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Antimicrobial performance of methanol extract of *Foeniculum vulgare* Mill. as a sanitizer agent

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In food industry, microbial contamination poses a big challenge. Chemicals used for disinfection compromise food safety and therby health. ofThere is an urgent need for effective safe sanitizers for the inhibition of pathogens in agricultural and food products. In this context, here, we investigated the possibility of using the *Foeniculum vulgare* methanol extract (ME) in the fight against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* as natural a sanitizer agent candidate. The components of *F. vulgare* ME were analyzed by GC-MS. Broth microdilution method and Surface disinfection test were used for antimicrobial activity and logarithmic inhibition, respectively. The main substances were anethole (50.44%), estragole (13.59%) and benzoic acid (13.58%). Minimum ınhibitory concentrations (MICs) of *F. vulgare* were 0.1 g/mL for *S. aureus* and *C. albicans* while it was >0.1 g/mL for *E. coli*. In surface disinfection test which investigated the survival of *E. coli*, *S. aureus* and *C. albicans* exposure to *F. vulgare* sanitizer (F-SAN: 10%), *F. vulgare* at 50, 100 and 150 µL caused an almost 8-log reduction in *E. coli* in clean condition (0.3 g/mL BSA). In *S. aureus*, 150 µL of *F. vulgare* caused about 4.8 and 4.7 log reduction in clean and dirty surface (3 g/mL BSA), respectively. The highest colony reduction was in *C. albicans* with >4.93 log reduction in both environments. The results suggest that *F. vulgare* methanol extract could be a strong natural sanitizer against pathogens.

Keywords: Fennel, Food industry, Natural sanitizer

Insufficient sanitation on food processing materials and surfaces causes long-term trapping of pathogens on the surface, cross-contamination, and the irreversible contamination of food[1,2]. Notably, critical pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* cause foodborne spoilage and diseases due to infection[3-5]. Often, surfaces are disinfected with some chemicals, but these chemicals exposure a concern for food safety and pose a threat to human health because of their residues[6]. Besides, microorganisms gain resistance against these chemicals over time and makes it difficult to achieve adequate hygiene[7]. There is an urgent need for effective safe sanitizers for inhibition of pathogens in agricultural and food products and for the hazard identification and critic control point (HACCP) system of food industry[8,9].

The importance of natural antimicrobial agents has increased in recent years, and the area of use has expanded. The researches have accelerated in order to use natural agents instead of chemicals in disinfection applications. Studies have indicated that natural antimicrobials are safer and cheaper than chemical antimicrobials. It has also been emphasized that it is suitable for both food and food processing hygienes since most natural agents are Generally Recognized as Safe (GRAS)[10,11]. Among them, *Foeniculum vulgare*, (fennel), belongs to Umbelliferae (Apiaceae) family, is commonly grown in the Mediterranean and western Asia. *F. vulgare* is a small, erect and aromatic herb, and has various chemical constituents, such as fatty acids, hydrocarbons, sterols and furocoumarins[12,13]. Its volatile oil is one of the indispensable flavours of the cosmetic, perfumery and food industry. Fennel seeds alone and in preparations are used to treat respiratory and gastrointestinal problems in traditional medicine[14,15]. Fennel extracts contain reliable antimicrobial components[16-19] besides antioxidant and anticarcinogenic ingredients and also rich in vitamins, such as E and C[20-23]. Methanolic extract of fennel has been shown to contain active antimicrobial agents for *E. coli*, *S. aureus*[24] and *C. albicans*[25]. However, there are not enough studies on the persistence of antimicrobial activity of fennel methanol extract and the use of this extract as a disinfectant.

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Hence, in this study, we investigated the antimicrobial activity of *Foeniculum vulgare* methanol extract (ME) in terms of a natural sanitizer agent and the tolerance or persistence levels in *E. coli*, *S. aureus* and *C. albicans* exposed to this extract based on the modified Kirby–Bauer disk diffusion method.

Materials and Methods

Materials and instruments

Mueller Hinton Agar (MHA), Sabouraud Dextrose Broth (SDB), Triptic Soy Broth (TSB) and Mueller Hinton Broth (MHB), were supplied from Merck (Darmstadt, Germany). *E. coli* (ATCC 25293), *S. aureus* and *C. albicans* were taken from Refik Saydam Hıfzıssıhha Centre (Ankara/Turkey). Dried *F. vulgare* was purchased from Mersin spice store TURKEY (2020).

