergamia* for all test bacteria is shown in Table 4. As shown in Table 4, the MIC value of the bacterial strains sensitive to the essential oil was in the range of 6.25 to 25 mg/ml. The lowest MBC was found for *Shigella dysenteriae* and *Shigella flexneri* (6.25 mg/ml), whereas the maximum MIC was found for *Ps. aeruginosa* (50 mg/ml).

**Table 2. Antibacterial activity of *Citrus bergamia* essential oil.**

	10 $\mu$ l	20 $\mu$ l	30 $\mu$ l	40 $\mu$ l	50 $\mu$ l	G.M	Control
<i>E. coli</i>	24.55 $\pm$ 0.25	29.66 $\pm$ 0.35	33.25 $\pm$ 0.5	36.15 $\pm$ 0.25	38.22 $\pm$ 0.25	21.23 $\pm$ 0.3	0
<i>K. pneumoniae</i>	25.25 $\pm$ 0.25	32.22 $\pm$ 0.29	36.25 $\pm$ 0.22	38.66 $\pm$ 0.63	39.25 $\pm$ 0.19	20.25 $\pm$ 0.2	0
<i>S. typhi</i>	14.15 $\pm$ 0.65	15.64 $\pm$ 0.25	17.75 $\pm$ 0.35	19.22 $\pm$ 0.45	25.66 $\pm$ 0.15	26.55 $\pm$ 0.3	0
<i>S. aureus</i>	24.95 $\pm$ 0.15	26.66 $\pm$ 0.15	27.55 $\pm$ 0.45	35.22 $\pm$ 0.25	38.15 $\pm$ 0.34	24.15 $\pm$ 0.2	0
<i>S. marcesens</i>	16.82 $\pm$ 0.35	18.83 $\pm$ 0.25	21.25 $\pm$ 0.45	23.83 $\pm$ 0.50	27.66 $\pm$ 0.65	21.75 $\pm$ 0.4	0
<i>S. dysenteriae</i>	14.44 $\pm$ 0.40	17.35 $\pm$ 0.65	22.66 $\pm$ 0.15	26.66 $\pm$ 0.40	28.66 $\pm$ 0.55	25.15 $\pm$ 0.2	0
<i>S. flexneri</i>	16.25 $\pm$ 0.50	21.22 $\pm$ 0.55	25.55 $\pm$ 0.40	30.44 $\pm$ 0.25	32.22 $\pm$ 0.30	22.65 $\pm$ 0.3	0
<i>E. aerogenes</i>	15.44 $\pm$ 0.15	17.85 $\pm$ 0.30	19.53 $\pm$ 0.25	22.44 $\pm$ 0.35	24.33 $\pm$ 0.44	20.55 $\pm$ 0.4	0
<i>P. vulgaris</i>	17.22 $\pm$ 0.65	19.73 $\pm$ 0.15	24.75 $\pm$ 0.65	25.55 $\pm$ 0.55	29.22 $\pm$ 0.50	22.25 $\pm$ 0.3	0
<i>P. aeruginosa</i>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	15.35 $\pm$ 0.6	0

**Table 3. MIC of the essential oil of *Citrus bergamia***

Bacteria	MIC mg/ml)							
	100	50	25	6.25	3.1	1.6	0.8	0.4
<i>E. coli</i>	-	-	-	-	-	+	+	+
<i>K. pneumoniae</i>	-	-	+	+	+	+	+	+
<i>S. typhi</i>	-	-	-	-	-	+	+	+
<i>S. aureus</i>	-	-	-	-	-	-	+	+
<i>S. marcesens</i>	-	-	-	-	-	-	+	+
<i>S. dysenteriae</i>	-	-	-	-	-	-	+	+
<i>S. flexneri</i>	-	-	-	-	-	-	+	+
<i>E. aerogenes</i>	-	-	-	-	-	+	+	+
<i>P. vulgaris</i>	-	-	-	-	-	-	+	+
<i>P. aeruginosa</i>	-	-	+	+	+	+	+	+

