Received: 19 November 2020 | Revised: 23 January 2021 | Accepted: 31 January 2021

DOI: 10.1111/jfs.12891

ORIGINAL ARTICLE

Journal of Food Safety WILEY

In vitro investigation of chemical composition and antibacterial activity of alcoholic, hydroalcoholic extracts, and essential oil of *Spinacia oleracea* leaves from Iran

Seyed Ali Issazadeh[1] | Samaneh Hatami[2] | Masoud Yavarmanesh[2]

[1]Department of Food Science & Microbiology, Quchan Branch, Islamic Azad University, Quchan, Iran

[2]Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

Correspondence
Seyed Ali Issazadeh, Department of Food Science & Microbiology, Quchan Branch, Islamic Azad University, Quchan, Iran.
Email: s.a.isazadeh@gmail.com

Abstract

This study is to investigate the antibacterial activity of the alcoholic and hydroalcoholic extracts of *Spinacia oleracea* leaves, on *Listeria monocytogenes* (ATCC 7644), *Salmonella enteritidis* (ATCC 13076), *Escherichia coli O157:H7* (NCTC 12900) and *Pseudomonas aeruginosa* (ATCC 15442), and to identify bioactive functional components including essential oil by GC–MS. Microbial analyses, including disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) were conducted, whose results were compared with some specific antibiotics. The results of the MIC and MBC analyses indicated that *S. enteritidis* and *L. monocytogenes* were more sensitive to the alcoholic extract than *P. aeruginosa* and *E. coli*. However, the hydroalcoholic extract showed no MIC and MBC for the tested pathogens. In the disc diffusion method, the alcoholic extract had a great effect on *L. monocytogenes*. The GC–MS analysis showed phenolic compounds (9.345%), unsaturated and saturated fatty acids (22.901%), alkaloids, and terpenes (58.57%), amino acids (6.279%), squalene (0.233%), and other compounds in the alcoholic extract of Spinach, which has functional roles in biological activities. Furthermore, the most important compounds of the essential oil of the Iranian spinach were durohydroquinone (34.73%), α-terpineol (12.98%), linalool (22.69%), and cineole (13.1%). According to the results, the alcoholic extract and the essential oil of *S. oleracea* leaves can be a good alternative for antibiotics and can be used in foods and animal feed as a preservative.

1 | INTRODUCTION

Medicinal plants have been used for several thousand years to cure different diseases. The recovering and restorative properties of different kinds of medicinal plants are significantly impressive and well known, Such plants are great sources of biological active molecules. Photochemical analysis has found several chemical compounds in plants, among which are flavonoids, tannins, alkaloids, saponins, glycosides, steroids, terpenoids, oils, proteins, and polysaccharides (Gamble, 1921). Each of these compounds has antibiotic and medicinal properties. Spinach is an important and nutritious member of the plants family. It is a green leafy plant that originates from the southwestern regions of Asia, especially Iran. The plant has been discovered about 2000 years ago and it currently grows in most regions of the world. Spinach is scientifically named *Spinacia oleracea*, which is often consumed as a food resource. Moreover, it has great medicinal values (Das & Guha, 2008). The plant can strengthen the body immune system (Akasaka et al., 2016). (Gaikwad, Shete, & Otari, 2010) studied at least 13 flavonoid compounds showing antioxidant and anticancer properties. By investigating the influence of the spinach extract on the cancer cells, these researchers claimed that the plant compounds slowed down the rate of the cancerous cell division in stomach. They also discovered some glycerolipids in the alcoholic extract of spinach that could inhibit the activity of α-polymerase in

the cancer cells in human and other mammals as well as preventing the proliferation of tumors (Gaikwad et al., 2010).

In late 1980s, a group of women in New Zealand were investigated and it was realized that the consumption of spinach was influential in slowing down the rate of breast-cancer cells division (Longnecker, Newcomb, Mittendorf, Greenberg, & Willett, 1997).

There are several gram-positive and gram-negative bacteria that cause dangerous and fatal diseases in humans and animals, among which *Escherichia coli* O157:H7 can be mentioned that produces enterotoxin and exotoxin which are two toxins secreted by pathogens, common in humans and animals, that cause dysentery (infectious diarrhea) and hemolytic-uremic syndrome in human. *E. coli* is the cause of a great portion of death in infants. This bacterium lives in the intestine of cow and is transferred to human through contaminated water and food in addition to raw milk (Karch, Tarr, & Bielaszewska, 2005). Being infected by this pathogenic bacterium may lead to dysentery and kidney disability. Several reports suggest that *E. coli* can kill old people, children under five and those with weak immune systems (Tamparo, 2011). Gastroenteritis is the most common and widespread *Salmonella*-based infection in human that is caused by the serotypes of *Salmonella*, including *Salmonella typhimurium* and *Salmonella enteritidis*. The main reason of gastroenteritis is the consumption of raw and under-cooked foods, especially meat, poultry, egg and their derivatives, in addition to eating in restaurants (Louis et al., 1988; Rabsch, Tschäpe, & Bäumler, 2001). *Pseudomonas aeruginosa* is one of the major pathogens that can cause folliculitis, otitis externa; eye Infection, post-trauma Infection, endocarditis, and respiratory tract infection. If the normal flora of body is destroyed due to the consumption of antibiotics, the serotypes of *Salmonella* will grow and distract the treatment process (Forbes, Sahm, & Weissfeld, 1998).

Listeria monocytogenes is the main cause of Listeriosis which is common between human and animal. In nonpregnant mature women, the bacterium may bring about Initial meningitis, encephalitis or toxic sepsis. However, older or prone individuals as well as those with weak immune systems (like receivers of organ-transplant, lymphoma patients and HIV-infected individuals) are more vulnerable to the above-mentioned diseases. *L. monocytogenes* tends to affect the central nervous system and causes severe illnesses which are highly fatal. Contaminated food may lead to 30–40% death in normal people and can also kill up to 75% of prone individuals (Jackson, Iwamoto, & Swerdlow, 2010).

