Antimicrobial effects of *Kelussia odoratissima* extracts against food borne and food spoilage bacteria "*in vitro*"

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ABSTRACT

The aim of this paper was to investigate the antibacterial potential of *Kelussia odoratissima* Mozff extract against Gram-negative and Gram-positive bacteria. Karafs-eKoohi with the scientific name of *Kelussia odoratissima* is an Iranian endemic edible plant in the middle region of Iran with enormous use as food, spice and medicinal herb. The antibacterial effect of the extracts was investigated using pour plate and disk diffusion methods. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also studied using the dilution method. Repeated measure ANOVA was used for data analysis. The results showed that in disk diffusion method all concentrations of ethanolic extract had inhibitory effect against *Bacillus subtilis* and *Staphylococcus aureus*. Minimum Inhibitory Concentration (MIC) of *Kelussia odoratissima* leaves of aqueous and ethanolic extracts for *Bacillus subtilis* and *Staphylococcus aureus* were 16 and 8 mg/ml, and for *Enterobacter aerogenes* were 32 and 16 mg/ml, respectively. Minimum Bactericidal Concentration (MBC) of *Kelussia odoratissima* leaves of aqueous and ethanolic extracts for *Bacillus subtilis* and *Staphylococcus aureus* were 32 and 16 mg/ml, and for *Enterobacter aerogenes* were 64 and 32mg/ml, respectively. The results showed that the extract of *Kelussia odoratissima* had a satisfactory antimicrobial activity and the ethanolic extract of *Kelussia odoratissima* leaves had greater inhibitory effects on the strains studied compared to aqueous extract in vitro. A significant correlation was also observed between zone of inhibition and concentration of extracts.

Keywords: *Kelussia odoratissima*; Aqueous and Ethanolic extracts; Antibacterial effects.

INTRODUCTION

Diseases caused by bacteria are widespread worldwide. The treatment of these infections is mainly based on the use of antibiotics. As antibiotics are increasingly used and misused, the bacterial strains become resistant to antibiotics rapidly. In recent years, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes [1]. In addition to this problem, antibiotics are sometimes associated with adverse effects including hypersensitivity, immune-suppression and allergic reactions [2]. Therefore, screening of antibacterial activity of alternative antibacterial drugs for the treatment of infectious diseases such as medicinal plants is very important since vast number of medicinal plants have been used for centuries as remedies for human diseases [3, 4].

Undoubtedly, medicinal plants are the prime source of drugs in both developing and developed nations, as drugs or herbal extracts for various chemotherapeutic purposes. Recently, researchers have estimated that there are about 400,000 species of plants worldwide, including about a quarter or a third have been used by companies for medicinal purposes [5]. For thousands of years, plant products and their modified derivatives have been rich sources for clinically useful drugs. Even today, most of the world's population relies predominantly on plants and plant extracts for health care [6]. Aromatic plants have been known about for a very long time and owing to their aromatic and antiseptic properties they are used as spices and natural food preservatives, in the perfume industry, for aromatherapy and for different medical purposes [7].

In Iran, a favorable climate and geography have contributed to a diversity of medicinal plants, and many endemic plant species exist. The Umbelliferae family contains approximately 275 genera and 2,850 species [8]. Among the aromatic plant species, the genus *Kelussia* occupies a special position. *Kelussia* is one of the newest genera of this family and is represented by only one species, *Kelussia odoratissima* Mozaff which is solely found in Iran [9].

Kelussia adoratissima with the local names "keloss*"* or "Karafs-ekoohi" , are in some resources reported with other scientific names such as "*Amirkabiria odoratissima*", "*Apium graveolens*" and "*Opopanax* sp" [9,10]. It is one of the most valuable plants which are used frequently in Iran and therefore at risk of extinction. Local communities which have long been used in various forms of *Kelussia odoratissima*, believe that this plant has analgesic effects, anti- inflammatory, sedative and anti-cough. New scientific findings also confirm that the flavonoid compounds as a main part of the plant has anti-inflammatory, anti-viral, anti-diabetic, anti-cancer and anti-toxin effects which is accumulated mainly within the seeds, stems and inflorescence of the plant [11,12,13]. *Kelussia odoratissima* is native to central Zagros Mountains especially in Chaharmahal and Bakhtiari province. Keloss natural habitats are seen on shallow to very deep soils with medium to heavy textures that have high water hold in capacity and lack salinity and alkalinity.

