Original Article

marA efflux pump gene expression in *Salmonella* Enteritidis strains treated with *Artemisia tournefortiana* hydroalcoholic extract and comparison with commercial efflux pump inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP)

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Received: 2 April, 2018; Accepted: 24 August, 2018

Abstract

Background: *Salmonella enterica* subsp. *enterica serovar* Enteritidis is a food-borne pathogenic bacterium that has recently become resistant to most quinolone antibiotics. The *MarA* efflux pump plays a significant role in the development of ciprofloxacin resistance in S. Enteritidis strains. The aim of this study was comparative evaluation of anti-efflux activity of *Artemisia tournefortiana* extract and commercial efflux inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on *marA* efflux pump gene expression in S. Enteritidis clinical strains. **Materials and Methods:** In this experimental study, *Artemisia tournefortiana* extract was prepared using maceration method. Subsequently, *MarA* efflux pump was detected in 20 clinical strains of S. Enteritidis via cartwheel and PCR methods. Finally, after treatment of strains with subMIC concentration of extract and 20 µg/L and CCCP, their anti-efflux activity against *MarA* efflux pump was studied using Real Time PCR. **Results:** The results of cartwheel and PCR methods indicated that all of ciprofloxacin resistant strains had *MarA* efflux pump. Subsequently, after treatment of strains with subMIC concentration of extract and CCCP, results show that both component have the ability to inhibit the *MarA* efflux pump, significantly. **Conclusion:** Considering the results of *MarA* efflux inhibition by A. *tournefortiana* and CCCP, it seems that this plant can be used as a potential source of drug use as a suppository pump inhibitor instead of CCCP.

Keywords: *Salmonella* Enteritidis, efflux pump, *MarA*, *Artemisia* tournefortiana, gene expression

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Please cite this article as Khosravani M, Soltan Dallal M M, Norouzi M. marA efflux pump gene expression in Salmonella Enteritidis strains treated with Artemisia tournefortiana hydroalcoholic extract and comparison with commercial efflux pump inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Arch Med Lab Sci. 2018;4(1):10-17.

Introduction

Salmonella enterica subsp. *enterica serovar* Enteritidis is a food-borne pathogenic bacterium that has recently become resistant to most quinolone antibiotics (1). S. Enteritidis strains with high-level drug resistance have caused several problems in treatment so that the unnecessary use of antibiotics is one of the reasons for antibiotic resistance in S.

Enteritidis strains. Ciprofloxacin-resistance rate in S. Enteritidis strains is increasing, which are gradually become resistant to all antibiotics. Resistance to ciprofloxacin has also occurred following the administration of ciprofloxacin to the treatment of salmonelosis, so that there have been cases with 100% resistance to this antibiotic (2). Generally, there are various mechanisms for antibiotic resistance in S. Enteritidis strains, including the inhibition of drug accumulation inside the cell by efflux systems. The efflux pumps extrude toxic compounds such as antibiotics to extracellular, and the presence of efflux pumps is a faculty of this bacterium to become resistant to antibiotics (3). Totally, based on the sequence and similarity of amino acids, bacterial efflux pumps are classified into five main groups. Efflux pumps are clinically related to resistance-nodulation-division (RND) efflux pumps that contribute to the removal of antibiotics by proton-motive force (4). RND efflux system is one of the most important efflux systems in S. Enteritidis strains. *MarA* efflux pump is an important member of this family, and some studies show that the *MarA* efflux pump can bind to certain promoter regions of acrAB efflux pump gene as an important efflux pump of *Salmonella*, increasing the expression of acrAB gene (5). More studies have shown that up-regulation of *marA* gene can cause resistance to fluoroquinolone antibiotics such as ciprofloxacin in clinical strains of S. Enteritidis (6). Nowadays, many researchers are attempting to use alternative methods to inhibit the efflux pumps. Chemical compounds such as Chloronyl Cyanide Chlorophenyl Hydrazine (CCCP) have been used to inhibit the efflux pump activity. These agents block the efflux pump by affecting oxidative phosphorylation and gradient of membrane proton motive-force. Recently, herbal extracts have been used to investigate anti-efflux pump effects (7).

