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Original Article

Evaluation of antibacterial activity of some medicinal plants against isolated *Escherichia coli* from diseased laying hens

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Summary. Plant extracts and their phytochemicals with antibacterial properties can be influential on infection treatment. In the present study, inhibitory activity of aqueous and ethanol 90% extracts of *Zataria multiflora*, *Thymus vulgaris*, *Mentha pulegium*, *Mentha piperita*, *Ocimum basilicum*, *Cassia senna*, and *Carthamus tinctorius* on the growth of *Escherichia coli* isolated from diseased chickens were tested *in vitro* using disk diffusion method. Inhibition zone diameters of bacterium were calculated in the presence of the plants extract. All the ethanol 90% extracts with concentration of 0.2-1.0 mg/mL effectively inhibited the growth of the bacterium, while aqueous extracts showed no activity against *E. coli*. Inhibition zone diameters (IZD) of the bacteria treated with different concentrations of ethanol 90% extracts of the plants raging between 14.6-41.0 mm. *Zataria multiflora* and *M. pulegium* with concentration of 1.0 mg/mL exhibited highest antibacterial activity compared with other plants with IZDs of 41.0 ± 0.0 and 39.0 ± 0.0 mm, respectively. According to the results, the order of inhibitory effect of plants extract with concentration of 1.0 mg/mL against *E. coli* was *Z. multiflora*> *M. pulegium*> *T. vulgaris*> *M. piperita*> *C. angustifolia*> *C. tinctorius*> *O. basilicum*. Our findings revealed that the tested plants could be considered as suitable source of phytochemicals for prevention of *E. coli* growth. The extracts of the examined plants are suitable supplementary alternative for control of *E. coli* in laying hens.

Key words: antibacterial, *E. coli*, *Z. multiflora*, *M. pulegium*

Introduction

One of the commensal bacteria in animal intestine is *Escherichia coli*; however, some strains associated with respiratory, intestinal, urinary infections. Intestine is the most prominent reservoir of avian pathogenic *E. coli* (APEC). Inhalation of feces-contaminated dust is the main route of bacterium entry in the respiratory tract; afterwards, the bacteria colonise in the air sacs and lungs. In the next step of infection, bacteria penetrate to the blood and colonise in the heart, spleen, and liver (1, 2). APEC caused colibacillosis, multiple organ lesions including pericarditis, peritonitis, sacculitis, synovitis, osteomyelitis or yolk sac infection, one of the principal causes of poultry morbidity and mortality worldwide (2). Occurrence of *E. coli* in human and animal waste considered a public health problem, since disposal of the waste in inadequate and potable water cause serious health hazard to people especially infants and elderly. Consumption of contaminated food especially meat dishes evaluated major cause of illness (3, 4) by food-borne pathogens.

Plants with direct antimicrobial activity or resistance modification can overcome increasing incidence of bacteria resistance to conventional antibiotics (5, 6). Alcoholic extract of *Zataria multiflora* inhibits production of verotoxin in *E. coli* (7). In addition, essential oils of *Z. multiflora* and *Thymus vulgaris* suppresses growth of food borne pathogens like *Staphylococcus aureus*, *E. coli*, *Salmonella typhimorium*, *Bacillus cereus* and *Listeria monocytogenes* (8-10). Essential oil components are lipophilic in nature therefore interact with cell membrane leading to substantial morphological damage, changing permeability for cations like Na^+ and K^+ (11). Ethanol extract of *Mentha arvensis* dramatically potentiated effect of gentamycin on *E. coli*, although, the extract has not showed antibacterial activity (12). Antibacterial activity of juice of *Mentha piperita* leaf and stem were examined against several isolated bacteria from different clinical specimens using disk diffusion method. Fresh juice (100 µL) of leave and stem of the plant exhibited antibacterial activity against *E. coli* with inhibition zone diameters (IZDs) of 16.38 ±3.56 and 16.14 ± 3.70 mm, respectively (13). N-hexane extract of *Cassia senna* at concentration of 300 µg/disk moderately inhibited *E.* coli with IZD of 18 mm, which possess less effect in contrast to kanamycin (IZD: 32 mm) (14). The exploration of naturally occurring antimicrobial attracts attention of scientist due to the growing concern of microbial resistance towards conventional antibiotics. The aim of this research work was to find out possible antimicrobial action of aquoeus and ethanol 90% extracts of *Zataria multiflora*, *Thymus vulgaris*, *Mentha pulegium*, *Mentha piperita*, and *Ocimum basilicum*, which all belong to Lamiaceae along with *Cassia senna*, a species of Fabaceae family, with *Carthamus tinctorius*, a flowering plant of Asteraceae on the growth of *E. coli* isolated from diseased chickens *in vitro*.

