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Synergistic Effect of *Cinnamomum camphora* and *Origanum vulgare* Essential Oils against *bla* $_{CTX-M}$ Producing *Escherichia coli* Isolated from Poultry Colibacillosis

Saba Pouyan [1], *Khatereh Kafshdouzan* [2*], *Ashkan Jebelli* [3]

[1] *Student Research Committee, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran.*

[2] *Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran.*

[3] *Department of Food Hygiene, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran.*

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ABSTRACT

Background: The transmission of antibiotic resistance through the food chain is one of the major health challenges, worldwide. A combination of essential oils with synergistic or additive effects to enhance the antimicrobial activity, is an applied approach to improve food safety.

Methods: In this study, 93 *E. coli* isolated from the viscera of broilers, suspected to colibacillosis, were examined for detection of ESBL by the combined disk method according to Clinical and Laboratory Standards Institute. CTX-M was detected by PCR. Antibacterial activity of cinnamon and oregano essential oils were studied against *bla* $_{CTX-M}$ harboring isolates by broth microdilution method and fractional inhibitory concentration index.

Results: According to the results of this study, 32/93 (34.4%) of tested samples produced ESBL, and 10/32 (31.2%) harbored CTX-M. All the CTX-M producing *E. coli* investigated by broth microdilution assay, were sensitive to cinnamon and oregano essential oils in the range of 400 to 3200 and 800 up to 1600ppm, respectively. Fractional inhibitory concentration indices ranging from 0.5 to 1.5, suggested synergistic, and additive inhibitory effect of cinnamon and oregano essential oils.

Conclusion: The results of this study indicated that bla CTX-M might be transmitted to humans through chicken meat. The combination of cinnamon and oregano can be suggested as a safe bio-preservative which leads to growth inhibition of antibiotic resistant *E. coli*. However, further studies should concern the potential interaction between essential oils and food matrices.

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*Corresponding Authors: Khatereh Kafshdouzan, Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran.

Tel: +98-23-33654215, *E-mail:* kafshdouzan@semnan.ac.ir

Introduction

Nowadays, an increase of extra-intestinal pathogenic *Escherichia coli* (ExPEC), showing resistance to beta-lactamase, is a substantial challenge worldwide (1). Extended-Spectrum Beta-Lactamases (ESBLs) are plasmid-encoded enzymes, produced by some bacteria such as Enterobacteriaceae members that hydrolyse and inactivate a wide variety of beta-lactam antibiotics. Among ESBL genes, *bla* $_{\text{CTX-M}}$ is the most frequent variety of such enzymes and has become disseminated, globally (1). Similar characteristics between ExPEC in human and poultry has led to the hypothesis of the zoonotic potential of poultry strains. The zoonotic risk to humans from chicken-source is not fully elucidated. However, recent studies have suggested that the contaminated chicken meat and meat products can be a source of ExPEC strains transmission to humans (2). Antibiotic-resistant bacteria and antibiotic-resistant genes (ARGs) can easily spread at each stage of the food production chain and can cause infections in humans. Therefore the emergence of antibiotic resistance (ABR) in the food chain is considered a cross-sectoral problem, as antibiotics are widely used in aquaculture, livestock production, and crop culture (3).

Synthetic preservatives have been widely used to eliminate bacteria and prolong the shelf-life of food products. However, synthetic preservatives may cause health problems for consumers in the long term period. Thus, searching for new and potential natural antimicrobial agents from different sources such as microbial metabolites, plant, and spice extracts for use in the food industry, has been increasing significantly. Essential Oils (EOs) with some proven biological properties such as anti-bacterial, anti-parasitic, anti-fungal, anti-oxidants, and anti-inflammatory activities, are the appropriate alternative methods, applied in this context (4, 5).

Cinnamomum camphora (Cinnamon), from *Lauraceae* family, is a traditional herbal medicine that is widely distributed in China, India, and Australia. Due to antimicrobial, antioxidant, and anti-carcinogenic activities, it has been widely applied in food seasonings, cosmetic, and medical industries. The antimicrobial activity of both cinnamon EO and its major compositions have been previously evaluated. Several studies have shown the main compound of cinnamon, such as cinnamaldehyde and eugenol, inhibits the growth of both Gram-positive and Gram-negative bacteria as well as foodborne pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli* (6, 7, 8, 9).

