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Research Article

Investigating the antimicrobial activity of extract prepared by ultrasound against Escherichia coli isolated from poultry stool

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Abstract

Background: The aim of this study was to investigate the antimicrobial activity of *Mentha piperita*, Withania somnifera and *Rosmarinus officinalis* extract on antibiotic resistant *Escherichia coli* isolated from poultry stool in Zabol city.

Materials & Methods: The extract of *Mentha piperita*, Rosmarinus officinalis and *Withania somnifera* was prepared by rotary machine. Escherichia coli strains were isolated from poultry stool samples. Minimum inhibitory concentration and minimum bactericidal concentration were determined by micro dilution method.

Results: The results of this study showed that *Mentha piperita* extract showed the highest inhibitory ratio compared to other extracts. The minimum inhibitory concentration was 3.1 ppm, with 5 strains inhibited at this concentration.

Discussion: The results of this study could be an effective strategy for production and preparation of medicinal plants to help treating *E. coli* infections.

Keywords: *mentha piperita*; rosmarinus officinalis; withania somnifera; antimicrobial activity; escherichia coli

Introduction

In recent decades, antibiotics have been widely used in the treatment of bacterial infections in humans and animals as well as growth promoters in agriculture [1]. Increasing the percentage of antibiotic-resistant bacterial species in different environments can lead to problems in the selective treatment of bacterial infections [2]. The most important reason for the increase in bacterial resistance to antibiotics in Iran is due to the overuse of antibiotics [3]. *Escherichia coli* is one of the most important members of the natural intestinal flora of warm-blood animals. The importance of this bacteria is due to the presence of pathogenic strains that cause intestinal diseases and food poisoning for humans. Among the E. coli strains that cause diarrhea, *E. coli* strains producing shiga toxin (STEC) are distinguished for their ability to cause severe human diseases [4]. Infection with by bacterium can cause gastroenteritis and may be followed by manifestations such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenia purpura (TTP). Most cases of hemorrhagic colitis epidemics and hemolytic uremic syndrome are attributed to O157 STEC strains [5]. Although antibiotic treatment is not required for this bacterium, many strains of this bacteria had shown multiple resistance to various antibiotics, which can be a major health concern [6]. As the rate of resistance of this bacterium is generally increasing, different strains of this bacterium can cause diseases across the globe [7]. Mint species are scattered throughout the world, but especially in Mediterranean areas. Lavender, Thymus, Common sage (*Salvia officinalis*) and rosemary (*Rosmarinus*) are major Mediterranean plant species and are also found in the African and Indian regions [8]. In recent years there has been a great deal of research into the inhibitory effects of natural substances against microorganisms. In this regard, it is essential to use compounds that are non-toxic to humans and have no side effects. *Rosmarinus officinalis* is a plant of the mint family, which is made of durable small shrubs with aromatic leaves and small blue flowers. It blooms in early spring and late winter. The height of this plant varies from 50 cm to 1 m; also has woody stems, permanent bilateral green leaves with narrow, long, pointed, and rough edges [9]. Antimicrobial compounds such as phenolic compounds are abundantly found in it. Rosemary essential oil is used in cosmetics [10]. This plant contains essential oils, oleoresin and tannin. *Rosemary* essential oils include 1 - 8 cineol, pinene, camphor, bornyl acetate, D- limonene, borneol, myrsene, terpineol, camphene, linalool caryophyllene and rosemaren. Other substances in this plant are amirine, epi-α-carnosic, carnosol, cryptothancione, epirozmanol, isorozmanol, napin, ramadial and rosmarinic acid. The leaves contain 0.5 to 2.5% volatile oils. The main constituents of oils include monoterpene α and β pinene hydrocarbons, camphene, limonene, camphor (10-20%), buneol, cyanol, linalool and veronyl. Rosemary actually contains varying amounts of aromatics and volatiles. Flavonoids include diosmetine, diosmin, genoquinone, luteolin, hepsidoline, and apigenin. Other terpenoids found in rosemary include oleanolic acids and ursolic acids and diterpene carnosols. Phenols in

