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Role of *Foeniculum vulgare* oil on the Antimicrobial Activity of Some Antibiotics against Resistant Pathogenic Gram-negative Bacteria

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ABSTRACT

In this manuscript, the antimicrobial efficacy of fennel oil alone and combined with antibiotics were studied by the agar diffusion method. Fifty samples from edible food were collected from supermarkets in the El-Giza governorate, Cairo, Egypt. The presence of multi drug resistant bacteria in the edible food is reported. The aim of this work focuses the sensitivity improvement of the most five resistant Gram negative bacterial isolates due to the presence of fennel oil in the agar medium. Isolation was carried out by classical method using a selective medium followed by biochemical tests. The results showed that the identified isolates include 50 pathogenic bacteria like *Staphylococcus* spp. 10 isolates (40%), *Micrococcus* spp. 1 isolate (4%), *E. coli* 11 isolates (44%), *Citrobacter freundii* 1 isolate (4%), *Enterobacter species* 1 isolate (4%), *Enterobacter cloacae* 1 isolate (4%) and *P. aeruginosa* 1 isolate (4%). There are significant improvements in the antimicrobial activity of tetracycline against *E. coli* isolated from milk due to the fennel oil contained medium. In addition, the sensitivity of *Enterobacter* spp. to meropenem was significantly increased in the medium containing fennel oil. Furthermore, the sensitivity of *E. coli* and *Enterobacter* spp. had significantly decreased to ciprofloxacin in the medium containing fennel oil.

INTRODUCTION

Many species of Gram negative bacteria are pathogenic and cause various infections as urinary tract infections, bacteremia, septicemia and pneumonia [1]. Most species of the *Enterobacter* genus is considered to be pathogenic like *Enterobacter agglomerans, Enterobacter aerogenes* and *Enterobacter cloacae* [2]. Prolonged uses of antibiotics in therapeutic doses lead to bacterial resistance [3]. The multi-drug resistance bacteria shows resistance to most of the antibiotics classes [4]. Plant alkaloids may be considered as efflux pump inhibitors [5]. On other hand, many plant alkaloids cause the increase in the sensitivity of resistant bacteria to antibiotics [6].

The mechanism of antimicrobial resistance in bacteria is mainly mediated by the interaction between specific transporters of antibiotics and efflux pump, so the plant compounds could act through modulation of these efflux pumps which increase the antibiotic sensitivity of the bacteria [7]. Also, Garvey *et al.* stated that some medicinal plants have efflux inhibitory activity against bacteria [8]. The efflux pumps are a major cause of multi-drug resistance. These multidrug effluxes pump presented in the bacterial cell membrane eliminate antimicrobial agents from bacterial cells [9]. Finally, antimicrobial activities of plant products have increased the interest of scientists due to the resistance to antibiotics that some bacteria have acquired [10].

This study was undertaken to detect the resistance pattern of Gram-negative bacteria obtained from food in El Giza governorate and the effect of medicinal plants oil on these strains.

MATERIALS AND METHODS

Bacterial isolation from food samples

Fifty samples from edible food were collected from supermarkets in El-Giza governorate, Cairo, Egypt, from January until December 2015. Samples were collected according to the method recommended by previous workers [11].

Growth Media

Nutrient Broth, Nutrient agar, Mueller–Hinton agar, Eosin methylene blue Agar.

Identification of bacteria

Vitec II was used as biochemical identification for identification of the isolated genera to the species level.

Oil extraction and analysis

The medicinal plants oils were obtained from Phytochemistry Department, Applied Research Center for Medicinal Plants, National Organization for Drug Control and Research "NODCAR" Giza, Egypt. The oil extraction was done of hundred grams of each plant part powder were covered with sufficient water in a flask and subjected to steam distillation according to the method described in the British Pharmacopoeia for 4 h to obtain essential oil. The oils were dried over anhydrous sodium sulfate and stored in black vials at 5°C. All the tested oils were complying with British Pharmacopoeia specifications [12].