In this research, we have studied for the first time a native Iranian medicinal herb called *Artemisia tournefortiana* from a phytochemical and biological point of view. *Artemisia* belongs to Asteraceae (Compositae) family and in Iran, there are 34 types of *Artemisia* (8). Considering the fact that no study has been conducted on anti-efflux pump activity of *Artemisia* tournefortiana, the aim of this study was to investigate the effect of A. *tournefortiana* extract on gene expression of *MarA* efflux pump in ciprofloxacin resistant S. Enteritidis strains isolated from outbreaks in Iran and to compare it with CCCP as a commercial anti-efflux agent.

Methods

Extraction preparation. *Artemisia tournefortiana* was purchased from Iran's Biological Reserve with Herbarium No. P 1000632 and dry powder dissolved in the solvent (70% ethanol).The solvent was removed by a rotary using vacuum evaporation method.

Total phenolic and flavonoid content measurement. Total phenolic content was measured by a spectrophotometer using the Folin-Ciocalteau reagent (9). In addition, the flavonoid content of the extract was measured by the Council of Europe method (10). It should be noted that these tests were performed in triplicate.

DPPH test. To investigate antioxidant activity of the extract, 2, 2-diphenyl-1-picry-hydrazyl (DPPH) free radical inhibition method was used (11).

Sample collection, culture, and detection of Salmonella Enteritidis isolates. In this experimental study, 1200 clinical samples were collected from clinical feces specimens during a period of approximately 6 months (during 2015-2016). S. Enteritidis strains were identified by microbiological and serological tests. After biochemical tests, serotyping was performed to determine O and H antigens with specific anti-sera for identification of species (Staten Serum Institute, Copenhagen, Denmark).

Antibiotic susceptibility of isolates. After isolation and identification of S. Enteritidis strains, their susceptibility to different antibiotics was investigated using disc diffusion method according to standards of Clinical and Laboratory Standards Institute (CLSI) (12). Sensitivity of S. Enteritidis isolates to ceftazidime (30µg), cefotaxime (30µg), streptomycin (10µg), ceftriaxone (30µg), tetracycline (30µg), trimethoprim/sulfamethoxazole (5µg), amoxicillin (10µg), meropenem (10µg), chloramphenicol (30µg) and imipenem (10µg) (MAST, UK) was examined on Mueller-Hinton agar (Merck, Germany). It should be noted that the S. Enteritidis ATCC 13076 was used as a positive control

(standard strain) for ciprofloxacin resistance (containing *marA* gene) in all experiments. Moreover, ciprofloxacin resistant rate was determined using minimum inhibitory concentration (MIC) method.

Phenotypic study of MarA efflux pump. Ethidium bromide-agar (Cartwheel method) was used for phenotypic study of efflux pump in S. *Enterica* isolates. Firstly, ciprofloxacin-resistant strains of S. Enteritidis were cultured on a line from the center to edge of nutrient agar plates containing different concentrations of ethidium bromide (0.25-2.5 mg/L). The plates were incubated for 24 hours at 37°C and the fluorescence rate of each isolate was measured using Gel doc system. The strains without fluorescence include an efflux pump (13).

DNA Extraction and PCR for marA efflux pump gene. DNA extraction was performed manually based on phenol-chloroform method. PCR reaction was done to examine the presence of *marA* efflux pump gene in ciprofloxacin-resistant isolates of S. Enteritidis. To amplify *marA* gene, PCR reaction was conducted in a final volume of 25µL containing 1 µL of extracted DNA as the template (100 ng), 0.5 µL of forward primer, 0.5µL of reverse primer (0.4 mM), 12.5µL of master mix (1x) (Cinnagen, Iran), and 10.5µl of double distilled water. Furthermore, PCR reaction was performed for *marA* gene using forward primer (GACCCGGACGTTCAAAAACTAT) and reverse primer (TCGCCA TGCATATTGGTGAT) (14) with an appropriate thermal profile over 35 cycles (Table 1).