rosemary include caffeic, chlorogenic, labiatic, neochlorogenic and rosmarinic acid. Rosemary contains large amounts of salicylates [11]. In traditional medicine, this herb is used for anti-asthmatic, digestive, sedative, headache relief [12] circulatory disorders, and increased visual acuity, anti-rheumatism and memory stimuli [13]. Various pharmacological effects have been reported for this plant including antioxidant effects, stimulation of nerve growth factor, antimicrobial and antiviral activity [14] and inhibition of hepatotoxicity [15]. Studies in China have shown that extracts of mint plants have strong antimicrobial activities. Peppermint, scientifically named *Mentha pipertia* is a member of Lamiaceae family, and is one of the aromatic herbs that has many medicinal, nutritional, cosmetic, and health benefits [16]. The compounds in this plant have antioxidant, fungicidal and insecticidal properties. Its antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* has been confirmed [17]. Antimicrobial properties of the essential oil of this plant have been studied on a large number of pathogenic microorganisms. According to the results of these studies which conducted on peppermint essential oil, its antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* has been demonstrated. The main constituents of the essential oil of this plant are menthol and menthon and have antioxidant properties and are capable of trapping free radicals. Withania is a plant with the scientific name *Withania somnifera* from the Solanaceae family [18]. This plant grows in Africa, the Mediterranean area and India and is used as an antibacterial, antioxidant, anti-inflammatory and liver diseases [19]. Much of the plant's use is roots, which contain 35 known chemical compounds. The most important of these substances are alkaloids (such as vitamin A, somnifrin, sominin and anferin), steroidal lactones (such as vitanolides and iethaphrines), saponins, and iron. Withania roots are used as a somnifacient, treating hiccups, female disorders, bronchitis, rheumatism, skin infections, and skin diseases. The leaves and seeds of this plant have medicinal use. Fruits contain an enzyme that is used for clotting milk and make cheese [20]. The aim of this study was to investigate the antimicrobial activity of the extract of these herbs on antibiotic resistant Escherichia coli isolated from poultry stool in Zabol city.

Materials & Methods:

Extract preparation

The plant which used in this study was collected from Baluchistan region (Sistan and Baluchestan province) and dried in natural conditions in shade and then crushed. For preparing the extract, 40 grams of dried powder of herb were placed in a half-liter Erlenmeyer flask containing 300 ml of chloroform and extracted for 24 hours at room temperature using a Shaker machine (at 130 rpm) then filtered with Whatman No. 2 paper. The solvent separated from the extract by rotary apparatus using vacuum pump (vacuum distillation). The weights of the extracts were measured and then dissolved in DMSO solvent. They were kept in the refrigerator at 4 ° C until use in antimicrobial tests.

Bacteria strains

Different strains of *Escherichia coli* used in this study were isolated from urine samples of Zahedan patients and cultured on Nutrient *agar* medium. The purified strains were identified on artificial media using genes - specific tests.

Antibiotic activity

Ten pure strains of *Escherichia coli* were examined by Kirby-Bauer antibiogram method and their susceptibility to antibiotics was evaluated. Antibiotics used in this study included gentamicin, amoxiclav, azithromycin, and amikacin (developed by Padtan teb Company). After 24 hours of incubation at 37 ° C, each zone of growth inhibition were measured and the sensitivity and resistance of the strains were determined, then their results compared with standard NCCLS table.

Determination of Minimum Inhibitory Concentration and Minimum Lethal Concentration of Medicinal Plant Extracts:

To determine the minimum inhibitory concentration of the plant extract, 100 microliters of Müller Hinton broth (Merk-German Company) was added to each well of microtiter plate. In the first well, 100 µL of the 20 mg / ml extract was added and after mixing, 100 µL was removed from the first well and added to the second well and continued to the last well. Afterward 10 microliter of each bacterial suspension (cfu = 1/5108 per ml, half McFarland) added to wells contents. DMSO was added (without extract) to the negative control well, then the microtiter plate incubated for 24 h at 37 ° C. The minimum inhibitory concentration was defined as the lowest concentration needed to stop the growth of bacteria at the end of 22 h of incubation. To determine the minimum bactericidal concentration, In the end of 24 h of incubation, 10 microliter of wells contents were cultured on a Nutrient agar medium (Merk-German Company) and the plates were examined for bacterial growth after 24 hours of incubation. The lowest concentration of extract which inhibited 99.9% of bacterial growth was considered as MBC. All antimicrobial tests were repeated at least 3 times.

