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Antibacterial and Antioxidant Characteristics of Zataria multiflora Boiss Essential Oil and Hydroalcoholic Extract of Rhus coriaria L.

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HIGHLIGHTS

- Phenolic content in sumac extract (305.65 mg/g) was higher than Zataria multiflora Essential Oil (ZEO) (179.42 mg/g).
- Sumac extract had more antioxidative activities than ZEO.
- ZEO showed more antibacterial activities than Sumac extract.

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Acronyms and abbreviations

MIC=Minimal Inhibitory Concentration MBC=Minimal Bactericidal Concentration FIC=Fractional Inhibitory Con-ZEO=Zataria multiflora Essential GC-MS=Gas Chromatography-Mass Spectrometry DPPH=2, 2-diphenyl-1picrylhydrazyl

ABTS=2, 2'-azino-bis 3ethylbenzothiazoline-6sulphonic-acid RSA=Radical Scavenging Activi-

BHT=Butylated hydroxytoluene

ABSTRACT

Background: The increasing demand for natural preservatives results in their extended usefulness. The objective of the present study was to investigate the physicochemical and antioxidative characteristics of Rhus coriaria L. (sumac) fruit and comparison of its antioxidative and antibacterial activity with Zataria multiflora Essential Oil (ZEO) as native Iranian natural additives.

Methods: Antioxidant activities of Z. multiflora Boiss and sumac were analyzed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2, 2'-azino-bis 3ethylbenzothiazoline-6-sulphonic acid (ABTS). Reducing power tests were used for measuring antioxidant activity. Total phenolic content of extract and essential oil were studied as well. The Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC), and Fractional Inhibitory Concentration (FIC) of a hydroalcoholic extract of sumac and ZEO against of Salmonella Typhimurium and Listeria monocytogenes were studied. Statistical analysis of data was performed using the SPSS software.

Results: The phenolic content in sumac extract (305.65 mg/g) was significantly (p < 0.05) higher than ZEO (179.42 mg/100 g). The highest level of antibacterial activity was demonstrated by ZEO with the MICs of 0.625 for S. Typhimurium and 1.25 mg/ml for L. monocytogenes.

Conclusion: Sumac extract showed more potent antioxidative activity than ZEO. However, based on the results of antibacterial activity, ZEO had more potent than sumac extract, significantly.

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Introduction

Nowadays, chemical preservatives are being used to control the microbial population and as well as retard the oxidation reactions in food. The consumers are unsatisfied from different synthetic preservatives because of

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their side effects. The increasing demand for natural preservatives results in their extended usefulness. Generally, replacement of essential oils instead of chemical preservatives is so important. It has been proved that this alternative may reduce the adverse effects of chemical preservatives (Mojaddar Langroodi et al., 2018; Prakash et al., 2015).

Sumac plant belonging mainly to the genus Rhus, grows mostly in the tropics and subtropics but also into the temperate areas of the world (Rayne and Mazza, 2007). It has been stated that sumac (Rhus coriaria L.) some natural antimicrobial compounds contains (Chorianopoulos et al., 2004; Shabbir, 2012). Sumac is famously used in the Mediterranean region and Middle East as a spice (Rayne and Mazza, 2007). Many studies have recognized sumac to contain phenolic compounds such as anthocyanins, hydrolysable tannins, and gallic acid (Kosar et al., 2007), flavones, such as, myricetin, quercetin and kaempferol (Mehrdad et al., 2009), malic, palmitic, stearic, oleic, and linoleic acids (Kizil and Turk, 2010) and also organic acids such as citric acids. Antibacterial activity of sumac is clear and tannins are an important part of sumac extract. Except for tannins, other compounds should also have a role in antimicrobial effect of sumac (Kosar et al., 2007; Wang and Zhu, 2018). Also anthocyanin and hydrolysable tannins have power for inhibition of lipid peroxidation and scavenging activity (Kosar et al., 2007).

Zataria multiflora Boiss belongs to Lamiaceae family that grows widely in warm and mountainous parts of Iran, Pakistan, and Afghanistan (Hosseinzadeh et al., 2000). Lamiaceae family has more than 200 genus and 2000-5000 species of aromatic bush and short shrubs. Due to the presence of thymol and carvacrol, Zataria multiflora Essential Oil (ZEO) can show some antioxidant, antibacterial, and antifungal characteristics (Ettehad and Arab, 2007).

