

Original Research

Anti-bacterial effect of essential oil from *Xanthium strumarium* against shiga toxin-producing *Escherichia coli*

J. Sharifi-Rad^{1,2}, L. Soufi³, S. A. M. Ayatollahi^{4,5}, M. Iriti⁶, M. Sharifi-Rad⁷, E. M. Varoni⁸, F. Shahri⁹, S. Esposito¹⁰, K. Kuhestani¹¹, M. Sharifi-Rad^{11*}

¹ Zabol Medicinal Plants Research Center, Zabol University of Medical Sciences, Zabol, Iran

² Department of Pharmacognosy, Faculty of Pharmacy, Zabol University of Medical Sciences, Zabol, Iran

³ Guilan University of Medical Sciences, Rasht, Iran

⁴ Phytochemistry Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁵ Department of Pharmacognosy, School of Pharmacy, Shahid Beheshti University of Medical Sciences Tehran, Iran

⁶ Department of Agricultural and Environmental Sciences, Milan State University, Milan, Italy

⁷ Department of Range and Watershed Management, Faculty of Natural Resources, University of Zabol, Zabol, Iran

⁸ Department of Biomedical, Surgical and Dental Sciences, Milan State University, Milan, Italy

⁹ Department of Optometry, School of Rehabilitation, Zahedan University of Medical Sciences, Zahedan, Iran

¹⁰ Pediatric High Intensive Care Unit, Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

¹¹ Zabol University of Medical Sciences, Zabol, Iran

Abstract: Shiga toxin-producing *Escherichia coli* (STEC) serotype O157:H7 is one of the most important human pathogenic microorganisms, which can cause life-threatening infections. *Xanthium strumarium* L. is a plant with anti-bacterial activity against gram-negative and gram-positive bacteria. This study aims to demonstrate *in vitro* efficacy of the essential oil (EO) extracted from *Xanthium strumarium* L. against *E. coli* O157:H7. Using the agar test diffusion, the effect of *Xanthium strumarium* L. EO (5, 10, 15, 30, 60, and 120 mg/mL) was verified at each of the four different growth phases of *E. coli* O157:H7. Cell counts of viable cells and colony forming unit (CFU) were determined at regular time points using Breed's method and colony counting method, respectively. No viable cell was detectable after the 1 hour-exposure to *X. strumarium* EO at 30, 60, and 120 mg/mL concentrations. No bacterial colony was formed after 1 h until the end of the incubation period at 24 h. At lower concentrations, the number of bacteria cells decreased and colonies could be observed only after incubation. At the exponential phase, the EO at 15 mg/mL was only bacteriostatic, while from 30 mg/mL started to be bactericidal. *X. strumarium* EO antibacterial activity against Shiga toxin-producing *E. coli* O157:H7 is dependent on EO concentration and physiological state of the microorganisms tested. The best inhibitory activity was achieved during the late exponential and the stationary phases.

Key words: Shiga toxin-producing *E. coli* (STEC), *Xanthium strumarium* L., agar diffusion test, medicinal Plant.

Introduction

Escherichia coli is a Gram-negative bacteria, usually living in human and animal gut. Although, most of *E. coli* strains do not cause any diseases, the shiga toxin-producing *E. coli* (STEC) can cause serious and life-threatening infections in humans, including severe dysentery, hemorrhagic colitis, and hemolytic uremic syndrome. Children and elderly adults are at the highest risk of infection, which usually occurs by ingestion of contaminated food or water throughout oro-fecal transmission (1). *E. coli* O157:H7 is the most common, but not the unique, entero-hemorrhagic serotype of STEC, harboring and expressing the genes for Shiga toxin type 1 (Stx1) and 2 (Stx2). Shiga toxin acts against endothelial cells and it is composed by two subunits: B subunit binds a component of the cell membrane to form a complex, which is internalized, then the A subunit, an N-glycosidase, inactivates the RNA of ribosomes, impairing protein biosynthesis and compromising cell survival.

Herbal medicine, ancient medical cure in human history, could be considered one of the former candidates of the modern pharmaceutical, currently contributing to treatment of certain diseases (2-6), though clinical evi-

dence of efficacy is still demanding in the majority of cases. Herbal medicine uses, among the other natural compounds, essential oils (EO), complex mixtures of lipophilic, volatile and aromatic plant secondary metabolites (7, 8). Pre-clinical evidence exists supporting EO activities against a plethora of pathogens, related to clinically relevant diseases (9-14).

