JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 3 (2016) 107-113

Comparative Evaluation of Phytochemical, Antioxidant, and Antibacterial Properties from the Essential Oils of Four Commonly Consuming Plants in Iran

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HIGHLIGHTS

- Clove had more antioxidant properties comparing to cumin, origanum, and anise Essential Oils (EOs).
- *Bacillus cereus* and *Escherichia coli* had the highest and lowest susceptibility to the EOs, respectively.
- Studied EOs can be regarded as antioxidant and antimicrobial agents in Iranian food industries.

Article type
Original article

Keywords
Oils, Volatile
Antioxidants
Food Safety

Article history
Received: 23 Mar 2016
Revised: 1 May 2016
Accepted: 28 May 2016

Acronyms and abbreviations
EO=Essential Oil
DPPH=2,2-diphenyl-1-picrylhydrazyl
BHT=Butylated hydroxytoluene
GC/MS=Gas Chromatography/Mass Spectrometry
MIC=Minimum Inhibitory Concentration
BHI=Brain Heart Infusion

ABSTRACT

Background: This study aimed at investigation of the chemical composition, antimicrobial activity, and antioxidant properties of clove, cumin, origanum, and anise Essential Oils (EOs).

Methods: Chemical compositions of the EOs were identified using Gas Chromatography/Mass Spectrometry (GC/MS). The antibacterial activities of EOs against four important food-borne bacteria were assessed by disc diffusion, agar well diffusion, and broth micro-dilution assays. Evaluation of antioxidant properties of the EOs was carried out by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, β-carotene-linoleic acid bleaching test, and total phenolic contents as well. Statistical analysis of data was performed using SPSS, Inc, Chicago, IL software.

Results: Eugenol (69.26%) was the main constituents of studied EOs. Although, all five tested bacteria were sensitive to EOs, but *Bacillus cereus* and *Escherichia coli* had the highest and lowest susceptibility to the antibacterial activity of EOs (p<0.05), respectively. Remarkable antioxidant capacity was observed in all EOs; however, clove EO had the highest antioxidant properties (p<0.05).

Conclusion: Clove, cumin, origanum, and anise EOs could be regarded as potential sources of natural antioxidant and antimicrobial agents in Iranian food industries and the best results was belonged to clove EO.

Introduction

Both natural and also synthetic antioxidants are widely applied for food preservation (Kamkar et al., 2014). The most common synthetic antioxidants in food are includeing butylated hydroxyl anisole, Butylated hydroxytoluene (BHT), propyl gallate, and tertiary butyl hydroquinone

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To cite: Raeisi M., Hashemi M., Aminzare M., Sadeghi M., Jahani T., Keshavarzi H., Jebelli Javan A., Mirahahidi M., Tepe B. (2016). Comparative evaluation of phytochemical, antioxidant, and antibacterial properties from the essential oils of four commonly consuming plants in Iran. *Journal of Food Quality and Hazards Control*. 3: 107-113.

Journal website: http://www.jfqhc.com

that have been suspected to case harmful effects to the health (Kamkar et al., 2014). Nowadays, demand for reducing use of synthetic food preservatives have increased throughout the world. Therefore, substitution of synthetic antioxidants by natural agents has caused great interest in food research (Hur et al., 2014).

Reactive oxygen and nitrogen species are constantly produced in human and are controlled by some endogenous enzymes, e.g. glutathione peroxidase, superoxide dismutase, as well as catalase. When these radicals are overproduced, vital biomolecules may be damaged (Oke et al., 2009). Antioxidants have been reported to prevent oxidative damage and may prevent the occurrence of some diseases such as cancer. They can interfere with the oxidation process and diminish them by scavenging free radicals or chelating catalytic metals. A large number of vegetables and plants Essential Oils (EOs) are known to be rich sources in antioxidants (Kamkar et al., 2014; Oke et al., 2009). Fruits, vegetables, and plants contain some antioxidant compounds, including phenolic compounds, carotenoids, anthocyanins, and tocopherols (Hur et al., 2014; Naczk and Shahidi, 2006). The EOs of vegetables and plants are liquid volatile oily and aromatic with density less than water. They are widely used as food preservatives in order to control the growth of pathogenic and spoilage microorganisms in food (Abdollahzadeh et al., 2014; Alboofetileh et al., 2014; Azhdarzadeh and Hojjati, 2016; Azizkhani et al., 2013; Dashipour et al., 2015; Khorasany et al., 2016; Miguel, 2010; Shojaee-Aliabadi et al., 2013). There are various herbs in Iran in some other parts of the world that are used in traditional medicine and food preservation such as clove (*Eugenia caryophyllata* Thunberg), cumin (*Cuminum cyminum* L.), origanum (*Origanum vulgare*), and anise (*Pimpinella anisum*) (Al-Bayati, 2008; Dhandapani et al., 2002; Johri, 2011; Moradi et al., 2014; Shojaii and Abdollahi Fard, 2012; Singh et al., 2012).