According to a study carried out by Sun, Yan, Wang, and Wei (2017), it was shown that the antibacterial activity of the spinach polyphenols against gram-negative bacteria was stronger than that against the gram-positive ones. In addition, the effects of pH, temperature and NaCl concentration on the antibacterial stability of the spinach polyphenols were also discussed (Sun et al., 2017).

Adapa, Sushanth, Prashant, and Mohamed (2018) investigated the influence of the alcoholic extract of spinach on mutated *Staphylococcus* (an important cause of tooth decay) and *Lactobacillus acidophilus*. They found out that the alcoholic extract of spinach had a greater inhibitory effect on the latter (Adapa et al., 2018).

Jaime et al. (2015) also extracted the spinach essential oil using the solvent soluble method and supercritical CO_2 Higher concentrations of carotenoids and lower contents of phenolic compounds were observed in the supercritical CO_2 extracts, whereas the aqueous and/or ethanol PLE[1] extracts presented lower amounts of carotenoids and higher concentrations of phenolic compounds (Jaime et al., 2015).

The diseases caused by contaminated food are really important issues threatening public health. The unfavorable consequences of some antibiotics, increase in the resistance of bacteria due to the consumption of such drugs and the life-threatening dangers of contaminated foodborne diseases are the reasons for natural compounds to be used as antimicrobial agents. It seems that using such compounds is an influential method in controlling pathogenic bacteria and extending the shelf-life of processed foods. Herbal essential oils and extracts obtained from aromatic plants have antibacterial, antifungal, antioxidant and anticancer properties. They can also inhibit the growth of pathogens and the production of toxin by microorganisms (Das & Chatterjee, 2013).

Since most consumers do not trust the safety of the foods with synthetic preservatives, they tend to consume natural foods in which some alternatives to chemical preservative compounds are used. Therefore, the main purpose of this study was to investigate the antibacterial impact of the alcoholic and hydroalcoholic extracts and the essential oil of spinach on four dangerous pathogens, spread between humans and animals. Moreover, the herbal extracts were compared with three prevalent antibiotics, including gentamicin, erythromycin and chloramphenicol.

2 | MATERIALS AND METHODS

2.1 | Collection of microorganisms

Pathogenic microorganisms, including *L. monocytogenes* (ATCC 7644), *S. enteritidis* (ATCC13076), *E. coli O157:H7* (NCTC 12900), and *P. aeruginosa* (ATCC 15442) were obtained from bacteriology department, Ferdowsi University of Mashhad, Iran. To determine the bacterial concentration, optical density, as the indicator of the bacterial growth, was determined using a spectrophotometer. The standard solutions of the microbial suspensions with an optical density of 0.5 McFarland were provided (Agrawal, Kotagiri, & Chaitanya Kolluru, 2018).

2.2 | Collection and preparation of *S. oleracea* leaves

Spinach leaves were collected from Iran (Khorasan Razavi, Mashhad) with herbarium code of 1076 in spring and their scientific name was verified by Institute of Botany of Ferdowsi University of Mashhad.

2.3 | Alcoholic and hydroalcoholic extraction of *S. oleracea* leaves

The alcoholic and hydroalcoholic extracts were prepared using the same amount of spinach powder. To that end, the plant was washed

with tap water and then dried, powdered and stored in the dark. About 5 g of the powdered spinach were extracted by180 ml of alcohol 96% through the automatic Soxhlet method. In the case of the hydroalcoholic extract, 5 g of the powder were extracted by 90 ml of alcohol 96% and 90 ml of distilled water using the same method. The collected solvent was then evaporated to dryness with a rotary vacuum evaporator (model Strike 202) at 40°C to afford a thick residue. In order to determine the initial concentration, the dry bulk density of the samples was calculated (3,900 and 21,625 ppm) (Adapa et al., 2018).

2.4 | Essential oil extraction of *S. oleracea* leaves

For the extraction of essential oil from the hydroalcoholic extract of spinach by hydrodistillation under optimal operating conditions, a quantity of 50 ml of the hydroalcoholic extract was added to 800 ml of distilled water in a 2-L flask. The set was placed in a balloon heater for 8 hr to ensure condensation of the essential oil. At the end of the distillation, two phases were observed; an aqueous phase (aromatic water) and an organic phase (essential oil) which was less dense than water. Of note, 1 μl of the essential oil was obtained, which was injected into the GC/MS apparatus. Since the amount of the spinach essential oil was not enough for the quantification of its antibacterial properties, its antibacterial properties were predicted by analyzing its compounds (de Araújo et al., 2017).

2.5 | Disc diffusion method

The disc diffusion assay was performed according to the Kirby-Bauer method. The antibiotic discs (gentamicin, erythromycin, and chloramphenicol) containing 10, 15, and 30 μg of each antibiotic, as the positive controls, were obtained from Research and Production Laboratory of Roshd (Afzal, Ullah, Hussain, & Rukh, 2017; Bauer, 1966).

2.6 | Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by microbroth dilution. First, the mass of the dried substance was calculated in order to provide the main concentration for the alcoholic and hydroalcoholic extracts. Accordingly, the highest concentration of the alcoholic extract of the spinach leaves was calculated to be 3,900 ppm. Then, 95 μl of Muller Hinton broth for *S. enteritidis* (ATCC 13076), *E. coli O157:H7* (NCTC 12900) and *P. aeruginosa* (ATCC 15442) and BHI broth for *L. monocytogenes* (ATCC 7644) was injected into the wells and 100 μl of the alcoholic extract (main concentration)-the highest concentration of the examined extract was added to the first well. Next, 100 μl of the substances was taken from the first well and added to the second one. This procedure was repeated for all the wells other than the 11th one (positive control). In the next stage, 5 μl of the bacterial suspension (with a concentration of 1.5×10^8 cfu/ml) was added to all the wells except the 12th one (negative control). All the above stages, except the addition of the bacterial suspension, were repeated in order to obtain the control values. After that, the micro-plate was located inside an incubator at 37°C for 24 hr and subsequently, its opacity was measured by the Elisa Reader ELX808 at 620 nm. The intersection point between the absorbance of the extract containing the microbial suspension and that of the control was considered the beginning of the MIC zone Agrawal et al., 2018). This was done precisely for the hydroalcoholic extract with the initial concentration of 21,625 ppm; and for the essential oil with the same ratio between Tween, as the emulsifier, and the culture medium. All the steps were performed in triplicate (de Araújo et al., 2017).