Xanthium strumarium L. (common name: cocklebur) is an annual plant belonging to the *Asteraceae*. Different parts of this plant have been proposed as remedies for treating a plethora of infectious diseases because of overwhelming activities including antiviral (15), antistaphylococcal (16), antileishmanial (17), antitrypanosomal (18), antihelmintic (19), anti-inflammatory (20, 21) effects. Antiulcerogenic (22), diuretic (23), and hypoglycemic (24) activities further promoted this plant for potential use in the management of chronic diseases,

Received June 2, 2016; Accepted August 24, 2016; Published September 19, 2016

* Corresponding author: Mehdi Sharifi-Rad, Zabol University of Medical Sciences, Zabol, Iran. E-mail: mehdi_sharifirad@yahoo.com

Copyright: © 2016 by the C.M.B. Association. All rights reserved.

from peptic ulcers to diabetes or arterial hypertension.

This study investigates the anti-shiga toxin activity of *X. strumarium* L. EO against *E. coli* O157:H7, using time kill assay in a batch culture.

Materials and Methods

Plant material

The *Xanthium strumarium* L. leaves were collected between August-September 2013 from area of Hamun Lake of Zabol, Sistan and Baluchestan Province, Iran. The plant was taxonomically identified at the Department of Botany of Shahid Beheshti University of Medical Sciences, Tehran, Iran, where a voucher specimen was conserved.

Essential Oil extraction

Fresh leaves (1 kg) were detached from the stem and dried in the shade for 96 h. Then, they were chopped and hydro-distilled for 3 h utilizing an all-glass Clevenger-type apparatus. The distillate was saturated with sodium chloride (NaCl) (Merck, Darmstadt, Germany) and the *X. strumarium* L. EO was extracted with n-hexane (Merck) and dichloromethane (Merck). The EO obtained was dried over anhydrous sodium sulphate (Sigma-Aldrich, St. Louis, MO, USA) and stored at 4 °C for future assays. Full chemical characterization of the *X. strumarium* L. EO is described in our previous work (13).

Bacterial strains and culture condition

Shiga toxin-producing *E. coli* O157:H7 (Gen-Bank accession number JX161807 and JX161808) was kept on nutrient agar at 4 °C. Before usage, bacteria subcultures were performed thrice on a fresh nutrient agar plate and incubated at 37 °C overnight. Single colonies were transferred to 10 mL of nutrient broth, were grown overnight in this broth at 37 °C with shaking (180 rpm) and bacterial cell suspension were, then, homogenized with mixing. By preparing 10-fold serial dilutions of the suspension which each dilution (0.1 mL) was spread on the surface of nutrient agar plate, the total CFU/mL (\log_{10} CFU/mL) were determined after 24 h incubation at 37 °C (25-27).

Antibacterial activity

The agar diffusion method allowed determining the antibacterial activity of *X. strumarium* L. EO, as recommended by Clinical and Laboratory Standards Institute (28). Briefly, 100 of the bacterial suspension were inoculated on the nutrient agar. The filter discs (Whatmann No. 1) were saturated with 20 μ L of *X. strumarium* EO, then put on the agar plates which had been inoculated with 100 μ L *E. coli* O157:H7. The negative control was a filter disc without any sample and the positive reference was Chloramphenicol disc (30 μ g/disc). The plates were 24 h incubated at 37 °C. Finally, antibacterial activity was determined by measuring the diameter of the growth inhibition zones (mm) compared to the control (29).

Standard growth curve determination of *E. coli* O157:H7

The growth curve was determined according to the

Clinical and Laboratory Standards Institute (28). Briefly, 0.5 mL of *E. coli* O157:H7 suspension was mixed with 49.5 mL nutrient broth in a sterile flask and was shaken to mix well. A 10-fold serial dilution was prepared with sodium chloride 0.85% (w/v) solution (from 10^{-1} till 10^{-10}) and 100 μ L of culture. Then 100 μ L from each dilution were used for direct cell count using Breed's method (a laboratory technique for counting the living and dead bacteria [total number of bacteria] with methylene blue staining) and colony count using the colony counting (a technique for counting the number of colony forming units of bacterial suspension). The flask was incubated 24 h in a shaker at 37 °C, and total cell count and CFU were determined at 1, 4, 8, 12, 16, 20, and 24 h after the incubation. Time-kill curves showed microbial growth depending on both time and EO concentration and it has been recommended to evaluate interaction between bacteria and anti-bacterial agent (30). The killing curves are described as the \log_{10} CFU/mL versus time, up to 24 h (31).