Gas chromatography

GC analyses of the obtained essential oil was carried out using HP5890 Series II Gas Chromatograph, HP 5972 Mass Selective Detector and Agilent 6890 Series Autosampler (Agilent Technologies, USA). A supelco MDN-5S 30m by 0.25mm capillary column with a 0.5 µm film thickness was used with helium as the carrier gas at a flow rate of 1.0ml/min. The GC oven temperature was programmed at an initial
temperature of 40 ºC for 5 minutes, then heated up to 140 ºC (5ºC /min) and held at 140 ºC for 5 min, then heated to 280ºC (9ºC /min) and held for 5 additional minutes. Injector and detector temperatures were set at 250 ºC.

Antibiotic susceptibility test

A sterile cotton wool swab dipped into the bacterial suspension was spread on the surface of previous Mueller–Hinton agar plates. The inoculated plates were allowed to dry before placing the diffusion antibiotic disks. Susceptibility of 5 tested isolates to various tested antibiotics was performed by disk diffusion method as described by the Clinical and Laboratory Standards Institute [13]. Using commercially available antibiotic disks, purchased from Oxoid Ltd. Co. revealed to many antibiotic groups (fluoroquinolones, quinolones, β-lactams, β-lactamase inhibitor combination, 2nd generation cephalosporens, 3rd generation cephalosporins, aminoglycosides, folate pathway inhibitors and tetracycline) were placed on the surface of the inoculated MHA plates with Gram negative bacteria. The inoculated plates were then incubated at 37°C for 24 h. Inhibition zone diameters were measured inclusive of the diameter of the disks. Results were expressed as sensitive, intermediate and resistant according to the CLSI.

Antibacterial activity of fennel oil

Antibacterial activity of fennel oil against various tested clinical bacterial isolates was studied by agar well diffusion method according to Perez *et al.* using (100 µl) of each oil was added to fill the well of 10 mm diameter [14]. After 24h incubation at 37ºC, all plates were observed for zones of growth inhibition, and the diameters of these zones were measured in millimeters. Less than 14 mm was considered resistant organism.

Detection of synergetic interaction between plant oils and antibiotics

Detection was carried out according to Moussaoui and Alaoui with some modification [15], the plates inoculated with 0.5 ml oil/50ml Mueller–Hinton agar (MHA) then allowed drying before placing the diffusion antibiotic disks. Susceptibility of the tested isolate to various tested antibiotics was performed by disk diffusion method as described by CLSI.

RESULTS AND DISCUSSION

Fifty samples from edible food were collected from supermarkets in El-Giza governorate, Cairo, Egypt. The results indicated that there are many pathogenic bacteria like *Staphylococcus aureus* 10 isolates (40%), *E. coli* 11 isolates (44%), *Citrobacter freundii* 1 isolate (4%), *Enterobacter species* 1 isolate (4%), *Enterobacter cloacae* 1 isolate (4%) and *Pseudomonas aeruginosa* 1 isolate (4%).

Gram negative bacteria isolation and characterization

The detection of the strain was done by Vitec to determination the biochemical tests.

Antimicrobial effect of fennel oil against pathogenic Gram-negative bacteria

The Gram-negative bacteria strains were susceptibility tested to 13 antibiotics and Fennel. The antimicrobial activities of the fennel oil against *E. coli*, *Citrobacter freundi, E. coli, Enterobacter cloacae* and *Enterobacter* spp. are presented in **Table 1** indicated that all strains were resistant to fennel oil except (*E. coli* from milk and *Citrobacter freundi* from milk were sensitive).

Table 1. Antibacterial effect of fennel oil using well diffusion method inhibition zone (mm). (R) Resistant, (S) sensitive.

Source	Milk	Milk	Cheese	Egg	Milk
Oil	*Citrobacter freundi*	*E. coli*	*E. coli*	*Enterobacter cloacae*	*Enterobacter* spp.
Fennel	19	18	-(R)	-(R)	-(R)

In this respect, medicinal plant oils are natural components used as antimicrobial that makes many scientists to screen plants and studying their antimicrobial activities in therapeutic aspects [16]. From previous results, the fennel oil was considered the weak antimicrobial oil against tested strains.

