



The Antibacterial Activity of Barberry Root and Fennel Seed Extracts Individually and in Combination with Nisin and Sodium Diacetate Against *Escherichia coli* O157:H7

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Abstract

Background: One of the most important health challenges is to deal with infectious diseases due to their high prevalence. Since resistance of pathogens to antimicrobial compounds is rising, more studies are performed to develop new antimicrobial agents. Herbs are one of the best sources to achieve the goal.

Objectives: The current study aimed at examining the possible antibacterial activity of the barberry root and fennel seed extracts individually and in combination with sodium diacetate and nisin against *Escherichia coli* O157:H7.

Methods: Extract of the barberry root and fennel seed were prepared by soaking in ethanol 80% and analyzed by high-performance thin-layer chromatography and gas chromatography-mass spectrometry, respectively. Antimicrobial activity of the extracts was detected by disk diffusion and broth microdilution methods in triplicate.

Results: Nisin showed the highest antibacterial activity against *E. coli* O157:H7 followed by the extracts of barberry root and fennel seed, and sodium diacetate. The data were consistent with the results of the disk diffusion test. Moreover, a significant difference was observed between antibacterial activity of nisin and sodium diacetate individually, and in combination with the extracts.

Conclusions: The results of the current study showed that the inhibitory effects of sodium diacetate and nisin combined with barberry root and fennel seed extracts were higher compared to their individual effects; it leads to the application of lower amounts of sodium diacetate and nisin in combination with herbal extracts and highly prevents their individual side effects such as toxicity and organoleptic changes.

Keywords: Antibacterial Activity, Barberry Root, Fennel Seed, Nisin, Sodium Diacetate, *Escherichia coli* O157:H7

1. Background

According to evidence of ancient time, early man used herbs as medicine to treat diseases. With the advancement of science, application of chemical compounds drew the attention of researchers to develop medicine, but it did not last long; side effects and inefficiency of such agents made scientists focus on herbal compounds to treat diseases (1). Fighting against infectious diseases is the most important treatment challenge due to their high prevalence. Misuse of antimicrobial drugs led to increased resistance against different antibiotics in most bacteria (2). With increased antimicrobial resistance in most bacteria, researchers performed more studies to develop new antimicrobial agents. Herbs are the best sources to achieve this goal due to being natural, causing lower complications, easy access, and being economical compared to syn-

thetic agents (3, 4). *Foeniculum vulgare* Mill. (*F. vulgare* Mill.) is a perennial flavored herb belonging to the family Umbelliferae (5). The plant has oval or spindle-shaped seeds and studies indicated antimicrobial activity of fennel and spices containing fennel (6, 7). *Berberis vulgaris* (*B. vulgaris*) is a thorny herb belonging to the family Berberidaceae found in different parts of the world. History of the application of this plant as a medicinal herb dates back to 3000 years (8). Due to containing alkaloid compounds, it has significant effects on the treatment of many diseases including leishmaniasis; it also prevents bacterial growth, reduces inflammation, stimulates bile secretion, reduces blood pressure, etc. (8, 9). Following laboratory analyses, various alkaloids of this plant were identified including berberine, oxycontin, jatrorrhizine, berbamine, etc. (8, 10, 11). Nisin is a polypeptide produced by some strains of *Lac-*

tococcus lactis. The World Health Organization (WHO) permitted the application of nisin as a food preservative and it is currently used in more than 50 countries (12). Nisin has inhibitory effects against several bacteria (13). Sodium diacetate is a natural food preservative with the trade name Alvita that is permitted as a food additive by Food and Drug Administration and WHO and no poisonous effect is reported with its permitted values. Sodium diacetate is usually added to food products and uses as a growth inhibitor for molds (14). *Escherichia coli* are considered a poisonous factor in most foods. Several strains of *E. coli* are identified as potential foodborne pathogens of which *E. coli* O157:H7 is one of the most important ones that can cause gastrointestinal symptoms in human. It is a well-known serotype of enterohemorrhagic *E. coli* (EHEC) and the main cause of hemorrhagic colitis and hemolytic uremic syndrome (HUS) in human (15, 16). Occurrence of resistance against chemical microbial agents and their devastating effects paved the way to use herbal medicines as food preservatives or treatment of infectious diseases. On the other hand, their synergistic effects in concomitant use of various antimicrobial agents may reduce the administered doses of drugs and preservatives.

