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**Original article**

# Antibacterial Activity of Dill (*Anethum graveolens*) Essential Oil and Antibiofilm Activity of Cumin (*Cuminum cyminum*) Alcoholic Extract

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**Abstract**

**Background:** Emergence of drug-resistant bacteria has highlighted the need to identify new and more efficient antibacterial agents. The aims of this study were to evaluate the antibacterial activity of dill (*Anethum graveolens*) seeds essential oil and to investigate the effect of cumin (*Cuminum cyminum*) seeds alcoholic extract on biofilm formation ability of *Klebsiella pneumoniae*.
**Materials and methods:** This experimental study was carried out at the Faculty of Medicine of Kurdistan University of Medical Sciences in 2014. Activity of dill seeds essential oil was evaluated based on the inhibition zone diameter and minimum inhibitory concentration (MIC) against some important pathogenic bacteria including: *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Furthermore, the effect of sub-inhibitory concentrations of cumin seeds alcoholic extract was evaluated on biofilm formation ability of *K. pneumoniae*. The biofilms were formed on semi-glass lamellas and observed by a scanning electron microscope.
**Results:** Dill essential oil showed a good to moderate activity against the tested strains. The highest antibacterial activity was observed against *S. aureus* (inhibition zone of 15 mm and MIC of 0.62 mg.mL$^{-1}$) and *V. cholerae* (inhibition zone of 14 mm and MIC of 0.7 mg..mL$^{-1}$). The cumin alcoholic extract had no effect on biofilm formation ability of *K. pneumoniae*.
**Conclusion:** The results of this study showed the presence of antimicrobial compounds in dill extract. The cumin alcoholic extract was not able to inhibit biofilm formation ability of *K. pneumoniae*. Because of the medicinal plants properties, it is valuable to search for promising herbs and novel chemical compounds.

*Keywords:* Antibacterial activity, Medicinal plants, Dill, Cumin, Biofilm

## 1. Background

Uncontrolled use of antimicrobial chemicals plays an important role in the emergence of antimicrobial resistant strains. Multi-drug resistant pathogenic strains have been reported worldwide (1-3). These problems have caused the pharmaceutical industries to gradually remove anti-bacterial chemicals and to search for natural alternatives. In recent years, extensive research has been conducted to discover new antimicrobial drugs from natural sources such as plants. Plants synthesize many compounds with complex molecular structures, containing antimicrobial properties (4). Antimicrobial activity of several plants extracts and their derivatives has been reported by many researchers (5-9). Dill (*Anethum graveolens*) is an annual plant belonged to the family Apiaceae. Its leaves and seeds have been used as a spice since ancient times. Dill seeds are used to flavor cakes and sweets, soups, salads, potatoes, meat, and pickles. Also, its seeds have some medicinal properties including antispasmodic, carminative, and stomachic properties (10). Essential oils are rich sources of active compounds; thus, it is possible to use them as antimicrobial food additives (6). Cumin (*Cuminum cyminum*) is an aromatic plant belonged to the family Apiaceae. Its fruits called as seeds are the crescent-shape and yellow to brown with 4-5 mm length. Its seeds have a distinctive strong and warm bitter flavor (11). Cumin plant has numerous medicinal properties. It is used in the treatment of bronchopulmonary disorders such as cough and disorders in digestive tract. It is also carminative, eupeptic, anticonvulsant, appetite stimulant, and analgesic (12). Our earlier research revealed that ethanolic extract of cumin seeds is able to reduce the thickness of polysaccharide capsule in *K. pneumoniae* (9). In addition to protection against phagocytosis and serum bactericidal effect, the capsule as an important virulence factor in *K. pneumoniae* is involved in biofilm formation (13). Biofilms are the complex aggregation of microorganisms, in which cells become embedded within a sticky extracellular matrix composed of exopolysaccharides (14). It is estimated that 65% of all infections are caused by biofilms (15). It has also been found that bacteria growing in biofilms are much more resistant to antibiotics than bacteria growing planktonically in vitro. Within a host, biofilm formation is thought to enhance antibiotics resistance and host defense mechanisms (13). Considering the increase in the emergence of antibiotic resistant bacteria, the use of compounds with inhibitory effect on biofilm formation could be an attractive strategy to reduce bacterial pathogenicity.

