

***Kelussia odoratissima* Essential Oil: Biochemical analysis and Antibacterial Properties against pathogenic and probiotic bacteria**

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Abstract

Kelussia odoratissima Mozaff, is an endemic plant in Iran and its known as *Keluss* or *Karafs-e- Bakhtiari* in Persian. This has been used as a sedative and vegetable in Bakhtiari folk. Phytochemical component and antibacterial potential of *K. odoratissima* essential oil was studied. The *Kelussia odoratissima* seeds essential oil was obtained by hydro-distillation and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The antibacterial activities of this EO was investigated against Food born pathogenic bacteria (*Salmonella thyphimurium*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*) and probiotic bacteria (*Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus rammus*) by standard Microplate serial dilution method. GC/MS analysis of the EO showed that α Caryophyllene (22.60%), α Humulene (20.00%), Tetracyclononan-3-one (CAS) Triasteranone (16.04%) and Cyclopropane (11.54%) were the major compounds. Most of the probiotic bacteria showed a relatively high resistance. Among the pathogenic bacteria, *S. aureus* was the most sensitive bacteria followed by *L. monocytogenes*. The MIC and MBC values for essential oil ranged between 1250-10000 ppm. Among the probiotic bacteria tested, the *L. rammus* was the susceptible (MIC 1250 ppm and MBC 2500 ppm value) and *L. casei* and *L. plantarum* were the resistant (MIC 5000 ppm). It can be said that the *K. odoratissima* EO could be used as natural preservative agents in foodstuff.

Keywords: GC/MS analysis, *Kelussia odoratissima*, Essential oil, Probiotic.

1. Introduction

Food spoilage is one of the most important issues facing the food industry. In fact, food-borne illnesses are a global problem, even in developing and developed countries.

Food spoilage or deterioration is predominantly caused by the growth of microorganisms. Many pathogenic microorganisms, including *Escherichia coli*, *S. aureus*, *L. monocytogenes*, and *Salmonella*

spp. have been identified as the causal agents of food-borne diseases or food spoilage [1].

Antibiotic resistance has become a global concern. In recent years there is increasing incidence of multiple resistances in human pathogenic microorganisms, largely due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. In addition, concerns over the inevitable side effects of chemical food preservatives, these had forced scientist to search for new antimicrobial substances from various sources like medicinal plants [2]. Search for new antibacterial agents should be continued by the screening of many plant families. In Iran, a favorable climate and geography have contributed to a diversity of medicinal plants, and many endemic plant species exist. The *Apiaceae* family contains approximately 275 genera and 2850 species. Coumarins, polyacetylenes, flavonoids, sesquiterpenes and phthalides are among the important chemical constituents of this family along with biologically active essential oil [3,4].

Kelussia is one of the newest genera of this family and is represented by only one species, *Kelussia odoratissima* Mozaff, which is found only in Iran [5]. *Kelussia* locally called “*karafse Bakhtiari*” or “*kelus*” [6,7]. It is a wild rebus, erect, glabrous, perennial aromatic herb, which grows in high altitude of western Zagros mountains, Iran. *Kelussia* is traditionally consumed as a medicinal plant to treat hypertension, inflammation, ulcers and cardiovascular diseases [8]. The *K. odoratissima* seeds have long been used as a remedy for various ailments, particularly to treat head cold and stomachache, *K. odoratissima* seeds could be rich sources of phenolics components. The EO isolated by hydrodistillation from the seeds of *K. odoratissima* was found to be a pale yellow liquid, The Iranian medicinal plants such as *Kelussia odoratissima* has been utilized as traditional medicines by the indigenous people of Chaharmahal va Bakhtiari in Iran [5].

Lactobacillus strains have been utilized as dairy starter and may act as both probiotic and bioprotective culture (one possible mode of action for probiotics is the production of antimicrobial compound) as well as fermenting agent in

fermented products. Studies showed the inhibitory effect of these bacteria against the growth of various foodborne bacterial pathogens e.g. *S. aureus*. Probiotic species such as *Lactobacillus* spp. have been safely used for more than 70 years. These probiotic are available in conventional foods and dietary supplements [9].

The present study was undertaken to investigate the chemical composition and effectiveness of the EO isolated from *Kelussia odoratissima* Mozaff on gram positive and negative food borne pathogenic and probiotic bacteria.