X. strumarium EO effects on growth of *E. coli* O157:H7 in a batch culture

The inhibiting or killing effect of *X. strumarium* EO against *E. coli* O157:H7 was evaluated by measuring the reduction in the CFU/mL at different time of bacteria growth using time-kill assay. Briefly, six concentrations (5, 10, 15, 30, 60, and 120 mg/mL) of *X. strumarium* EO were used to evaluate its anti *E. coli* O157:H7 activity. Then nutrient broth, bacterial culture and *X. strumarium* EO were added, mixed, and incubated in a shaker at 37 °C for a period ranging from 5 minute to 24 h. Colonies formed on the nutrient agar and, then, CFU at different time points were counted. Killing curves were represented by plotting the \log_{10} CFU/mL versus time up to 24 h. All assays were performed in triplicate and repeated for at least thrice (31-33).

X. strumarium EO effects on *E. coli* O157:H7 at different growth phases

Basing on results from the previous screening with agar diffusion test, two concentrations (15 and 30 mg/mL) of *X. strumarium* EO were selected to evaluate the effect of them on *E. coli* O157:H7 at different growth phases (i.e. exponential, late exponential and stationary phase), in a batch culture. Three sets of experiments were performed to verify antibacterial effects during the different growth phase of bacteria. In each experiment, three flasks of each selected concentration (15 and 30 mg/mL) were used. In all of them, at first, nutrient broth and bacterial suspension were mixed in a flask and the initial total number of viable cells and CFUs were determined using Breed's method and colony counting method, respectively. Then, plant EO was added immediately to the desired final concentration of 15 and 30 mg/mL and let in incubation. Total and viable counts were determined at regular time points, after the exposure to EO.

X. strumarium EO effects on *E. coli* O157:H7 at exponential phase

In the first experiment, the total count and viable count were determined at 2 and 4 h. Then plant EO was added immediately to a desired final concentration of 15

and 30 mg/mL. Total count and viable count were then determined at 4, 8, 12, 16, 20 and 24 h.

***X. strumarium* EO effects on *E. coli* O157:H7 at late exponential phase**

In the second experiment, total count and viable count were determined at 4, 6, and 8 h. Then plant EO was added immediately to a desired final concentration of 15 and 30 mg/mL. Total count and viable count were then determined at 10, 12, 16, 20 and 24 h.

***X. strumarium* EO effects on *E. coli* O157:H7 at stationary phase**

In the third experiment, total count and viable count were determined at 16 h. Then plant EO was added immediately to a desired final concentration of 15 and 30 mg/mL. Total count and viable count were then determined at 16, 20, and 24 h.

The bacterial growth reduction assessment was determined by comparing viable cell counts (\log_{10} CFU/mL) between the control and treatments at specific time and to the initial inoculums.

Bactericidal activity was defined as a reduction of 99.9% ($\geq 3 \log_{10}$) of the CFU/mL in the original inoculums. Bacteriostatic activity was defined as maintenance of the original inoculums concentration or a reduction of less than 99.9% ($< 3 \log_{10}$) of the CFU/mL in the original inoculums. We used from two controls (A negative control = (Plant EO + Nutrient broth without the bacterial) (Positive control = Nutrient broth containing bacterial suspension) to ensure from our results validity and to rule out any broth contamination (34).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 11.5, SPSS Inc., Chicago, IL). Statistical significance was determined by Chi square test. Statistical significance was set at $p < 0.05$.

Results

Antibacterial activity

The agar diffusion assay was used to assess antibacterial activity of the *X. strumarium* EO against *E. coli* O157:H7. At the concentration of 20 μ L of *X. strumarium* EO /disc, the diameter of bacterial inhibition zone was 25.5 ± 0.2 mm, near to the chloramphenicol inhibition zone at 30 μ g/disc (26.8 ± 0.1 mm).

***E. coli* O157:H7 standard growth curve**

The pathogen grew logarithmically following a sigmoidal curve as shown in Fig. 1. The four growth phases were defined, i.e. lag phase (0-1 h), exponential phase (1-4 h), late exponential phase (4-8 h) and stationary phase (8-20 h).

***X. strumarium* EO effects on *E. coli* O157:H7 in a batch culture**

The EO concentrations at 30, 60, and 120 mg/mL completely killed all bacteria in one hour of exposure: no bacterial colony could be detected. At lower concentrations (5 and 10, mg/mL) of EO and longer incubation some colonies were observed, though in lesser extent

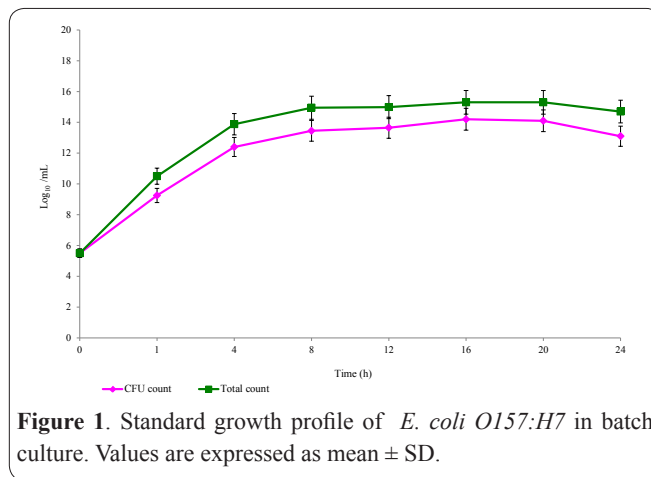


Figure 1. Standard growth profile of *E. coli* O157:H7 in batch culture. Values are expressed as mean \pm SD.