## 2. Objectives

The aims of the present study were to evaluate the antibacterial activity of dill seeds essential oil and to investigate the effect of the cumin alcoholic extract on biofilm formation in *K. pneumoniae*.

## 3. Materials and methods

### *3.1. Plant samples and extraction*

This experimental study was carried out at the Faculty of Medicine of Kurdistan University of Medical Sciences in 2014. Dill and cumin seeds were collected from the Agricultural Research Center of Tehran, Iran. Dill seeds essential oil was prepared using the hydrodistillation method by Clevenger apparatus. Briefly, 50 grams of powdered dill seeds were placed in the Clevenger system with 1 liter of distilled water, and essential oil extraction was performed for





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3 h. The essential oil was then removed and stored at 4 °C until used for antibacterial testing (16).

To prepare an alcoholic extract of cumin seeds, 50 grams of powdered seeds were added to a flask containing 250 mL of 95% ethanol and mixed for 72 h at room temperature on a rotary shaker machine (100-120 rpm). The obtained extract was centrifuged in the 3000 rpm for 10 min and filtered. The extract was then concentrated by evaporation using a rotary evaporation equipment at 60 °C (17). After evaporation of solvent, the concentrated extract was stored at 4 °C until used.

*3.2. Test strains*

Bacterial strains of *E. coli* ATCC25922, *S. aureus* ATCC25923, mucoid cystic fibrosis isolate of *P. aeruginosa* 8821M, clinical *V. cholerae* (from Mashhad University of Medical Sciences, Iran), clinical *K. pneumoniae* (from the Baqiyatallah Hospital, Iran), and *K. pneumoniae* ATCC13883 were examined. Strains were cultured on Nutrient agar (Hispanlab, Spain) and incubated at 37 °C for 18 h.

*3.3. Determination of antibacterial activity of dill essential oil*

Antibacterial activity of dill essential oil was determined against the four strains of *E. coli* ATCC 25922, *S. aureus* ATCC25923, *P. aeruginosa* 8821M, and clinical *V. cholerae* by agar well diffusion (18) and micro broth- dilution (19) methods. At first, the essential oil was dissolved in 95% ethanol -Mueller Hinton Broth (Merck, Germany). The solution obtained was then added to Mueller Hinton Broth and serially diluted two-fold. The final concentration of ethanol was less than 1%, and thus did not affect the bacterial growth (20).

In the agar well diffusion method, two-fold serial dilutions of essential oil (1/2, 1/4, 1/8) were used to evaluate the inhibitory zones. Of each strain suspension [0.5 McFarland, $10^8$colony-forming units (CFU).$mL^{-1}$], a volume of 0.1 mL was spread on the surface of Mueller-Hinton agar (Merck, Germany). Wells with a diameter of 6 mm were then made on plates, and 40 µL of the undiluted oil and various dilutions of oil (1/2, 1/4, 1/8) were added to the wells. Control well contained 40 µL of 95% ethanol. The plates were incubated at 37 °C for 18 h. The halos around each well were then observed, and their diameter was measured.

To determine the minimum inhibitory concentration (MIC) of the dill essential oil, the micro broth dilution method was used (19). A volume of 100 µL of Mueller Hinton Broth was added to each well of the microtiter plates. In the first well, 100 µL of diluted oil with a concentration of 1/64 was added. Then, 100 µL of the first well content was removed and added to the second well, and serial two-fold dilutions were made. About 100 µL of each bacterial suspension ($10^6$ CFU.$mL^{-1}$) was added to the wells. The well containing the inoculated medium was served as positive growth control. Negative control well contained 95% ethanol. The microtiter plate was then incubated for 22 h at 37 °C. The MIC was defined as the lowest concentration needed for inhibition of bacterial growth after 22 h of incubation. To determine the MBC (the minimum bactericidal concentration), each well showing growth inhibition after 22 h, was sub-cultured onto an agar plate. Then 10 µL of the well content were cultured on the Nutrient agar medium (Oxoid,England), and plates were incubated for 22 h to determine the bacterial growth. The minimal concentration of essential oil needed to kill equal to or more than 99.9 % (≥3 $\log_{10}$ drop in CFU.$mL^{-1}$) of the organisms was considered as the MBC. All antimicrobial experiments were repeated three times.