**Table 4. MBC of the essential oil of *Citrus bergamia*.**

Bacteria	MBC (mg/ml)							
	100	25	12.5	6.25	3.1	1.6	0.8	0.4
<i>E. coli</i>	-	-	-	+	-	-	-	-
<i>K. pneumoniae</i>	-	+	+	+	+	+	+	+
<i>S. typhi</i>	-	-	-	-	+	+	+	+
<i>S. aureus</i>	-	-	-	-	+	+	+	+
<i>S. marcesens</i>	-	-	-	-	+	+	+	+
<i>S. dysenteriae</i>	-	-	-	-	-	-	+	+
<i>S. flexneri</i>	-	-	-	-	-	-	+	+
<i>E. aerogenes</i>	-	-	-	-	+	+	+	+
<i>P. vulgaris</i>	-	-	-	+	+	+	+	+
<i>P. aeruginosa</i>	-	+	+	+	+	+	+	+

## DISCUSSION

Plant extracts are extensively used for traditional medicine in Iran. Essential oils have been used as flavoring agents in food and beverages and, due to the presence of antimicrobial compounds, they have a potential as natural agents for food preservation (Helander et al., 1998). Moreover, researchers have been interested in biologically active compounds isolated from plant species for the elimination of pathogenic micro-organisms because of the resistance they have developed to antibiotics (Cordell, 2000).

Recently, many studies have focused on the antibacterial activity of the essential oil or extracts of *Bergamot* species. These studies have revealed that the genus has antimicrobial activity against human, food and plant pathogens (Ciani et al., 2000; Baser et al., 2004;

Ozcan and Erkmen, 2001; Gulluce et al., 2003; Adiguzel et al., 2006), due to the presence of phenolic components such as thymol and carvacrol. On the other hand, a large number of studies have reported that the essential oils of *Bergamot* species are among the most potent essential oils regarding their antimicrobial properties (Sahin et al., 2003; Skocibusic et al., 2006; Vagionas et al., 2007). The essential oils of many species of the genus *Bergamot* are known to possess antibacterial and fungicidal properties, such as *S. brownie*, *S. montana*, *S. hortensis* and *S. thymbra* (Ozcan and Boyraz, 2000; Ozcan and Erkmen, 2001; Ciani et al., 2000; Sahin et al. 2003; Vagionas et al., 2007). To our knowledge, no data have been published on the biological properties of *Citrus bergamia* essential oil, and there is a lack of information on its antimicrobial activity. Here, the *in vitro* antimicrobial activity of *Citrus bergamia* essential oil against the microorganisms employed and its activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters, MIC and MBC values. The results of the present study indicated that the essential oil of *Citrus bergamia* have good antimicrobial effect on human pathogenic bacteria, which could be attributed to the high content of the compounds' essence with known antimicrobial activity, such as phenol (37.36%), thymol (22.65%) and *p*-cymene (19.29%). The essential oil extract did not display any antibacterial activity against *Ps. aeruginosa*. The basis of the varying degree of sensitivity of the test organisms of bacteria may be due to the intrinsic tolerance of microorganisms, and the nature and combinations of phytochemicals present in the essential oil (Ozturk and Ercisli, 2007). In earlier investigations, other *Bergamot* species such as *Bergamot hortensis*, *Bergamot icarica*, *Bergamot pilosa* and *Bergamot boissieri* were studied with respect to essential oil composition, and have been shown to be rich in components such as carvacrol,  $\gamma$ -terpinene, thymol and *p*-cymene (Gulluce et al., 2003; Adiguzel et al., 2006; Azaz et al., 2002), which was comparable to that of our study. Essential oils rich in phenolic compounds such as cavaracrol are widely reported to possess high levels of antimicrobial activity (Aligiannis et al., 2001; Baydar et al., 2004), which has been confirmed and extended in the present studies. This is the first study to provide data which show that the extracts of *Bergamot bakhtiarica* plants evaluated against a wide range of microorganisms possess potential antibacterial.

## CONCLUSION

Based on these results, it is possible to conclude that the usage of the essential oil of *Bergamot bakhtiarica* as a natural antibacterial has a strong and broad spectrum of

antibacterial activity against many human pathogenic bacteria, because the oil possesses strong antibacterial activity. The antibacterial property of the essences of *Bergamot bakhtiarica* is mostly attributable to the phenolic compound thymol and *p*-cymene. Further study is needed in order to obtain information regarding the practical effectiveness of *Citrus bergamia* essential oil in order to prevent the growth of human pathogenic bacteria as antimicrobial agents in new drugs for the therapy of infectious diseases in humans.

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**Abbreviations:** GC-MS, Gas chromatography-mass spectrometry; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; RI, retention indices; RT, retention times; MHA, Muller Hinton Agar; NIST, National Institute of Standards and Technology; NBS, National Bureau of Standards; DMSO, dimethyl sulfoxide; SD, standard deviation