# Evaluation of Antibacterial Properties of Edible Oils and Extracts of A Native Plant, *Ziziphora Clinopodioides (Mountains' Kakoty)*, on Bacteria Isolated From Urinary Tract Infections

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**Abstract:** Essential oils, plant extracts and their constituents have known anti-bacterial effects. Wild Kakoty is a genus of Ziziphora and breed of mint. Despite the heavy use of plants in the mint family flavors in Iran, systematic research have been performed on antibacterial effects of the mountains' Kakoty's extract on pathogenic bacteria. This study aimed to investigate the antibacterial activity of essential oil and methanol extract of mountains' Kakoty on some pathogenic bacteria in laboratory culture and determination of its minimum inhibitory concentrations and its ability to kill bacteria. The results showed that the minimum inhibition concentration for mountains' Kakoty essence were 250 microgram/ml for most of the gram-negative bacteria unless for Pseudomonas aeruginosa. Also the gram-negative bacteria; Klebsiella pneumoniae had more sensitivity to this essence comparing to the other species of gram-negative bacteria and the minimum inhibitory concentration of gram-positive bacteria such as Staphylococcus aureus was 250 microgram/ml even though, for other species of Staphylococcus (coagulase negative) it was 500 micrograms per milliliter. The results of the MIC determination of methanol extract of mountains' Kakoty showed that the essence has inhibitory and germicidal effect on all the under test bacteria except Pseudomonas aeruginosa. These observations indicate that the minimum germicidal concentration of the methanol extract of mountains' Kakoty was 2000 microgram/ml for most of the gram-negative bacteria and less than this amount for the gram-positive ones. The results of this study showed that, with comparison of the inhibitory effect and germicidal effects of the essence and extract of mountains' Kakoty; we can conclude that the essence of this plant compared to its extract and in its low concentrations is able to inhibit the growth of under study bacteria.
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**Key words:** antibacterial effect, Ziziphora clinopodioides, Kakoty, essence, extract, UTI

## 1. Introduction

Essential oils, plant extracts and their constituents have known anti-bacterial effects. Wild Kakoty is a genus of Ziziphora and breed of mint. Among the important species of this breed mint, thyme and dried marjoram, lavender, marjoram and Kakoty can be named (Zargari, 1995). Kakoty is an herbaceous plant; with one year old lifetime, with short stems, 5-15 cm tall and thin, sharp leaves that are scattered in many parts of Iran (Zargari, 1995). This plant groves in wild state and in vast areas of Iran like mountainous regions of Alborz, west Iran, Karaj, Pole Jajrud, southwest of Tehran, Dushan tape, Isfahan, khorasan, Damghan, Semnan, Azna, Qom, Hamedan, Baluchestan and northern mountainous regions like Manjil, Azarbijan provinces, especially in mountains of Tabriz (Zargari, 1995; Baser et al., 1991). Four species of plant called Ziziphora clinopodioides (mountains' Kakoty) Ziziphora capitata, Ziziphora persica and Ziziphora tenuir have been identified in Iran (Zargari, 1995). Among the healing properties of this plant sputum collection, carminative and stomach reinforcement can be named. In some areas the dust of its grains mixed with honey is used to treat dysentery (Zargari, 1995). In different areas, the plant's powder is used as a garnish on yogurt and dairy products (Sajadi et al., 2003). Also, it is used for treatment of diseases of the stomach and as an antiseptic to relieve colds (Babakhanloo et al., 1998). Despite the heavy use of plants in the mint family flavors in Iran, systematic research have been performed on antibacterial effects of the mountains' Kakoty's extract on pathogenic bacteria. This study aimed to investigate the antibacterial activity of essential oil and methanol extract of mountains' Kakoty on some pathogenic bacteria in laboratory culture and determination of its minimum inhibitory concentrations and its ability to kill bacteria.

## 2. Materials and Methods

Collecting plants used in the study: wild plants of Kakoty been collected in the hills around the city of Shabestar in East Azerbaijan province when flowered in April and May and the species of herbarium group were determined in aromatic and medical plants' department in Islamic Azad University of Shabestar. After collecting the plants, the leaves were dried in appropriate circumstances and in shadow and were crushed by mill in order to prepare extracts and essence.