*3.4. Determination of cumin alcoholic extract effect on K. pneumoniae biofilm formation*

Biofilm assay was done based on O' Toole and Kolter method with minor modifications (21). Semi-glass lamellas were cut to equal diameters and sterilized by autoclaving. To create a biofilm on the surface of the lamellas, the method for determining MIC was performed, and the lamellas were placed inside the tubes. A broth dilution technique was used to determine the susceptibility of *K. pneumoniae* strain ATCC13883 and clinical isolates (from the Baqiyatallah Hospital, Iran) to cumin alcoholic extract. The broths containing serially two-fold diluted amounts of cumin alcoholic extract (the extract was diluted in Tween 80) were inoculated with $10^6$ CFU of bacterial cells. The tube containing only growth medium was considered as the negative control. The untreated *K. pneumoniae* cells were used as positive control. The test tubes were incubated for 48 h at 37 °C. Bound cells to the lamellas were then stained by a 1% aqueous solution of crystal violet for 2 min. After washing with distilled water, the bound dye was released using 95% ethanol, and the absorbance at 595 nm was read by a spectrophotometer.

To further investigate the ability and microscopic images of biofilm formation, scanning electron microscopy (SEM) was used. Specimens were dried and mounted on aluminum stubs. The specimens were coated with a layer of gold by sputter coating and examined with a scanning electron microscope XL 30 (Philips, The Netherlands), operated at an acceleration voltage of 20 KV and ZAF software. All tests were repeated three times.

## 4. Results

*4.1. Antibacterial activity of dill essential oil*

The highest antibacterial activity was observed against *S. aureus* (with 15 mm inhibitory zone of undiluted oil) and *V. cholerae* (with 14 mm inhibitory zone of undiluted oil). Sensitivity of *E. coli* and *P. aeruginosa* strains to oil was similar (both with 12 mm inhibitory zones of undiluted oil). Control well (95% ethanol) did not show any inhibitory effect.

Using MIC method as a useful complementary method for assessing the antibacterial activity of essential oil, it was found that *S. aureus* was the most susceptible strain (with a MIC of 0.62 mg.$mL^{-1}$), followed by *V. cholerae*, *E. coli*, and *P. aeruginosa*. The MIC and MBC results were the same. Control treatment had no inhibitory effect. The results of MIC and inhibitory zones for dill seed oil against the four tested strains are shown in Table1.

**Table 1. Antibacterial activity of dill seed oil based on agar well diffusion and micro broth- dilution methods.**

| Tested Strains | Zones of Inhibition (mm) Formed by Dill Seed Essential Oil | | | | MIC (mg.$mL^{-1}$)[a] |
|---|---|---|---|---|---|
| | The undiluted oil | 1/2 Dilution | 1/4 Dilution | 1/8 Dilution | |
| *S. aureus* ATCC25923 | 15 | 13 | 11 | 9 | 0.62 |
| Clinical *V. cholerae* | 14 | 12 | 10 | 9 | 0.7 |
| *E. coli* ATCC 25922 | 12 | 10 | 9 | NC[b] | 1.25 |
| *P. aeruginosa* 8821M | 12 | 10 | 8 | NC | 1.5 |

[a] MIC: Minimum inhibitory concentration [b] NC: Not clear





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*4.2. Effect of cumin alcoholic extract on biofilm formation ability of K. pneumoniae*

The ability of *K. pneumoniae* to form biofilm in different concentrations of cumin alcoholic extract is shown in Fig. 1. The decrease in the absorbance of dye dissolved in ethanol is associated with a decrease in the number of bacteria forming biofilm on lamellas. Strains exposed to sub- inhibitory concentrations of the extract did not show a significant decrease in the OD595 nm absorption compared to the control (Fig. 1). SEM images confirmed the results (Fig. 2). In biofilm formed by *K. pneumoniae* control cells (untreated), high density of cells was observed (Fig. 2a), and in the sub-inhibitory concentration of the extract, dense aggregates of cells were observed (Fig. 2 b and 2 c).