Table 1. Effects of *Xanthium strumarium* L. essential oil on the growth of *E. coli* O157:H7. (+: Bacterial growth, -: No growth).

Concentration (mg/mL)	Exposure Time (min)	Bacterial growth
5	0	+++++
	1	+++
	4	+++
	8	++++
	12	++++
	24	+++++
	Control *	+++++
10	0	+++++
	1	+++
	4	+++
	8	+++
	12	+++++
	24	+++++
	Control	+++++
15	0	+++++
	1	+
	4	+
	8	+
	12	++
	24	+++
	Control	+++++
30	0	+++++
	1	-
	4	-
	8	-
	12	-
	24	-
	Control	+++++
60	0	+++++
	1	-
	4	-
	8	-
	12	-
	24	-
	Control	+++++
120	0	+++++
	1	-
	4	-
	8	-
	12	-
	24	-
	Control	+++++

than the positive control. Based on this result 15 mg/mL and 30 mg/mL were selected for the future study.

***X. strumarium* EO effects on *E. coli* O157:H7 at different growth phases**

Our results showed that *X. strumarium* L. EO at 30

mg/mL killed all bacteria after 1 h, while at 15 mg/mL only decreased the number of bacteria. Therefore, for assessment of EO effectiveness on bacterial growth at different phases in a batch culture, both of these concentrations of EO were used.

***X. strumarium* EO effects on *E. coli* O157:H7 at exponential phase**

By adding 15 mg/mL of the *X. strumarium* EO to an exponential phase culture of the bacteria (after 2 h), the total cell counts and CFU decreased rapidly. A significant difference ($P < 0.05$) was found between the EO treatment and control. In the treated culture CFU began to decrease to zero in 8 h then increased to 10^{11} within 24 h (Fig. 2A). Total count also began to decrease in 1 h till 10^3 at 8 h; after that, it increased up to 10^{12} until the end of the incubation period, at 24 h.

By adding 30 mg/mL of the *X. strumarium* EO, CFU decreased to zero in 4 h then increased to 10^8 in 24 h (Fig. 3A). Overall, the final CFU in EO treated cultures at 30 mg/mL concentration was 3-log lower than in cultures treated with EO at 15 mg/mL. Total