2. Materials and Methods

2.1. Plant materials. The seeds of *K. odoratissima* were collected in July-August 2011 from Zardkooh mountain which is located in Charmahal Bakhtiari province (south western of Iran). The plant identity was confirmed by Herbarium of the faculty of Pharmacy, University of Tabriz, Tabriz, Iran.

2.2. Essential oil preparation. Plant material was hydrodistilled in a Clevenger-type apparatus for 3 h. The volatile oil was dried over anhydrous sodium sulfate and stored in sterile sealed vial at 4°C until analysis [10]. The yield of oil was calculated based on the dried weight of the plant material.

2.3. GC-MS Analysis. The essential oil was analysed by GC (Agilent 6890, USA). The chromatograph was equipped with HP-5MS capillary column (30 × 0.25 mm ID × 0.25 mm film thickness) and the data were acquired under the following conditions: initial temperature 50 °C; program rate 15 °C min⁻¹; final temperature 300 °C (holding for 20 min) and injector temperature 290 °C. The carrier gas was Helium and the split ratio was 0.8 mL min⁻¹. The essential oil was also analysed by GC-MS (Agilent 6890 gas chromatography equipped with Agilent 5973 mass selective detector, USA) and the same capillary column and analytical conditions indicated above. The MS was run in the electron ionization mode, using ionization energy of 70 eV.

2.4. Identification of components. Identification of the components of the EO was based on GC retention indices relative to n-alkanes and computer matching with the Wiley 275 L mass spectra library. In addition, the analysis included comparisons of the fragmentation patterns of the MS to those reported in the literature [11].

2.5. Bacterial strain. The essential oil was individually tested against two Gram negative and two Gram positive bacteria including *S. aureus* ATCC6538, *L. monocytogenes* ATCC19118, *S.thyphimurium* ATCC13311 and *E.coli* ATCC43894: rephrased: The EO was individually tested against two gram negative (*S.thyphimurium* ATCC13311 and *E.coli* ATCC43894) and two Gram positive (*S. aureus* ATCC6538, *L. monocytogenes* ATCC19118) bacteria. Lyophilized cultures of these organisms were obtained from the culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran. Subcultivation and preparation of these organisms were conducted according to the Parsaeimehr et al. 2010 [12].

Commercial lyophilized cultures of the probiotic *L. casei* ATCC3939, *L. acidophilus* ATCC4356, *L. plantarum* ATCC 4142 and *L. ramnus* ATCC 5031 were obtained from the Iranian Organization of Industrial Research. Sub cultivation and preparation of the probiotic bacteria were conducted according to standard method [13].

2.6. Micro-well dilution assay. The MIC and MBC values were studied for the bacterial strains in microplate. The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Essential oil dissolved in 10% dimethylsulfoxide (DMSO) were first diluted to the highest concentration (10000 ppm) to be tested, and then serial two-fold dilutions were made in a concentration range 10000-312 ppm in 10 ml sterile test tubes containing nutrient broth. MIC values of EO and extract against pathogenic bacteria strains were determined based on a micro-well dilution method. The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µl of the inoculum. A 100 ppm aliquot from the stock solutions of essential oil initially prepared at the concentration of 10000 ppm were added into the first wells. Then, 100 ppm from their serial dilutions was transferred into 5 consecutive wells. The last well containing 195 µl of nutrient broth without compound and 5 ppm of the inoculum on each strip was used as the negative control. The final volume in each well was 200 ppm. The plates were covered with a

sterile plate sealer. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by absorbance at 600 nm using the EL_800 universal microplate reader (Biotek Instrument inc, Highland Park, Vermont, USA) and confirmed by plating 5 µl samples from clear wells on nutrient agar medium. These EOs tested in this study were screened two times against each organism [14].

3. Statistical analysis

Statistical analysis was performed using SPSS 17, all the tests were conducted in triplicate, and the data obtained were analyzed by Analysis of Variance (ANOVA). The Statistical significance was determined at P<0.05.