2.7 | Determination of minimum bactericidal concentration

In the MIC test, 10 μl of the wells without turbidity was cultured on nutrient agar medium (Merck, Germany), and the first concentration at which bacterial growth was not observed, was considered to be minimum bactericidal concentration (MBC; de Araújo et al., 2017).

2.8 | GC/MS analysis

The chemical composition of the essential oil of *spinach* was identified by a gas chromatograph (Agilent Technologies 6,890) integrated with a mass spectrometer (5975 C inert MSD Agilent Technologies) equipped with HP-5MS 5% phenyl methyl silox 325C°. The temperature of pendulum varied from 50 to 290°C at a rate of 5°C/min. The detector temperature was 260°C and the ratio of the mixture was 1–50. Injection was done with the split method using Helium as the career gas at a speed of 1 ml/min. Compound retention time and mass spectrometry were employed to identify the compounds, and the identifications were confirmed using standard compound mass spectrums and the information in the Database/Wiley7n.1 library on the GC/MS computer. The relative percentages of each of the oil constituents were obtained with respect to the area under its curve in the chromatogram spectrum (Chandra & Shamli, 2015). Considering the high dilution of the hydroalcoholic extract, the percentage of its compounds could not be quantified by Gas Chromatography-Mass Spectrometry (GC/MS).

2.9 | Data analysis

The experiments were conducted in triplicate, and the average and *SD* were calculated. The graphs for determining the MIC range were drawn by Slide Write Software (Figures 1 and 2). Using the Slide Write software, the MIC spectra of the alcoholic and hydroalcoholic extracts were drawn. Each experiment was triplicated and the average and *SD* were calculated. Afterwards, the area under the MIC curve

was determined by comparing the opacity of the culture media with the control curve (bacterium-free culture medium). Indeed, MIC is the point at which the lines cross each other (cutoff point). Duncan's test at $p < .05$ was done to determine significant differences in the disc diffusion method.

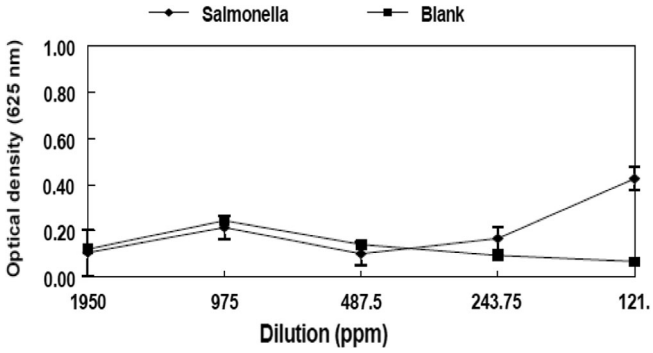

FIGURE 1 The zone of MIC of alcoholic extract of *Spinacia oleracea* L. on *Salmonella enteritidis*. *Notes*: Effect of alcoholic extract of *Spinacia oleracea* L. is shown at different dilutions on bacterial growth (turbidity) in broth micro dilution test; the dilution at which bacterial growth is less than the blank is considered to be MIC

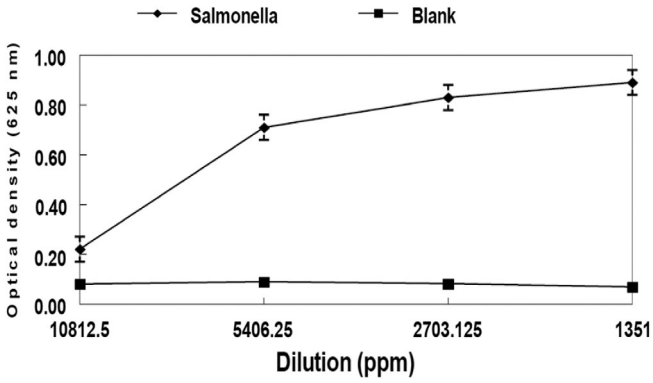

FIGURE 2 The zone of MIC of hydroalcoholic extract of *Spinacia oleracea* L. on *Salmonella enteritidis*

2.10 | Ethical approval

This study does not involve any human or animal testing.

3 | RESULTS

3.1 | Disc diffusion analysis

According to Table 1, the longest inhibition zone diameter (10.5 mm) was gained for the alcoholic extract of spinach against *L. monocytogenes* at the high concentration of 3,900 ppm, while *S. enteritidis*, *L. monocytogenes*, and *P. aeruginosa* were more resistant. The alcoholic extract was used at two concentrations (1950 and 975 ppm), which showed similar antimicrobial activities. Furthermore, all these bacteria were more resistant to the hydroalcoholic extract. The effect of the alcoholic extract is compared with different antibiotics in Table 1. Chloramphenicol (30 μg) showed the longest inhibition zone diameter (14 mm) on *S. enteritidis*. The differences were significant at $p < .05$.

3.2 | Minimum inhibitory concentration and minimum bactericidal concentration

The MIC of the alcoholic extract of spinach for *S. enteritidis* and *L. monocytogenes* was observed at the fourth concentration, 487.5 ppm, while MIC was determined at the first concentration for *P. aeruginosa*, 1950 ppm. However, no MIC was observed for *E. coli* in the alcoholic extract. The highest antibacterial activity was observed for the alcoholic extract on *S. enteritidis* and *L. monocytogenes* (Figure 1). The hydroalcoholic extract did not show any inhibitory influence on the investigated bacteria (Figure 2). It was also concluded that the MBC of the alcoholic extract at the first and second concentrations, 1950 and 975 ppm, for *S. enteritidis*, *L. monocytogenes*, and *P. aeruginosa*, was equal to 1950 ppm. However, this extract had no bactericidal effect on *E. coli*. The hydroalcoholic extract did not also show any fatal impact on the studied bacteria (Table 2).