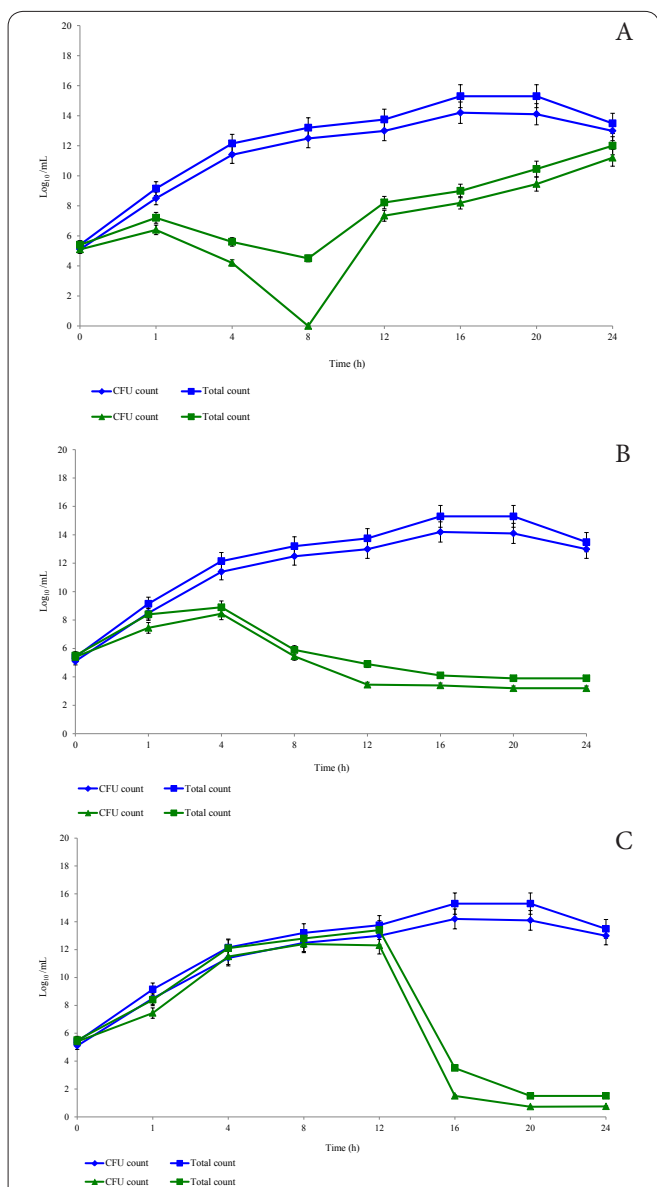


Figure 2. Effects of 15 mg/mL *Xanthium strumarium* L. essential oil on *E. coli* O157:H7 growth at exponential phase (A), late exponential phase (B), and stationary phase (C). Data are presented as the mean ± SD. Values are significantly different ($P < 0.05$).

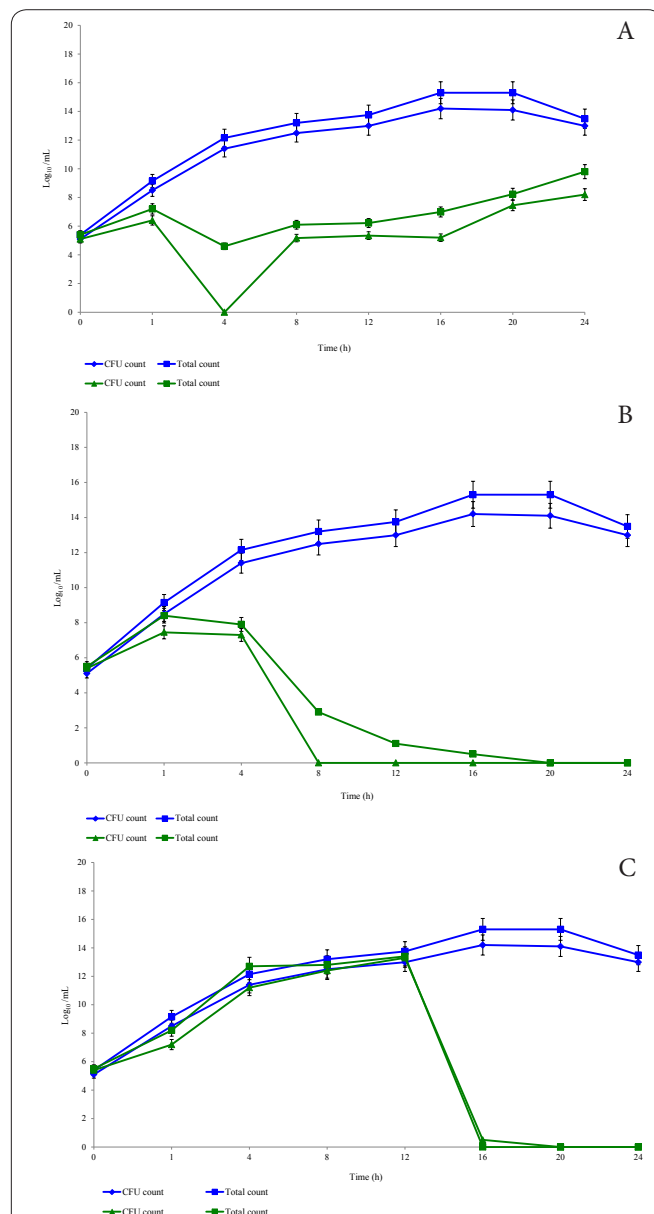


Figure 3. Effects of 30 mg/mL *Xanthium strumarium* L. essential oil on *E. coli* O157:H7 growth at exponential phase (A), late exponential phase (B), and stationary phase (C). Data are presented as the mean ± SD. Values are significantly different ($P < 0.05$). (n=3).

count began to decrease within 1 h till 10^4 at 4 h; then, it increased from 10^4 in 4 h to 10^9 until the end of the incubation period, at 24 h.