Table1. The PCR thermal program.

Program	Temperature (ºC)	Time
Initial denaturation	95	5 min
Denaturation	95	30 S
Annealing	55	30 S
Extension	72	30 S
Final extension	72	5 min

Determining minimum inhibitory concentration (MIC) of the hydroalcoholic extract. In order to determination of S. Enteritidis strains susceptibility to A. *tournefortiana* extract, the Minimum Inhibitory Concentration (MIC) method was used. MIC testing was done in triplicate using micro dilution in 96-well plates at 0.97-250µg/mL concentrations (15).

Phenotypic study of active efflux pump. In order to determine efflux pump activity, EtBr solution was poured into wells (2–250µg/ml) and an amount of 5µL of ciprofloxacin-resistant and intermediate S. Enteritidis strains (with 0.5 McFarland concentration) was added to all wells. Subsequently, carbonyl cyanide3- chlorophenyl hydrazine (CCCP) (with a concentration of 20µg/mL) was added as an inhibitor of the efflux pump (16).

marA gene expression analysis in S. Enteritidis isolates. To investigate the expression of *marA* gene in strains treated with extract and commercial CCCP using Real-Time-PCR, the ciprofloxacin-resistant strains were cultured for 24 hours in nutrient broth medium at 37°C in subMIC concentration of the extract and CCCP (20 µg/mL, optimal concentrations to inhibit efflux pumps). Subsequently, RNA extraction was performed using an RNA extraction kit (Cinnagen, Iran) according to the instructions, and cDNA was synthetized by Quanti Tect Reverse Transcription Kit (Takara, Japan). Finally, the concentration of synthesized cDNA was determined by NanoDrop. Quantitative Real-Time-PCR (qRT-PCR) was performed using SYBR green master mix (Ampliqon, Denmark) to evaluate the expression of *marA* gene. The ingredients in final volume of 25µL included 5µL of cDNA (5ng), 0.15 µM of forward and reverse primers, 12.5 µL(1x) of SYBR Green-containing master mix and qPCR reaction was carried out in Corbett device (Australia). The qPCR thermal profile was as follows: 95°C for 5 minutes, 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, which was performed in 40 cycles. Also, the 16S rRNA gene was used as an internal control (17). Finally, the relative expression of *marA* gene was calculated by ΔΔCt method. It should be noted that the primers used in this section were *marA* F 5/-GACCCGGACGTTCAAAAACTAT -3' and *marA* R-5'-TCGCCATGCATATTGGTGAT-3' as well as 16S rRNA F5'- CGTGTTGTGAAATGTTGGGTTAA-3' and 16S rRNA-R5'-CCGCTGGCAACAAAGGATAA -3'.

Statistical analysis. In this study, statistical analysis was done by SPSS software version 21, and Real Time PCR data were analyzed by one way ANOVA. The P<0.05 was considered statistically significant.

Results

Total phenolic test. Gallic acid was used as a standard for the evaluation of phenolic compounds. Absorbance of various concentrations of Gallic acid (25, 50, 75, 100, 125, and 150µg/mL) was determined to draw the calibration curve. The absorbance of samples was read at 765 nm wavelength. Based on the Gallic acid equation (y=0.0067x-0.0194 R2=0.9919), total phenol value based on mg Gallic acid/gr was 5.06 in the hydro alcoholic extract.

Flavonoid content. Based on the standard equation of quercetin (y=0.002x+0.0227, R^2 = 0.9975), the content of flavonoids was 4.7 mg quercetin per gram of hydroalcoholic extract.