4. Results

The yellowish oil of *K. odoratissima* was obtained by hydro-distillation in the yield of 0.40 to 0.48% (w/w). The chemical constituents were identified by GC/MS and are presented in the Table1. The essential oil of *K. odoratissima* consists of 45 components, which represent 96.75% of the total essential oils. The main constituents of the EO are α Caryophyllene (22.60%), α Humulene (20.00%), Tetracyclononan-3-one (CAS) triasteranone (16.04%), Cyclopropane (11.54%) and Trans-Geraniol (5.00%).

Table 1. EO compositions of *Kelussia odoratissima* by GC/MS Analysis

NO.	Constituent	RT*	Percent
1	1,6-Octadiene	15.36	0.90
2	Cyclopropane	17.4	11.54
3	Cyclohexanol, 2-methyl-acetate	29.78	1.02
4	Alpha Caryophyllene	34.87	22.60
5	Alpha Humulene	35.40	20.00
6	1,6-Octadien-3-ol, 3,7-dimethyl	36.18	2.87
7	Cyclobutene, 4,4-dimethyl	37.88	0.94
8	5,9-Tetradecadiyne	39.86	1.33
9	Alpha Farnesene	40.73	2.58
10	4-Methyl-4-phenyl-diepoxy cyclohexanone	40.86	0.85
11	Trans-Geraniol	41.92	5.00
12	3-isobenzofuranon	46.06	2.98
13	Tetracyclononan-3-one (CAS) Triasteranone	48.56	16.04

*Retention time (min)

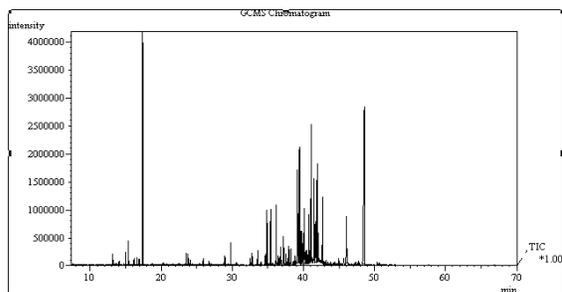


Figure 1. Chromatogram of *Kelussia odoratissima* EO

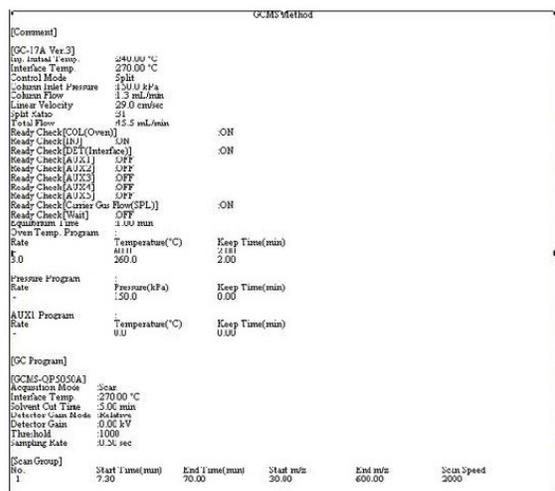


Figure 2. GC-MS methods

4.1. Antibacterial test. The growth inhibition value of essential oils on microbial strains is shown in table 2. The results showed significant differences ($P < 0.05$) was found in the susceptibility of food borne pathogenic bacteria to antimicrobial activity of this essential oil. Among the microorganisms tested, *S. aureus* and *L. monocytogenes* showed the highest susceptibility to the EO antimicrobial activity (Table 2). Among the probiotic bacteria tested, *L. ramosus* showed the susceptible and *L. casei* and *L. plantarum* were the resistant ($P < 0.05$) to the antibacterial activity of the *K. odoratissima* essential oil (Table 3).

Table 2. MIC and MBC value *K. odoratissima* EO (ppm) against food born pathogenic bacteria

Microorganisms	MIC (ppm)	MBC (ppm)
<i>S. aureus</i>	1250	5000
<i>L. monocytogenes</i>	5000	10000
<i>E. coli</i>	NE*	NE*
<i>S. typhimurium</i>	10000	NE*

*NE: Not effect

Table 3. MIC and MBC value *K. odoratissima* EO (ppm) against probiotic bacteria

Microorganisms	MIC (ppm)	MBC (ppm)
<i>L. acidophilus</i>	2500	10000
<i>L. casei</i>	5000	NE*
<i>L. ramosus</i>	1250	2500
<i>L. plantarum</i>	5000	NE*

NE*: Not effect

5. Discussion.

The fact that infectious dose of many foodborne pathogens is low, has motivated the researchers to conduct extensive investigations on finding new pharmaceutical compounds with high bactericidal potential, towards this goal, using oil compounds extracted from plants and spice is of great importance in providing healthy food products [15].