The objective of the present study was to investigate the physicochemical and antioxidative characteristics of *Rhus coriaria* L. (sumac) fruit and comparison of its antioxidative and antibacterial activity with ZEO as native Iranian natural additives.

Materials and methods

Preparation of sumac extract

Fresh sumac fruits were provided from local markets and identified at the Institute of Medicinal Plants, Karaj, Iran. In this study 250 g of sumac powder were added to 700 ml alcohol and 300 ml distilled water, and this mixture was left in a shaker for 24 h. Then, it was left in a

water bath at 40 °C for 1 h with occasional stirring. After cooling and filtration through a filter paper, the obtained extract was concentrated using a rotary evaporator under reduced pressure at 45 °C to eliminate the solvent. The hydroalcoholic extract was stored at 4 °C until use (Mojaddar Langroodi et al., 2018).

Extraction of ZEO and Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Z. multiflora Boiss was collected from Shiraz (a city in Iran) and was transported to the Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. Then, it was authenticated by Institute of Medicinal Plants, Karaj, Iran. Dried leaves were powdered using an electric device and the essential oil was prepared by hydrodistillation for 2-3 h using a Clevenger-type apparatus. The ZEO was dried by anhydrous sodium sulfate followed by filtering. Then, it was kept in glass tube covered with parafilm and aluminum foil at refrigerator temperature (Moradi et al., 2012). It was analyzed using GC-MS (Model 6890N; Hewlett-Packard, Palo Alto, USA) with a column HP-5MS (length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 µm) in addition to a Mass Spectrometer (MS) (Model 5973N; Hewlett-Packard, Palo Alto, USA). Column temperature program was formulated in this way: the initial temperature of oven was 50 °C, injector chamber temperature was 290 °C, and helium was used as a carrier gas at a rate of 1.5 mm/min. Mass spectrometer with 70 eV of ionization voltage was used. The individual compounds were confirmed with those of authentic samples and with available library data of the GC-MS system (WILEY 2001 data software; John Wiley and Sons, New York, USA) (Marriott et al., 2001).

Chemicals and reagents

Total phenol content was evaluated using spectrophotometr. Antioxidant activity was evaluated using three different methods, including reducing power assay, scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and 2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS). The results were compared with synthetic antioxidants, Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT) (Sanchez-Moreno et al., 1999). BHT and DPPH, disodium hydrogen phosphate (Na₂HPO₄), sodium dihydrogen phosphate, ferric chloride, potassium ferricyanide [K₃Fe(CN)₆], trichloroacetic acid, acid galic, and ABTS were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Antioxidant analysis

-ABTS

ABTS assay of ZEO and sumac extract were determined according to the method by Han et al. (2008) and Re et al. (1999). Briefly, ZEO samples dissolved in methanol and sumac extract dissolved in methanol and distilled water; afterward, ABTS solution and potassium persulfate (K₂S₂O₈) were mixed and incubated in a dark place for 16 h at room temperature. The final solution was diluted with methanol, insofar as absorbance of it reached to 0.7±0.02 in 734 nm. Different concentrations of samples were mixed with ABTS solution and after 6 min, the absorbance was read at 700 nm. ABTS and methanol were used for positive control sample and ethanol as a control to zero the spectrophotometer. In this method percentage of Radical Scavenging Activity (RSA) was obtained from the following equation:

 $RSA (\%) = [(AC - AS)/(AC)] \times 100$

Where AC is the absorbance value of ABTS and methanol and AS is the absorbance value of ABTS and samples (ZEO or sumac extract).

-Total phenolic assay

For this purpose, 2.25 ml distilled water was dissolved with different concentration of ZEO (dissolved with methanol) and sumac extract (dissolved with methanol and distilled water). Afterward a volume of 250 µl of Folin-Ciocalteu reagent was added with gallic acid as a standard (Siripatrawan and Harte, 2010). The mixture was mixed by vortex for 1 min and incubated for 5 min at room temperature before addition of 2 ml of a 7.5% sodium carbonate (Na₂CO₃) solution. After incubation in a dark chamber at room temperature for 2 h, the absorbance was read at 760 nm. The amount of total phenolic compounds is expressed as gallic acid tantamount. Gallic acid standard curve was plotted to determine the total phenolic contents. The equation of the gallic acid standard curve to calculate the total phenol content was y=5.7923 x+0.1696 (R2=0.9816). The phenolic content of the extracts was measured according to this equation.