Preparing *F. vulgare* extract and GC-MS analysis

To prepare *F. vulgare* methanol extract (ME), the 10% fennel prepared in methanol was kept at 25°C in a shaking incubator for 24 hours, and methanol was evaporated usinga rotary evaporator and stored at 4°C for experiments. Application and Research Center by GC-MS 7890A-(5975C-MSD) instrument equipped with an Agilent 19091S-433 column (30 m X 250 µm film X 0.25 µm) and used helium as a carrier gas. The extract was eluted for 64 minutes of retention time using the following temperature program. The first temperature of 60°C for 5 min, then it was gradually raised to 150°C by an increase of 3°C/min for 2 min, then by 3°C/min to 200°C and by 4°C/min to 240°C. Characterization of *F. vulgare* ME components were performed based on the mass spectra library (Wiley Registry 9th-NIST 2011, W9N11.L)[26].

Antimicrobial screening

The inoculums of *S. aureus*(ATCC 25923), *E. coli* (ATCC 25293), and *C. albicans* (ATCC 90028) were prepared in 4 mL TSB for bacteria and 4 mL SDB for yeasts and incubated at 37°C, overnight. After 24 h, the microorganism suspensions were adjusted to 0.5 McFarland Standard Turbidity and stored at +4°C until experiments.

Broth Microdilution Method

The 50 µL of MHB medium was added into 96-well microtiter plates, and two-fold serial dilutions of 50 µL *F. vulgare* ME solution (10%) was made x-axis along from 2nd to 10th columns and used columns 11 (only MHB and microbe) and 12 (only MHB) as the negative controls. Then, 5 µL of microorganism culture were inoculated on the wells except negative control. Finally, all plates were incubated at 37°C for 24 h. Finally, MIC was determined as the lowestconcentration where no visible turbidity was observed in each row of the 96-well plate[27].

Modified Tolerance Disc (*TD*) *test*

It was evaluated the tolerance or persistence levels of *E. coli*, *S.aureus* and *C. albicans* against fennel extract were used by TD (Tolerance Disc) test based Kirby–Bauer disk diffusion method. The disc-diffusion antimicrobial activity test assesses only the concentration at which microorganism stops reproduction, namely the degree of resistance. The tolerance of microorganisms against antimicrobial agents and their persistence can not be determined by this test.

TD test consists of two steps: First, the MIC values of fennel extract were used for the agar well diffusion method. For this, microbial cultures at the stationary phase of incubation were spread onto MHA plates, and 6 mm diameter wells were drilled into the middle of plates. The 50 µL (0.1 g/mL) of fennel extract was placed in the wells and all plates incubated at 37°C for 24 hours. In the second stage, 50 µL glucose (10%) was placed in the well, which discharged because of the diffusion of the extract into the agar media. The alteration in the zone regions of the petri plates re-incubated during 37°C for 24 h was measured and compared with the clear zone in the primary step. TD test, which fennel extract was replaced with the glucose solution, allows detection of the resurviving bacteria on the agar surface. According to this method, it is interpreted as tolerant strain if colonies inside the clear zone and susceptible strain if inhibition zone (IZ) were found around the well after glucose addition (Fig. 1)[28,29].

Surface disinfection test

Surface sanitizer tests were performed using the EN 13697:2015 standard “Chemical disinfectants and antiseptics. Quantitative non-porous surface test for the evaluation of microcidal activity of chemical disinfectants used in many industries such as food, cosmetics, and agriculture. Test method and requirements without mechanical action”. The microcidal activity of *F. vulgare*ME as natural sanitizer (F-SAN) was evaluated on glass lam by modified Falco’s study[30].Briefly, lams were sterilized at 121°C for 15 min in an autoclave before each assay. *E. coli* and *S.aureus* and *C. albicans* suspensions were diluted (ratio 1:1) with 0.3 and

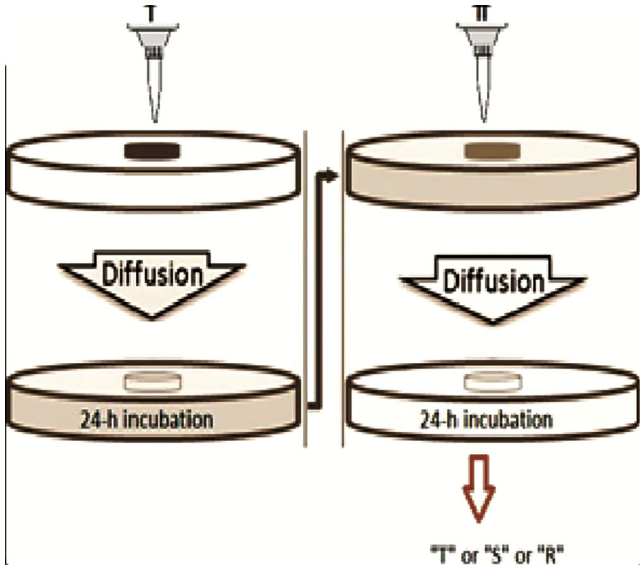

Fig. 1 — The diagram of modified TD test. Application of *F. vulgare* ME on the agar well (I), Replacing the extract with glucose solution (II). "T", "S", "R" Susceptible strain (S): no colonies in the inhibition zone around well after glucose addition. Tolerant strain (T): Colony observation in inhibition zone after glucose.