Results

The results of this study showed that resistance to antibiotics such as ceftazidime (80%), gentamicin (20%), azithromycin (20%) and amoxiclav (10%), while sensitivity to antibiotics such as gentamicin was 70%, amoxiclav (60%), azithromycin (60%) and amikacin (30%).

The results of this study showed that *Rosmarinus officinalis* extract was the most inhibitory. The minimum inhibitory concentration was 3.1 ppm, with 5 strains inhibited at this concentration. The highest inhibitory concentration of *Rosmarinus officinalis* extract was 25 ppm, which is unilaterally inhibited in this concentration (Table1).

The results of this study showed that the lowest concentration of *Mentha piperita* was 3.1 ppm, in which the two strain were inhibited. While the maximum inhibitory concentration was 25 ppm, one side of this concentration was inhibited (Table1). The minimum Bactericidal concentration of *Rosmarinus officinalis* extract was 6.25 mg / ml, which was inhibited by 5 strains. (Table2). The minimum Bactericidal concentration of *Mentha piperita* extract was 3.1 mg / ml, with 2 strain inhibited. (Table2)

	Rosmarinus officinalis MIC	***Mentha piperita MBC***	***Withania somnifera MBC***
1	3.1	6.25	25
2	25	12.5	3.1
3	3.1	12.5	12.5
4	3.1	3.1	6.25
5	6.25	12.5	25
6	6.25	6.25	12.5
7	3.1	3.1	12.5
8	12.5	12.5	50
9	6.25	6.25	12.5
10	3.1	25	12.5

Table 1: The minimum inhibitory concentration of plant extracts against Escherichia coli bacteria (MIC)

	Rosmarinus officinalis MBC	*Mentha piperita MBC*	*Withania somnifera MBC*
1	6.25	12.5	50
2	50	25	6.25
3	6.25	25	25
4	6.25	6.25	12.5
5	12.5	25	50
6	12.5	12.5	25
7	6.25	6.25	25
8	25	25	100
9	12.5	12.5	25
10	6.25	50	25

Table2: The minimum Bactericidal concentration of plant extracts against Escherichia coli bacteria (MBC)

Discussion

In Tahmasebi's study, the contamination of ornamental birds of Yazd with *Escherichia coli* O157: H7 was investigated. This was a cross-sectional descriptive study.180 fecal samples from ornamental birds, 150 fecal samples from canary (Serinus canari) and 30 samples from love birds (Agapornis personata) were collected from different parts of Yazd and bacteriological and PCR methods were used to search for *Escherichia coli* O157: H7. The rate of *E. coli* infection in canary fecal samples was 72.7% (109 of 150), but it was not found in love birds. *Escherichia coli* O157: H7 was not found in any of the Agapornis personata's samples [21].

In a study by Ramey et al., Results showed that migratory birds from Alaska were infected with *Escherichia coli* [22].

In a study by Umar et al., Which examined the antibiotic resistance pattern, the results showed that *Escherichia coli* was resistant to tetracycline (42.6%), sulfonamide (24.5%), ampicillin (22.9%), gentamicin (19.6), nalidixic acid. (18.03%) and streptomycin (16.3%) [23].

In the study by Radhouani et al, the highest resistance to streptomycin and tetracycline was 75% while 60% strains were resistant to ampicillin; E.coli strains antibiotic resistence to Ciprofloxacin, Amikacin, cefozetin , tubramycin and Chloramphenicol was about 41.7-50% [24].

In the study of Ahmadi et al., Interactions showed that at concentrations of 1, 1.2 and 1.4, the inhibitory effect of Lavender on five different bacteria was greater than *Rosemary*. Comparison of mean interactions of inhibition zone diameter between bacteria and concentrations of two plants showed that concentration of 1 essential oil had the greatest inhibitory effect on Proteus mirabilis. Also comparison of different effects of Lavender and Rosemary essential oils on five different bacteria showed that at concentration of 1, 1.2 and 1.4, lavender essential oils had the greatest inhibitory effect on Proteus mirabilis than other bacterias and its inhibitory effect was more than *Rosemary* extract [25].