-Reducing power

Reducing power of ZEO and sumac extract was performed according to the method of Pourmortazavi et al. (2017) with slight modifications. ZEO samples were dissolved in methanol and sumac extract was dissolved in methanol and distilled water. One ml of each sample with different concentration was mixed with one ml buffer phosphate and one ml potassium ferricyanide; then, the mixture was allowed to stand at 50 °C for 20 min. Next, one ml of trichloroacetic acid (10%) was added to the

mixture and centrifuged for 10 min. Afterward, all the upper layer (1 ml) was mixed with 0.5 ml of distilled water and 0.5 ml of ferric chloride. The tubes were then incubated at room temperature for 10 min under dark conditions and the absorbance was measured at 700 nm. For positive control sample, BHT was used. The reducing power of the sample was indicated by the increase in absorbance of the reaction mixture.

-DPPH radical scavenging activity

The potential antioxidant capacity of ZEO and sumac extract was assessed by the scavenging activity of stable free radicals of DPPH. First, ZEO samples were dissolved in methanol and sumac extract was dissolved in methanol and distilled water. Then, all of the samples were mixed with 2 ml of DPPH (2.4 mg in 100 ml ethanol). The mixture was mixed by vortex and incubated in the dark at ambient temperature for 60 min. The absorbance was read at 517 nm by spectrophotometer (Model Novaspec II; Pharmacia LKB, Uppsala, Sweden). When the DPPH solution was combined with the sample, a stable non radical form of DPPH was obtained with contemporaneous change of the violet to pale yellow color (Lin et al., 2009). The percentage of DPPH free radical quenching activity was determined using the following equation:

DPPH scavenging effect %=AD-AS (Ad) × 100

Where AD is the absorbance value at 517 nm of the methanolic solution of DPPH and AS is the absorbance value at 517 nm for the sample extracts. All samples were assayed three times and results were reported as mean±SD of triplicates.

Antimicrobial analysis

-Bacterial strains

Standard strains of *S.* Typhimurium (PTCC 1609) as well as *L. monocytogenes* (PTCC 1163) were obtained from Laboratory of Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Iran. The bacteria were stored at -70 °C.

-Determination of Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC), and Fractional Inhibitory Concentration (FIC) of sumac extract and ZEO

MIC values were determined by micro dilution assay. MICs were assessed for the sumac extract, ZEO as well as their combination. Subsequently, MBC and FIC was evaluated. Dimethyl sulfoxide (10%) was used as a solvent for ZEO (6400 mg/ml). Afterward, dilutions of ZEO and sumac extract (5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.78,

0.39, 0.19 mg/ml) were prepared (Cho et al., 2011). In each well, 160 μl of Luria-Bertani broth (Merck, Germany), 20 μl of bacterial suspension, and 20 μl of different concentration of ZEO or sumac extract were inoculated, until dose of bacteria in each well was adjusted to 5×10⁵ CFU/ml. Then, microplate was left in a shaker for 30 s in 2500 rpm and was incubated at 35 °C for 24 h. Finally, MIC was determined according to the method by Rohani et al. (2011). According to MIC of samples, MBCs were determined; concentrations that had no bacterial growth were reported as MBC values (Yu et al., 2004).

To determine the combined effects of ZEO and sumac extract, FIC was used. Eight dilutions of ZEO and sumac extract were prepared similar to MIC. In each well, 140 μl of BHI medium, 20 μl of bacterial suspension, and 20 μl of different concentration of ZEO and sumac extract were inoculated, and sumac extract and ZEO in any dilutions were combined. The final concentration in each well was adjusted to 5×10⁵ CFU/ml. Afterward, microplate was left in a shaker for 30 s in 2500 rpm and was incubated at 35 °C for 24 h. After all, FIC values were determined according to visual and turbidity method.

Statistical analysis

All experiments were done in triplicate and results were reported as mean \pm standard error. Statistical analysis of data was performed using the SPSS, Inc, Chicago, IL software (IBM SPSS statistics 20). Tukey test and analysis of variance was used to assess differences between groups. The significance level was considered at p<0.05.