2. Objectives

The current study aimed at investigating the antimicrobial effect of the *F. vulgare* and *B. vulgaris* extracts individually and combined with nisin and sodium diacetate on *E. coli* O157:H7.

3. Methods

3.1. Preparation of Extracts and Chemical Analysis

Barberry root (*B. vulgaris*) and fennel seed (*F. vulgare* Mill.) were collected and the taxonomic identification of plant materials was performed by the Herbarium of Medicinal Plants, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Extracts of the barberry root and fennel seed were prepared by maceration in 80% ethanol (Merck, Germany). The extracts were then condensed and dried by rotary and freeze drying machines, respectively. Fennel seed extract was analyzed by gas chromatography-mass spectrometry (GC-MS) and barberry root extract was analyzed using high-performance thin-layer chromatography (HPTLC) (7, 17). In the current study, silica gel 60 F₂₅₄ was employed for the stationary phase studying and butanol (Merck, Germany): acetic acid (Merck, Germany): water (8:1:1) solvent system for mobile phase. Then to standardize the extract, serial dilution of berberine in methanol solvent was prepared and the absorbance of the solution was recorded at 346 nm wavelength by a spectrophotometer and the calibration curve was drawn. Also, a standard

concentration of the barberry extract in methanol was prepared and its absorption was measured in the calibration curve of berberine (17).

3.2. Bacterial Strain, Media, and Supplements

Lyophilized *E. coli* O157:H7 (ATCC 43895) was purchased from Pasture Institute of Iran (Tehran, Iran). It was cultured at 37°C in brain-heart infusion (BHI) broth (Merck, Germany), and stored at -20°C with 10% glycerol. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) media were obtained from Quelab Chemical Co. (Canada). Nisin (2.5% w/w) and sodium diacetate were purchased from Sigma Co. (the USA). Also, 2.5% pure nisin was suspended at 20 mg/mL in MHB containing 0.02 M HCl for activation (containing 500 µg/mL pure nisin); then it was serially diluted to investigate the antibacterial activity.

3.3. Disk Diffusion Test

Disk diffusion method was employed to examine the antibacterial activity according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (18). For this purpose, 30 µL of each extract (12 mg/disk), nisin (3, 7.5, and 15 µg/disk) and sodium diacetate (12 and 24 mg/disk) were put on standard sterile blank disks (Padtan Teb, Iran) with 6 mm diameter and after drying aseptically, were placed on the inoculated MHA with 0.5 Mcfarland turbidity of the bacterium. Some standard antibiotic disks (i.e., erythromycin, gentamicin, streptomycin, and ciprofloxacin) were employed as positive controls and sterile distilled water as well as the solvent of extracts (Tween 80) as negative controls. Then, the inoculated plates were incubated at 37°C for 24 hours and the growth inhibition zones were measured in millimeter to evaluate antibacterial activity. All the experiments were performed in triplicate.

3.4. Broth Microdilution Test

Broth microdilution method was employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), according to CLSI guidelines in triplicate, with some modifications (19, 20). Bacterial inoculants (5×10^6 CFU/mL) were prepared with two overnight cultures at 37°C for 20 hours; then a serial dilution was prepared to enumerate bacteria and afterwards, each dilution was plated on MHA in triplicate. Broth microdilution was performed in 96-well microplates with consecutive dilutions of the extracts (0.2 to 360 mg/mL), nisin (0.12 to 400 µg/mL), and sodium diacetate (10 to 720 mg/mL) in MHB and then a certain amount of the initial bacteria was inoculated into each well. Firstly, different concentrations of the barberry root and fennel seed extracts were set up in MHB supplemented with 2% Tween 80 (Samchun, South Korea) as the solvent of the extracts. Then, 160 µL of MHB, 20 µL of different concentrations of

the extracts, nisin or sodium diacetate, and 20 μL of the initial bacterial inoculum (5×10^6 CFU/mL) were added into each well to achieve a concentration of 10^5 CFU/well. Negative (uninoculated MHB) and positive (inoculated MHB without the extracts, nisin, or sodium diacetate) control cultures were also designed. Contents of the 96-well microplates were mixed on a plate shaker and incubated at 37°C for 24 hours. The MIC is defined as the lowest concentration that prevents the growth of the bacteria. Therefore, the concentration of the first well without turbidity was considered as MIC. The MBC is the lowest concentration of the solution required to kill bacteria. To determine combination effects, the abovementioned procedure was employed, but 140 μL of MHB, 20 μL of different concentrations of nisin (0.005 - 100 $\mu\text{g}/\text{mL}$), or sodium diacetate (0.25% - 56%) plus 20 μL of equivalent to 1/2 MIC of the extracts and 20 μL of the bacterial inoculum were added to each well.