However, the effect of plant extracts and EOs on cell wall structure has been supported by Burt (2004) [16]. The hydrophobic activity of essential oils leads to their penetration into cell membrane lipids and increase of their permeability and this causes dysfunctions in all vital activities associated with cell membrane, removal of ions and vital compounds, and finally cell death [16]. Due to the fact that the *K. odoratissima* mainly found in Iran, so there are limited studies examining the pharmacological properties of this plant. For example, Chahrmahal va Bakhtiari, Kohkiluyeh va Boyerahmad recent publications on the antioxidant activity of methanolic extract of *K. odoratissima* [17], and the sedative property of EO and hydroalcoholic extract of *K. odoratissima* [9], which may be mediated by the bioactive phthalides contained in this plant. The results of recent study by Asadiyeh Shojaei et al. (2011) [18] showed that major compositions of EO of aerial parts of the three ecotypes (Koohrang, Bazoft and Samsami) of wild populations of *K. odoratissima* are z-Ligustilide and 3-e-butyl phthalide. The essential oil from the aerial part of *K. odoratissima* contains 23 kinds of different valuable components, of which the major compound is z-Ligustilide. However, ligustilide and butyl phthalide are the major compounds in *K. odoratissima* essential oil, that has been reported to have a positive impact on the nervous system, blood pressure, and cholesterol [19]. The essential oil yield and their components in medicinal and aromatic plants is related to genetic, climate, elevation and topography [20] and genotyp, growing conditions and their interaction.

Chemical polymorphisms or chemotypes have been reported for many medicinal plants [21]. Recent findings showed that some of the medicinal plant characteristics can be affected by genetic and ecological factors such as precipitation, temperature, plant competition and nitrogen content in the soil [22]. So, the differences in the quantity or quality of the oils composition of the present and previous studies [6, 8, 18, 19]. may be because of the chemotypes, phenological stage, drying conditions, mode of distillation and geographic and climatic factors. Further studies are needed to see if the changes of chemical composition in the studied oils of *K. odoratissima* are on the account of different environmental conditions of both localities or the chemotypes are *K. odoratissima* genetically fixed.

The presented results show that the antimicrobial activities of the *K. odoratissima* EO varied in relation to the test organisms. Gram-positive food born pathogenic bacteria (*S. aureus* and *L. monocytogenes*) were more sensitive than Gram-negative bacteria (*E. coli* O157:H7 and *S. typhimurium*) (Table 2). Shan et al., 2007 [23] reported that Gram-positive bacteria (*L. monocytogenes*, *S. aureus* and *B. cereus*) were generally more sensitive to the tested extracts than Gram-negative (*E. coli* and *S. anatum*). *S. aureus* was the most sensitive, while *E. coli* was the most resistant.

A possible explanation for these observations may lie in the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess an outer membrane and a unique periplasmic space not found in Gram-positive bacteria [24]. The resistance of Gram-negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules. It is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside [25]. Gram positive bacteria do not possess this type of outer membrane and cell wall structure. Antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic

membrane and result in a leakage of the cytoplasm and its coagulation [26].

Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects [27]. There has been no large scale, systematic investigation of the relationship between bacterial inhibition and the total phenolic content of spices and herbs. A highly positive linear relationship exists between antioxidant activity and total phenolic content in some spices and herbs [23].