Results

GC-MS analysis of the ZEO was performed and 36 compounds were determined. Percentages of components of the essential oil (as determined by GC and GC-MS) are summarized in Table 1. As the results, the main component of the essential oil was carvacrol (46.82%).

The results of DPPH assay showed that increased scavenging of free radicals depended on the concentration, while this variation did not occur in the control sample and increasing concentration (Figure 1). There was no significant difference in radical scavenging effect (p>0.05). The antioxidant activity of sumac extract was significantly (p<0.05) higher than ZEO. Both sumac extract and ZEO showed the highest percentage of radical scavenging activity at concentrations of 250 µg/ml. In 62, 31, and 15 µg/ml concentrations, BHT showed more antioxidant ability than sumac extract and ZEO, but anti-

radical activity of BHT at concentrations of 250 and $125 \mu g/ml$ was less than sumac extract.

According to the reducing power assay, increased scavenging of free radicals in sumac extract and ZEO depended on the concentration. At all concentrations, sumac extract was more reducing capacity than BHT whereas the reducing power of ZEO was significantly less than BHT (p<0.05) and sumac extract (Figure 2).

The level of total phenolics in sumac extract and ZEO was 305.65 ± 61.94 and 179.42 ± 80.40 mg/g, respectively. Results of phenolic content showed that there is a significant correlation (p<0.05) between total phenolic compounds and antioxidant properties. Total phenol of sumac extract was significantly more than ZEO (p<0.05).

The results of ABTS radical scavenging power is shown in Table 2 in terms of percentage of inhibition and antioxidant capacity of ascorbic acid. It was found that antioxidant activity of BHT was more than sumac extract and ZEO in all concentrations and showed a statistically significant difference (p<0.05). Overall, the percentage of radical scavenging activity of sumac extract was more than ZEO in all concentrations. Concentration dependent increase in radical scavenging was observed for ZEO and sumac extract. This situation did not apply for BHT.

The sumac extract as well as ZEO showed different inhibitory capabilities towards the tested bacteria. S. Typhymurium with MIC 0.625 and 2.5 mg/ml was more sensitive than L. monocytogenes with MIC 1.25 and 5 mg/ml for ZEO and sumac extract, respectively. The MIC combination values were 0.322 and 0.161 mg/ml for sumac extract and ZEO, respectively. The highest level of antibacterial activity and the minimum bactericidal concentration against both bacteria was demonstrated by ZEO for S. Typhymurium and L. monocytogenes. Also, S. Typhymurium and L. monocytogenes had FIC values of 0.14 and 0.18 mg/ml, respectively. Based on the results of antibacterial activity, ZEO had significantly more potent than sumac extract (p<0.05). FICs of the ZEO and sumac extract in combined form showed clearly anti-Listeria and anti-Salmonella effect as synergistic.

Discussion

Results of GC-MS analytical data of compounds in ZEO showed that ZEO is rich in monoterpene phenols, especially thymol and carvacrol that have antibacterial and antioxidant properties. The amount of these compounds is related to season of growth, plant age, weather, soil type, drying plant method, and extraction method (Mehdizadeh et al., 2018).

In the present study, the hydroalcoholic extract of sumac and ZEO were evaluated for their radical scavenging activities by means of the DPPH assays. The current

Table 1: Chemical composition of Zataria multiflora Boiss essential oil

Compounds	KI ^a	Area (%)
α-Pinene	935	0.71
3-Octanone	986	0.78
3-Octanol	1002	0.27
Para-Cymene	1031	0.89
Limonene	1052	0.15
1,8-Cineole	1035	0.69
Linalool	1099	12.71
2-Nonanol	1107	0.42
Borneole	1178	0.27
α-Terpineol	1203	1.34
α-Terpinolene	1191	0.11
Caryophyllene oxide	1592	0.94
β-Caryophyllene	1437	1.09
β-Myrcene	1004	0.71
Aromadendrene	1472	0.94
Gamma-Terpinene	1081	0.59
Hotrienol	1151	0.32
Thymol,methyl ether	1237	0.82
Trans-Sabinene hydrate	1081	0.42
Thymol	1151	18.34
Carvacrol	1237	46.82
Carvacrol methyl ether	1081	1.51
Carvacryl acetate	1271	0.52
Linalyl acetate	1031	1.03
Aromadendrene	1252	0.72
Spathulenol	1394	1.21
Trans-Caryophyllene	1271	1.43
Veridifloren	1461	0.52
Cis-Linaloloxide	1584	0.71
Terpinene-4-ol	1446	0.94
Geraniol	1504	0.32
(1,3,8-p) Menthatriene	1107	0.32
Bicyclogermacrene	1172	0.24
(ar) Curcumene	1276	0.36
(3-) Octanol acetate	1147	0.06
Geranyl acetate	1512	0.27
Total		99.49