3.5. Statistical Analysis

All data were expressed as mean \pm standard deviation (SD) and analyzed using SPSS version 16. One-way ANOVA and the Duncan test (P value < 0.05) were employed to examine the effect of various factors on *E. coli* O157:H7.

4. Results

The chemical composition of the barberry root and fennel seed extracts was analyzed by HPTLC and GC-MS. According to the obtained results, the main component was berberine in the barberry root (*B. vulgaris*) extract. Also, bis (2-ethylhexyl) phthalate and trans-anethole were the main components in the fennel seed (*F. vulgare* Mill.) extract. The results of antibacterial activity of the extracts, nisin, and sodium diacetate on *E. coli* O157:H7 based on the disk diffusion test results are presented in Table 1. The antibacterial activity of the extracts (MIC and MBC), individually and combined with nisin and sodium diacetate, based on the results of broth microdilution test are presented in Tables 2 and 3, respectively. Lower amounts of MIC and MBC indicate higher antibacterial activity. Statistical analysis using one-way ANOVA indicated a significant difference between antibacterial activities of the extracts against *E. coli* O157:H7. In comparison of the individual effects of studied factors, nisin showed the highest antibacterial activity followed by barberry root extract, fennel seed extract, and sodium diacetate. The findings conformed to the results of the agar diffusion test. The effect of sodium diacetate combined with the studied extracts on *E. coli* O157:H7 showed significant differences with its individual effect ($P < 0.05$). The combined effect of sodium diacetate and the *B. vulgaris* extract was stronger. Regarding nisin, there was no significant difference between combined effects of nisin and the

extracts in MIC, compared with that of the nisin individually on *E. coli* O157:H7 ($P > 0.05$), but in terms of MBC, a significant difference was observed. It indicated a more significant bactericidal effect of nisin combined with the extracts. The combined effect of nisin and the *B. vulgaris* extract in MIC showed a higher antibacterial activity. In MBC, the combined effect of nisin and fennel was higher on *E. coli* O157:H7 indicating their higher synergistic bactericidal activity, which may be due to the difference in the bactericidal mechanism of the extracts.

Table 1. Comparison of Antibacterial Activity of Studied Compounds and Standard Antibiotics Against *E. coli* O157:H7 Based on the Disk Diffusion Test Results

Treatment	Diameter of Growth Inhibition Zone, mm
<i>Berberis vulgaris</i> 12 mg/disk	15
<i>Foeniculum vulgare</i> 12 mg/disk	12
Nisin, $\mu\text{g}/\text{disk}$	
15	23
7.5	20
3	18
Sodium Diacetate, mg/disk	
24	R
12	R
Erythromycin 15 μg	R
Streptomycin 10 μg	16
Cyproflaxazine 5 μg	34
Gentamicin 10 μg	20
Tween 80 30 μL	R

Abbreviation: R, resistant.

5. Discussion

It is evident that application of medicinal herbs has always been the oldest approach towards treatment of diseases and during the development of human civilization, there was always a close relationship between human and herbs. Since drug resistance and complications related to chemical antibacterial agents are rising, scientists' attentions are drawn towards natural resources including antibacterial activity of herbs. Results of the current study indicated that nisin had the highest antibacterial activity against *E. coli* O157:H7, followed by extracts of *B. vulgaris* and *F. vulgare* Mill, while sodium diacetate had the lowest activity. Also, previous studies showed that *B. vulgaris* and *F. vulgare* Mill had antibacterial activities (21, 22). For example, Dashti et al., in a study on the antibacterial activity of *B. vulgaris* extract against some Gram-positive and -negative bacteria found that *B. vulgaris* has antibacterial activity, particularly against *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *E. coli* (21). Also in a study, antimicrobial activity of some medicinal herbs was investigated on different microbial strains, in which the extract of *F. vulgare* Mill showed a growth inhibitory effect on *Staphylococcus aureus*, *E. coli*,

Table 2. Comparison of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the Extracts, Nisin, and Sodium Diacetate Against *E. coli* O157:H7 Based on Broth Microdilution Test Results^a

Treatment <i>E. coli</i>	Nisin, $\mu\text{g/mL}$	Sodium Diacetate, mg/mL	<i>Berberis vulgaris</i> , mg/mL	<i>Foeniculum vulgare</i> , mg/mL
MIC	0.41 \pm 0.14	200.0 \pm 0.0	40.0 \pm 0.0	93.33 \pm 23.09
MBC	20.0 \pm 0.0	586.6 \pm 46.1	200.0 \pm 0.0	266.67 \pm 23.09

^a Values are expressed as mean \pm SD.