DPPH test results. To calculate of IC50 value (the concentration of sample that inhibits 50% of DPPH radicals), a curve was plotted using concentrations of extract and their oxidation inhibition rates and IC50 was calculated. IC50 for hydroalcoholic extracts was 102.5±0.61µg/mL, while, the IC50 value of standard vitamin E was 14.23µg/ml (Figures 1).

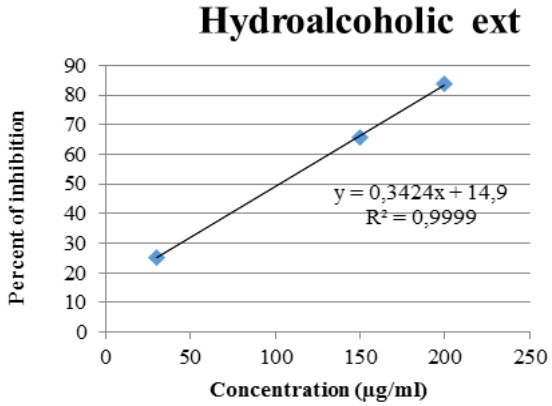

Figure 1. The curve of DPPH radical inhibition by different concentrations of hydroalcoholic extract.

Isolation of S. Enteritidis strains and antibiotic susceptibility test. In this study, out of 1200 fecal samples, 60 S. Enteritidis strains were isolated using microbiological methods. In this study, antimicrobial resistance analysis of S. Enteritidis strains showed the highest resistance to ciprofloxacin (R: 15% and I: 18%) (Table 2). The percentage of antibiotics susceptibility for all tested antibiotics are as follow: Sulfamethoxazole trimethoprim (8%), Amoxicillin (7%), Tetracycline (5%), Streptomycin (3%), Ceftazidim, Ceftriaxone and Cefotaxime (2%). No resistance has been seen for chloramphenicol, imipenem and meropenem (0%).

Table2. MIC of ciprofloxacin, EtBr and EtBr + CCCP in different strains.

Strain NO.	MIC Ciprofloxacin (µg/mL)	Resistance pattern	EtBr (µg/ml)	EtBr + CCCP (µg/ml)
2	0.5	I	125	**62.5**
3	0.5	I	125	**62.5**
4	0.25	I	125	**62.5**
5	1	R	125	**62.5**
6	0.5	I	125	**62.5**
9	1	R	125	**62.5**
13	0.25	I	125	**62.5**
14	0.5	I	125	**62.5**
15	0.25	I	125	**62.5**
16	1	R	125	**62.5**
19	2	R	125	**62.5**
22	0.5	I	125	**62.5**
28	0.5	I	125	**62.5**
31	2	R	62.5	**31/2**
32	1	R	62.5	**31/2**
33	0.5	I	125	**62.5**
38	1	R	125	**62.5**
40	0.25	I	62.5	**31/2**
41	1	R	62.5	**31/2**
66	2	R	62.5	**31/2**
C+	2	R	62.5	**31/2**

Positive control: C+ (Salmonella enteritidis ATCC 13076)

Detection of efflux pump by Cartwheel test. In this study, 20 S. Enteritidis strains were resistant to ciprofloxacin, which were studied by Cartwheel method to investigate the presence of efflux pump. The results of Cartwheel test showed that all ciprofloxacin-resistant strains possessed efflux pump, so that the strains bearing the efflux pump ejected ethidium bromide to the outside, but those lacking the

efflux pump did not have this ability, ethidium bromide entered into their cells, and they showed fluorescence (Figure 2).