Materials and Methods

Plant materials

Aerial parts of the plants including *Z. multiflora*, *T. vulgaris*, *M. pulegium*, *M. piperita*, *C. senna*, and *O. basilicum* along with flowers of safflower *C. tinctorius* were collected from Abarkuh city, Yazd province, Iran in spring of 2013. The samples of the plants were cleaned of sand or dust and dried at room temperature (20-22°C) in the shade. Plant materials were powdered using mortar and pestle and extracted with distilled water (DW) and ethanol 90% three times by percolator apparatus each time 48 h (100 g for each). All the extracts were dried in the airflow using table fan. Extracts were consequently dissolved in DW to get different concentrations including 0.2-1.0 mg/mL. In the case of dissolution difficulty Tween 80 (Merck, Germany) was used for better solubility of the ethanol 90% extracts.

Bacterial strain

Escherichia coli strain was obtained from laying hens with colibacillosis symptoms like stunted growth, lameness, inactivity, lack of appetite and water consumption. The strain was isolated from heart blood and cultured in MacConkey agar. The media were incubated at 37°C for 48 h. The smear of lactose fermenting colonies were observed using Zeiss microscope and rod-shaped bacterium were referred to as coliform bacteria, which were used for evaluation of antibacterial activity of the plants extracts (15).

Antibacterial activity test

Antibacterial test were carried out against isolated strain of *E. coli* from diseased hen using disk diffusion method (14). The suspension of the bacterium was prepared in normal saline and the turbidity adjusted to 0.5 McFarland with absorbance of 0.08-0.1 at 600 nm. Sterile cotton swab was used to spread microbial suspension on Mueller-Hinton agar (Merck, Germany). Sterile paper discs (6 mm in diameter) were impregnated with 10 µL of ethanol 90% extracts with concentrations of 0.2-1.0 mg/mL individually and concomitantly were placed on the inoculated plates. All the plates were incubated for 16 h at 37°C. All the tests were performed in triplicate.

Statistical analysis

Growth diameters of the bacteria at different concentrations of all the extracts were compared using SPSS software. All data were expressed as mean ± standard deviation (SD) and statistical significances were assessed by analyzing of variance (ANOVA) along with Duncan post hoc test for multiple comparisons and $p< 0.05$ implies significance.

Results

Using disk diffusion method, inhibition zone diameters of *E. coli* isolated from diseased chicken in the presence of ethanol 90% and aqueous extracts of some medicinal plants were successfully investigated in the present study. The results of this study indicated that all the ethanol 90% extracts significantly inhibited *E. coli* growth with concentrations of 0.2-1.0 mg/mL (Table 1). However, aqueous extracts of the plants did not showed any such inhibition activity against growth of *E. coli* at any concentrations. Inhibition zone diameters of *E. coli* treated with the ethanol 90% extracts (1.0 mg/mL) ranged between 24.0-41.0 mm. The ethanol 90% of *Z. multiflora* with concentrations of 0.8 and 1.0 mg/mL substantially inhibited *E. coli* with no significant difference with IZD values of 40.6 ± 0.5 and 41.0 ± 0.0 mm, respectively. In addition, ethanol 90% extracts of *T. vulgaris*, *M. pulegium*, and *M. piperita* with concentrations of 0.8 and 1.0 mg/mL demonstrated comparable antibacterial activity toward isolated *E. coli* ($p> 0.05$). Highest concentration of *C. angustifolia* and *C. senna* extract with concentration of 1.0 mg/mL was effective against *E. coli* similar to *T. vulgaris* and *M. pulegium* with concentration of 0.4 mg/mL. The concentrations of 0.8 and 1.0 mg/mL of *C. tinctorius* revealed inhibitory activity toward the bacterium with respective IZDs of 25.6 ± 0.5 and 26.0 ± 1.0 mm, same as *Z. multiflora* and *C. angustifolia* with concentrations of 0.2 and 0.8 mg/mL, respectively ($p> 0.05$). Although, the ethanol 90% extract of *O. basilicum* inhibited the growth of *E. coli*, it showed lower activity against isolated bacterium in comparison to the other examined plants ($p< 0.05$). According to the results, the order of inhibitory effect of the plants extract with concentration of 1.0 mg/mL against *E. coli* was *Z. multiflora*> *M. pulegium*> T. *vulgaris*> *M. piperita*> *C. angustifolia*> *C. tinctorius*> *O. basilicum*.