Table 3. Comparison of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the Extracts Combined With Nisin and Sodium Diacetate Against *E. coli* O157:H7 Based on Broth Microdilution Test Results^a

Treatment <i>E. coli</i>	Nisin, $\mu\text{g/mL}$ + 1/2 MIC of <i>Berberis vulgaris</i>	Sodium Diacetate, mg/mL + 1/2 MIC of <i>Berberis vulgaris</i>	Nisin, $\mu\text{g/mL}$ + 1/2 MIC of <i>Foeniculum vulgare</i>	Sodium Diacetate, mg/mL + 1/2 MIC of <i>Foeniculum vulgare</i>
MIC	0.16 \pm 0.07	53.3 \pm 11.5	0.33 \pm 0.14	80 \pm 0.0
MBC	2.08 \pm 0.72	120 \pm 0.0	1.08 \pm 0.14	560 \pm 0.0

^a Values are expressed as mean \pm SD.

and *Salmonella typhi* (23). Moreover, Saeidi et al., indicated antibacterial activity of the ethanolic extract of *F. vulgare* Mill against *S. aureus*, *Bacillus subtilis*, *E. coli* (PTCC:1074), and *S. typhi*. They observed that *F. vulgare* extract has the higher inhibitory effect on *E. coli* (22). In the current study, chemical analysis showed that the main component of the *B. vulgaris* extract is berberine. The antimicrobial activity of the *B. vulgaris* extract could be associated with berberine, although other compounds might be important. The mechanism of action of berberine is related to the inhibition of DNA, RNA, and protein synthesis, destabilization of the structure of bacterial cell membrane, and promotion or inhibition of enzymes activity (24, 25). It seems that bis (2-ethylhexyl) phthalate and trans-anethole, the main components of the *F. vulgare* Mill extract, are predominantly responsible for most of their antibacterial activities and pharmaceutical effects (26-28). Increased application of extracts or essential oils in oral products as antimicrobial agents can change their taste and flavor (29). Thus, preservation by multiple preservatives in fewer amounts is much better than the application of a high amount of one preservative (30). Therefore, to determine the efficiency of natural antibacterial agents, they should be examined individually and in combination with other antimicrobial agents to determine the synergistic effects. In many studies, synergistic effect between extracts, essential oils, and other antimicrobial agents is confirmed. For example, the antimicrobial activity of concomitant use of *Mentha spicata* and nisin against *Listeria monocytogenes* was investigated. The study indicated that their concomitant use led to a significant decrease in the number of bacteria (31). In a study, concomitant use of nisin and sodium diacetate as natural preservatives in French ketchups was investigated and it was found that nisin at 200 and 250 ppm and sodium diacetate at 750 ppm could keep the microbial load of the product within permissible range during preservation pe-

riod and also it could maintain pH at standard levels (32). Also in a research, antimicrobial activity of nisin combined with *Zataria multiflora* extract against *L. monocytogenes* was examined and the results showed that the inhibitory effect of *Z. multiflora* obviously increased when used in combination with nisin (33). Moreover, a synergistic inhibitory effect was observed on *E. coli* O157:H7 when using a combination of monolaurin and spearmint oil (34). In the current research, synergistic inhibitory effect of the extracts combined with nisin or sodium diacetate on *E. coli* O157:H7 was obtained. Therefore, the studied extracts could be recommended, especially in combination with other antimicrobial agents such as nisin and sodium diacetate, as pharmaceutical materials to develop new antimicrobial agents.

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Footnotes

Authors' Contribution: Developing the protocol and writing of the manuscript: Maryam Ekhtelat and Shima Arzani Birghani. Developing the original idea, protocol, abstract, and analyzing data: Maryam Ekhtelat. Developing the protocol: Forough Namjoyan and Abdolghani Ameri.

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