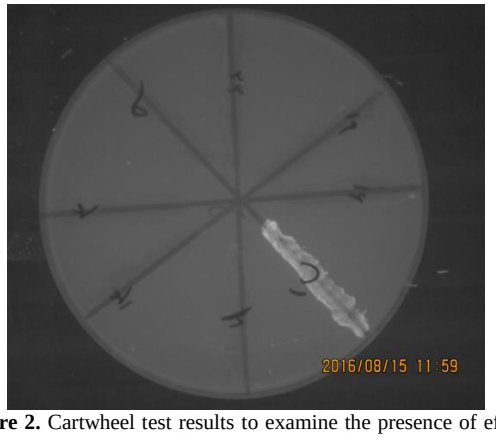

Figure 2. Cartwheel test results to examine the presence of efflux pump. Strains lacking efflux pump were fluorescent but those having the efflux pump did not show fluorescence.

marA gene amplification. In PCR technique, specific primers for *marA* efflux pump gene were used to investigate the presence of this gene in S. Enteritidis isolates, and it was expected to detect a 150 bp band in gel electrophoreses, which was observed in gel electrophoresis (Figure 3). *marA* gene was observed in all the ciprofloxacin-resistant strains (20 samples), and there was a significant correlation between *marA* gene and resistance to ciprofloxacin among the strains (P<0.05).

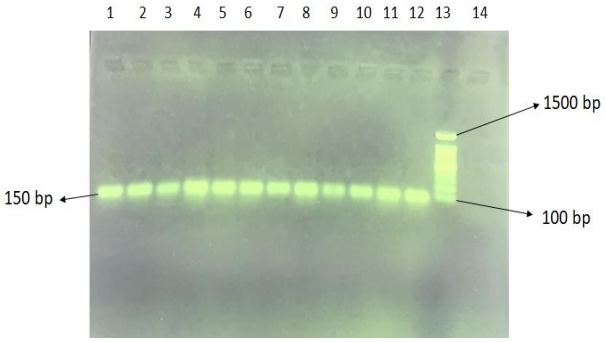

Figure 3. Electrophoresis of marA gene PCR product in different strains. Lane 1 to 11: Positive samples, lane 12: Positive control, Lane 13: DNA Marker (100 bp), Lane 14: Negative control.

MIC test. The results showed that the hydro alcoholic extract had MIC in the range of 1.95-3.9µg/ml (Table 3) and MIC EtBr were in the range of 62.5-125µg/ml and MIC EtBr together with CCCP were in the range of 31.2-62.5µg/ml (Table 2).

Table3. MIC and sub MIC values of A. tournefortiana hydroalcoholic extract in different strains. C+: Positive control.

Strain NO.	MIC (µg/mL)	SubMIC (µg/mL)
2	3.9	1.95
3	3.9	1.95
4	1.95	0.97
5	1.95	0.97
6	1.95	0.97
9	3.9	1.95
13	1.95	0.97
14	3.9	1.95
15	3.9	1.95
16	3.9	1.95
19	3.9	1.95
22	3.9	1.95
28	3.9	1.95
31	3.9	1.95
32	3.9	1.95
33	1.95	0.97
38	1.95	0.97
40	1.95	0.97
41	1.95	0.97
66	3.9	1.95
C+	3.9	1.95

Analysis of marA gene expression in ciprofloxacin-resistant strains. In this study, the relative expression of *marA* efflux pump gene in SubMIC concentration of hydro alcoholic extract as well as a standard 20µg/mL concentration of CCCP were assessed in ciprofloxacin-resistant isolates using Real Time PCR (qRT-PCR). Specific amplification, non-pairing of primers, and lack of non-specific amplification were determined using the melting curve (Figure 4). The results showed that different strains had changing expressions of *marA* under the influence of the extract in subMIC concentration as well as under the effect of CCCP, and there was a significant correlation between the *marA* gene expression and 16S rRNA gene (P<0.05). The results of *marA* gene

expression in ciprofloxacin-resistant strains treated with extract and in ciprofloxacin-resistant strains affected by CCCP are presented in Figure 5.