Effect of fennel oil and antibiotic combination against pathogenic Gram-negative bacteria

The fennel oil interaction with the tested antibiotics is presented in **Table 2.** which showed that addition of fennel oil in the tested agar medium did not give any significant change on antimicrobial activity of all tested antibiotics for *Citrobacter freundi* from milk. Same results were reported for *E. coli* from milk except tetracycline had significantly increased in agar medium contained fennel oil as exceeded the antimicrobial activity of the control.

In the case of *E. coli* from cheese addition of fennel oil in the tested agar medium gave a significant improvement for the antimicrobial activity of nalidixic acid, cefoperazone and

sulphamethazole/trimethoprim. But fennel oil in the tested agar medium gave significantly reduction the sensitivity of tested strain to Ciprofloxacin. While In the case of *Enterobacter cloacae* from egg addition of fennel oil in the tested agar medium gave a significant improvement for the antimicrobial activity of nalidixic acid, ciprofloxacin, meropenem, gentamycin and amikacin.

In the case of *Enterobacter* spp. from milk addition of fennel oil in the tested agar medium gave a significant improvement for the antimicrobial activity of Nalidixic acid, Meropenem, Gentamycin and Amikacin. Finally, addition of fennel oil in the tested agar medium gave significantly reduction the sensitivity of tested strain to ciprofloxacin.

Table 2. The antibacterial response to combinations between antibiotics and fennel oil using disk diffusion method. Inhibition zone measured in (mm), (R) Resistant, (S) sensitive, (I) intermediate.

microorganism and source	*Citrobacter freundi* from milk		*E. coli* from milk		*E. coli* from cheese		*Enterobacter cloacae* from egg		*Enterobacter* spp. from milk	
	control	with oil	control	with oil	control	with oil	control	with oil	control	with oil
nalidixic acid 30µg	20(S)	21(S)	20(S)	21(S)	18(I)	30 (S)	16(I)	20(S)	16(I)	25(S)
ciprofloxacin 5 µg	29(S)	30(S)	29(S)	30(S)	30(S)	28(S)	30(S)	32(S)	30(S)	20(R)
pipracillin/tazobactam110 µg	19(I)	19(I)	20(I)	20(I)	16(R)	16(R)	18(I)	18(I)	14(R)	14(R)
ampicillin/sulbactam 10 µg	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)
cefoperazone 75µg	15(R)	15(R)	15(R)	15(R)	15(R)	18 (I)	-(R)	-(R)	-(R)	-(R)
cefaclor 30 µg	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)
ertapenem 10µg	10(R)	10(R)	13(R)	13(R)	10(R)	10(R)	9(R)	9(R)	8(R)	8(R)
meropenem 10µg	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)	20(I)	37(I)	-(R)	38(R)
gentamycin 10µg	15(S)	15(S)	22(S)	22(S)	22(S)	22(S)	15(S)	20(S)	15(S)	30(S)
amikacin 30µg	22(S)	22(S)	22(S)	22(S)	25(S)	25(S)	20(S)	28(S)	20(S)	32(S)
doxycycline 30µg	9(R)	9(R)	-(R)	-(R)	15(S)	15(S)	-(R)	-(R)	15(S)	15(S)
tetracycline 30µg	13(I)	13(I)	-(R)	12(I)	-(R)	-(R)	-(R)	-(R)	-(R)	-(R)
sulphamethazole/trimethoprim 25µg	25(S)	25(S)	16(S)	16(S)	-(R)	25 (S)	-(R)	-(R)	-(R)	-(R)

The results obtained from **Fig. 1** revealed that the fennel oil in the tested medium did not give any significant change on antimicrobial activity of tested antibiotic against *Citrobacter freundi* strain isolated from milk.

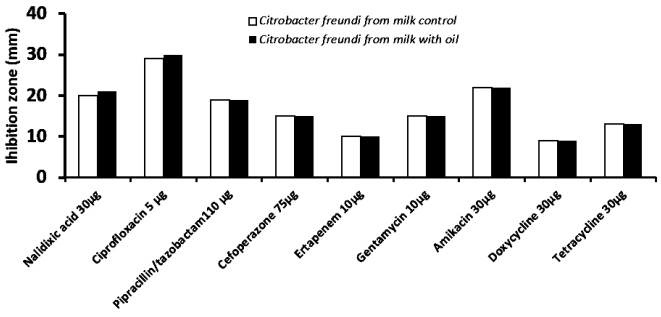


Fig. 1. Effect of fennel oil on antimicrobial activity of some antibiotics against *Citrobacter freundi* isolated from milk.