According to recent studies, to produce optimal antimicrobial results, a large amount of essential oils is needed to use in food systems, which cause adverse effects on food flavors. To achieve the similar antibacterial effects as those obtained in in vitro assays, todays, the simultaneous application of EOs and/or their isolated components, are new approaches to enhance the efficacy of essential oils (Eos) in foods (4, 10, 11). Combination effects of two or more EOs usually assessed by the Fractional Inhibitory Concentration (FIC) method, which is a criterion to determine the interaction between two or more drugs predesignate to be used in combination (12).

The inhibitory effects of EOs are usually studied on culture collection strains in which the antibacterial susceptibility profile is not known. Therefore, our current knowledge about the antibacterial effects of EOs on resistant strains is limited. Because of the importance of the transmission of bacterial resistance through the animal food around the world and the increasing desire of using herbal EOs for inhibition of the bacterial growth, this study aimed to evaluate the frequency of ESBL producing *Escherichia coli* in chicken suspected to colibacillosis and evaluate the antibacterial activity of cinnamon and oregano essential oils in different concentration, against *bla*$_{\text{CTX-M}}$ producing *Escherichia coli* and

determination of their synergistic or antagonistic effects by FIC index.

Materials and Methods

Isolation of Escherichia coli

From February 2016 to April 2017, 100 isolates were collected from poultry farms and veterinary laboratories in Semnan, Iran. Samples were isolated aseptically from the heart, liver, and air sacs lesions of broilers suspected to colibacillosis and identified as *Escherichia coli* by standard biochemical tests. *E. coli* isolates were stocked in BHI broth (Merk; Germany) with 15% glycerol at -20°C until the next procedures.

Extended-Spectrum Beta-Lactamase Detection by Combined Disk Method

Bacterial suspensions were prepared in normal saline solution at a concentration equal to 0.5 McFarland opacity tube for culture on Mueller Hinton agar (Merck, Germany). Antibiotic susceptibility was studied against chloramphenicol (30 µg), ampicillin (10 µg), cefepime (30 µg), gentamicin (10 µg), tetracycline (30 µg) and ciprofloxacin (5 µg) (Padtan Teb, Iran). To confirm ESBL producing isolates, resistant phenotypes related to ESBLs were detected by performing Combined Disk Method (MAST® D67C). Disks including Ceftazidime 30 µg, Ceftazidime 30/Clavulanic Acid 10 µg, Cefotaxime 30 µg, Cefotaxime 30/Clavulanic Acid 10 µg, Cefpodoxime 10 µg, Cefpodoxime 10/Clavulanic Acid 1 µg, placed onto the inoculated medium, with sufficient space between them. Plates incubated at 35°C for16-18 hours. Any zone of inhibition that was observed, measured, and recorded. An increase in the zone diameter (≥5mm) between the zone of an antibiotic disk and their respective antibiotic- clavulanate disk, confirms as ESBL producing *E.coli*. Results were interpreted according to CLSI guidelines 2017. *Escherichia coli* ATCC 25922 was used as the quality control strain (1).

DNA Extraction and Polymerase Chain Reaction

Genomic DNA was extracted from the colonies of ESBL-producing organisms. Individual colonies on EMB agar were inoculated in3ml Luria-Bertani broth at 37° C, overnight. 30µl of bacterial culture was added to 270 µl of TE buffer and boiled for 10min. The supernatant was used as a DNA template (13). The presence of *bla* $_{\text{CTX-M}}$ was detected by a specific primer and PCR master mix (Takapou Zist Co, Iran) (1). Description of primer and amplification program, summarized in Table 1. PCR products (688bp) were detected by electrophoresis through 1.5% agarose gel.