TABLE 1 Inhibition zone of alcoholic extract of spinach and antibiotics on different pathogen bacteria using disc diffusion method

Concentration (ppm)		*Salmonella enteritidis*	*Listeria monocytogenes*	*Escherichia coli*	*Pseudomonas aeruginosa*
Alcoholic extract	3,900	7.5*mm	10.5**mm	–	8*mm
	1950	7*mm	7*mm	–	7*mm
	975	–	–	–	–
Antibiotics	Gentamicin (10 μg)		9*mm		
	Erythromycin(15 μg)		11.5**mm		
	Choloramphenicol (30 μg)	14***mm		12**mm	11.5**mm

Note: Values are the means of triplicate determinations. (*), (**), (***) Data with the same letter for zone of inhibitions are not significantly different ($p < .05$). Erythromycin, gentamicin, and chloramphenicol antibiotics were used for *Listeria monocytogenes* ATCC (7644), *Salmonella enteritidis* ATCC (13076), *Escherichia, coli O157:H7* NCTC (12900) *and Pseudomonas aeruginosa* ATCC (15442) as a positive control. (–): no antimicrobial activity.

TABLE 2 MIC and MBC of alcoholic and hydroalcoholic extracts of *Spinacia oleracea L*

	Alcoholic extract		Hydroalcoholic extract	
Microorganisms	**MIC**	**MBC**	**MIC**	**MBC**
Salmonella enteritidis	487.5 ± 0.33	975	NS	NS
Listeria monocytogenes	487.5 ± 0.33	975	NS	NS
Escherichia coli	NS	NS	NS	NS
Pseudomonas aeruginosa	1950 ± 0	1950	NS	NS

Note: Values are the means of triplicate determinations. Results are presented as mean ± *SD*.
Abbreviations: MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; NS, not seen.
MIC, MBC (ppm).

TABLE 3 GC/MS analysis of spinach essential oil

Compounds name	Molecular composition	Activity	% (W/W)	Known mechanism of action	Retention time
Cineole	$C_{10}H_{18}O$	Antiseptic properties and antibacterial	13.1	Inhibits cell wall or membrane	10.87
Linalool	$C_{10}H_{18}O$	Antibacterial	22.69	Inhibits cell wall or membrane	12.80
a-terpineol	$C_{10}H_{18}O$	Antibacterial	12.98	Quorum sensing/ biofilm inhibitor and inhibits cell wall or membrane	15.70
Linalyl anthranilate	$C_{17}H_{23}NO_2$	Antibacterial	2.89		17.04
Geraniol	$C_{10}H_{18}O$	Antibacterial	4.99	Synergic	17.14
Terpinyl acetate	$C_{12}H_{20}O_2$	Antibacteria and antiyeasts	5.08		19.82
2,6-octadien-1-ol.3,7-dimethyl-acetate	$C_{12}H_{20}O_2$		3.55		20.54
Durohydroquinone	$C_{10}H_{14}O_2$		34.73	Destroy the bacterial cell wall and membrane	24.16

Note: Main compounds of spinach essential oil (% W/W and RT) and their activities are shown.

3.3 | Chemical composition of essential oil

According to the results (Table 3), GC–MS analysis detected eight compounds in terms of the extraction method (Clevenger method, hydrodistillation). Durohydroquinone (34.73%), linalool (22.69%), cineole (13.1%), and α-terpineol (12.98%) were the dominant allocated compounds in the essential oil obtained from the hydroalcoholic extract.

3.4 | Chemical composition of alcoholic extract

Over 194 compounds were detected in the alcoholic extract using GC/MS, including phenolic compounds (9.345%), unsaturated and saturated fatty acids (22.901%), alkaloids, CHO and terpene (58.57%), vitamins (0.567%), heterocyclic compounds (2.09%), amino acids (6.279%), and squalene (0.233%), which have functional roles in biological activities (Table 4).

4 | DISCUSSION

For years, the antimicrobial activity of medicinal plants such as *S. oleracea* L. has been demonstrated. The content of phenolic compounds, unsaturated and saturated fatty acids, alkaloids, CHO, terpenes, vitamins, heterocyclic compounds, and amino acids along with the extracts of this plant are the main reasons for its antibacterial effects (Sun et al., 2017). Therefore, more inhibitory effect of *S. oleracea* L. on pathogenic bacteria can essentially be evaluated according to the variables in this study.

In the MIC method, the highest antibacterial activity was observed for the alcoholic extract on *S. enteritidis* and *L. monocytogenes*, whereas *E. coli* was identified as the most resistant one. However, the hydroalcoholic extract showed no MIC and MBC for the tested pathogens. Furthermore, in the disc diffusion method, *L. monocytogenes*, as a gram-positive bacterium, was the most sensitive one to the alcoholic extract. It seems that the bioactive compounds and their amounts govern the antibacterial activity of the