**2.1. Preparation of plant extracts and essential oils**

A specialized laboratory in faculty of agriculture in Islamic Azad University of Shabestar used maturation method to prepare extractions of medical plants. For this purpose 50 gram of each sample was soaked in 80% methanol and 48 hours later it was smoothed by filter paper. Extracts obtained using rotary machine at 40 to 50°C, concentrated and dried at the same temperature for 2 days and was gradually dried (Manna and Abalaka, 2000; Shariff, 2000). For production of oil, water distillation method using Clevenger apparatus was applied.

**2.2. Reviewed bacteria**

The examined bacteria in this study included 9 strains of bacteria which were collected from UTI referees to clinics of Tabriz in 2011. In the following table, bacterial strains were isolated from urine sample of patients with UTI are given:

| | **Name of isolated bacteria** |
|---|---|
| 1 | *Escherichia coli* |
| 2 | *Staphylococcus epidermidis (coagulase-negative)* |
| 3 | *Citrobacter frundii* |
| 4 | *Klebsiella pneumonia* |
| 5 | *Staphylococcus saprophyticus (coagulas-negative)* |
| 6 | *Staphylococcus aureus (coagulase-positive)* |
| 7 | *Enterobacter aerogenes* |
| 8 | *Pseudomonas aeruginosa* |
| 9 | *Proteus vulgaris* |

**2.3. Preparation of microbial suspension**

For preparation of such suspension; a 24 hours culture of each bacterium is needed. Hence, 24 hours before the test; the stored cultures were inoculated into nutrient agar medium and incubated for 24 h at 37°C. The colonization of the medium was washed with normal saline solution and bacterial suspensions were diluted with normal saline and their turbidity was set equivalent to turbidity of standard tube 0.5 McFarland. The test suspension contained $1.5\times10^8$ CFU/ml (Babayi et al., 2004; Naderinasab et al., 1997).

**2.4. Antibacterial effects of extracts and essential oil of mountains' Kakoty**

**2.4.1. Diffusion method or Disk Diffusion**

To evaluate the antibacterial effects of essential oils; disk diffusion in agar method was used. It should be explained that the disks containing extracts are prepared from sterile blank discs manufactured in Padtan Teb Company. Thus the blank disks were placed in tubes containing diluted oil extracts and after 30 to 50 minutes and following the complete absorption by disk, the disks were placed at 37°C until to completely dry and get ready for disk (Inouye et al., 2001; Vander and vlietinck, 1991). Then all the under test bacterial strains of suspension for which the microbe of 0.5 McFarland with 100 ml of suspension were prepared, were cultured separately on the surface of Muller Hinton agar medium. Then using sterile forceps discs impregnated with extraction of essential oils of mountains' Kakoty were placed with certain distance from each other and from the edge of the plate in the medium and were fixed with little pressure on the environment. Then the plates were incubated for 24 h at 37 ° C and the results of antibacterial activity was recorded by measuring the diameter of inhibition zone around the discs. To make sure, the experiment was repeated three times for each strain. The mean inhibition zone diameter in the final three repeats was registered as the final diameter (Rezai and Rasooli, 2000; Quinn et al., 1994). Also, the standard antibiotic ampicillin (10 micrograms per disc) was used as positive controls.

**2.4.2. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

Using a dilution method, the minimum inhibitory concentration (**MIC)** and minimum bactericidal concentration (MBC) of each extract was determined in separate. To determine the MIC, for each extract; a series of 10 test tubes were used. 8 tubes were used for testing the different dilutions of each extract, one tube for positive control and one for negative control. Each extract was tested with different dilutions from tube number one containing dilution of 8 mg/ml up to tube 8 with concentration of 5.62 mcg/ml of extract's oil in BHI medium broth with 1 ml antibiotic suspension which contains $1.5\times10^8$ CFU/ml bacterium. Simultaneously a tube containing 9 ml medium plus 1 ml of extract are for control extract and a tube containing 9 ml medium as well as 1 ml of bacteria's suspension were prepared as control bacteria. All test tubes were placed at 37°C for 24 hours. After the incubation period the tubes' inoculated turbidity were studied due to bacterial growth. This method was repeated 3 times for each extract and every type of bacteria. The extract

dilution containing lowest concentration and maximum inhibition turbidity due to bacterial growth was considered as the MIC of that extract. Also, a sample was taken from all the tubes in which no bacterial growth was observed and in order to determine the minimum lethal concentration of the extracts, they were cultured by pure plate method. For this purpose, 1 ml of each tube were mixed with 20 ml of BHI agar in 45° C and in Petri dishes and after closing the agar and incubation for 24 hours, the incubated plates were controlled in the presence of microbial growth. Dilution plates containing the lowest concentration of the extract and no colonies if bacteria was found; were selected as MBC of that extract (Vanden and Vlietinck, 1991; Sindambiwe et al., 1999).