In the study of khodaei and colleagues who examined the antimicrobial effect of garlic and rosemary, the results showed that at 50% of concentration there was no significant difference between garlic and rosemary essential oils and garlic essential oil showed the least effect. Significant differences were observed between the two essential oils in 10% ($p < 0.002$) and 30% ($p < 0.007$) concentration,which rosemary showed stronger antibacterial effect in both cases. On the other hand, the results of analysis of variance of mean growth zone diameter for different concentrations of each essential oil showed that for rosemary essential oil had a significant difference at concentration of 10% and 50% ($p < 0.01$).

In the case of garlic, there was a significant difference between all three concentrations ($p < 0.01$). Antibacterial activity was increased in both essential oils by increasing the concentration [26].

In another study, Seydim et al. showed the antimicrobial effects of *rosemary* on Gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* [27].

Tsai et al. investigated the effects of ethanolic and aqueous extracts of rosemary extract on Streptococcus subrinus, they have determined the MIC of this plant was 42.1 mg / ml [28].

Rozman investigated the effect of rosemary extract on different Listeria species and MIC ranged from 625 to 5000 µg / ml. It was also found that the resistance of Listeria to rosemary extract depends on the selected extract, Listeria species and different concentrations of extract [29].

In a study by Yazdani that examined the antimicrobial properties of peppermint, menthol, neomentyl acetate, and menthofuran were the most important constituents of peppermint essential oil samples which collected from Marivan, respectively; The essential oil of this plant was effective on Gram-positive bacteria such as *Staphylococcus epidermidis*, *Bacillus subtilis* and *Staphylococcus aureus* and Gram-negative bacteria such as *Shigella dysentery* and *Klebsiella pneumoniae*. Also the essential oil of this plant had a good antioxidant activity compared to BHT standard (Butylated Hydroxyl Toluene) [30].

In the Mimica-Dukic study, menthol was identified as the main ingredient of peppermint essential oil [31].

In a study by Sujana et al., Which investigated the antimicrobial activity of peppermint leaf, the results showed that it is a potent inhibitor of *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli* [32].

In another study, the results showed that aqueous extract of peppermint was an inhibitor of *Pseudomonas aeruginosa* [33].

In a study by Laggoune it was shown that *E. coli* and *Proteus mirabilis* were sensitive to peppermint extract [34]. Dhiman show that acetone, methanol, ethanol and aqueous extracts inhibit *Bacillus cereus* bacteria [35].

In a study by Bokaian et al., Which investigated the antimicrobial activity of Withania extracts on *E.coli*, Antibiotic resistance to antibiotics such as erythromycin was (52.94%), tetracycline (76.47%), ceftazidime (41.17%), cefaxime (35.29%) Ampicillin (58.82%) and nalidixic acid (41.17%) were %). The highest inhibitory concentration was 200 ppm and the lowest was 50 ppm. Three strains were inhibited at this concentration [36].

In a study by Kumar et al., Which investigated the antimicrobial activity of Withania root extract on *Escherichia coli*, the results showed that the greatest inhibition of bacterial growth was at 1: 8 concentration [37].

In the study of Jaina et al., The results showed that methanolic extract of Withania produced an inhibition zone with 38, 36, 15, 38 mm against

Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans [38].

In the study of Aqeel et al., Which investigated the antimicrobial activity of Withania extract on E. coli, the results showed that the inhibitory zone of Withania extract against *Escherichia coli* was 28/15±2/39mm [39].

In the study of Khatak et al., who examined the antimicrobial activity of ethanolic extract of Withania, the results showed that at concentration of 20 mg / ml produced inhibition zone wirh 44, 45 and 42 mm against Pseudomonas aeruginosa, Escherichia coli and Candida albicans [26].

Conclusion

The results of the study showed that the extract of *Withania* plant is a strong inhibitor of Escherichia coli bacteria and can be used in the treatment of poultry infection.

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Footnotes

- **Authors' Contribution:** All authors had an equal role in study design, work, statistical analysis, and manuscript writing.
- **Conflict of Interests:** The authors declare no conflict of interest.
- **Patient Consent:** The written informed consent was obtained.

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