Preparation of cinnamon and oregano essential oils

EO of *Cinnamomum camphora* was purchased from Barij essence CO (Kashan, Iran) and kept at 2-8 °C in sealed brown vials until required. Air-dried aerial parts of oregano were purchased from local retail markets and transported to the laboratory. It was identified and approved by botanists at Semnan Agriculture and Natural Resources Research Center, Iran. The dried aerial parts of oregano were ground, and essential oil was extracted through steam distillation, using the Clevenger-type apparatus. Then, it was dried by sodium sulfate and was stored in dark glass containers at 4°C.

Preparation of bacterial inoculum

Stock cultures were propagated through two consecutive 24 h growth cycles on BHI broth at 35° C. Bacterial suspensions were adjusted to an optical density of 0.1 at 600 nm, using a spectrophotometer. Then, 1 ml of the suspension was transferred into a tube containing 9 ml of 0.1 %(w/v) peptone water to prepare successive

dilutions of up to 10^{-6}. Then, 100 µl of each dilution was aseptically transferred to plates containing BHI agar and enumerated after duplicate plating from tenfold serial dilutions, incubated at 35°C for 24 h. The Exact number of inoculated bacteria was calculated through co-culture and colony counting (13). The stock cultures of the bacteria wereere transferred to tubes containing 10 ml of brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) and wereere incubated at 37°C for 24h. The same procedure was repeated one more time. For short time preservation, a loop of the bacteria from the second culture was inoculated on slant BHI agar and was incubated under the same condition and kept at 4°C. A loop of bacteria from the second culture was grown on BHI broth and incubated at 37°C for 6h and then was used to prepare inoculums; the bacterial suspension was adjusted to an optical density of 0.1 at a wave length of 600 nm using a spectrophotometer andspectrophotometer and enumerated by duplicate plating from 10-fold serial dilutions on BHI agar (Merck, Darmstadt, Germany) (14).

Determination of the Minimum Inhibitory Concentration (MIC)

Broth microdilution method recommended by the CLSI (2017), with some modification, was used for the determination of the inhibitory effect of EOs against *bla* $_{\text{CTX-M}}$ producing *Escherichia coli*. Then, 200 µL of different concentrations of cinnamon ((0, 100, 200, 400, 800 and 1600ppm) and oregano(EO) (0, 100, 200, 400, 800, 1600 and 3200 ppm) in sterile Mueller Hinton broth (Merck, Germany), which contained 10% Dimethyl Sulfoxide (DMSO) were prepared in 96-well microplate (300 µl capacity, round–button wells) and 20 µl of bacterial suspension was aseptically inoculated to each test well (final bacterial concentration was 5×10^5 CFU/ml bacteria per well). All experiments were performed in triplicate. A positive control containing the bacterial culture and DMSO without the EO and a negative control containing only the sterile medium were performed as well.

The contents of wells were gently mixed with a microplate reader equipped with a shaker (BioTek® Instruments, Inc., Winooski, Vt., U.S.A.) for 2 min and absorbance (0 h) was immediately read at 630 nm (OD630). The plates were aerobically incubated at 35°C for 24 h.

The turbidity, if any, was observed with the naked eye, and the absorbance (24h) was read using the microplate reader. An increase in absorbance ≥0.1 against 0 h demonstrated bacterial growth and turbidity. The first single or combined concentration of the essential oils without turbidity was defined as the MIC (14-15).

Interaction of clove and cinnamon essential oils

The combination effect of *C. camphora* and *O. vulgare* essential oils was evaluated by using the FIC index. Serial, twofold dilutions of the essential oils were prepared using the same solvents as in the MIC tests. 100µl of each cinnamon EO dilution was added to the wells of a 96-well plate in a vertical orientation, and 100 µl of each clove EO dilution was added in a horizontal direction so that the dish would contain various concentration combinations of the two compounds. Then, each well was inoculated with 20 µl (final bacterial concentration was 5×10^5 CFU/ml bacteria per well) of bacterial suspensions and incubated at 35°C for 24h. The MIC of combined cinnamon and clove EOs was defined as described above, and their interactive effect was measured by using the Fractional Inhibitory Concentration (FIC).