**2.5. Statistical Analysis**

The SPSS software was used for statistical computing and the Tukey test was for the comparison of samples. Also, in order to determine which samples have significant mean differences, ANOVA with equal frequency was used. It is crucial that the statistical methods used in the comparison between MBC and MIC of plant extracts and essential oils was descriptive statistics.

**3. Results**

**A)** The results of the bacterial effects of essential oils and Kakoty extracts in disc diffusion method are given in table (1):

**Table 1**: The average diameter of inhibition halos of bacteria are in mm

| Name of isolated bacteria | Essence (10µl/disk) | Extract (10µl/disk) | Ampicillin (10µl/disk) |
|---|---|---|---|
| *Pseudomonas aeruginosa* | - | - | - |
| *Escherichia coli* | 21 | 20 | 13 |
| *Citrobacter frundii* | 20 | 19 | 4 |
| *Enterobacter aerogenes* | 19 | 17.5 | 11 |
| *Staphylococcus aureus (coagulase-positive)* | 22 | 19 | 13 |
| *Klebsiella pneumonia* | 29 | 26 | 19 |
| *Staphylococcus epidermidis (coagulase-negative)* | 20 | 19.5 | 14 |
| *Proteus vulgaris* | 19 | 17 | 9 |
| *Staphylococcus saprophyticus (coagulase-negative)* | 21 | 19 | 14 |

**Note**: (-) Indicator of the growth inhibition zone

The results show that the essences and extract of mountains' Kakoty has no effect on Pseudomonas aeroginosa under the effect of tested concentration. In 5% level of confidence; there is no significant difference between antibacterial effect of essence and the plant's extract on E.coli, Citrobacter frundii, Enterbacter aerogenes but there is a significant difference between other bacteria. Also the most antibacterial effect was on Klebsiella pneumoniae. Compared with positive control (Ampicilin); in most of the cases except Pseudomonas aeruginosa, the essence and extract show higher antibacterial activity and this fact is more evident in the case of essence.

**B)** The growth inhibitory effect of essential oils and plant extracts of Kakoty are shown in tables bellow:

**Table 2**: shows the results of minimum inhibitory concentration (MIC) of different concentrations of mountains' Kakoty's essential oil.

| | Essence concentration (µg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Bacterial species** | **62.5** | **125** | **250** | **500** | **1000** | **2000** | **4000** | **8000** |
| Enterobacter aerogenes | + | + | - | - | - | - | - | - |
| Pseudomonas aeruginosa | + | + | + | + | + | + | + | + |
| Escherichia coli | + | + | - | - | - | - | - | - |
| Staphylococcus epidermidis (coagulase-negative) | + | + | + | - | - | - | - | - |
| Staphylococcus saprophyticus (coagulase-negative) | + | + | + | - | - | - | - | - |
| Staphylococcus aureus (coagulase-positive) | + | + | - | - | - | - | - | - |
| Citrobacter frundii | + | + | - | - | - | - | - | - |
| Klebsiella pneumonia | - | - | - | - | - | - | - | + |
| Proteus vulgaris | - | - | - | - | - | - | + | + |

**Note**: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence growth of bacteria.

**Table 3:** shows the results of minimum inhibitory concentration (MIC) among different concentrations of the methanol extract of mountains' Kakoty

| | **Extract concentration (μg/ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Bacterial species** | **62.5** | **125** | **250** | **500** | **1000** | **2000** | **4000** | **8000** |
| Enterobacter aerogenes | + | + | + | + | + | - | - | - |
| Pseudomonas aeruginosa | + | + | + | + | + | + | + | + |
| Escherichia coli | + | + | + | + | + | - | - | - |
| Staphylococcus epidermidis (coagulase-negative) | + | + | + | + | + | - | - | - |
| Staphylococcus saprophyticus (coagulase-negative) | + | + | + | + | - | - | - | - |
| Staphylococcus aureus (coagulase-positive) | + | + | + | - | - | - | - | - |
| Citrobacter frundii | + | + | + | + | - | - | - | - |
| Klebsiella pneumonia | + | + | - | - | - | - | - | - |
| Proteus vulgaris | + | + | + | - | - | - | - | - |