The objectives of this study were (1) to determine the chemical composition of hydro-distilled EOs of clove, anise, cumin, and origanum, (2) to investigate the antimicrobial activity of these EOs against *Escherichia coli*, *Salmonella thyphimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, as well as *Bacillus cereus*, (3) to evaluate antioxidant capacity of these EOs.

Materials and methods

Plant materials and EOs preparation

In this experimental study, the aerial parts of *E. caryophyllata*, *P. anisum*, *O. vulgare*, *and C. cyminum* were purchased from local markets in Urmia, West Azarbayjan, Iran. The plants were confirmed at Faculty of Agriculture and Natural Resources of Urmia University, Urmia, Iran. Extraction of EOs was performed using a Clevenger type apparatus for 3 h. The obtained EOs were dehydrated over anhydrous sodium sulfate, filtered by 0.22 µm filters, and were stored at 4 °C for further experiments (Hashemi et al., 2013; Raeisi et al., 2012).

Identification of the EOs components

According to Moradi et al. (2014), Gas Chromatography/Mass Spectrometry (GC/MS) analysis of EOs was performed using Hewlett Packard 5890 equipped with an HP-5MS capillary column (30×0.25 mm ID×0.25 mm film thickness). Flow rate of helium was 1 ml/min. The column temperature was initially 50 °C and then gradually was increased to 120 °C at a 2 °C/min rate, held for 3 min, and finally increased to 300 °C. The MS procedure was operated with ionization energy of 70 eV. The compounds were identified by comparing their retention indices with those of credible samples and mass spectral data available in the Wiley library (Wiley-VCH 2001 data software, Weinheim, Germany).

Determination of antibacterial activities of EOs

Two Gram-negative bacteria, including *E. coli* (PTCC 1533) and *S. thyphimurium* (PTCC 1730); and three Gram-positive bacteria, including *S. aureus* (PTCC 1015), *L. monocytogenes* (PTCC 1298), and *B. cereus* (PTCC 1665) were obtained from microbial collection of Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

For disk diffusion method, an amount of 0.1 ml of 18 h grown bacterial cultures (1.5×10^6 CFU/ml) were spread on plates containing Mueller-Hinton agar (Merck Darmstadt, Germany) and sterile paper disks (Padtan Teb, Iran) with 6 mm diameter were impregnated with 10 µl EOs and placed on the agar media. The plates were then incubated at 37 °C for 24 h and diameters of inhibition zones were measured in mm (Moradi et al., 2014).

To do well diffusion method, an amount of 1 ml of 18 h broth culture of bacteria was added to 100 ml of molten Mueller-Hinton agar (Merck Darmstadt, Germany), which were cooled to 45 °C, completely mixed for 2 min and poured into sterile plates. When agar was set, four wells were cut in each plate using a sterile cork-borer and 10 µl EOs were poured in each well. The plates were incubated at 37 °C for 72 h and the diameter of inhibitory zones was measured in mm (Boyanova et al., 2005).

Micro-well dilution assay was used to determine the Minimum Inhibitory Concentration (MIC) of EOs for the bacterial strains (Hashemi et al., 2013). Bacterial suspensions were prepared from 18 h broth cultures (1.5×10^6 CFU/ml). EOs was dissolved in 10% dimethyl sulfoxide. Then, the solutions firstly were diluted to the highest

concentration (100000 µg/ml) as a stock solution, and then serial two-fold dilutions were made in a concentration range from 100000 to 1562.5 µg/ml in nutrient broth. Aliquots of 160 µl Brain Heart Infusion (BHI) broth (Merck Darmstadt, Germany) and 20 µl inoculums were dispensed into 96-well micro plate. Amount of 20 µl EOs concentrations was then added into each well. Positive control (180 µl BHI broth+20 µl inoculums) and negative controls (180 µl uninoculated BHI broth+20 µl EOs) were considered in the last wells. The ultimate volume in each well was 200 µl, the final concentrations of EOs were in a range between 10000 to 156.2 µg/ml and final bacterial suspensions in each well was approximately 1.5×10^5 CFU/ml. The lowest concentration with no visible bacterial growth was regarded as the MIC values of EOs.