3 g/L bovine serum albumin (BSA) to mimic dirty and clean working conditions (as in EN 13697:2015), respectively. The 50 µL of resulting inocula (8 log CFU/mL) were spotted into sterile lams and dried at 25°C for 15 min. Later, 50, 100, and 150 µL of 10% F-SAN aprepared and slowly poured onto the lams and mixed. After incubated for 30 min at 25°C, all samples were carefully transferred to the tube by mixing with a pipette and swap. The serial dilutions of samples were performed using peptone water and inoculated on MHA and finally incubated 24 h at 37°C to obtain colony forming units (CFU/mL). Colonies were counted, and the logarithmic reduction was calculated by compared to the control. Over 300 colonies were kept equivalent to the control. As a negative control, water was used instead of F-SAN, but other conditions were the same. The experiments were done twice.

Statistical analysis

Statistical analyses and significance were evaluated by Tukey test in one way analysis of variance for MICs and IZ using SPSS 25. Differences were considered significant at $P \leq 0.05$.

Results

Chemical composition of *F. vulgare*

The components of methanol extracts from *F. vulgare* were detected by comparing the data library. The results of the chemical composition of fennel were presented in Table 1. *F. vulgare*contained predominanly anethole (50.44%), estragole (13.59%), and benzoic acid (13.58%). The other components were 1-heptadecanol (6.22%), DL-limonene (4.56%), (4-methoxy-phenyl)-(2-nitrocyclohexyl)-methanol (3.69%) and L-fenchone (1.16%).

Table 1 — Chemical composition of *Foeniculum vulgare* methanolic extract

RI	Compound	% RA
1028	DL-limonene	4.56
1088	L-fenchone	1.16
1203	estragole	13.59
1225	benzoic acid	13.58
1267	benzaldehyde, 4-methoxy- (CAS)	3.48
1293	anethole	50.44
1607	(4-Methoxy-phenyl)-(2-nitrocyclohexyl)-methanol	3.69
2177	1-Heptadecanol	6.22
	Total	96.72

[RI, Retention Index; and RA, Relative area (peak area relative to the total peak area)]

Table 2 — MIC and IZ (mm) of *Foeniculum vulgare* ME against *E. coli*, *S. aureus* and *C. albicans.*

	MIC (g/mL)	IZ (24 h)	IZ (48 h)-Res
E. coli	$>0.1^{a}\pm0.01$	$3.10^{a}\pm0.03$	$1.50^{a}\pm0.08$-S
S. aureus	$0.1^{a}\pm0.02$	$4.88^{a}\pm0.02$	$3.27^{b}\pm0.02$-S
C. albicans	$0.1^{a}\pm0.1$	$6.12^{a}\pm0.01$	$5.50^{b}\pm0.03$-T

[Res, Response of microorganisms in step 2 according to TD test; S, Susceptible strain; and T, Tolerant strain. Average MICs are expressed with the standard deviation (±) and significance level (ANOVA, 25; 0.05, Tukey test). Values on the same column with different superscript letters differ statistically at the 0.05 level]

Antimicrobial activity and response of microorganisms according to TDTest

The results showed that *F. vulgare* ME was effective against *S. aureus, E. coli* and *C. albicans* by broth microdilution and agar well diffusion method (Table 2). There was no statistically significant difference between the MICs of *F. vulgare* against the pathogens. While MICsof fennel extracton *E. coli* was >0.1 g/mL, it was 0.1 g/mL for either *S. aureus* and *C. albicans*. Although it wasn't seen any statistically significant difference between IZs at the end of the 24-h incubation, there was a considerable difference among 48-h IZs (P <0.05). The highest IZ was found with 6.12 mm on *C. albicans* while the lowest IZ with 3.10 mm on *E. coli* and also it was 4.88 on *S. aureus* at the end of the 24-h incubation by agar well diffusion test (P <0.05).