Discussion

The results of the present study indicated that all the concentrations of the ethanol 90% extracts of the plants effectively prohibited growth of *E. coli*. While, aqueous extracts of the plants showed no inhibition activity against the bacterium. Aqueous extracts could not diffuse through the membrane of the bacterium regarding to the hydrophilic nature of their composition and therefore could not interfere and suppress the growth of the bacteria (16). Among the tested extracts, the ethanol 90% extracts of *Z. multiflora* and *M. pulegium* prohibited the bacterium growth stronger than others did. *Mentha piperita* and *T. vulgaris* were placed in the second position of antibacterial activity compared to the other plants. The antibacterial activity of *C. angustifolia*, *C. tinctorius*, and *O. basilicum* toward isolated *E. coli* were decreased sequentially in contrast with each other tested plants. Previous findings mostly

Table 1. Prevalence of overweight and obesity in the examined school children

Plants Name	**Inhibition zone diameter (mm)**				
	Concentrations (mg/mL)				
	0.2	0.4	0.6	0.8	1.0
Z. multiflora	26.6 ± 2.5	33.6 ± 1.1	37.3 ± 0.5	40.6 ± 0.5	41.0 ± 0.0
T. vulgaris	24.0 ± 1.7	32.3 ± 1.1	35.0 ± 0.0	38.7 ± 0.5	38.6 ± 1.1
M. pulegium	29.0 ± 0.0	32.3 ± 0.5	33.6 ± 0.5	38.6 ± 0.5	39.0 ± 0.0
M. piperita	28.3 ± 0.5	30.3 ± 0.5	34.0 ± 1.7	36.6 ± 0.5	37.6 ± 0.5
C. tinctorius	16.0 ± 1.0	17.0 ± 0.0	21.3 ± 0.5	25.6 ± 0.5	26.0 ± 1.0
C. angustifolia	15.3 ± 0.5	16.0 ± 1.0	19.6 ± 1.1	25.3 ± 2.8	31.6 ± 1.1
O. basilicum	14.6 ± 0.5	15.3 ± 0.5	20.3 ± 0.5	21.3 ± 1.5	24.0 ± 0.0

evaluated antibacterial activity of *Z. multiflora* essential oil form different parts of Iran against gram-positive and gram-negative bacteria as well as *E. coli* (17-19). Extracts of the plant revealed weaker anti-bacterial activity comparing to the essential oil. Lipophilic compounds present in the oils cause morphological damage of the cell membrane resulting in permeability change and release of cellular content (11). Antibacterial activity of polar extracts could be regarded to the presence of various types of compounds like flavonoids and phenolic compounds. Flavonoids, phenolic compounds, and rosmarinic acid in the polar extract of *Z. multiflora* may be contributing in the antibacterial activity of the plant (16). Aqueous and alcoholic extracts of *T. vulgaris* were examined against two pathogens, *E. coli* and *Staphylococcus aureus*, in the previous study. Unlike our findings, aqueous extract of *T. vulgaris* showed higher antibacterial activity compare to its alcoholic extract, where IZDs of *S. aureus* and *E. coli* treated with the aqueous extract with concentration of 25 mg/mL were 21 and 15 mm, respectively. Qualitative tests revealed presence of flavonoids, carbohydrates, condensed tannins, catechol, loquanthucyandin, saponins and phenolic acids in the *T. vulgaris* extracts (20). However, the results of earlier study revealed sensitivity of *E. coli* to methanol 80% extract of *T. vulgaris* (21). Combination of methanol extract of *T. vulgaris* and *Pimpinella anisum* enhanced antibacterial activity of the plants against tested pathogenic bacteria. The antibacterial activity of the *T. vulgaris* may regards to the phenolic compounds like carvacrol, thymol, γ-terpinene, and *p*-cymene (22). Dietary treatment of laying hens with 0.1 and 0.5% of *T. vulgaris* significantly improved feed conversion ratios and egg production. Although, the count of coliform in the feces did not differ by any treatments, feeding with 0.1 and 0.5 % of the plant had significantly decreased count of *E. coli* (23). Plant extracts containing carvacrol, cinnamaldhyde and capsaicin reduced total number of *E. coli* count in the intestine of broilers chickens (24). Moreover, mixture of garlic, anise, cinnamon, rosemary, and thyme significantly reduced *E. coli* in the digestive tract of pigs (25). It has been reported that essential oil of *M. pulegium* was active against several bacteria, while methanol extract of the plant was remained inactive. Methanol extract of *M. pulegium* was reach in flavonoids and phenolic compounds (256.2 mg catechin equivalent/100 g, 204.7 gallic acid equivalent/100 g dry weight, respectively) (26). Nafcillin resistant *E. coli* was considerably susceptible to the both methanolic and ethanolic extracts of *M. pulegium* with concentration of 4-24 mg/mL in which IZDs ranged between 12 to 14 mm. While, the results of other experiment showed that methanol extract of *M. pulegium* was not active against *E. coli* (27). Regarding to the different effect of the plant against microorganisms, it can be point out that environmental factors influenced chemical composition of the plants (28). Diverse secondary metabolites were found in *M. pulegium* like tannins, flavonoids, phenolic compounds, resins, pectins, and essential oil that may responsible for antibacterial property of the plant (27, 29, 30). In the previous survey, different extracts of *O. basilicum* demonstrated anti-*Candida* and antibacterial activity with MIC values ranged between 62.5 to 500 µg/mL, while no antifungal property was observed (31). Aqueous extract of *Ocimum gratissimum* were also active against several medicinally important bacteria that cause gastrointestinal disorders like *Aeromonas sobria*, *E. coli*, *Plesiomonas shigelloides*, *Salmonella typhi*, and *Shigella dysenteriae* (32). Rosmarinic acid, ester of caffeic acid, was constitutively found in the most plants of the Labiateae family probably contribute with their antimicrobial activity. The hairy root culture of *O. basilicum* secrete rosmarinic acid in the presence of pathogen attack suggesting that the compound possibly performed microbial inhibition against pathogens. Intercellular pH of the cells changed due to the uptake of rosmarinic acid resulting in cell wall modification and pilferage (33).