**Note**: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence of bacteria.

**C)** Bacteria killing power of essential oils and plant extracts of Kakoty are given in tables (4, 5):

**Table 4:** shows the results of the minimum bactericidal concentration (MBC) of different concentrations of essential oil of mountains' Kakoty.

| | **Essence concentration (μg/ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Bacterial species** | **62.5** | **125** | **250** | **500** | **1000** | **2000** | **4000** | **8000** |
| Enterobacter aerogenes | + | + | - | - | - | - | - | - |
| Pseudomonas aeruginosa | + | + | + | + | + | + | + | + |
| Escherichia coli | + | + | - | - | - | - | - | - |
| Staphylococcus epidermidis (coagulase-negative) | + | + | + | + | + | + | - | - |
| Staphylococcus saprophyticus (coagulase-negative) | + | + | + | - | - | - | - | - |
| Staphylococcus aureus (coagulase-positive) | + | + | + | - | - | - | - | - |
| Citrobacter frundii | + | + | - | - | - | - | - | - |
| Klebsiella pneumonia | + | + | - | - | - | - | - | - |
| Proteus vulgaris | + | + | - | - | - | - | - | - |

**Note**: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence of bacteria.

**Table 5**: shows the results of the minimum bactericidal concentration (MBC) of different concentrations of methanol extract of mountains' Kakoty.

| | **Extract concentration (μg/ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Bacterial species** | **62.5** | **125** | **250** | **500** | **1000** | **2000** | **4000** | **8000** |
| Entrobacter aerogenes | + | + | + | + | + | - | - | - |
| Pseudomonas aeruginosa | + | + | + | + | + | + | + | + |
| Escherichia coli | + | + | + | + | + | - | - | - |
| Staphylococcus epidermidis (coagulase-negative) | + | + | + | + | - | - | - | - |
| Staphylococcus saprophyticus (coagulase-negative) | + | + | + | + | - | - | - | - |
| Staphylococcus aureus (coagulase-positive) | + | + | + | + | + | - | - | - |
| Citrobacter frundii | + | + | + | + | + | - | - | - |
| Klebsiella pneumonia | + | + | + | + | - | - | - | - |
| Proteus vulgaris | + | + | + | + | + | - | - | - |

**Note**: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence of bacteria.

The results showed that the minimum inhibition concentration for mountains' Kakoty essence were 250 microgram/ml for most of the gram-negative bacteria unless for Pseudomonas aeruginosa (table2). Also the minimum concentration of germ-killing of this essence for above mentioned bacteria was equal to the minimum inhibition concentration of them (Baser et al., 1991; Babakhanloo et al., 1998). But the essence was not affecting Pseudomonas aeruginosa. In another hand,

the minimum inhibitory concentration of gram-positive bacteria such as Staphylococcus aureus was 250 microgram/ml even though, for other species of Staphylococcus (coagulase negative) it was 500 micrograms per milliliter (table 2).

Overall, these results indicated that among the gram positive bacteria, Staphylococcus aureus respectively have the most and coagulase-negative species of this bacterium least sensitivity against the used concentrations of mountains' Kakoty essence in our study. Also among the 7 gram-negative bacteria; Klebsiella pneumoniae had more sensitivity to this essence comparing to the other species of gram-negative bacteria.

The results of the MIC determination of methanol extract of mountains' Kakoty showed that the essence has inhibitory and germicidal effect on all the under test bacteria except Pseudomonas aeruginosa (Table 3).

The minimum bactericidal concentration of this essence for most isolates were equivalent with its minimum inhibitory concentration, and had only inhibitory effect on some of the bacteria in the range of under test concentration (Tables 2,4).

These observations indicate that the minimum germicidal concentration of the methanol extract of mountains' Kakoty was 2000 microgram/ml for most of the gram-negative bacteria and less than this amount for the gram-positive ones. Also, the minimum inhibitory concentration of the extract for both gram-negative and gram-positive bacteria was often germicidal in minimum concentrations (Tables 3,5).

The results of this study showed that, in general, with comparison of the inhibitory effect and germicidal effects of the essence and extract of mountains' Kakoty; we can conclude that the essence of this plant compared to its extract and in its low concentrations is able to inhibit the growth of under study bacteria.