In this respect, indifference and antagonism between essential oils and different antibiotics have been also reported [17]. The results obtained from **Fig. 2** revealed that the fennel oil in the tested medium give significant change on antimicrobial activity of tetracycline antibiotic against *E. coli* strain isolated from milk.

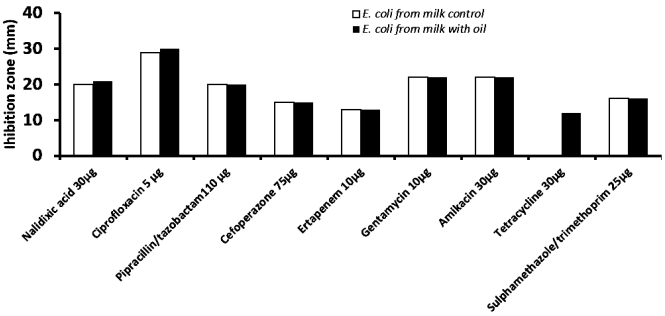


Fig. 2. Effect of fennel oil on antimicrobial activity of some antibiotics against *E. coli* isolated from milk.

In this respect, Tetracycline mode of action thought inhibition the growth of bacteria as entering the microbial cell, binding to bacterial ribosomes, and stopping protein synthesis as it binds strongly to a single site on the 30S ribosomal subunit and the 7S ribosomal protein appears to form part of the binding site [18]. Also, there is 3 mechanisms of microorganism resistance to tetracycline as (limiting the access of tetracycline antibiotic to the bacterial ribosomes, ribosome alteration to prevent binding of tetracycline to the ribosome and the bacteria producing tetracycline inactivating enzymes) all of these types of resistance may be found in one bacterial strain [19]. Finally, our results agree with Hung *et al.* where the resistance to doxycycline was less than tetracycline [20] as doxycycline is considered as a 2nd generation tetracycline differing structurally from the tetracycline and make it more lipid soluble [21]**.**

The results obtained from **Fig. 3. and Fig. 4.** revealed that the fennel oil in the tested medium give significant change on antimicrobial activity of sulphamethazole/ trimethoprim antibiotic against *E. coli* strain isolated from cheese.

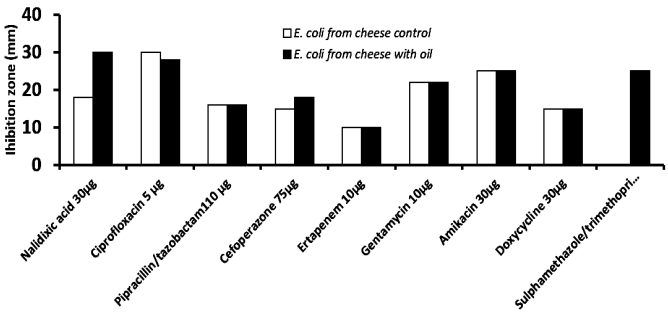


Fig. 3. Effect of fennel oil on antimicrobial activity of some antibiotics against *E. coli* isolated from cheese.

Fig. 4. Effect of fennel oil on antimicrobial activity of sulphamethazole/-trimethoprim against *E. coli* isolated from cheese using the disk diffusion method. Left is sulphamethazole/-trimethoprim antibiotic only while right is sulphamethazole/-trimethoprim antibiotic with fennel oil.

In this respect, both sulfonamides /trimethroprim antibacterial mode of action is by folate biosynthesis pathway, so mutations of the DHFR (dihydropteroate synthase and dihydrofolate reductase) enzymes cause affinity reduction for sulfonamides and trimethroprim respectively; causing antibiotic resistance [22]. Both sulfonamide and trimethroprim resistant enzymes encoding genes are presented on plasmids causing resistance spread [23]**.** The results obtained from **Fig. 5.** revealed that the fennel oil in the tested medium give significant change on antimicrobial activity of nalidixic acid, meropenem, gentamycin and amikacin antibiotics against *Enterobacter cloacae* isolated from edible egg.