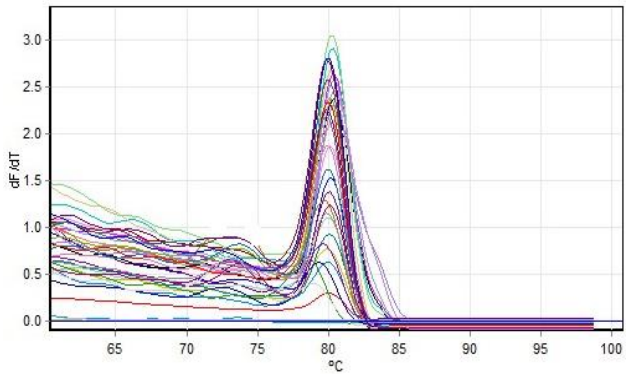

Figure 4. Analysis of marA gene melting curve affected by CCCP.

Also, the results of gene expression showed that inhibition of the *marA* gene in the presence of the extract was similar to CCCP.

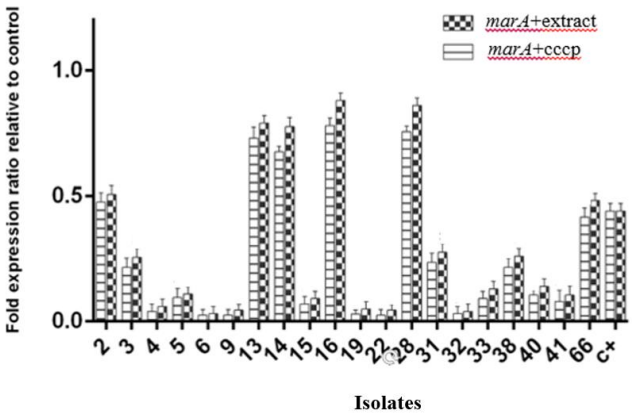

Figure 5. Diagram of marA gene expression in different ciprofloxacin-resistant S. Enteritidis strains treated with extract and CCCP.

Discussion

In recent years, the traditional medicine has become widespread in the world. Today, there are numerous research and clinical centers in developed countries that specifically deal with the treatment of infectious diseases in accordance with the teachings of traditional medicine. The use of natural compounds in nature, including medicinal herbs, is a component of traditional medicine. Acute gastroenteritis due to *Salmonella* infections is one of the most important food-borne infectious and microbial diseases, which accounts for a significant percentage of human infections each year, especially among children and the elderly (18). Resistance to fluoroquinolones, especially ciprofloxacin, is one of the most common and important types of resistance in *Salmonella*, and it seems that detecting and preventing the spread of resistant bacteria reduces the use of drugs and antibiotics. Efflux pumps are the most important resistance mechanisms in ciprofloxacin-resistant S. Enteritidis strains. Antibiotic expulsion systems in S. Enteritidis are of great importance in antibiotic resistance, and the use of efflux pump inhibitors along with antibiotics is an approach to control these bacteria (19). In this study, *Artemisia* tournefortiana, which is a native plant of Iran belonging to Asteraceae family, was used for its anti-efflux pump activity. Subsequently, the antioxidant activity of A. *tournefortiana* extract was determined using DPPH test. DPPH is a stable free radical and used for determination of antioxidant properties of compounds. The IC50 value of A. *tournefortiana* extract was 102.5 µg/mL. Termz and et al 2008. reported the antioxidant activity of A. vulgaris using DPPH assay with IC50=11.4 µg/mL (20). In current study, we evaluated the total phenolic of A. *tournefortiana* based on mg Gallic acid/gr was 5.06 in the hydro alcoholic extract. Sengul and 2011, investigated total phenolic contents of *Artemisia* santonicum extract, the result of this study showed that the total phenolic contents of A. santonicum was 8.86 µg GAE/mg dry weight basis (21). Totally, there was a positive correlation between the total phenolic content and antioxidant activity.