**4. Discussion**

Though thousands of years, the inhibitory effect of spices, herbal extracts and essential oils are known; but in recent years the effect of aromatic extracts, essential oils and herbal ingredients of these oils on Pathogenic bacteria and microorganisms causing food spoilage is of great interest (Ali et al., 1999; Valero and Giner, 2006). The effect of these substances on important food-borne isolates like E. coli, (Schaechter et al., 1989), Salmonella enteritidis (Nazer et al., 2005; Sokmen et al., 2004; Tassou et al., 2000), Bacillus cereus (Valero and Giner, 2006) and (Delgado et al., 2004), Staphylococcus aureus (Sokmen et al., 2004; Tassou et al., 2000), Listeria monocytogenes (Smith et al., 2001; Quinn et al., 1994) represents the efforts of researchers to replace the natural preservatives derived from plant, animal, and microbial sources instead of chemical preservatives. Analysis of essential oils from different plants showed the presence of different combinations. The original composition of the essential oils of mint family's plants is Thymol and carvacrol. The strong anti-microbial effect of carvacrol has been expressed by the researcher (Chami et al., 2004; Aghajani et al., 2008). Ozturk and Ercisli, 2006 review showed that the essence of mountains' Kakoty is formed of 31.86 percent Poligon, 12.21 percent Senion, 10.48% Limonen, 9.13% Menthol, 6.88% beta-pinene, 6.73% Menton, 3.5% Peperitnon, 4.18% Peperiton. The main component of the essential oils of some the mint family's plants including Kakoty, were Poligon. Poligon has antibacterial and antifungal properties and is particularly effective for the different isolates of Salmonella (Amiri, 2009). According to this study, Kakoty's essence showed more antibacterial impact compared to the methanol extract of it and probably this antibacterial activity is more associated with Poligon which is an essential component of mountains' Kakoty's essence. Results of Salehi, et al. study (2005) which was conducted on the antimicrobial effect of Kakoty's extract showed that mountains' Kakoty's extract can inhibit the growth of gram-negative bacteria Klebsiella pneumoniae and Escherichia coli. Besides lack of antibacterial activity observed against Pseudomonas aeruginosa in mountains' Kakoty's extract in studies of above people are consistent with results of the present study (Salehi et al., 2005). Results from Salehi, et al., 2005 study also suggests that the extract can inhibit the growth of Staphylococcus epidermidis and Bacillus subtilis. Studies of Ercili and Ozturk's 2006 and 2007, also showed that mountains' Kakoty extract and persica Kakoty are capable to prevent growing a wide range of gram-positive and gram-negative pathogenic bacteria. In this study, the essence of mountains' Kakoty has inhibitory and germicidal effect on most of the gram-negative bacteria but has no effect on Pseudomonas aeruginosa which are in agreement with results of Baser et al., 1991 in which the experiment was done on the Kakoty which is the native plant of Turkey. Their results also showed that the essence of Kakoty can prevent the growth of gram-negative bacteria Escherichia coli and Enterobacter Aorogenous and had no effect on P. aeruginosa. Also, the above results are the same as results of Salehi et al., (2005) on antibacterial effect of mountains' Kakoty essence. Their study showed that essential oils of mountains' Kakoty can prevent the growth of gram-negative bacteria Klebsiella pneumoniae and Escherichia coli and the lack of antibacterial activity of mountains'Kakoty essence

against Pseudomonas aeruginosa has been observed. The results of this study showed that the mountains' Kakoty essence has good anti-bacterial effect on under test gram-negative bacteria. Based on Baser et al., 1991 the anti-bacterial effect of Kakoty essence native for Turkey has been observed on gram-positive bacteria, Staphylococcus aureus and Bacillus subtilis. The results of Salehi, et al.'s research shows that mountains' Kakoty essence can prevent the growth of gram-positive bacteria, Bacillus subtilis and Staphylococcus aureus.

Most studies suggest that the susceptibility of gram-negative bacteria against antibacterial compounds are less than gram-positive ones which may be due to the presence of outer membrane in the structure of their cell walls. Gram-positive bacteria have a large amount of mucopeptide compositions in their cell wall while gram-negative bacteria have only a thin layer of mucopeptide and much of their cell wall's structure are made of lipoprotein and lipopolysaccharide (LPS) and it seems that for this reason they are more resistant to anti-bacterial subastances and these results are consistant with the results obtained in this study (Schaechter et al., 1989).

**5. Conclusion**

In this study it was found that the essential oil and methanol extracts of mountains' Kakoty have anti-bacterial effects on under test bacteria except Pseudomonas aeruginosa; therefore, it seems that the above mentioned compounds can be used as antibacterial agent against a broad spectrum of bacteria causing urinary tract infections.

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