Table 2: Percentage of inhibition and radical scavenging of Zataria multiflora Boiss Essential Oil (ZEO), sumac extract and Butylated Hydroxytoluene (BHT)

Concentration (mg/ml)		Radical scavenging Percentage of inhibition (ascorbic acid as mg/ml)	
	Sumac	0.05±0.01 ^{aA}	53.09±2.5 ^{aA}
0.25	ZEO	0.01±0.00 b	7.92±0.54 bA
	BHT	0.19±0.02 °	93.53±2.47 ^{cA}
0.5	Sumac	0.08±0.00 aB	74.48±1.67 aB
	ZEO	0.01±0.00 b	11.39±0.67 bA
	BHT	0.19±0.00 °	94.66±6.39 cA
1	Sumac	0.14±0.01 ^{aC}	85.91±1.15 ^{aC}
	ZEO	0.02±0.00 b	15.68±1.14 bB
	BHT	0.19±0.02 °	95.73±5.45 ^{cA}
2	Sumac	0.17±0.01 ^{aD}	93.40±0.98 ^{aD}
	ZEO	0.01±0.00 b	20.36±0.96 bC
	BHT	0.20±0.02 °	98.93±2.32 ^{aA}
4	Sumac	0.19±0.02 ^{aE}	97.22±1.51 ^{aD}
	ZEO	0.06±0.01 b	34.11±1.83 bD
	BHT	0.20±0.04 a	100/0±0/00 ^{aA}

⁻ Different capital letters indicate a statistically significant difference between different concentrations of sumac or ZEO (p<0.05) - Different lowercase letters indicate a statistically significant difference between sumac and ZEO (p<0.05)

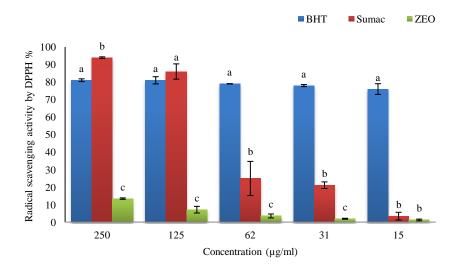


Figure 1: 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of *Zataria multiflora* Boiss Essential Oil (ZEO), sumac extract and Butylated Hydroxytoluene (BHT). Different letters indicate a statistically significant difference (p<0.05)

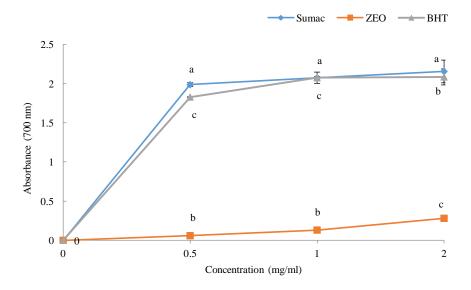


Figure 2: Reducing power of Zataria multiflora Boiss Essential Oil (ZEO), Sumac extract and Butylated Hydroxytoluene (BHT). Different letters indicate a statistically significant difference (p<0.05)

results of DPPH are in agreement with Zangiabadi et al. (2012) results but it is not match with Bazargani-Gilani et al. (2014). These differences may be due to changes in culture, harvest, and drying conditions that lead to diversity in antioxidant activity. The plant in different areas can show different combinations, features, and proper-

ties. Also, type and techniques of extraction can play important role in the antioxidant and antibacterial activity of plant, *in vitro* (Anzabi, 2015). Various studies have reported that inhibition of DPPH free radical by extracts is concentration-dependent and the inhibitory effect increases with increasing concentration (Kil et al., 2009;