Due to growing medical plants and the presence of biologically active compound find in Keloss plant and above all, the presence of this plant in Chaharmahal and Bakhtiari, we decide to investigate the antimicrobial activity of *Kelussia odoratissima* extract against *Enterobacter aerogenes, Staphylococcus aureus* and *Bacillus subtilis.*

MATERIALS AND METHODS

Preparation Plant

The aerial parts of *Kelussia odoratissima* were collected from the central Zagros region of western Iran in March 2012 and Taxonomic identification was performed by the Faculty of Science Herbarium, Ferdowsi University of Mashhad, Iran. Then the plant was dried and pulverized into fine powder by mill laboratory (Waring model). Their aqueous and ethanolic extracts were prepared using the maceration technique. Fifty gram sample was extracted with 250 ml ethanol 96˚and sterile distilled water for 24 h. The extracts were filtered using paper filters (Whatman No.1) and then centrifuged in 3000g for 15 minutes. Then the extracts were concentrated to dryness at 80 C in the vacuum oven [1].

Bacterial strains

A total of 3 strains of frequently reported food borne pathogens and food spoilage bacteria, including *Enterobacter aerogenes* (ATTC 13048), *Staphylococcus aureus* (ATTC 25923) and *Bacillus subtilis* (PTCC 1720) were used in the study.

Preparation of microbial suspension

Twenty four hours before experiments, the culture was inoculated on Muller Hinton agar slopes storage medium. Concentrated bacterial suspensions using Ringer's solution of the bacterial growth on agar slopes were obtained. Then the turbidity of the suspension was measured by a spectrophotometer at a wavelength of 530 nm. Turbidity of solution was diluted to equality of the Ringer solution with 0.5 (1.5×10^8CFU /ml) McFarland standard solution [14, 15].

Antimicrobial activity testing

Antibacterial activity of bacterial strains was assayed using all plant extracts according to the method of Collins et al. (1995) and disc diffusion methods. In Collins et al. (1995) method 0.2 gram of aqueous and ethanol extracts, were added to 5 ml of sterile distilled water. Then it was stirred with vortex system in order to be steady. Then 1 ml of this solution was added to sterile plates. In the next step, Mueller Hinton agar (Merck-Germany) medium was steriled and added to the plates, and placed at room temperature. One loop of each standard strain culture media was cultured inoculums on these medium. The plates were incubated for 24 hours at 37°C. The culture with extract and without bacteria were used as control [2,16]. In the disk diffusion method 1.5×10^8 CFU /ml (equivalent to 0.5 McFarland standards) of standard culture of each strain was cultured on agar surface at the first step, then it was spread on the surface of agar by sterile glass spreader. After the

inoculated plates had dried sufficiently the discs were kept over the agar plates using sterile forceps at various concentrations (20, 40, 60 and 80 mg/ml). Antibacterial activity was observed as inhibition zone on Petri plates. Size of the inhibition zone was measured in millimeters using a metric ruler [3, 17].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum bactericidal concentration (MBC)

For each extract a set of 9 sterile test tubes was used, 8 test tubes for different dilutions of each extract (2, 4, 8, 16, 32, 64, 128, 256 mg /ml) and 1 test tube as a negative control were used. An aliquot of 1ml of the bacterial suspension was inoculated into each tube. The control tubes were inoculated with the same quantity of extracts. All tubes were incubated at 37 C for 24hrs. The lowest concentration that did not permit any visible growth when compared with the control was considered as the Minimum Inhibitory Concentration.