Determination of antioxidant properties of EOs

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed as previously described with minor modification (Aminzare et al., 2015). The amount of 50 µl from various concentrations of EOs and a reference antioxidant (BHT) were added to 2 ml methanolic solution of DPPH (24 µg/ml). The mixture was shaken and maintained for 60 min at room temperature in a dark place. Then, the absorbance was measured at 515 nm against a blank sample, a solution without any antioxidant, using a spectrophotometer (LKB Novaspec II; Pharmacia, Sweden). The capability of EOs to scavenge the DPPH radicals was calculated using the following equation:

$I\% = (A_{blank} - A_{sample}/A_{blank})\times100$

EOs concentration providing 50% inhibition (IC_{50}) was measured according to the curve of inhibition percentage of each sample.

β-carotene-linoleic acid bleaching test was carried out as previously described by Miraliakbari and Shahidi (2008) with minor modification. In order to prepare stock solution of β-carotene–linoleic acid, approximately 0.5 mg β-carotene (type I synthetic, Sigma–Aldrich) was dissolved in chloroform (1 ml) in a flask. Then, 20 µl linoleic acid (Sigma–Aldrich) and 200 mg tween 40 (Sigma–Aldrich) were added into the flask. The chloroform was removed completely using a rotary evaporator (Heidolph laborta 4003, SchwaBach, Germany) at 40 °C and 100 ml distilled water was added and vigorously shaken. Aliquots of this mixture (2.5 ml) were pipetted in test tubes containing 350 µl EOs (concentration: 2 mg/ml). The same procedure was repeated with BHT and a blank. The absorbance of each tube was measured at 470 nm immediately at zero time and after a two-hour period; the tubes were kept in a water bath at 50 °C. The capacity of EOs to protect against oxidation of β-carotene was determined as following equation:

$I\% = (A_{\beta\text{-carotene after 2 h assay}}/A_{\text{Initial }\beta\text{-carotene}})\times100$

Total phenolic contents of EOs were carried out using Folin-Ciocalteu reagent assay with gallic acid as a standard (Aliakbarlu et al., 2013). Briefly, 0.5 ml EO (concentration: 2 mg/ml) was mixed with 2.25 ml distilled water and 250 µl Folin-Ciocalteu reagent, and completely vortexed. The mixtures were allowed to react for 5 min; and then, aliquots of 2 ml Na_2CO_3 solution (7.5%) were added. After incubation period of 120 min at room temperature, absorbance of the mixtures was measured at 760 nm and data were expressed as mg of gallic acid equivalent per g of EOs, relative to the values obtained with a standard curve prepared using known concentrations of gallic acid.

Statistical analysis

All assays were performed in triplicate. Statistical analysis of data was carried out using SPSS, Inc, Chicago, IL software (v. 16.0). Tukey's test was used to compare differences among mean values obtained from the experiments ($p<0.05$).

Results

The main constituents of EOs are summarized in Table 1. GC/MS analysis of the clove, cumin, origanum, and anise EOs identified 7, 13, 25, and 17 components representing 98.10, 99.27, 97.23, and 96.35% of total content of studied EOs, respectively. Eugenol (69.26%), cuminalaldehyde (38.27%), thymol (21.83%), and also trans-anethole (59.67%) were determined as the main constituents of clove, cumin, origanum, as well as anise EOs, respectively.

Results of *in vitro* antibacterial activities of EOs against tested food-borne bacterial strains were assessed using disk diffusion, well diffusion, and broth micro-dilution methods, as shown in Table 2 to Table 4, respectively. All EOs had antibacterial effect against tested Gram-positive and Gram-negative bacteria. *B. cereus* as well as *E. coli* had the highest and lowest susceptibility to the antibacterial activity of EOs ($p<0.05$), respectively. On the other hand, inhibition pattern of antibacterial effect of applied EOs on each bacterium was compared and the following order was observed in all tested bacteria, clove>origanum>cumin>anise.