FIC index =

$$\frac{MIC\ of\ C.zeylanicum\ essential\ oil\ with\ O.vulgare\ \ essential\ oil}{MIC\ of\ Cinnamon\ essential\ oil\ alone} + \frac{MIC\ \text{of}\ O.vulgare\ \ essential\ oil\ combined\ with\ C.zeylanicum\ essential\ oil}{MIC\ of\ O.vulgare\ essential\ oil\ alone}$$

Theoretically, the FIC index < 1, defined as a synergistic interactive effect of the essential oils,

while FIC = 1, 1< FIC< 2, and FIC > 2, classified as an additive, neutral and antagonistic effect, respectively (12).

Result

Phenotypic characterization of antimicrobial resistance

Out of 100 collected samples, 93% (93) showed positive culture results for *E. coli*. As demonstrated in table 2, different rates of resistance to antibiotics were detected. The most prevalent resistance was related to tetracycline, whereas the lowest rate was shown for cefepime. Furthermore, 34.4% (32/93) isolates confirmed as ESBL producing *Escherichia coli* by the combined disk method.

CTX-M harboring Escherichia coli

Out of 32 strains producing ESBL, in 31.25% (10) *bla* CTX-M CTX-M *bla* gene was detected by a polymerase chain reaction.

MIC determination by broth microdilution method

Table 3 presents the results of MIC determination for the cinnamon and oregano essential oils and combination of these two essences against the 10 aforementioned bacterial isolates, using the broth microdilution method. As shown, the lowest and highest MIC of oregano was 800 and 1600 ppm respectively and for cinnamon was 400 and 3200 ppm. Finally, as shown in Table 4, on both essential oils, MIC50 was observed at 1600 ppm.

Determination of the FIC indices

The combined effect of the essential oils was investigated by calculating the FIC index. The FIC indices ranging from 0.5 to 1.5 were listed in Table 3. FIC index of these two essential oils in combination is suggesting their synergistic inhibitory effect on 5 (50%) isolates and additive inhibitory effect on 4 (40%) isolates.

Discussion

The potential risk of *E. coli* and significant importance of antibacterial resistance transmission through the food chain caused ESBL producing *Escherichia coli* has been studied in chicken as well as in humans (16, 17). Gundran et al. 2019 reported out of 78 selected poultry farms in four provinces in Philippine, 52 were involved by ESBL, and 44.23% *Escherichia coli* producing ESBL were isolated. Also, the most prevalent ESBL encoding gene was CTX-M (18). In a similar study, Falgenhauer et al. (2019), reported out of 140 broilers and 54 children, 29% and 61% harbored ESBL producing *E. coli* respectively, and CTX-M-15 was the most prevalent ESBL type among human (97%) and chicken (96%). While in some countries, studies have demonstrated low prevalence (0-2%) of CTX-M (19).

In the present study, 32/93 (34.4 %) tested isolates were confirmed as the ESBL-producing *E. coli* using the combined disk method, and the frequency of *bla* $_{\text{CTX-M}}$ was 31.25%. The variation between the rates of ESBL producing *E. coli* reported in different studies may be attributed to the sample size, study design, bio-security management or history of antibiotic usage in a farm, and the geographical area.

As regards, several studies have reported that the CTX-M, is the most widespread type of ESBL in human and some livestock animals, it seems that the main route of transmission of ESBL resistant genes is through the food chains (18, 19). Therefore the use of a safe method to combat ESBL producing bacteria in food is a global challenge. Application of EOs with antimicrobial potency as a flavoring bio- preservative is well documented in the last decade (5-7). However, to our knowledge, this is the first report of the combined effect of cinnamon and oregano

essential oils against *bla* CTX-M producing *Escherichia coli* isolated from poultry colibacillosis.