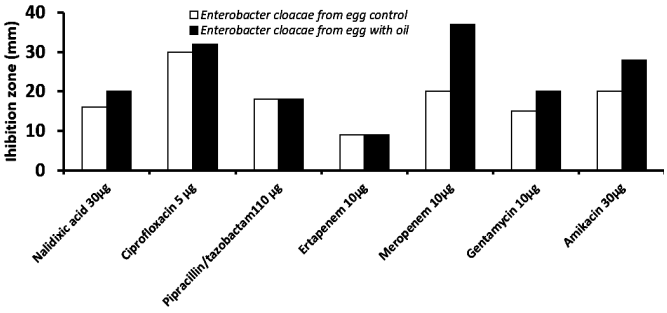


Fig. 5. Effect of fennel oil on antimicrobial activity of some antibiotics against *Enterobacter cloacae* isolated from edible egg.

Antibacterial effects of quinolone antibiotics are by binding to complexes that form between DNA and DNA gyrase. Shortly after binding, the quinolones cause a molecular change to the DNA gyrase enzyme [24]. In this respect, mutations of gyrase or topoisomerase IV enzymes cause quinolone resistance [25] which may be the cause of the intermediate resistance of *E. coli* from cheese, *Enterobacter cloacae* from egg and *Enterobacter* spp. from milk to Nalidixic acid as the mutations responsible for reduce the affinity of the enzyme-DNA complex to quinolones [26]. The results obtained from **Figs 6** and **7** revealed that the fennel oil in the tested medium did give significant change on antimicrobial activity of nalidixic acid, meropenem, gentamycin and amikain antibiotics against *Enterobacter* spp. isolated from milk.

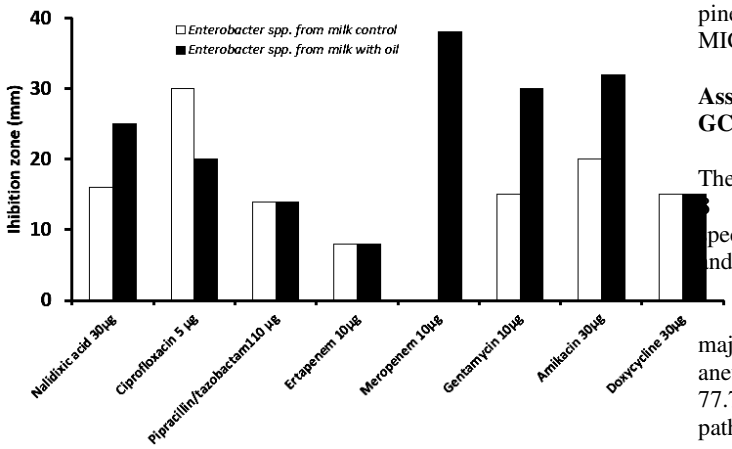


Fig. 6. Effect of fennel oil on antimicrobial activity of some antibiotics against *Enterobacter* spp. isolated from milk.

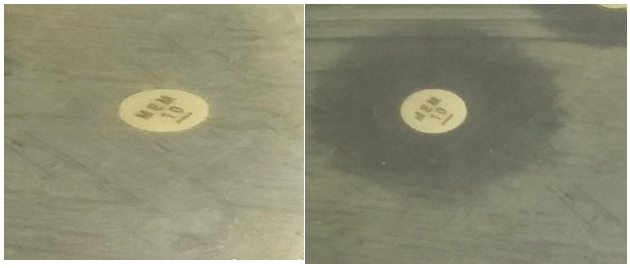


Fig. 7. Effect of fennel oil on antimicrobial activity of meropenem against *Enterobacter* spp. isolated from milk using disk diffusion method. Left is control while right is supplemented with fennel oil.

In this respect, the main cause of aminoglycoside resistance is mainly due to aminoglycoside modifying enzymes [27]. Also, the majority of the enzymes belong to the APH (3′) subfamily are widespread among pathogenic microorganisms [28]. In this respect, Amikacin antibiotic is more effective in the treatment of resistant bacteria to other aminoglycosides antibiotics [29]. Amikacin antibiotic is a semisynthetic antibiotic manufactured from kanamycin A, this modification leading to amikacin become less susceptible to the harmful action of many aminoglycoside-modifying enzymes [27].