Another objective of this study was to investigate the anti-efflux pump effects of A. *tournefortiana* extract in clinical ciprofloxacin-resistant isolates of *Salmonella* (22). As mentioned, the presence of efflux pumps in S. Enteritidis strains is one of the most important reasons for resistance to antibiotics. In S. Enteritidis bacterium, *MarA* efflux pump plays a significant role in resistance to ciprofloxacin, which is a therapeutic option for S. Enteritidis strains. Overexpression, destruction, and elimination of an efflux pump may affect the resistance to various antibiotics in this bacterium. Purification of the proteins of efflux pump and clinical investigation of the inhibitory effects of various compounds can accurately reflect the anti-efflux pump

effects (23). Researchers are now attempting to find natural compounds to inhibit the efflux pumps in bacteria, so that plant extracts are among the natural choices for controlling the efflux pumps .In this study, S. Enteritidis strains bearing *MarA* efflux pump were first identified using phenotypic method of Cartwheel and genetic method of PCR. In Cartwheel method, strains having the *MarA* efflux pump ejected the substrate (ethidium bromide) outside the cell but those lacking *MarA* pump were not able to pump out ethidium bromide and were fluorescent. Moreover, the strains having *MarA* efflux pump were confirmed using the PCR method. In the next step, after treatment of ciprofloxacin-resistant strains with SubMIC concentration of A. *tournefortiana* extract and 20µg/mL concentration of CCCP efflux pump inhibitor, the expression of *marA* efflux pump gene was also analyzed by Real Time PCR to examine anti-efflux pump effects of the extract. The results showed that *marA* gene expression was significantly reduced compared to 16S rRNA as the reference gene, indicating significant anti-efflux pump effects of the extract. It should be noted that the inhibitory effect of extract on efflux pump was different among different strains, so that some of the strains had a more significant reduction in the expression of *marA* gene in reaction to the extract, and in fact, the extract showed a higher anti-efflux pump effect in them. Totally, there was no significant relationship between extract and CCCP in *marA* gene expression. It seems that compounds with ring structure and functional groups in the extract of this plant possess anti-efflux pump activity. Various studies have been conducted to investigate natural compounds with anti-efflux pump activity and their effect on the expression of efflux pump genes in different bacteria (23). In 2015, Masuria and colleagues studied the anti-efflux pump activity of Acer saccharum Marshall Extract on Escherichia coli ATCC 700928, Proteus mirabilis ATCC HI4320, and Pseudomonas aeruginosa ATCC 15692. The results of this study showed that the extract of this plant inhibited the efflux of ethidium bromide in the three above-mentioned bacteria, which was comparable to the effect of CCCP (24). Jyoti M et al in 2016 evaluated the extracts of five plants of Allium sativum (Amaryllidaceae), Syzygium aromaticum (Myrtaceae), Berberis aristata (Berberidaceae), Rhus cotinus (Anacardiaceae), and Phyllanthus emblica (Phyllanthaceae) in synergy with ciprofloxacin to inhibit the efflux pump of *Salmonella* Typhimurium, which showed that the extracts of these plants had the inhibition ability of efflux pump (25).

Overall, by comparing the results of our study with those of other researchers, it can be concluded that naturally occurring compounds, especially of plant origin, have the potential to inhibit efflux pumps, and it is suggested to study A. *tournefortiana* extract in combination with other antibiotics to treat drug-resistant S. Enteritidis infections.

Conclusion

Regarding the anti-efflux pump effects of A. *tournefortiana* extract and their comparison with commercial CCCP, it is recommended to conduct further studies on biological properties of this plant's compounds in order to further clarify its medical significance to be offered as an inhibitor of efflux pump and an eventual promising drug supplement to clinical centers.

Conflicts of Interest

There is no conflict of interest among authors.

Acknowledgment

This study was supported by Vice-Chancellor for Research grant (no.31320) of Tehran University of Medical Sciences (Tehran, Iran). This study is part of the dissertation of Mrs. Marjan Khosravani in Shiraz Islamic Azad University. We herewith appreciate the contribution of colleagues in Microbiology Laboratory in Faculty of Health and Traditional Medicine Center of Medical University (Ahmadieh Health Center), as well as all those who collaborated in this project.

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