According to the results of our study, all CTX-M producing *E. coli* were investigated by the broth microdilution method, were sensitive to the cinnamon and oregano EOs in the range of 400-3200 and 800-1600 ppm, respectively. Ghabraie et al. (2015) evaluated 32 different essential oils, including Cinnamomum cassia and Cinnamomum verum on *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O: 157: H7, *Salmonella typhimurium* and *Pseudomonas aeruginosa* that were isolated from prepared meat. Their results showed *Cinnamomum cassia* EO had the best effect at 470 ppm concentration and inhibited the growth of *Staphylococcus aureus* and *E. coli* (6). Man et al (2019) studied the antibacterial activity of six essential oils against some gram-positive and negative bacteria. Their results showed oregano oil had up to 64 times lower MICs/ MBCs than ethylic alcohol (20).

In another study, Becerril et al. investigated the susceptibility of 48 clinical and 12 reference isolates of gram-negative bacilli to oregano and cinnamon EOs and their combination. The results showed that both EOs and their combination inhibited all strains, independently of the antibiotic resistance profile (21). Sienkiewicz et al. (2013) investigated the antibacterial effects of basil (*Ocimum basilicum*) and rosemary (*Rosmarinus officinalis*) essential oils against *E. coli* producing broad-spectrum beta-lactamases. In 18 strains of 22 strains of *E. coli* producing broad-spectrum β-lactamases, the Basil essential oil inhibited the microbial growth at concentrations of 8.25-9.25 µL / mL, and rosemary inhibited the growth of these bacteria at concentrations of 18.0-19.0 µL / mL (22). In this study, all the *bla* CTX-M producing *Escherichia coli* investigated by broth microdilution assay were sensitive to cinnamon and oregano EOs in the range of 400 to 3200 and 800 to 1600ppm respectively. Also, the calculation of the FIC index showed that the combined effect of these two EOs had a synergistic, additive, and ineffective effect on 50%, 40%, and 10% of the samples.

Essential oils have generally strong hydrophobicity making them great alternatives to conventional antibiotics. Their positively charged side chains bind to the negatively charged surface of the bacterial membranes leading to disruption of the bacterial structure and eventually to cell death (23).

It is assumed that due to the antimicrobial effects of phytochemical components of these EOs such as cinnamaldehyde, carvacrol, and thymol, the growth of *bla* CTX-M producing *Escherichia coli* was generally inhibited. Carvacrol and thymol are the major constituents in the oregano EO (24). Thymol is a phenolic monoterpenoid, structurally very similar to carvacrol. Evidence has shown antibacterial activity of thymol and carvacrol, causing structural and functional damages to the cell membrane. Besides interaction with the cytoplasmic membrane, the mechanism of antibacterial activity of thymol and carvacrol is linked to their ability to interact with membrane protein and periplasmic enzymes. The antimicrobial effects observed in cinnamon essential oil are mainly related to cinnamaldehyde, which is one of the most important compounds of cinnamon essential oil.

Table 1. PCR- specific primer used for CTX-Mbla, thermal condition, and the size of product.

Primer	Sequence (5'-3')	PCR condition			Amplicon (bp)	Target gene	Reference
		Denaturing	Annealing	Extension			
CTX-M-1-F CTX-M-1-R	TTA GGA ART GTG CCG CTG YA CGA TAT CGT TGG TRGTRCCAT	94°C, 30 s	50°C, 30 s	72°C, 60 s	688	*Ctx-M -1* *Ctx-M -3* *Ctx-M -15*	2

Table 2. Details on antibiotic used, resistance breakpoint and resistance rates of *Escherichia coli* isolates.

Antimicrobial agent	Disk content	Resistance breakpoint	Rate of resistant (%) (n=93)
chloramphenicol	30 µg	≥12	28
ampicillin	10 µg	≥13	87
cefepime	30 µg	≥18	15
gentamicin	10 µg	≥12	30
tetracycline	30 µg	≥11	91
ciprofloxacin	5 µg	≥15	35

Table 3. MIC of cinnamon and oregano essential oils on *Escherichia coli* isolates (ppm) in separately and combinatorial approaches determined by using micro dilutions method.