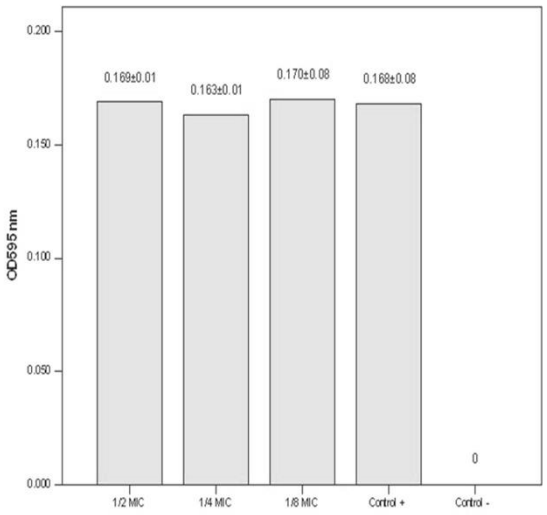


**Fig.1. Biofilm formation by *K. pneumoniae* exposed to sub-inhibitory concentrations of cumin alcoholic extract (OD 595 nm). Control +: Biofilms formed in a medium without the extract.**

## 5. Discussion

The emergence of multi-drug resistance in human pathogens has caused a broad range of clinical problems in the treatment of infectious diseases. Nowadays, pharmaceutical companies are seeking for drugs from other sources such as animals and plants. Medicinal plants are potential sources of new drugs due to the presence of various non-toxic or less toxic antimicrobial chemicals (17).

In addition to a variety of effects on the body's tissues, cumin as a medicinal plant has significant antimicrobial properties as well (9, 22-24). Sagdic and Ozcan (25) tested in vitro antibacterial activities of hydrosols of 16 spices. The hydrosols of cumin were reported to be active against *E. coli*, *Bacillus brevis*, and *Enterobacter aerogenes*. In Gachkar et al.'s (26) study, cumin essential oil exhibited strong antibacterial effects. They showed that *E. coli* is the most sensitive microorganism with the lowest MBC value (1 $\mu L.mL^{-1}$); *S. aureus* and *Listeria monocytogenes* were also sensitive with a MIC values of 1 and 2 $\mu L.mL^{-1}$, respectively (26). Wanner et al. (2010) tested the antimicrobial activities of cumin plants collected from different geographical locations. All essential oils were tested using agar diffusion and serial dilution methods against different gram-positive and gram-negative bacteria isolated from food and clinical specimens, as well as three different *Candida albicans* isolates. All cumin oils exhibited considerable inhibitory effects against all the organisms tested, except *Pseudomonas* species (27).