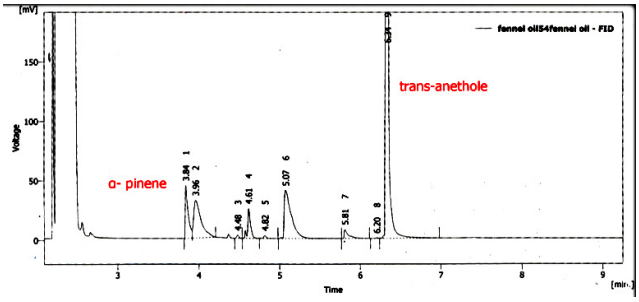
Detection of carbapenemase producering Gram negative bacteria is very important, as they are also associated with other antibiotics resistance, giving rise to multidrug resistance [30]. Also, the resistant pattern in our results agree with Bansal *et al.* as they stated that the increasing incidence of carbapenemase producing bacteria worldwide has posed a scientist challenge for diagnosing and treating bacterial infections as they hydrolyse β-lactam antibiotics including carbapenems, penicillins and cephalosporins their hydrolyzing activity is inhibited by sulbactam tazobactam and clavulanic [31].

From previous results, it could be summarized that Gram-negative bacteria foodborne strains appeared resistant to various antibiotics tested. In this respect, adequate sanitary measures cause decrease in cases of foodborne bacteria in developed countries [32]. Also, Infection due to foodborne bacteria becomes an important public health concern especially in developing countries [33]. Finally, Silva *et al.* stated that α-pinene when combined with different antibiotics will reduce the MIC of combined antibiotics [34].

Assay of α- pinene and trans-anethol in the fennel oil using GC analysis.

The data obtained from fennel oil GC analysis presented in **Fig.** indicate that it agrees with British Pharmacopoeia pecifications where the α- pinene 6.4% (must be 1% to 10%) nd trans-anethol 62.6% (must be 55% to 75%).

Our results agree with Acimovic *et al.* who stated that the major fennel composition is trans-anethol [35]. Also, trans-anethole in *Portuguese foeniculum* vulgare fruits was (7.9 – 77.7%) [36]**.** Finally, α-pinene has antimicrobial activity against pathogenic microorganisms [34].



A

Result Table (Uncal - fennel oil54\fennel oil - FID)

	Reten. Time [min]	Start Time [min]	End Time [min]	Start Value [mV]	End Value [mV]	Area [mV.s]	Height [mV]	Area [%]	Height [%]
1	3.837	3.817	3.917	1.798	2.049	113.046	44.365	6.4	7.3
2	3.957	3.917	4.203	2.049	2.769	199.096	31.390	11.2	5.1
3	4.480	4.440	4.533	1.880	1.855	4.655	2.627	0.3	0.4
4	4.613	4.537	4.750	1.855	1.808	65.193	24.895	3.7	4.1
5	4.817	4.750	4.977	1.808	1.758	6.031	2.592	0.3	0.4
6	5.073	4.980	5.767	1.758	1.750	246.035	40.276	13.9	6.6
7	5.807	5.767	6.110	1.750	1.800	27.014	7.490	1.5	1.2
8	6.203	6.127	6.237	1.801	1.833	0.680	0.336	0.0	0.1
9	6.337	6.237	6.977	1.833	2.046	1109.895	457.872	62.6	74.8
	Total					1771.646	611.843	100.0	100.0

B

Fig. 8. GC analysis illustrating fennel oil main composition (A) and their percentage concentration (B).

This study showed that antibiotic resistance concern becomes a big scientific problem and must take important consideration. In addition, combination between antibiotics and fennel oil has significant value as essential oils improve the antimicrobial effect of antibiotics against resistant Gram-negative bacteria as the volatile compounds may disrupt the microbial cell membrane, thus facilitating antibiotic penetration. Finally, fennel oil may reduce the sensitivity of *Enterobacter* spp. to ciprofloxacin so the complexity which generated when combining fennel and ciprofloxacin antibiotic must be studied.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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