Results of antioxidant activities of EOs, assessed by three basically different systems namely DPPH radical scavenging activity, β carotene-linoleic acid bleaching test, and total phenolic content assays has been shown in Table 5.

As it can be seen, results disclosed remarkable antioxidant capacity in all tested EOs using different methods. However, clove EO had the highest antioxidant properties (38.20 µg/ml for DPPH test, 211.80 mg gallic acid equivalent/g EO for total phenolic content as well as 93.03% for β carotene-linoleic acid bleaching test) among the tested EOs having significant difference (p<0.05).

Table1: Chemical components of clove, anise, cumin, and origanum EOs

Number	Compound	KI*	Concentration of chemical component (%) in EOs			
			Clove	Cumin	Origanum	Anise
1	α-thujene	927	0	1.79	0.26	0
2	α-pinene	935	0	2.12	3.15	3.26
3	1-octen-3-ol	981	0	0	0.42	0
4	3-octanone	987	0	0	1.21	0
5	β-myrcene	990	0	1.84	1.17	0
6	α-terpinene	1017	0	0	0.25	0
7	Limonene	1029	0	1.98	2.14	10.80
8	γ-terpinene	1055	0	16.12	2.13	2.56
9	β-linalool	1104	0	0	4.80	0.48
10	β-thujone	1122	0	0	0.43	0
11	Sabinol	1145	0	0	0.32	0
12	Borneol	1174	0	0	1.12	0
13	α-terpineol	1188	0	0	9.10	0
14	Citronellol	1230	0	0	10.76	0
15	Carvone	1248	0	0	0.45	0
16	Thymol	1296	0	0	21.83	0
17	Thymol acetate	1347	0	0	0.64	0
18	Carvacrol acetate	1364	0	0	0.31	0
19	Eugenol	1402	69.26	0	7.85	0
20	Caryophyllene	1453	0	0	9.90	0
21	α-humulene	1465	1.32	0	3.84	0
22	Germacrene-D	1487	0	0	5.22	0
23	γ-cardinene	1520	0	0	0.39	0
24	Myristicin	1574	0	0	0.21	0
25	Spathulenol	1587	1.06	0	9.33	0.41
26	α-Farnesene	1495	0.27	0	0	0
27	P-cymene	1029	0	0	0	0
28	Terpinene-4-ol	1182	0	0.15	0	0
29	2-caren-10-al	1009	0	4.28	0	0
30	Cuminaldehyde	1235	0	38.27	0	0
31	Myrtenal	1211	0	7.45	0	0
32	Camphene	915	0	0	0	0.48
33	β-pinene	945	0	12.21	0	1.85
34	β-myrcene	960	0	0	0	2.30
35	α-phellandrene	968	5.23	1.43	0	0.61
36	P-cymene	993	0	9.23	0	0.13
37	Methyl chavicol	1201	0	0	0	6.28
38	Trans-anethole	1285	0	0	0	59.67
39	Elemene	1335	0	0	0	0.72
40	β-phellandrene	1449	18.68	2.40	0	0.27
41	γ-himachalene	1479	0	0	0	4.60
42	Methyl eugenol	1490	2.28	0	0	1.74
43	Germacrene-D	1520	0	0	0	0.19
	Total content		98.10	99.27	97.23	96.35

*Kovats Indices (KI) calculated against n-alkanes on HP-5 column

Table 2: Antibacterial effect of EOs on tested bacterial strains based on disk diffusion method (mean±standard deviation)

EOs	Diameter of inhibition zone (mm)				
	E. coli	*S. thyphimurium*	*S. aureus*	*L. monocytogenes*	*B. cereus*
Clove	9.17±0.45 ^{a}	11.77±0.15 ^{b}	15.24±0.47 ^{d}	12.83±0.21 ^{c}	15.37±0.25 ^{d}
Cumin	6.90±0.47 ^{a}	9.37±0.21 ^{b}	9.57±0.40 ^{b}	9.53±0.31 ^{b}	10.53±0.31 ^{c}
Origanum	6.93±0.30 ^{a}	9.53±0.31 ^{b}	13.93±0.31 ^{d}	12.50±0.26 ^{c}	14.20±0.40 ^{d}
Anise	4.37±0.31 ^{a}	6.07±0.25 ^{b}	9.40±0.47 ^{c}	8.27±0.31 ^{d}	11.27±0.31 ^{e}