TABLE 4 GC/MS analysis of alcoholic extract of spinach

No	Compounds name	Retention time (min)	% (W/W)	Molecular composition
1	3,4-dimethyl-1H-Pyrazole	4.952	0.062	$C_5H_8N_2$
2	*N*-Methyl propionic acid amide	5.107	0.053	C_4H_9NO
3	3-Methyl-3-pyrazolin-5-one	6.652	0.114	$C_4H_6N_2O$
4	Propanoic acid, 2-(hydroxyimino)-, methyl ester	11.275	0.05	$C_4H_7NO_3$
5	Benzeneacetaldehyde	14.187	4.604	C_8H_8O
6	2-Pyrrolidinone	15.618	0.595	C_4H_7NO
7	3-Hydroxypyrrolidine	17.128	0.098	C_4H_9NO
8	Benzoic acid	20.132	0.067	$C_7H_6O_2$
9	Succinic acid, diethyl ester	20.688	0.181	$C_8H_{14}O_4$
10	Pyrollidine, 2,5-bis(imino)-	21.019	0.046	$C_4H_7N_3$
11	*N*-methyl piperazine	23.514	0.056	$C_5H_{12}N_2$
12	Glycine betaine	25.059	0.067	$C_5H_{11}NO_2$
13	Monoethylaminoethanol	26.175	0.138	$C_4H_{11}NO$
14	Thymol	26.312	0.078	$C_{10}H_{14}O$
15	2,5-Diethylphenol	26.661	0.116	$C_{10}H_{14}O$
16	Phenol, 2-methoxy-4-vinyl-	27.056	1.11	$C_9H_{10}O_2$
17	Acetaldehyde, phenyl-, diethyl acetal	27.52	0.147	$C_{12}H_{18}O_2$
18	Thiophene	29.974	0.089	C_4H_4S
19	Thiole	30.089	0.089	C_4H_4S
20	Ephedrin	30.764	0.098	$C_{10}H_{15}NO$
21	D-Valine	31.21	0.095	$C_5H_{11}NO_2$
22	Glutamic acid	32.767	1.944	$C_5H_9NO_4$
23	L(+)-isoleucine	33.385	0.108	$C_6H_{13}NO_2$
24	L-Isoleucine	34.958	0.53	$C_6H_{13}NO_2$
25	Formic acid, ethyl ester	36.629	0.129	$C_3H_6O_2$
26	(S)-(+)-Glutamic acid	39.192	0.053	$C_5H_9NO_4$
27	Pyrrolidin-1-acetic acid	39.65	0.139	$C_6H_{11}NO_2$
28	1-Methyl-5-D1-1,2,4-Triazole	39.765	0.314	
29	L-Proline, 5-oxo-	39.982	0.218	$C_5H_7NO_3$
30	Iron, tricarbonyl [(2, 3, 4, 5-.eta.)-2,3,4,5-tetrahydroxy-2,4-cyclopentadien-1-one]- (CAS)	40.6	0.119	
31	Myristinic acid	45.246	0.261	
32	Phthalic acid, dipropyl ester	48.548	0.203	$C_{14}H_{18}O_4$
33	Tetradecanoic acid	48.691	0.135	$C_{14}H_{28}O_2$
34	Lauric acid	51.884	0.196	$C_{12}H_{24}O_2$
35	Palmitic acid ethyl ester	53.154	0.465	$C_{34}H_{66}O_4$
36	Oleic acid	55.191	0.15	$C_{18}H_{34}O_2$
37	2-Phenylindole-Stabilizer I	55.5	0.063	$C_{14}H_{11}N$
38	Methyl linolenate-Linolenic acid, methyl ester	57.594	16.654	$C_{19}H_{32}O_2$
39	Stearic acid	58.327	3.561	$C_{18}H_{36}O_2$
40	1-Mercapto-4-methylbicyclo[2.2.2]octane	60.667	0.112	$C_9H_{16}S$
41	1-Eicosanol	67.407	1.935	$C_{20}H_{42}O$
42	Palmitic acid chloride	67.745	0.675	$C_{16}H_{31}ClO$
43	Squalene	75.87	0.233	$C_{30}H_{50}$
44	Stearol	79.269	0.2755	$C_{18}H_{38}O$

Note: Main compounds of alcoholic extract of spinach (% W/W and RT) are shown.

extract. Based on the results presented in Table 4, GC/MS indicated that heterocyclic compounds, including 3,4-dimethyl-1H-Pyrazole, 3-methyl-3-pyrazolin-5-one, and N-methyl propionic acid amide, accounted for 2.09% of the alcoholic extracted. Heterocyclic compounds display a broad spectrum of biological activities, including antimicrobial anticancer, antioxidant, antidepressant, and anticonvulsant activities (Agrawal et al., 2018). For example, 1,2,4-triazolopyrimidines have attracted growing interest due to their important pharmacological activities such as antitumor, antimalarial, antimicrobial, anti-inflammatory, and antifungal properties, as well as their potency in macrophage activation (Astakhov & Chernyshev, 2014). Also, thiazoles can be found in the drugs developed for the treatment of allergies, hypertension, inflammation, schizophrenia, bacterial infections, HIV, sleep disorders and more recently, for the treatment of pain, as fibrinogen receptor antagonists with antithrombotic activity, and as new inhibitors of bacterial DNA gyrase B (Abdelhamid, El Sayed, Hussein, & Mangoud, 2016).

(Abdelhamid et al., 2016) investigated the influence of different synthetic compounds such as 2-(5-(Furan-2-yl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-phenyl-5-(phenyldiazenyl)-thiazole and 3-Benzoyl-7,9-dimethyl-1-phenyl-5-(1-phenyl-3-(p-tolyl)-1H-pyrazol-4-yl)-7,9-and dihydropyrimido [4,5-d] [1,2,4] triazolo [4,3-a] pyrimidine-6,8(1H,5H)-dione on several bacteria, including *P. aeruginosa* and concluded that these compounds had desirable inhibitory impacts on the investigated bacteria. The findings of these researchers were consistent with ours.

Moreover, according to the GC/MS analysis, some of the phenolic compounds of the alcoholic extract of *spinach* with antibacterial effect were as follows: 2-phenylindole- stabilizer I (0.063%), phenol,2-methoxy-4-vinyl-(1.11%), thymol (0.078%), 2,5-diethylphenol (0.116%), and acetaldehyde, phenyl, diethyl acetal (0.147%). Due to binding with the cell wall of bacteria, the antibacterial mechanisms of phenolic compounds are presumably the enzymatic inhibition of the oxidized compounds, chemical reaction with the sulfhydryl groups and non-specific chemical reaction with the proteins (Delić, 2018). Using the GC/MS-based analysis, it was found out that the alcoholic extract of spinach contained alkaloids, CHO and terpenes (58.57%), amino acids (6.279%), and squalene (0.233%). It showed no effect on *E. coli* in the MIC and MBC tests, but it had desirable inhibitory and lethal effects on the other bacteria studied. Plant polyphenols have increasingly drawn scientists' attention due to their multiple functions in health care. According to a study conducted by Sun et al. (2017), it was shown that the antibacterial activity of the spinach polyphenols against gram-negative bacteria was stronger than against the gram-positive ones. In addition, the effects of pH, temperature, and NaCl concentration on the antibacterial stability of the spinach polyphenols were also discussed.