The contents of all tubes that showed no visible growth were cultured on Muller Hinton agar, incubated at 37C for 24hrs. The MIC was considered as the lowest concentration that could not produce a single bacterial colony and the MBC was defined as the lowest concentration of the extract at which 99.9% of the inoculated microorganisms were killed [18].

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance ($p \leq 0.05$) of the data obtained in all experiments. All results were determined to be within a 95% confidence level for reproducibility.

RESULTS

The results of the antimicrobial effects of extracts, by "method of Collins et al. (1995)" were show on in Tables 1 and 2.

The results of the antimicrobial effects of extracts, by "Disk diffusion method" were presented in Table 3.

Table 1: Antimicrobial effects of 2mg/ml *Kelussia odsoratissima* aqueous extract concentrations, on bacterial strains.

Microorganism	***Kelussia odsoratissima***
Enterobacter aerogenes	-
Staphylococcus aureus	+
Bacillus subtilis	+

Table 2: Antimicrobial effects of 2mg/ml *Kelussia odsoratissima* ethanolic extract concentrations, on bacterial strains.

Microorganism	***Kelussia odsoratissima***
Enterobacter aerogenes	-
Staphylococcus aureus	+
Bacillus subtilis	++

sign (+ +) indicates the failure of microbial growth on the medium and strong antimicrobial activity of ethanolic extract of *Kelussia odsoratissima.*
sign (+) indicates the failure of microbial growth on the medium and antimicrobial activity of aqueous and ethanolic extracts of *Kelussia odsoratissima.*
sign (-) indicates the absence of antimicrobial activity of aqueous and ethanolic extracts of *Kelussia odsoratissima*

Table 3: Average diameter (mm) of inhibition zone area of *Kelussia odsoratissima* extracts, on bacterial strains (Disk diffusion method).

		The concentration of *Kelussia odsoratissima* extracts (mg/ml)			
Type of extract	**Microorganism**	**20**	**40**	**60**	**80**
Ethanolic	***Enterobacter aerogenes***	-	11.80± 0.55	12.50± 0.53	14.10± 0.28
Ethanolic	***Staphylococcus aureus***	11.20±0.57	12.60±0.28	13.90 ±.55	15.200±0.55
Ethanolic	***Bacillus subtilis***	12.50±0.55	13.90 ±0.28	15.20± 0.57	17.50± 0.53
Aqueous	***Enterobacter aerogenes***	-	-	10.80±0.57	11.70±0.55
Aqueous	***Staphylococcus aureus***	-	8.80±0.28	10.90±0.55	11.50±0.57
Aqueous	***Bacillus subtilis***	-	9.90±0.53	11.30±0/28	12.90±0.52

- : No inhibition, n=3.

Table 4: Minimum inhibitory Concentration (MIC) and Minimum bactericidal Concentration (MBC) of *Kelussia odsoratissima* aqueous extract on bacterial strains.

Bacteria species	Concentration								
	2	4	8	16	32	64	128	256	Control
MIC									
Enterobacter aerogenes	-	-	-	-	+	+	+	+	-
Staphylococcus aureus	-	-	-	+	+	+	+	+	-
Bacillus subtilis	-	-	-	+	+	+	+	+	-
MBC									
Enterobacter aerogene	-	-	-	-	-	+	+	+	-
Staphylococcus aureus	-	-	-	-	+	+	+	+	-
Bacillus subtilis	-	-	-	-	+	+	+	+	-

+: Inhibition - : No inhibition, n=3.

Table 5: Minimum Inhibitory Concentration (MIC) and Minimum bactericidal Concentration (MBC) of *Kelussia odsoratissima* ethanolic extract on bacterial strains.

Bacteria species	Concentration								
	2	4	8	16	32	64	128	256	Control
MIC									
Enterobacter aerogenes	-	-	-	+	+	+	+	+	-
Staphylococcus aureus	-	-	+	+	+	+	+	+	-
Bacillus subtilis	-	-	+	+	+	+	+	+	-
MBC									
Enterobacter aerogenes	-	-	-	-	+	+	+	+	-
Staphylococcus aureus	-	-	-	+	+	+	+	+	-
Bacillus subtilis	-	-	-	+	+	+	+	+	-

+: Inhibition - : No inhibition, n=3.