Sample Number	Strain Number	Cinnamon EO MIC (ppm)	Oregano EO MIC (ppm)	MIC combination of Cinnamon and Oregano EO (ppm)	FIC index	Combination effect
1	47a	1600	800	400+800	1	Additive
2	34c	400	1600	800+100	0/75	Synergistic
3	33b	1600	1600	800+1600	1/5	Ineffective
4	44c	400	1600	800+200	1	Additive
5	32a	1600	1600	800+800	1	Additive
6	34a	1600	1600	200+800	0/62	Synergistic
7	77b	3200	1600	400+1600	0/75	Synergistic
8	37c	3200	1600	400+1600	0/75	Synergistic
9	32b	1600	800	400+800	1	Additive
10	34b	3200	1600	400+800	0/5	Synergistic

Table 4. The minimum inhibitory concentration (MIC) ranges and MIC50.

Essential oil	MIC_s Range (ppm)	MIC_{50} (ppm)	
		Value	NO
Cinnamon camphora	400- 3200	1600	5
Origanum vulgare	400-3200	1600	8

Several studies indicated that aldehyde groups could join to proteins or DNA structures and disrupt their functions. Consistent with this, cinnamaldehyde can inhibit enzymes such as ATPase and enzymes that are effective in cytokinesis, changes the lipid profile of the microbial membrane, and finally causes inhibition of the growth or death of the microbial cell by binding to Fstz protein and prevention of cell division (11).

Although antimicrobial activity of herbal essential oils is well documented, it is considered that to obtain an optimal antimicrobial effect in the food systems, as proved in the in vitro assay, the high concentration of EOs should be used in food. However, excessive consumption of an EO may have an adverse effect on the organoleptic properties. In addition, it is not economically affordable. A new approach to overcoming these problems is the application of EOs and or their constituents simultaneously. The use of combined essences that have synergistic or additive effects results in, use of fewer concentrations of the same essences alone, and increase the efficacy of EOs in food systems (5). A synergistic effect arises when a mixture of two EOs have a more antimicrobial activity than the total of the individual components. An additive interaction occurs when a blend of two EOs have a combined effect equal to the total of the individual compounds (11).

In the present study, the investigation of minimal inhibitory concentration of EOs by FIC index showed that the combination of EOs of cinnamon and oregano has synergistic or additive interactions on 90% of *Escherichia coli* producing *bla* $_{CTX-M}$. Pei et al. investigated the antibacterial activities of eugenol, cinnamaldehyde, thymol, carvacrol, and their combinations against *E. coli*. The results indicated that treatments with cinnamaldehyde/eugenol, thymol/eugenol, carvacrol/eugenol, and thymol/carvacrol revealed synergistic effects (25). A few reports have been published revealing the mechanism of the combined effect of essential oils or their purified components against microorganisms. (4).

The mechanism of synergistic or additive interactions between essential oils may be due to the improvement of bioavailability or solubility of them in combination with each other or targeting multiple sites in the bacterial cell. Subsequent inhibition of some biochemical pathways, inhibition of protective enzymes, and enhance the uptake of antimicrobial agents by increasing the number, and size of pores created by the binding of some components to proteins in the cell membrane, are also the acceptable hypothesis to explain these effects (5).

Considering the antimicrobial properties of the oregano and cinnamon EOs mentioned above, it can be concluded that additive and synergistic interaction observed in this study are attributed to thymol, carvacrol, and cinnamaldehyde as the main component of oregano and cinnamon. Oregano EO, by affecting the cytoplasmic membrane and increasing the membrane permeability, facilitates the penetration of cinnamon EO into the cytoplasm of bacteria. In other words, the disintegration of the outer membrane by thymol and carvacrol, lead to

increase the uptake of cinnamon. Therefore cinnamaldehyde easily achieves its site of action and inhibits bacterial growth. Additionally, it can be explained by their contribution to the disruption of the membrane and interacting with enzymes involved in the synthesis of ATP (5, 6, 20).

Conclusion

The results of this study indicate that the frequency of ESBL producing *Escherichia coli* in poultry is relatively high, and ARGs, such as CTX-M may be transmitted through the food chain to human beings. Thus, according to the desired antimicrobial effect of oregano and cinnamon singly and in combination, their use is recommended in the food industry. However, further studies are required to investigate the complicated food matrices.

Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors declare no competing financial interest.

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