Aqueous and methanol extracts of the leaves of *C. angostifolia* from different regions were tested against bacteria and fungi, in which both tested extracts, inhibited the tested microorganisms with concentration of 2000 µg/mL. The IZD of *E. coli* treated with the extracts ranged between 12-14 mm (34). Various anthraquinones and their glycosides presented in *C. angustifolia* including sennosides A, B, C, D, rhein, aloe-emodin, and emodin (35). The previous studies also indicated that aloe-emodin, sennosides A and B inhibited *E. coli* with MIC values of 62.5, 150 and 300 µg/mL, respectively (36, 37). However, the results of another survey indicated that the crude extract of *C.*

angustifolia was more active against several pathogenic fungi than a pure isolated triterpenoid saponin (38).

Antibacterial activity of *C. tinctorius* was previously surveyed against several gram-positive and gram-negative bacteria as well as *E. coli*. The results showed that the extract of the plant inhibited the bacteria with concentration of 1 g/mL (39). However, other studies indicated that growth of *E. coli* did not suppressed with extract of *C. tinctorius* (40). Moreover, the plant extract suppressed growth of other bacteria like *Propionibacterium acnes* and *S. aureus* with concentration more than 5 mg/mL that consider not active against bacteria comparing to other plants (41). Some microorganism including *Bacillus mycoides*, *Bacillus subtilis*, *Bacillus cereus*, *Geotrichum candidum*, *Aspergillus niger*, and *Penicillium expamsum* were found sensitive to aqueous extract of *C. tinctorius* regarding to the water soluble substances (42). Two quinochalcones, precarthamin and carthamin, from different stages of maturity of *C. tinctorius* were tested against *E. coli*, *S. aureus*, *B. cereus*, *Pseudomonas aeruginosa*, and *Candida albicans*. Carthamin revealed antibacterial activity against *E. coli* comparable to the gentamycin, while precarthamin exhibited lower activity. The activity of the both chalcones can be related to their hydrophobicity (43).

Comparison of the results of the present study with previous findings indicates that in some cases our findings are in consistency with them, while in other occasions our results are not in agreement with earlier outcomes. Hence, possible reason for differences in the antibacterial activity of the plants extracts can be due to the fact that growth region and climate conditions affect the physiology of the plants leading to diversity of their active constituents (44). Flavonoids and phenolic compounds especially rosmarinic acid, thymol and carvacrol with solidified antimicrobial activity may responsible for antibacterial activity of the tested plants in Lamiaceae family including *T. vulgaris, Z. multiflora*, *M. pulegium*, and *M. piperita* (22, 33). In addition, anthraquinones and quinochalcones were previously found responsible for antibacterial activity of *C. angustifolia* and *C. tinctorius*, respectively (36, 37, 43). Since, antimicrobial phytochemicals are divided in several categories; mixture of the plants may benefit us with all mentioned secondary metabolites. Our findings revealed that the tested plants are suitable source for control of *E. coli* in laying hens. Moreover, this study can be considered as a preliminary study for surveying potential consumption of the examined plants in laying hens as an alternative control of *E. coli*. Evaluation of synergistic activity of the examined plants in prevention of the bacterium growth *in vitro* and *in vivo* is recommended for the further studies.

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