In this study, it was applied the TD test with *F. vulgare* extract and discriminate by tolerance level (T) of *C. albicans* than the other bacteria at the end of

the 48-h incubation (Table 2). At the end of the 48-h, the IZs were found as 5.50 mm, 3.27 mm, 1.50 mm for *C. albicans*, *S. aureus*, and *E. coli*, respectively (P <0.05). It is clear that the extract had a persistent and robust antibacterial against *S. aureus*, a gram-positive bacterium. The sensitivities of *E. coli* and *S. aureus* can be presented as follows: *E. coli*< *S. aureus*. Finally, it was determined that *C. albicans* was tolerance to *F. vulgare* ME becauseof many colonies in the inhibition region (Fig. 2).

Evaluation of inhibition activity of F-SAN

Survival of *S. aureus*, *E. coli* and *C. albicans* was investigated by surface disinfection test exposured to F-SAN (10%) (Table 3). *F. vulgare* at 50, 100 and 150 µL caused an almost 8-log reduction in *E. coli* in clean condition (0.3 g/mL BSA); in other words, all colonies were inhibited. Whereas, in a dirty condition (3 g/mL BSA), no inhibition was seen in *E.coli* at 50 µL-concentration of *F. vulgare*. At 100 and 150 µL, the reduction was enhanced to 5.2 log and 6.3 log, respectively. In *S. aureus*, no reduction in colony number was seen at 50 and 100 µL of *F. vulgare* in dirty and clean condition, while 150 µL of *F. vulgare* caused about 4.8 and 4.7 log reduction in the clean and dirty surface, respectively. The highest colony reduction was in *C. albicans*. Because a remarkable decrease in the number of colonies was observed in all concentrations of the plant studied. At clean condition, the log reduction order was presented: 5.8 log (50 µL), 5.9 log (100 µL) and 6.4 (150 µL); at dirty condition, it was 4.93 log (50 mL), 5.1 log (100 µL) and 5.96 (150 µL) (Table 3).

Discussion

In this study, the main components of *F. vulgare* were mainly anethole, estragole, benzoic acid, the others were 1-heptadecanol, DL-limonene, (4-methoxy-phenyl)-(2-nitrocyclohexyl)-methanol, L-fenchone. Similarly, Mohamad *et al*.[20] showed that estragole, anethole and L-limonene were the most primary components in fennel methanol extract. In many previous studies, it was revealed that the main volatile components present in fennel were trans-anethole[31,32], a-pinene, limonene, fenchone, aphellandrene, estragole and methylchavicol[33-35].

Among microorganisms, *E. coli* was more resistant to *F. vulgare* ME because of the MIC of >0.1 g/mL than *S. aureus* and *C. albicans* (MICs = 0.1 g/mL) in this study. In an antimicrobial effect study on *E. coli* and *S. aureus* in water extraction of *F. vulgare* at different temperature conditions, they showed that all water extractions were more effective against *S. aureus*, a gram-positive bacterium[36]. They showed MICs of acetone and aqueous extracts on *S. aureus* as 5 and 60 mg/mL, respectively, whereas in this study, MIC was 0.1 mg/mL. This result shows that methanol extraction was more active from acetone and water. The essential oil of Fennel was reported to be active on *E. coli* and *S. aureus* with above 9 mm of IZs by Purkayastha *et al*.[37] and Ilic *et al*.[38]. In this study, IZs were determined lower for *E. coli* and *S. aureus* (3.10 mm and 4.88 mm, respectively). This may be the result of the interaction of the compounds in the extract with each other resulting in antagonistic effect. Ilic *et al*.[38] also determined that *C. albicans* was

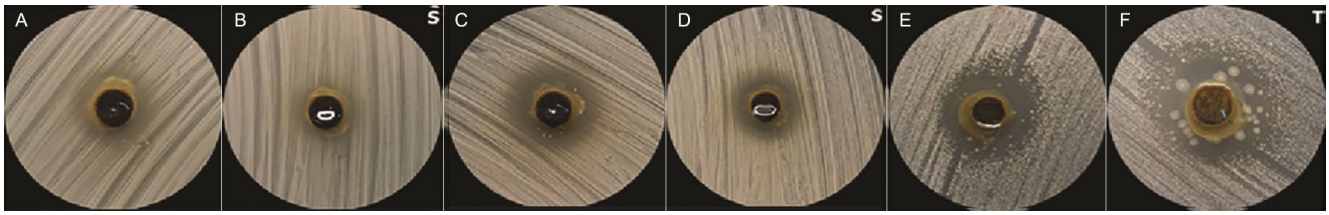

Fig. 2 — The images of tolerance and sensitivity levels of *E. coli*, *S. aureus* and *C. albicans* in exposure to *F. vulgare* ME. The first (A, C, E) and second (B, D, F) step of TD test, (S, Susceptible strain; and T, Tolerant strain)