Values followed by the same letter are not significantly different (p>0.05) according to Tukey's multiple range test

Table 3: Antibacterial effect of EOs on tested bacterial strains based on well diffusion method (mean±standard deviation)

EOs	Diameter of inhibition zone (mm)				
	E. coli	*S. thyphimurium*	*S. aureus*	*L. monocytogenes*	*B. cereus*
Clove	13.30±0.36 [a]	15.30±0.26 [b]	21.67±0.71 [d]	18.27±0.35 [c]	18.77±0.42 [d]
Cumin	11.47±0.32 [a]	11.77±0.38 [a]	14.17±0.25 [b]	13.60±0.30 [b]	14.20±0.36 [b]
Origanum	11.50±0.36 [a]	14.07±0.57 [b]	18.60±0.66 [d]	15.90±0.62 [c]	17.73±0.76 [d]
Anise	8.30±0.40 [a]	9.70±0.36 [b]	13.87±0.31 [c]	12.43±0.42 [c]	12.80±0.36 [c]

Values followed by the same letter are not significantly different (p>0.05) according to Tukey's multiple range test

Table 4: Antibacterial effect of EOs on tested bacterial strains based on micro-dilution method

EOs	Minimum inhibitory concentration (µg/ml)				
	E. coli	*S. thyphimurium*	*S. aureus*	*L. monocytogenes*	*B. cereus*
Clove	1250 [a]	625 [c]	312.5 [d]	312.5 [d]	625 [c]
Cumin	1250 [a]	625 [c]	625 [c]	625 [c]	625 [c]
Origanum	1250 [a]	625 [c]	312.5 [d]	625 [c]	625 [c]
Anise	2500 [b]	1250 [a]	625 [c]	625 [c]	625 [c]

Values followed by the same letter are not significantly different (p>0.05) according to Tukey's multiple range test

Table 5: Antioxidant capacity of tested EOs and BHT determined by different assays (mean±standard deviation)

Additive	DPPH IC_{50} (µg/ml)	Total phenolic content (mg gallic acid equivalent/g EO)	β carotene-linoleic acid bleaching test (%)
Clove	38.20±1.46 [a]	211.80±0.56 [c]	93.03±0.60 [d]
Cumin	63.30±0.95 [b]	172.97±2.93 [b]	86.77±0.55 [c]
Origanum	80.70±1.47 [c]	162.17±0.45 [b]	73.37±0.45 [b]
Anise	124.03±1.53 [d]	122.67±1.00 [a]	60.01±0.66 [a]
BHT	31.57±1.04 [a]	not examined	96.30±1.05 [d]

Values followed by the same letter are not significantly different (p>0.05) according to Tukey's multiple range test

Discussion

The major components of clove EO seen in the current study were orderly eugenol, β-caryophyllene as well as α-caryophyllene which were similar to components of native clove EO collected from India and also Argentina (Nunez and D'Aquino, 2012; Singh et al., 2012). Cuminaldehyde, γ-terpinene, β-pinene,o-cymene, and myrtenal were the major compounds of cumin EO that were confirmed in another study as well (Johri, 2011). The major compounds of origanum EO, including tymol, citronellol, caryophyllene, spathulenol, and α-terpineol were completely consistent with results of former studies (Mitchell et al., 2010; Teixeira et al., 2013). Also, we found that the major compounds of *P. anisetum* EO were orderly trans-anethole, limonene, methyl chavicol, and γ-himachalene. However, Shojaii et al. (2012) determined trans-anethole and estragol as major components of the oil belonging to *P. anisetum* collected from Iran. Results of other studies were completely consistent with the present study; although there were some differences in components and their quantities. This variation can be due to the various factors affecting on EOs chemical composition such as differences in climate, seasonal, and geographic conditions (Baydar et al., 2004).