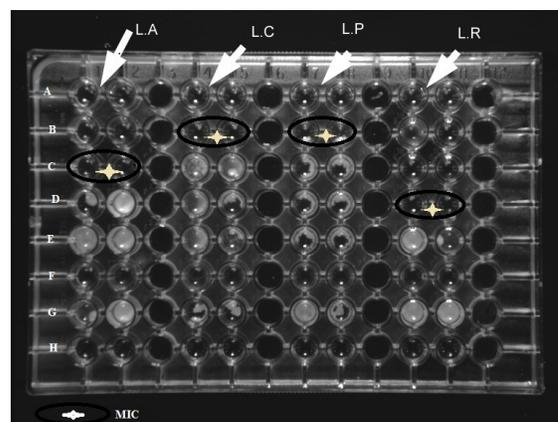


Figure 3. Effect of *Kelussia odoratissima* EO on growth probiotic bacteria by dilution assay (A-E: EO serial dilution: 10000, 5000, 2500, 1250, 625 ppm. F: culture without EO and probiotic bacteria, G: bacteria without EO and H: EO without bacteria)

Ghasemi et al. (2010) [2] studied the antimicrobial effects of some medicinal plants used in Iranian traditional medicine which were investigated against *E. coli* O157:H7, *Bacillus cereus*, *L. monocytogenes* and *Candida albicans* by agar disc diffusion and serial dilution assays. Most of the extracts showed a relatively high antimicrobial activity against all the tested bacteria and fungi. The MIC values for active extract and essential oil ranged between 0.039 and 10 mg/ml. They have reported that *C. albicans* was found to be the most sensitive bacteria to *K. odoratissima* essential oil, showing a MIC of 0.039 (mg/ml), while *B. cereus* and *L. monocytogenes* ranked next with 0.156 and 0.625 mg/ml followed by *E. coli* and with a MIC of 0.10 mg/ml.

In other study, antibacterial activity of ethanol extract and essential oil of 10 Iranian folklore herbs were investigated against of *S. aureus*, *Escherichia coli*, *P. aeruginosa* and *Klebsiella pneumoniae*. Most of the

extracts and essential oils showed relatively high antibacterial activity against all the tested bacteria. EOs of *H. lasiopetalum*, *K. odoretascima* and *A. kellalensis* showed promising antibacterial activities against *Escherichia coli*, *P. aeruginosa* and *Klebsiella pneumoniae* (The MIC values ranging between 0.039 and 10 mg/ml). The results obtained appeared to confirm the antibacterial potential of the plants investigated, In conclusion it can be said that the extract and EO of *K. odoretascima* could be used as natural antibacterial agents in the food preservation and human health [28]. Our findings also showed that the antimicrobial activity of *K. odoretascima* EOs were more effective against Gram-positive food born pathogenic bacteria (MIC and MBC value ranged 1250-10000 ppm).

EOs from plants and bacteriocins from probiotic bacteria (especially various species of *Lactobacillus*) have well-known antimicrobial effects which can substitute chemical preservatives to control and prevent the activity of foodborne pathogens. Besides, they exert positive effects on consumer's health [16]. Some research has shown the effect of plant-derived volatile oils on growth and viability of some lactic acid bacteria [29]. Kivanc et al. (1991) [30] also showed that EOs of *M. longifolia* and *Cuminum cyminum* in low concentrations lead to stimulation of growth and acid production; in high concentrations, they prevented *L. plantarum* growth. In a study conducted by Simsek et al. (2007), essential oils of *spearmint*, *thyme* and *garlic* had no inhibitory effects on growth and durability of lactic acid bacteria present in Ayran [31]. Different concentrations of herbal essential oils can influence the activity of starter bacteria in fermentative dairy products [10]. Mahmoudi et al. (2012) recently researched the Effects of *Mentha longifolia* L. EO on viability and cellular ultrastructure of *L. casei* during ripening of probiotic Feta cheese and them finding indicated that even the highest concentration this essential oil cased the highest viability of *L. casei* and the lowest pH value compared with other treatments ($P < 0.05$). Electron microscopy showed that EO was not harmful effect on *L. casei* cell membrane [10].

Among Gram-positive bacteria, lactic acid bacteria are often known as the most resistant species against antimicrobial agents of herbs [31].

While our finding (Table3) showed the *K. odoretascima* EO relatively high antibacterial activity against all tested probiotic bacteria, So that the maximum inhibitory effect was against *L. ramnus* (MIC: 1250 ppm; MBC: 2500 ppm).

6. Conclusion

These results indicated that the *K. odoretascima* seede EO could be considered as natural antimicrobial agents and so us as food preservatives and enhance the human health. Complementary investigations of individual compounds are necessary to assess the effectiveness of this EO in food system, perfumes and pharmaceuticals fields as natural antioxidant.

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Compliance with Ethics Requirements:

Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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