***X. strumarium* EO effects on *E. coli* O157:H7 at late exponential phase**

The EO at 15 mg/mL concentration was added during the late exponential phase of bacterial culture. The CFU and total count decreased rapidly to 10^5 in 8 h and 10^3 in 12 h and remained unchanged until the end of the incubation period at 24 h (Fig. 2B). Total count decreased rapidly until 10^6 in 8 h and 10^5 in 12 h, then kept on decreasing slowly up to 10^4 in 16 h. It remained unchanged until the end of the incubation period, at 24 h. After the addition of 30 mg/mL of the EO, both number of cells and CFU decreased. CFU was zero within 8 h and remained unchanged until the end of the incubation period, at 24 h (Fig. 3B). Total count decreased from 10^8 in 1-4 h to 10^3 in 8h, keeping on decreasing till 10^1 in 12 h and, finally, zero in 20 h. It remained unchanged until the end of the incubation period at 24

h. A significant difference ($P < 0.05$) was found between the EO treatment and control.

X. strumarium EO effects on *E. coli* O157:H7 at stationary phase

The EO at 15 mg/mL concentration was added to the bacteria culture, at which time culture was in the stationary phase. Results showed that CFU and total count dropped rapidly. CFU decreased from 10^5 at baseline to 10^{11} in 12h, and 10^2 in 16 h, to further decrease slowly until the end of the incubation period, at 24 h. In addition, total count decreased from 10^5 at baseline to 10^{12} in 12 h. After EO addition it was 10^3 in 16 h, to decrease slowly until the end of the incubation period at 24 h (Fig. 2C). Meanwhile both total counts and CFU were decreased. CFU increased from 10^5 to 10^{11} in 12 h, then decreased nearly to zero in 16h and then slowly to zero until the end of the incubation period at 24 h. Total counts increased from 10^5 to 10^{12} in 12 h, then decreased nearly to zero in 16h and then slowly to zero until the end of the incubation period at 24 h (Fig. 3C). A significant difference ($P < 0.05$) was found between the treatments and controls.

Discussion

Our study showed that *X. strumarium* EO at 30, 60, and 120 mg/mL completely killed the organism in one hour of exposure. No bacterial colony was formed from 1 h up to 24 h, after the exposure, suggesting bactericidal effect of the compound. At lower concentration the number of bacteria was decreased and some colonies were observed after longer incubation, suggesting bacteriostatic effect of the compounds at this concentration. Results of our study showed that the EO of *X. strumarium* at both concentrations (15 and 30 mg/mL) could not completely kill the cells at the exponential phase, and with longer incubation, until the end of the incubation period at 24 h, resistant cells could proliferate. At 30 mg/mL EO could completely kill cells at the late exponential and stationary phases. Therefore, data confirmed the EO at 15 mg/mL was bacteriostatic, while at 30 mg/mL was bactericidal against *E. coli* O157:H7.

Yakob (27) studied the effect of *Ocimum basilicum* EO against anti-shiga toxin producing *E. coli* O157:h7. Our data are consistent with those on *O. basilicum* EO: Yakob showed that the latter could completely kill cells at the late exponential and stationary phases. McKay and Portnoy (35) also demonstrated that stationary-phase of bacteria is adequate phase for response to aminoglycosides. Dhanya *et al.* (36) in a study on nimbolide from *Azadirachta indica* and its derivatives plus first-generation cephalosporin antibiotics described that significant reduction in the number of CFUs in the time kill assay was between 4 and 12 h. Also, in a study on the resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime, Authors showed that during the exponential phase of bacterial growth up to the stationary phase, the resistance of bacteria decreased and antibacterial effect of these drugs increased. This may be due to availability and consumption rate of nutrient and oxygen and density of bacteria population (37). Flandrois *et al.*, (38) research on early stages of in vitro killing curve of LY 146032 and

vancomycin for *Staphylococcus aureus* demonstrated a decrease of the bacterial population from 10^5 to 10^4 CFU within 8 h with vancomycin. This is approximately the same as observed in previous studies on *S. aureus*. In their study, the killing of bacteria by LY146032 increased from early stages of the bacterial growth up to the older phases. In addition, killing of bacteria was faster at the higher concentrations (34).

Nonetheless, the time killing curves of Burt and Reinders (29) in a study on antibacterial activity of selected plant EOs against *E. coli* O157:H7 were similar to ours.

Our study showed that the *X. strumarium* EO at 15 mg/mL was only bacteriostatic, while at 30 mg/mL was bactericidal. The activity of the fraction is dependent on plant EO concentration and physiological state of the organism. It has been observed the best inhibition effect in late exponential phase and stationary phase during the growth curve of bacteria. Therefore, the antibacterial properties of the EO were more effective towards the older culture of *E. coli* O157:H7, which are in active dividing phase. Finally, the *X. strumarium* EO was effective against anti-shiga toxin of *E. coli* O157:H7 and it could be further explored as natural antibacterial drug.

Acknowledgements

Proficient Iran-Italy collaboration.