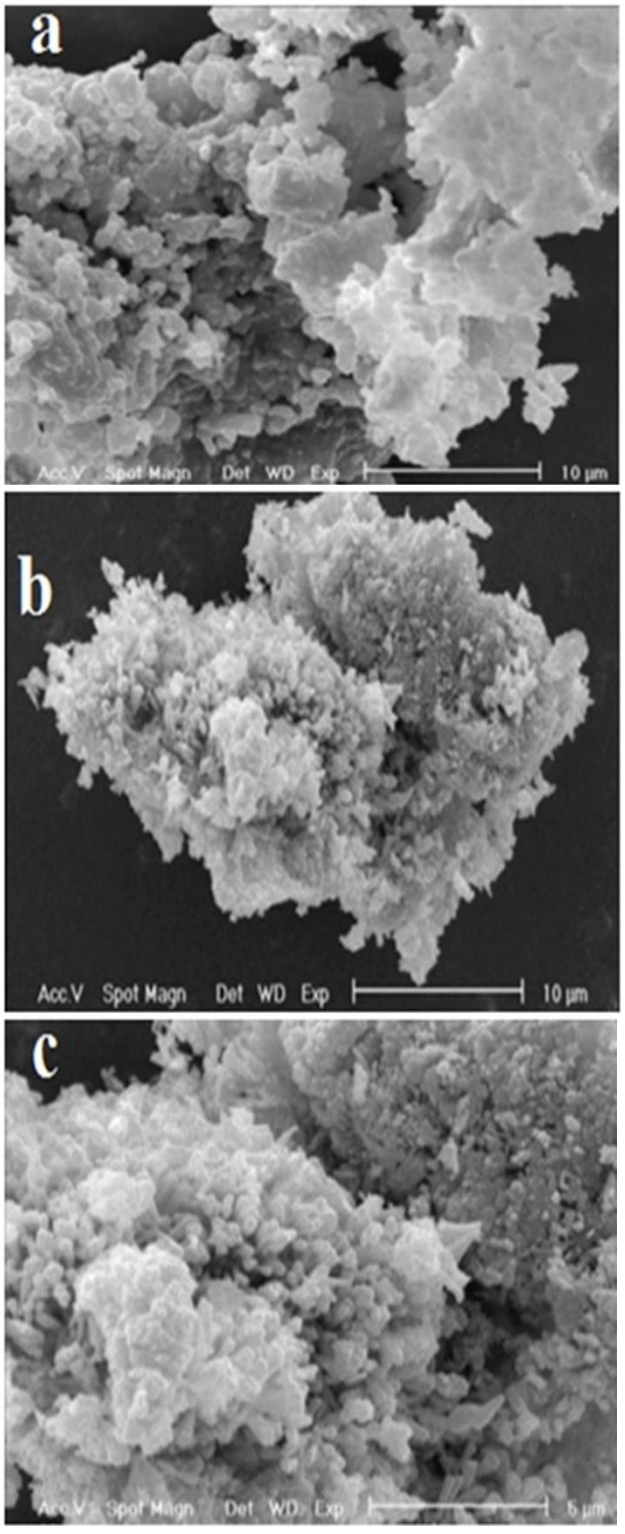


**Fig. 2. SEM images of biofilm formation in *K. pneumoniae* exposed to sub-inhibitory concentrations of the cumin alcoholic extract. a) Control biofilm formed in a medium without extract; b and c) Biofilm formed in the presence of sub-inhibitory concentrations of the extract.**



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Although there are many studies conducted on the antimicrobial properties of cumin, very few reports are available on the antibiofilm activities of cumin extracts. Packiavathy et al. (2012) showed that *C. cyminum* extract interferes with acyl homoserine lactone regulated physiological functions coupled with biofilm formation such as exopolysaccharides production. It promotes the destruction of biofilm structure and strongly inhibits in vitro biofilm formation in *P. aeruginosa* PAO1, *Proteus mirabilis*, and *Serratia marcescens* at sub-MIC levels (14). Since "cell to cell communication" or "quorum sensing" is involved in biofilm formation, Ganesh and Rai (2015) evaluated the anti-quorum sensing activity of *C. cyminum* essential oil. They demonstrated the anti-quorum sensing activity of the essential oil against the wild strain of *Chromobacterium violaceum* CV12472 (28).