Table 3 — Log reduction in *E. coli*, *S. aureus* and *C. albicans* exposed to *F. vulgare* on a glass surface clean (0.3 g/mL BSA) and dirty (3 g/mL BSA) conditions

	Log reduction (CFU/mL)								
BSA	*E. coli*			*S. aureus*			*C. albicans*		
g/mL	50 µL	100 µL	150 µL	50 µL	100 µL	150 µL	50 µL	100 µL	150 µL
0.3	8	8	8	0	0	4.8	5.8	5.9	6.4
3	0	5.2	6.3	0	0	4.7	4.93	5.1	5.96
Control*		~1.5×10^8			~1.5×10^8			~1.5×10^8	

[*Starting population]

resistant to fennel oil unlike this study (tolerant). The studies about fennel oil[39], and ethanol extract[40] of *F. vulgare* were found to be active on *E. coli* and *S. aureus* were among the other current researches.

The most common element of *F. vulgare* essential oil was anethole, as in this study. Anethole, an aromatic compound, is a significant leading not only of fennel oil but also of *Croton zehntner*[41] and *Illicium verum*[42]. In a previous study, it was shown that anethole has antibacterial and antifungal effects and other pharmacological properties such as anti-inflammatory and analgesic activity[42,43].In Ghasemian's study, trans-anethole in essential oils from *F. vulgare* seeds obtained from Kerman, Golestan, and East Azerbaijan regions were dominant component (above 49%), and the EOs have antimicrobial activity on *E. coli*, *S. aureus* and *C. albicans*[44]. In this case, it can be said that the main actor of the antimicrobial effect determined in this study is the anethole.

TD test has made it possible to detect tolerant and persistent microorganisms by encouraging the growth of the surviving microorganisms in the inhibition zone after the antimicrobial agent has spread[29]. Glucose was added to the inhibition zone formed around the disc diffusion well as a result of the *F. vulgare* effect, and the incubation was extended to 48 h. At the end of the experiment, *C. albicans* tolerance level was evident compared to other bacteria. (Fig. 2). In literature, Kotkova et al.[45] tried persistence or tolerance of *S. aureus* strain against 1 µg oxacillin, and they showed that *S. aureus* was tolerant to this antibiotic. Also, this test was suitable for persistent/tolerant screening for clinic strains. However, in this study, unlike the previous TDtest method, the agar well diffusion method was used instead of disk, and *S. aureus* was susceptible to fennel extract. No colony was found even in the 48-h incubation.

Natural sanitizer studies using plant extracts have been tried on *E. coli*, *S. aureus* and *C. albicans* before. Ramli *et al*.[46] found that the extract of *S. polyanthum* reduced the microflora in grapes fruit (0.50% of the extract at 5 min) and also inhibited to *E. coli* and *S. aureus* strongly. In another study which was evaluated the antimicrobial efficacy and safety of Pure Hands Herbal Hand Sanitizer in healthy volunteers, the fewer pathogens such as *E. coli*, *Proteus mirabilis*, *Staphylococcus aureus* in people using this hand sanitizer were found than those who do not use[47]. Tayel & Tras[48] demonstrated that pomegranate peel extracts (methanol, ethanol, and water) had strongly inhibited *C. albicans* as sanitizer agents. In addition, essential oils of *Lippia alba* and *Cymbopogon citratus* were evaluated as natural sanitizers on some pathogens including *Staphylococcus aureus* and *Escherichia coli*[49]. In this case, it can say that the herbal sanitizer has a high inhibition potential for *E. coli*, *S. aureus*, and *C. albicans*. In literature, no surface sanitation studies have been found regarding fennel extracts. According to this study, with the modified surface sanitizer test measured at the 30 min, the 150 µl of *F. vulgare* extract caused at least 50% reduction in *E coli*, *S. aureus* and *C. albicans* pathogens.

Conclusions

In this study, the antimicrobial effect of *F. vulgare* ME on *S. aureus, E. coli*, and *C. albicans* was investigated. The highest effect was on *S. aureus*, and *C. albicans*. Whether this effect was permanent or tolerant was checked according to TD test method. *S. aureus* showed permanent sensitivity to fennel ME. In addition, it was shown for the first time that fennel extract significantly reduced the number of pathogens in clean and dirty conditions. The results showed that the fennel extract can be used as an antibacterial agent in industrial hygiene applications in combating many pathogens, especially *S. aureus*.

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Conflict of Interest

Authors declare no competing interests.

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