References

- Hunt JM. Shiga Toxin-Producing *Escherichia coli* (STEC). Clin Lab Med 2010; 30:21–45.
- Sharifi-Rad J, Hoseini-Alfatemi SM, Sharifi-Rad M, Miri A, Sharifi-Rad M. Phytochemical screening and antibacterial activity of *Prosopis farcta* different parts extracts against methicillin-resistant *Staphylococcus aureus* (MRSA). Minerva Biotechnol 2014; 26(4):287-93.
- Sahraie-Rad M, Izadyari A, Rakizadeh S, Sharifi-Rad J. Preparation of strong antidandruff shampoo using medicinal plant extracts: a clinical trial and chronic dandruff treatment. Jundishapur J Nat Pharm Prod 2015; 10(4):e21517.
- Raeisi S, Sharifi-Rad M, Young Quek S, Shabanpour B, Sharifi-Rad J. Evaluation of antioxidant and antimicrobial effects of shallot (*Allium ascalonicum* L.) fruit and ajwain (*Trachyspermum ammi* (L.) Sprague) seed extracts in semi-fried coated rainbow trout (*Oncorhynchus mykiss*) fillets for shelf-life extension. Food Sci Technol 2016; 65:112-121.
- Sharifi-Rad M, Tayeboon GS, Miri A, Sharifi-Rad M, Setzer WN, Fallah F, *et al.* Mutagenic, antimutagenic, antioxidant, anti-lipoxygenase and antimicrobial activities of *Scandix pecten-veneris* L. Cell Mol Biol 2016; 62(6):8-16.
- Sharifi-Rad M, Tayeboon GS, Sharifi-Rad J, Iriti M, Varoni EM, Razazi S. Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of *Veronica persica* phenolic-rich extracts. Cell Mol Biol 2016; 62(6):80-85.
- Sharifi-Rad J, Sharifi-Rad M, Hoseini-Alfatemi SM, Iriti M, Sharifi-Rad M, Sharifi-Rad M. Composition, cytotoxic and antimicrobial activities of *Satureja intermedia* CA Mey essential oil. Int J Mol Sci 2015; 16(8):17812-17825.
- Abdolshahi A, Majd MH, Rad JS, Taheri M, Shabani A, Teixeira da Silva JA. Choice of solvent extraction technique affects fatty acid composition of pistachio (*Pistacia vera* L.) oil. J Food Sci Technol 2015; 52(4):2422-2427.
- Reichling J, Schnitzler P, Suschke U, Saller R. Essential oils of

aromatic plants with antibacterial, antifungal, antiviral, and cytotoxic properties-An overview. *Forsch Komplementmed* 2009; 16:79-90.

10. Upadhyay RK, Dwivedi P, Ahmad S. Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains. *Asian J Med Sci* 2010; 2:152-158.

11. Sharifi-Rad J, Miri A, Hoseini-Alfatemi SM, Sharifi-Rad M, Setzer WN, Hadjiakhoondi A. Chemical Composition and Biological Activity of *Pulicaria vulgaris* Essential Oil from Iran. *Nat Prod Commun* 2014; 9(11):1633-1636.

12. Hoseini Alfatemi SM, Sharifi Rad J, Sharifi Rad M, Mohsenzadeh S, Teixeira da Silva JA. Chemical composition, antioxidant activity and in vitro antibacterial activity of *Achillea wilhelmsii* C. Koch essential oil on methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* spp. *3 Biotech* 2015; 5(1):39-44

13. Sharifi-Rad J, Hoseini-Alfatemi SM, Sharifi-Rad M, Sharifi-Rad M, Iriti M, Sharifi-Rad M, *et al.* Phytochemical compositions and biological activities of essential oil from *Xanthium strumarium* L. *Molecules* 2015; 20(4):7034-7047.

14. Sharifi-Rad J, Hoseini-Alfatemi SM, Sharifi-Rad M, Setzer WN. Chemical composition, antifungal and antibacterial activities of essential oil from *Lallemantia royleana* (Benth. In Wall.) Benth. *J Food Safet* 2015; 35(1):19-25.

15. Mandal SC, Dhara AK, Kumar CKA, Maiti BC. Neuropharmacological activity of *Xanthium strumarium* Linn. extract. *J Herbs Spices Med Plants* 2001; 8:69-77.

16. Rad JS, Alfatemi SMH, Rad MS, Iriti M. *In-vitro* antioxidant and antibacterial activities of *Xanthium strumarium* L. extracts on methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. *Ancient Sci Life* 2013; 33:107-111.

17. Lavault M, Landreau A, Larcher G, Bouchara JP, Pagniez F, *et al.* Antileishmanial and antifungal activities of xanthanolides isolated from *Xanthium macrocarpum*. *Fitoterapia* 2005; 76:363-366.