The results of this study showed all five tested bacteria were sensitive to EOs; however, using various assays confirmed that *B. cereus*, *L. monocytogenes*, as well as *S. aureus* were found more sensitive to EOs than *S. typhimurium* and *E. coli*. Results of previous studies showed that antibacterial effect of EOs against Gram-positive are higher than Gram-negative bacteria, because, polysaccharide part of lipopolysaccharides and divalent cations in the outer cell membrane of Gram-negative bacteria has hydrophilic properties that prevents the contact of the hydrophobic constituents of EOs with the bacterial cell. Results of the present study were completely consistent with results obtained by other researchers (Akhondzadeh Basti et al., 2014; Moradi et al., 2014; Nunez and D'Aquino, 2012; Shojaii and Abdollahi Fard, 2012). Hashemi et al. (2013) reported that Minimum Inhibitory Concentration (MIC) values of *Echinophora platyloba* EO against *L. monocytogenes* as well as *S. aureus* were 6250 and 12500 ppm, respectively. Tajik et al. (2015) reported that the MIC value of *Zataria multiflora* Boiss EO against *L. monocytogenes* was 625 µg/ml. Based on the results, clove EO had the highest antibacterial effect against all tested bacteria, probably due to the presence of high amount of eugenol (69.26%), which was a known phenolic agent (Burt, 2004). Due to existence of different chemical components, there was not a specific mechanism for antibacterial effects of EOs. Among the proposed mechanisms, including cell walls disruption, loss of cell contents, destroying of cytoplas-

mic membrane and membrane proteins, coagulation in the cytoplasm, and dysfunction of the system activated proton transfer; membrane damages are the most important inhibitory effect of EOs (Bakkali et al., 2008; Burt, 2004).

The DPPH assay is based on scavenging free radicals (Oke et al., 2009). The DPPH radical scavenging ability of EOs was compared together using IC_{50} value of each EO. The EOs lower IC_{50} value indicated higher antioxidant activity. The DPPH results showed antioxidant activity of the tested EOs orderly clove, cumin, origanum, and anise. Clove EO showed a remarkable capacity in scavenging of radicals, which was highly comparable with BHT. These results have been confirmed in another study carried out by Tepe et al. (2007). Regarding the basis of this test, the yellowish color of β-carotene disappears in β-carotene bleaching test due to reaction with radicals derived by linoleic acid oxidation as well as antioxidants prevent its oxidation and delay bleaching of β-carotene. The rate of this bleaching can be slowed down in presence of any antioxidant (Kulisic et al., 2004; Oke et al., 2009). The antioxidant activity measured by β-carotene bleaching assay showed similar results to those obtained by the DPPH assay. However, all tested EOs had a strong capacity in maintenance of β-carotene, but clove EO had the strongest capacity completely close to the BHT. The activated methylene groups of monoterpene hydrocarbons are major reason of their antioxidant capacity especially in β-carotene bleaching assay (Ruberto and Baratta, 2000). In this study, various monoterpene hydrocarbons in components of each EO may play major role in maintenance of β-carotene. The Folin-Ciocalteu procedure, known as a useful and rapid method, is used for estimating the phenolic content of plant EOs and extracts (Aliakbarlu et al., 2013). In this study, the highest total phenolic content was identified in clove EO, followed by cumin, origanum, and anise EOs. Ghasemzadeh et al. (2012) reported the level of phenolic compounds in methanolic extracts of the six different varieties of sweet potato (*Ipomoea batatas*) as 1.82±0.84 to 3.95±0.91 mg/g dry weight. This result is in agreement with those obtained by two other antioxidant assays as well as results of GC/MS analysis, showing that the major reason of antioxidant activity of these EOs may be due to their high total phenolic contents; however, the effect of minor constituents of EOs should be considered as well. According to these findings, there was a relationship between antioxidant activity and total phenolic contents of an antioxidant. Phenolic compounds are the main agents that can donate hydrogen to free radicals and thus block the chain reaction of lipid oxidation at the initiation step. These high potential of phenolic compounds to scavenge radicals may be due to their phenolic hydroxyl groups (Oke et al., 2009). Indeed, redox properties of phenolic compounds allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers as well as metal chelating ability (Oke et al., 2009).

Conclusion

In this study, the chemical composition, antibacterial activity, and antioxidant properties of four commonly consuming plants of Iran were analyzed. According to the obtained results indicating remarkable and strong antibacterial and antioxidant activities clove, cumin, origanum, and anise EOs could be regarded as potential sources of natural antioxidant and antimicrobial agents in Iranian food industries and the best results belonged to clove EO.

Conflicts of interest

All authors of this article declare that there is not any conflict of interest.

Acknowledgments

This investigation was financially supported by Golestan University of Medical Sciences, Gorgan, Iran.

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