Spinach has significant amounts of essential nutrients such as potassium, calcium, magnesium, phosphor, iron, copper, zinc, protein, fiber, phenol compounds, and essential unsaturated fatty acids (66%) like linoleic, linolenic, palmitoleic, hexadecatrienoic, and stearic acids, flavonoids and terpenes (Anzabi, 2014). These compounds have inhibitory effects on polymerase activity in prokaryotes. Moreover, these substances can influence the cell wall of gram-negative bacteria whose outer membrane includes lipopolysaccharide. Therefore, the substances can damage the cell wall and inhibit the proliferation of such bacteria (Hugo & Russell, 1998).

The antibacterial activity of unsaturated fatty acids (C18) along with the esterified fatty acids (C12) has been proved on gram-positive bacteria and some yeasts. The antibacterial mechanism of lipophilic glycerides and their derivatives is likely damaging the substrate transition into the bacterial cell due to more transition of protons across the bacterial cell membrane and consequently, the disruption of the cell membrane proton pump (Nazari, Yavarmanesh, & Khodaparast, 2016).

In this study, it was understood that the alcoholic extract contained 22.9% fatty acids. The low level of fatty acids probably prevented the alcoholic extract of Iranian spinach from influencing *E. coli* which is a gram-negative bacterium.

Alnashi, Hassouna, and El Dairouty (2017) studied the antibacterial and antioxidant effects of fresh Indian spinach on gram-positive and gram-negative microorganisms. They realized that the hydroalcoholic extract of spinach had an antibacterial effect on *Staphylococcus aureus*, while it had no effect on *E. coli*. However, the petroleum extract of spinach had a strong inhibitory effect on *E. coli*. They concluded that the different leaf extracts of *S. oleracea* might be due to one or more of the phytochemical constituents of spinach such as steroids, saponins, phenols, flavonoids, alkaloids, tannins, carbohydrates, amino acids, glycosides, carbonyl, and anthroquinone in the polar aqueous and ethanolic extracts, while the nonpolar petroleum ether extracted only the terpenoids, phenols and glycosides. Vergara et al. (2019) investigated the influence of the hydroalcoholic extract of *Mauritia flexuosa* leaves on gram-negative and gram-positive bacteria. They found out that the hydroalcoholic extract of *M. flexuosa* leaves did not inhibit the growth of the gram-negative bacteria, namely *Salmonella typhi* and *E. coli* (Vergara et al., 2019). Hydroalcoholic extracts usually impact gram-positive bacteria. It is known that hydroalcoholic extracts of plants release a large quantity of phenols, flavonoids, and other compounds, including a great diversity of secondary metabolites, which may explain this antibacterial action on gram-positive bacteria (Birur et al., 2015). The outer membrane of gram-negative bacteria which contains lipopolysaccharide creates a barrier to the penetration of these compounds and causes them not to have inhibitory effects on the gram-negative bacteria (Burt, 2004). In this study, the hydroalcoholic extract of spinach used in the MIC and MBC method did not affect the bacteria even *L. monocytogenes*. This is probably due to the low concentration of the hydroalcoholic extract.

Using GC/MS, eight effective antibacterial compounds were identified in the essential oil of spinach, extracted by the Clevenger and hydrodistillation methods. Durohydroquinone (34.73%), linalool (22.69%), cineole (13.1%), and α-terpineol (12.98%) were the most abundant compounds in the spinach essential oil. All of these compounds kill the bacteria by destroying their cell walls. The essential oil was obtained from concentrating the hydroalcoholic extract using rotary evaporation.

Chlorohexidine is a medicine generally used for hand disinfection and wound cleaning. (Şimşek & Duman, 2017) investigated 1,8 cineole and its antibacterial effect in combination with chlorohexidine on *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Candida albicans*. They declared that 1,8 cineole generated an intensifying and synergistic potential to kill the investigated bacteria. This bioactive compound was first discovered in eucalyptus oil (Şimşek & Duman, 2017).

Li et al. (2014) examined the influence of α-terpineol on *E. coli* using transmission electron microscopy. These researchers realized that this bioactive compound reduced the size of the bacterium cytoplasm and cell. Furthermore, it destroyed the cell membrane which caused the bacterium death. Hence, it was concluded that α-terpineol was a strong antibacterial compound (Li et al., 2014).

The addition of linalool to different oils and essential oils strengthens their antibacterial properties. High concentrations of the essential oils alter their organoleptic quality, which may lead to irritation and allergy. However, the addition of linalool intensifies the antibacterial properties of essential oils and reduces the possibility of irritation and allergy. (Herman, Tambor, & Herman, 2015) declared that this bioactive compound had intensified antibacterial influence on *P. aeruginosa*, *E. coli*, and *C. albicans* (Herman et al., 2015).

Ma et al. (2019) studied the mechanism of the antibacterial activity of hydroquinone. They suggested that this bioactive compound had a relatively strong antibacterial influence on *S. aureus* and the methicillin-resistant *Staphylococcus*. This compound can destroy the bacterium cell wall and membrane and affect the protein and cell expression. Then, the bacterium dies (Ma et al., 2019). Hassan et al. (2016) and Shi, Zhao, Firempong, and Xu (2016) demonstrated that, because of poor water solubility of Linalool and 1,8 Cineole, as classic essential oils, these compounds are not released in the hydroalcoholic extract, Due to their hydrophobic nature. For this reason, in this study spinach hydroalcoholic extract did not have an inhibitory effect on *E. coli*.