In our previous investigation, the effect of sub-inhibitory concentrations of the cumin alcoholic extracts (1/2× to 1/8×MIC) was studied against capsule of *K. pneumoniae*. The study showed a reduction or elimination in the capsule thickness compared to the control (9). Capsular antigens are the major determinants of pathogenicity in *K. pneumoniae*, and a large encapsulated strain may be more virulent than a smaller encapsulated strain (29). The capsule is considered to be involved in biofilm formation. Biofilm formation causes major problems associated with chronic and antibiotic resistant infections (13). Recent studies have suggested that biofilm formation may also be a major virulence factor in *K. pneumoniae* (30). *K. pneumoniae* is able to form strong biofilms on plastic surfaces and surfaces coated with host extracellular matrix proteins (31). A study by Boddicker et al. (2006) identified factors required for biofilm formation in *K. pneumoniae* strains (13). One of these factors was reported to be a gene cluster responsible for the synthesis of the capsule. In our study, despite a reduction in capsule thickness in the presence of the cumin alcoholic extract (9), no decrease was observed in biofilm formation, which can be attributed to the role of other genes and factors in addition to the capsule in the formation of biofilm in *K. pneumoniae*. Boddicker identified mutations in the *cps* capsule gene cluster, transcriptional regulators, fimbrial and sugar phosphotransferase homologues, and loci of unknown function, affecting formation of biofilm (13).

In recent years, many studies have been performed to discover natural compounds in order to partially or fully replace antimicrobial chemical additives in food. To this end, spices are promising alternatives. The inhibitory effect of spices and their derivatives on the growth of bacteria, yeasts, fungi, and microbial toxins has been reported by many researchers (7, 11, 22). In the present study, dill seed essential oil showed a good activity against *S. aureus* and *V. cholerae* as the two important food pathogens. In a study by Lopez and colleagues (2005) on the antimicrobial activity of dill seeds essential oil against four gram-positive bacteria (*S. aureus*, *Bacillus cereus*, *Enterococcus faecalis*, and *L. monocytogenes*), four gram-negative bacteria (*E. coli*, *Yersinia enterocolitica*, *Salmonella choleraesuis*, and *P. aeruginosa*), and three fungi (*C. albicans*, *Penicillium islandicum*, and *Aspergillus flavus*), the essential oil showed a significant antibacterial activity against *S. aureus* and *C. albicans* with inhibition diameters of 15 mm and 12 mm, respectively (6). Singh et al. (2002) also tested the antibacterial activity of dill essential oil against eight pathogenic bacteria, including *Corynebacterium diphtheriae*, *S. aureus*, *Streptococcus haemolyticus*, *Bacillus subtilis* (gram positive), *P. aeruginosa*, *E. coli*, *Klebsiella* species, and *Proteus vulgaris* (gram-negative); they also compared the antibacterial activity of dill essential oil to different antibiotics (22). The antibacterial activity of oil was good for all the bacteria, except for *C. diphtheriae.* The tested oil also showed better antibacterial activity compared with the standard antibiotics. Nanasombat and Wimuttigosol (2011) reported that the dill seeds essential oil revealed MICs of 6, 10, and 2 $mg.mL^{-1}$ for *S. aureus*, *E. coli*, and *A. flavus*, respectively (32).

Reports have suggested that essential oils react with the lipid parts of the cell membrane, where they create their effects and cause either an increase in ion permeability and leakage of cell constituents, or damage to the bacterial enzymes such as enzymes involved in polysaccharide synthesis (33). Studies have shown that, in general, the monoterpenes limonene and carvone are the major constituents of the dill seed oil (8), displaying potent antibacterial activity (10). Monoterpenes have lipophilic characters, allowing them to permeate into membrane structure and consequently increase membrane fluidity and inhibit membrane-embedded enzymes (34). As a result, the antibacterial activity of dill seeds essential oil in our study could, in part, be associated with their major constituents such as limonene and carvone.

## 6. Conclusion

The results of this study showed the presence of antimicrobial compounds in dill extract. Although cumin alcoholic extract was not able to inhibit biofilm formation in *K. pneumoniae*, the reduction in capsule thickness in the presence of the cumin alcoholic extract has previously been reported (9). Generally, plants show a wide range of antimicrobial and other activities which possibly would help discover new types of antibiotics and other drugs. Because of the properties of medicinal plants, it is valuable to search for promising medicinal herbs and novel chemical compounds for inhibiting the biofilm formation.

## Conflict of interest

There is no conflict of interest.

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## Author's Contribution

S. Derakhshan preformed the study. M. Navidinia and A. Ahmadi prepared the manuscript.

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