Shukla et al., 2009). Also, Liu et al. (2007) found that there was a relationship between the phenol content and antioxidant activity of Chinese herbs, but according to Verzelloni et al. (2007), no relation was found between the antioxidant characteristics and the phenolic content of traditional balsamic vinegar. Some studies confirm a direct relation between the amount of total phenol and antioxidant activity of medicinal herbs and spices (Cai et al., 2004; Liu et al., 2008; Shan et al., 2007). Aliakbarlu et al. (2013) evaluated that the phenol content for ZEO was 44.81 mg GAE/g of sample. Zangiabadi et al. (2012) showed that the total phenol content of ZEO was 0.322±0.029 mg GAE/ml. Bursal and Koksal (2011) showed that the free radical scavenging activity of water extract of sumac was 41.2% (at the dose of 30 mg/ml) and ethanol extract of sumac did not show considerable DPPH radical scavenging activity; but results of this study showed that hydroalcoholic extract of sumac had free radical scavenging activity. The present study showed a direct relation between reducing power and the amount of total phenol of extract and ZEO. Therefore, sumac extract with the most total phenol content had the most reducing power. The phenol contents are important vegetable antioxidant compounds, because their hydroxyl groups have the inhibitory potential for radicals. Many researchers have reported that there is a relation between the phenol content and antioxidant activity, but some researchers showed that, there may be no relation at all (Sharififar et al., 2007). Aliakbarlu et al. (2014) reported that reducing power of water extract of sumac (2 mg/ml) was 1.026; and in this investigation, it was 2.155 for hydroalcoholic extract of sumac. ABTS radical scavenging is one the best method of determining the measure of the antioxidant capacity, for essential oils and food extracts (Gliszczyńska-Świgło, 2006). Results of ABTS method in this study showed that antioxidant activity of sumac extract is more than ZEO.

We found that ZEO had the highest antibacterial activity. Similar to present study, Sharififar et al. (2007) reported that ZEO had a higher antibacterial activity for Gram-negative bacteria than Gram-positives. Antibacterial effects of essential oils and extract on the types of bacteria are still under discussion. Contrary to our results, Aliakbarlu et al. (2013) reported MIC values of 0.625 against Gram-positive bacteria and 1.25 mg/ml against Gram-negatives. It seems that the different antimicrobial activity in this work as compared to others is related to kind of effective substances in extracts and essential oils, methods of extraction, and kind of solvent even used techniques. In addition, various cultivation areas may affect the compositions of the plants. In current study, the MIC and MBC values were generally lower for the ZEO than sumac extract against both bacteria. Generally, the

antimicrobial efficacies of plants are related to the chemical structure of their components as well as the concentration. In the present work, similar to findings of Singh et al. (2003) and Anzabi (2015) which were done on anti-Listeria effects of thyme, clove, pimento, rosemary, and sage oil, it was found that ZEO had the most effect on Listeria spp. The main components with antimicrobial impress found in medicinal herbs especially on ZEO were phenol compounds, ketones, aliphatic alcohols, acids, trepans, aldehydes, and flavonoids. Chemical analysis of a range of these components showed that the major constituents included thymol, carvacrol, citral, and their precursors. Antimicrobial action of phenolic compounds was related to the inactivation of cellular enzymes, which depended on the level of penetration of the substance into the cell and on destruction of the permeability of cell membranes (Liu et al., 2008). Erturk (2006) reported the MIC value of 15 mg/ml for ethanolic extract of sumac. In the study by Fazeli et al. (2007), Bacillus cereus was found to be the most sensitive bacteria against sumac showing the MIC of 0.05%.

Conclusion

Results of this research showed that the sumac extract had more potent antioxidative activity than ZEO. Also, its total phenolic content was higher than ZEO. However, based on the results of antibacterial activity, ZEO had significantly more potent than sumac extract. Generally, ZEO exhibits its food preservative effects in low amounts. Its usage in food is limited due to the vigorous taste and aroma, so it cannot be directly used in high amounts as food preservative. However, sumac extract and its products can be directly used in various foods due to their wonderful taste and high palatability. It seems that the mentioned essential oils and extracts in food may be directly used as food preservative. However, additional studies are needed to investigate the toxicity, mode of action, and also sensory properties in various foods.

Author contributions

A.M.L. and H.T. designed the study; A.M.L. and T.M. conducted the experimental work and wrote the manuscript; T.M. analyzed the data. All authors edited and approved the revised manuscript.

Conflicts of interest

There is no conflict of interest regarding the publication of this paper.

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