5 | CONCLUSION

The results of this research showed that the alcoholic extract of the Iranian spinach has strong antibacterial substances. In this study, it was revealed that the amounts of phenolics, alkaloids, amino acids, terpenes, unsaturated, and saturated fatty acids in the alcoholic extract of *S. oleracea* leaves was able to have the greatest effect on the cell walls of *S. enteritidis* (ATCC 13076) and *L. monocytogenes* (ATCC 7644), as gram-negative and gram-positive bacteria. Moreover, the compounds of Durohydroquinone, linalool, cineole, and α-terpineol in the essential oil extracted from the hydroalcoholic extract have strong antibacterial properties. Since the alcoholic extract of spinach had antibacterial effects, especially on *L. monocytogenes* which causes contamination of livestock milk, it is recommended that this extract be used as livestock feed or in feed coatings.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance offered by the Molecular Genetics and Novel Technologies Laboratory at Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

INFORMED CONSENT

Written informed consent was obtained from the study participants.

ENDNOTE

[1] Pressurized liquid extraction.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. We agree to upload it to the system.

ORCID

Seyed Ali Issazadeh https://orcid.org/0000-0003-4355-4436
Masoud Yavarmanesh https://orcid.org/0000-0002-4771-5359

REFERENCES

Abdelhamid, A. O., El Sayed, I. E., Hussein, M. Z., & Mangoud, M. M. (2016). Synthesis and antimicrobial activity of some new thiadiazoles, thioamides, 5-arylazothiazoles and pyrimido [4, 5-d][1, 2, 4] triazolo [4, 3-a] pyrimidines. *Molecules*, *21*(8), 1072. https://doi.org/10.3390/molecules21081072

Adapa, S. B., Sushanth, V. H., Prashant, G. M., & Mohamed, I. (2018). In vitro antimicrobial activity of *Spinacia oleracea* against *Streptococcus* mutans and *Lactobacillus acidophilus*. *Journal of Indian Association of Public Health Dentistry*, *16*(3), 251. https://doi.org/10.4103/jiaphd.jiaphd_6_18

Afzal, J., Ullah, N., Hussain, Z., Rukh, S., Ayaz, M., Akbar, A., & Zaman, A. (2017). Phytochemical analysis andantibacterial potential of leaf extract of bauhinia Linn. *An ethnomedicinal plant*, *1*(2), 17–19. http://www.razipublishing.com/journals/matrix-science-pharma-msp/

Agrawal, P., Kotagiri, D., & Chaitanya Kolluru, V. (2018). Comparative analysis of antimicrobial activity of herbal extracts against pathogenic microbes. *journal of Advances in Biochemistry and Biotechnology*, *2*, 1–7.

Akasaka, H., Mizushina, Y., Yoshida, K., Ejima, Y., Mukumoto, N., Wang, T., & Sasaki, R. (2016). MGDG extracted from spinach enhances the cytotoxicity of radiation in pancreatic cancer cells. *Radiation Oncology*, *11*(1), 1–11. https://doi.org/10.1186/s13014-016-0729-0

Alnashi, B. A., Hassouna, H. Z., & El Dairouty, R. K. (2017). Evaluation of antimicrobial activity, total phenolic compounds, antioxidant activity and nutritional value of fresh spinach (*Spinacia oleracea*) extracts. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *7*(3), 1836.

Anzabi, Y. (2014). Evaluation of antibacterial activity of aqueous extracts of onion and some antibiotics on a number of important bacteria in terms of food hygiene. *Crescent Journal of Medical and Biological Sciences*, *1*, 136–142.

Astakhov, A. V., & Chernyshev, V. M. (2014). Molecular structure of 3-amino [1, 2, 4] triazolo-[4, 3-a] pyrimidin-5-one in various

tautomeric forms: Investigation by DFT and QTAIM methods. *Chemistry of Heterocyclic Compounds*, *50*(3), 319–326.

Bauer, A. W. (1966). Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology*, *45*, 149–158.

Birur, R. S., Ramachandra, Y. L., Subaramaihha, S. R., Subbaiah, S. G. P., Austin, R. S., & Dhananjaya, B. L. (2015). Antioxidant activities of leaf galls extracts of *Terminalia chebula* (Gaertn.) Retz.(Combretaceae). *Acta Scientiarum Polonorum Technologia Alimentaria*, *14*(2), 97–105. https://doi.org/10.17306/J.AFS.2015.2.11

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods—A review. *International Journal of Food Microbiology*, *94*(3), 223–253.

Chandra, J. H., & Shamli, M. (2015). Antibacterial, antioxidant and in silico study of *Ipomoea aquatica* Forsk. *Journal of Pure and Applied Microbiology*, *9*(2), 1371–1376.

Das, M. P., & Chatterjee, S. (2013). Evaluation of antibacterial potential of *Spinacia oleracea* L. against urinary tract pathogens. *International Journal of Pharmaceutical Sciences Review and Research*, *23*(1), 40.

Das, S., & Guha, D. (2008). CNS depressive role of aqueous extract of *Spinacia oleracea* L. leaves in adult male albino rats. *Indian Journal of Experimental Biology*, *46*(3), 185–190.

De Araújo, Í. D. R., de Aquino, N. C., de Aguiar Guerra, A. C. V., de Almeida Júnior, R. F., Araújo, R. M., de Araújo Júnior, R. F., … Andrade, V. S. (2017). Chemical composition and evaluation of the antibacterial and cytotoxic activities of the essential oil from the leaves of Myracrodruon urundeuva. *BMC Complementary and Alternative Medicine Journal*, *17*(1), 1–8.

Delić, A. (2018). *The nutritive quality of the New Zealand spinach hydroponically cultivated under different nitrogen concentrations*. (Doctoral dissertation), University of Zagreb. Faculty of Agriculture. Department of Agricultural Technology, Storage and Transport. https://urn.nsk.hr/urn:nbn:hr:204:514641

Forbes, B. A., Sahm, D. F., & Weissfeld, A. S. (1998). *Scott & Bailey's diagnostic microbiology*. St. Louis Missouri: Mosby.