18. Talakal TS, Dwivedi SK, Sharma SR. In vitro and in vivo antitrypanosomal activity of *Xanthium strumarium* leaves. *J Ethnopharmacol* 1995; 49:14-15.

19. Sharma SR, Singh D, Khan FA, Swarankar CP, Bhagwan PSK. Anthelmintic activity of *Xanthium strumarium* against *Haemonchus contortus* infection in sheep. *Indian J Anim Sci* 2003; 73:342-344.

20. Kim IT, Park YM, Won JH, Jung HJ, Park HJ, Choi JW, Lee KT. Methanol extract of *Xanthium strumarium* L. possesses anti-inflammatory and anti-nociceptive activities. *Biol Pharm Bull* 2005; 28:94-100.

21. Anjoo K, Kumar SA. Phytopharmacological review of *Xanthium strumarium* L. (Cocklebur). *J Pharm Pract Res* 2010; 4:129-139.

22. Favier LS, María AOM, Wendel GH, Borkowski EJ, Giordano OS, *et al.* Anti-ulcerogenic activity of xanthanolide sesquiterpenes from *Xanthium cavanillesii* in rats. *J Ethnopharmacol* 2005; 100:260-267.

23. Nieves JL, Padilla L, Del Carmen M, Rodríguez HR, Simón GG, Freixas GLC. Efecto diurético del *Xanthium strumarium* L. (Guizajo de Caballo). *Rev. Cubana. Plant Med* 1999; 1: 22-25.

24. Hsu FL, Chen YC, Cheng JT. Caffeic acid as active principle

from the fruit of *Xanthium strumarium* to lower plasma glucose in diabetic rats. *Planta Med* 2000; 66:228-230.

25. Gehring AG, Irwin PL, Reed SA, Tu SI, Andreotti PE, Akhavan-Tafti H, *et al.* Enzyme-linked immunomagnetic chemiluminescent detection of *Escherichia coli* O157:H7B. *J Immunol Methods* 2004; 293(1-2):97-106.

26. Mei GY, Tang J, Carey C, Bach S, Kostrzynska M. The effect of oxidative stress on gene expression of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and non-O157 serotypes. *Int J Food Microbiol* 2015; 215:7-15.

27. Yakob HK. Anti-Shiga Toxin Producing *Escherichia coli* O157:H7 Effect of *Ocimum basilicum* L. Essential Oil Analyzed Using Time Kill Assay in a Batch Culture. *Br Microbiol Res J* 2015; 8(6):624-634.

28. Wayne PA. Clinical and Laboratory Standards Institute, CL-SIM7-A7. 7th ed. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 2006; 22(15):1-30.

29. Burt SA, Reinders RD. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. *Lett Appl Microbiol* 2003; 36:162-167.

30. Mueller M, de la Peña A, Derendorf H. Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC. *Antimicrob Agents Chemother* 2004; 48(2):369-77.

31. Wei GX, Campagna AN, Bobek LA. Effect of MUC7 peptides on the growth of bacteria and on *Streptococcus mutans* biofilm. *J Antimicrob Chemother* 2006; 57:1100-1109.

32. Rukholm G, Mugabe C, Azghani AO, Omri A. Antibacterial activity of liposomal gentamicin against *Pseudomonas aeruginosa*: a time-kill study. *Int J Antimicrob Agents*. 2006; 27(3):247-52.

33. Hassan IJ, Stark RM, Greenman J, Millar MR. Activities of beta-Lactams and Macrolides against *Helicobacter pylori*. *Antimicrob Agents Chemother* 1999; 43(6):1387-1392.

34. Jothy SL, Zakariah Z, Chen Y, Sasidharan S. In Vitro, in Situ and in Vivo Studies on the Anticandidal Activity of *Cassia fistula* Seed Extract. *Molecules* 2012; 17:6997-7009.

35. McKay SL, Portnoy DA. Ribosome hibernation facilitates tolerance of stationary-phase bacteria to aminoglycosides. *Antimicrob Agents Chemother* 2015; 59:6992-6999.

36. Dhanya SR, Kumar SN, Sankar V, Raghu KG, Dileep Kumar BS, *et al.* Nimbolide from *Azadirachta indica* and its derivatives plus first-generation cephalosporin antibiotics: a novel drug combination for wound-infecting pathogens, *RSC Adv* 2015; 5:89503-89514.

37. Desai M, Buhler T, Weller PH, Brown MRW. Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during the exponential phase. *J Antimicrob Chemother* 1998; 42:153-160.

38. Flandrois JP, Fardel G, Carret G. Early Stages of In Vitro Killing Curve of LY146032 and Vancomycin for *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1988; 32(4):454-457.