DISCUSSION

Anecdotal evidence and the traditional use of plants as medicines provide the basis for indicating which essential oils and plant extracts may be useful for specific medical conditions. Historically, many plant oils and extracts have been used as topical antiseptics, or have been reported to have antimicrobial properties [19].

Since the main habitat of this plant is in the province of Chahar Mahal and Bakhtiari, especially the Zardkooh Mountains, the studying of antimicrobial effects of *Kelussia odsoratissima* population was a priority in the area. Therefore, this study explored the antibacterial effects of this plant.

The result of one-way ANOVA analysis at $p < 0.05$ showed that increasing the concentration of the *Kelussia odsoratissima* aqueous and ethanolic extracts results in a significant increase in the inhibition zone (Table 3). Sagdic et al (2005) studied the Antimicrobials effects of Black tea, Fennel, Sage, wild tea and wild mint. Their research also showed that increasing the concentration level for each plant extract tested had a significant ($p<0.05$) inhibitory effect on all bacterial strains [20]. We also observed that the minimal inhibition is related to the effect of aqueous extract on *Enterobacter aerogenes* and maximum inhibition is related to the effect of ethanolic extract against *Bacillus subtilis* (Table 3,4,5).

The findings of this study show that antimicrobial effect of *Kelussia odsoratissima* ethanolic extract on the studied microorganisms was better than *Kelussia odsoratissima* aqueous extract (Table 3). The traditional medicine practitioners use mainly water as the solvent, but we observed that plant extracts prepared in ethanol as solvents provided more consistent antimicrobial activity. These observations seems reasonable in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in different media used in the assay [21]. In attempting to study the antibacterial activity of 9 medicinal plants against

Urinary Tract Infection pathogens, Sharma et al. (2009) also observed that ethanolic extract of plant exhibited broad spectrum activity against isolates as compared to aqueous extract.

The present study showed that the *Kelussia odsoratissima* aqueous and ethanolic extracts had more effect on the Gram-positive bacteria than the Gram-negatives bacteria (Table 3,4,5). These results also supported by the study of Hoque et al (2008) who investigated antimicrobial activity of Cloves and Cinnamon extracts against food-borne pathogens and spoilage bacteria. They reported that aqueous and ethanolic extracts of Cloves and the ethanolic extract of cinnamon were more effective against Gram-positive bacteria than Gram-negative bacteria *in vitro* [22]. The variant effect of the extracts on Gram-positive and Gram-negative can be related to cell structure.

Hydrophilic surface of the outer membrane of Gram-negative bacteria is rich in lipopolysaccharide molecules which functions as a barrier to the penetration of antibiotic as well as the existence enzymes in Periplasmic space able to break molecules coming into this space. However, the anti-bacterial substances easily destroy Cytoplasmic membrane of Gram-positive bacteria for lack of outer membrane [23].

The results of recent studies by Asadiyeh Shojaei et al. (2011) and Rabbani *et al* (2011) showed that major compositions of essential oil of aerial parts of *K. odoratissima* are z-Ligustilide and 3-e-butyl phthalide [24, 25]. Salimi et al (2010) showed that the main compounds identified in the essential oil of *Kelussia odsoratissima* are Phthalides, particularly E-ligustilide, that constitute about 70% of *Kelussia odsoratissima* essential oil [26]. Z-ligustilide, the pure isolated phthalide has shown antifungal effects [27].

CONCLUSION

In conclusion, it can suggest that *Kelussia odsoratissima* extract in "*in vitro*" has considerable antimicrobial ability over the studied strains. In addition, to identify the effective dose of the extract on the microorganisms, and finally introduce the extract as a natural and novel antimicrobial compounds, more studies are needed in "*in situ*"
be done.

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