Gaikwad, P. S., Shete, R. V., & Otari, K. V. (2010). *Spinacia oleracea* Linn: A pharmacognostic and pharmacological overview. *International Journal of Research in Ayurveda and Pharmacy (IJRAP)*, *1*(1), 78–84. https://www.researchgate.net/publication/215648126_Spinacia_oleracea_Linn_A_pharmacognostic_and_pharmacological_overview

Gamble, J. S. (1921). *Flora of the presidency of Madras: Rubiaceae to Ebenaceae/JS Gamble*. London: Adlard & Son.

Hassan, B., Soumya, E., Moulay, S., Mounyr, B., Hajar, M., & Koraichi, I. S. (2016). Evaluation of hydrophobic-hydrophilic properties and anti-adhesive potential of the treated cedar wood by two essential oil components against bioadhesion of *Penicillium expansum* spores. *Journal of Animal and Poultry Sciences*, *16*(8), 372–379. https://doi.org/10.3923/jas.2016

Herman, A., Tambor, K., & Herman, A. (2015). Linalool affects the antimicrobial efficacy of essential oils. *Current Microbiology*, *72*(2), 165–172.

Hugo, W. B., & Russell, A. D. (1998). *Pharmaceutical microbiology*. 6th edition, by Blackwell Science Ltd a Blackwell Publishing company.

Jackson, K. A., Iwamoto, M., & Swerdlow, D. (2010). Pregnancy-associated listeriosis. *Epidemiology & Infection*, *138*(10), 1503–1509. https://doi.org/10.1017/S0950268810000294

Jaime, L., Vázquez, E., Fornari, T., López-Hazas, M. D. C., García-Risco, M. R., Santoyo, S., & Reglero, G. (2015). Extraction of functional ingredients from spinach (*Spinacia oleracea* L.) using liquid solvent and supercritical CO_2 extraction. *Journal of the Science of Food and Agriculture*, *95*(4), 722–729.doi: https://doi.org/10.1002/jsfa.6788. Retrieved from https://europepmc.org/article/agr/ind601259102

Karch, H., Tarr, P. I., & Bielaszewska, M. (2005). Enterohaemorrhagic *Escherichia coli* in human medicine. *International Journal of Medical Microbiology*, *295*(6–7), 405–418. https://doi.org/10.1016/j.ijmm.2005.06.009

Li, L., Shi, C., Yin, Z., Jia, R., Peng, L., Kang, S., & Li, Z. (2014). Antibacterial activity of α-terpineol may induce morphostructural alterations in *Escherichia coli*. *Brazilian Journal of Microbiology*, *45*(4), 1409–1413. https://doi.org/10.1590/S1517-83822014000400035

Longnecker, M. P., Newcomb, P. A., Mittendorf, R., Greenberg, E. R., & Willett, W. C. (1997). Intake of carrots, spinach, and supplements containing vitamin A in relation to risk of breast cancer. *Cancer Epidemiology and Prevention Biomarkers*, *6*(11), 887–892.

Louis, M. E. S., Morse, D. L., Potter, M. E., DeMelfi, T. M., Guzewich, J. J., Tauxe, R. V., … Greenspan, J. R. (1988). The emergence of grade a eggs as a major source of *Salmonella enteritidis* infections: New implications for the control of salmonellosis. *Jama*, *259*(14), 2103–2107.

Ma, C., He, N., Zhao, Y., Xia, D., Wei, J., & Kang, W. (2019). Antimicrobial mechanism of hydroquinone. *Applied Biochemistry and Biotechnology*, *189*(4), 1291–1303. https://doi.org/10.1007/s12010-019-03067-1

Nazari, H., Yavarmanesh, M., & Khodaparast, M. H. H. (2016). In vitro study to evaluate the antibacterial effect of *Pistacia khinjuk* stocks oil as compared with olive oil on food borne pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*). *Journal of Essential Oil Bearing Plants*, *19*(1), 125–133. https://doi.org/10.1080/0972060X.2014.971067

Rabsch, W., Tschäpe, H., & Bäumler, A. J. (2001). Non-typhoidal salmonellosis: emerging problems. *Microbes and Infection*, *3*(3), 237–247. https://doi.org/10.1016/s1286-4579(01)01375-2

Shi, F., Zhao, Y., Firempong, C. K., & Xu, X. (2016). Preparation, characterization and pharmacokinetic studies of linalool-loaded nanostructured lipid carriers. *Pharmaceutical Biology*, *54*(10), 2320–2328. https://doi.org/10.3109/13880209.2016.1155630

Şimşek, M., & Duman, R. (2017). Investigation of effect of 1, 8-cineole on antimicrobial activity of chlorhexidine gluconate. *Pharmacognosy Research*, *9*(3), 234. https://doi.org/10.4103/0974-8490.210329

Sun, F. L., Yan, Y. R., Wang, F. J., & Wei, T. A. N. G. (2017). Investigation of Antibacterial Activity and Stability of the Polyphenol Crude Extract from Spinacia oleracea. 2nd International Conference on Advanced Materials Science and Environment Engineering (AMSEE 2017). ISBN: 978-1-60595-475-2.

Tamparo, C. (2011). *Diseases of the human body* (5th ed., p. 61). Philadelphia, PA: F.A. Davis Company.

Vergara, A. S., Córdova, M. F., Castillo, M. L. S., Haro, I. M. R., & Rodríguez, A. P. V. (2019). Antibacterial effect of the hydroalcoholic extract of *Mauritia flexuosa* leaves on gram-negative and gram-positive bacteria. *F1000Research*, *8*, 1487. https://doi.org/10.12688/f1000research.19151.1

How to cite this article: Issazadeh SA, Hatami S, Yavarmanesh M. In vitro investigation of chemical composition and antibacterial activity of alcoholic, hydroalcoholic extracts, and essential oil of *Spinacia oleracea* leaves from Iran. *J Food Saf*. 2021;e12891. https://doi.org/10.1111/jfs.12891