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Antimicrobial Effects of the Nanoemulsion of Rosemary Essential Oil against Important Foodborne Pathogens

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Background: The purpose of this study was to determine the effect of rosemary essential oil (REO) nanoemulsion against some important food borne pathogens.
Methods: Antibacterial effects of REO and REO nanoemulsion were determined using Agar disc diffusion, Broth microdilution and Steam phase diffusion methods against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shewanella SP*, *Listeria monocytogenes* and *Salmonella enteritidis*.
Results: Antibacterial effect of REO and REO nanoemulsion was increased with concentration enhancing of REO. There was no significant antibacterial activity in the effectiveness of nanoemulsion on the studied bacteria in comparison with REO in both disk diffusion and steam phase diffusion methods. MIC and MBC analysis of REO and prepared REO nanoemulsion showed that REO and its nanoemulsion have inhibited all studied bacteria. REO showed better inhibitory effects. REO and nanoemulsion of rosemary essential oil have the greatest effect on *Shewanella SP.*, *L. monocytogenes*, *S. aureus*, *S.enteritidis*, *E. coli* and *P. aeruginosa*, respectively.
Conclusion: In total, it can be said that REO and its nanoemulsion are desirable to inhibit the growth of food borne pathogens and can be a good choice as antimicrobial agents in food industry to enhance safety and extend foods' shelf life.

1. Introduction

Recently, there has been growing demand for safe foods across the world due to the increased prevalence of severe foodborne diseases, which are caused by the consumption of food products contaminated with food pathogens. The Center for Disease Control and Prevention (CDC) has reported that approximately 48 million individuals are affected by foodborne diseases each year in the United States. Several methods have been developed to increase food safety, such as thermal processing and use of chemical preservatives [1,2].

Each of these methods has specific advantageous and limitations; for instance, heating may corrupt foodborne pathogens and spoilage bacteria, while it could also decrease the nutritional value of food products.
There have been growing concerns about consumers due to the long-term use of chemical preservatives in large quantities as it may cause various disorders (e.g., cancer) [2]. Therefore, trends to use natural preservatives have extended as an alternative by manufacturers and consumers since they increase the shelf life and safety of food products with no side-effects [2]. Essential oils (EOs) and herbal extracts are considered to be excellent natural

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preservatives for food products. These compounds are aromatic, oily liquids, which are extracted from plants and commonly used as flavoring agents in food [3].Numerous studies have confirmed the antimicrobial effects of EOs against foodborne pathogens and spoilage microorganisms [4,5]. In this regards, the scientific community and food industry have attempted to use potential preservatives of natural origins in various food products.

Rosemary essential oil (REO) is a bioactive compound with remarkable antifungal, antibacterial, and hepatoprotein properties. REO is extracted from rosemary (*Rosmarinus officinalis*), which is a perennial herb of the Lamiaceae family and widely used for the preservation and flavoring of food products [6,7]. However, direct addition of aromatic plants or their derivatives to food as preservatives is mainly limited due to their flavor, which influences the organoleptic attributes of food at bioactive effective doses. On the other hand, the dispersion of EOs in water-based products is rather difficult [7]. Special methods have been developed in order to overcome these limitations, such as encapsulation and nanotechnology.

Nano-scale manipulation of materials has created novel opportunities for new research and interdisciplinary scientific cooperation [8]. The particle size of bioactive compounds changes to less than 100 nanometers by nanotechnology, which could also be applied to carry EOs, compromise their adverse effects, and optimize the interactions of EOs with food components [9].

In recent years, nanotechnology has expanded rapidly in food industry [9]. This technology is highly capable of extending the shelf life of food products, production of new foods, and optimizing the taste, texture, and sensory attributes of food [10]. Furthermore, nanoemulsions, which are in the form of oil in water emulsions with the droplet size of less than 100 nanometers, have been identified as a transmission system for the bioactive compounds that are produced by nanotechnology with unique features such as transparency, stability, and high performance [8].

Given the importance of EOs in the prevention of foodborne diseases and food spoilage, and since no reports have been published regarding the antimicrobial effects of REO nanoemulsion against foodborne pathogens, the present study aimed to determine the effects of REO nanoemulsion against some important foodborne pathogens.

2. Materials and Methods

2.1. Essential Oil Extraction

Rosemary plant (*Rosmarinus officinalis*) was purchased a local market in Zanjan, Iran. The plants were washed and dried in shade at room temperature for seven days. REO was extracted from the grinded, dried plants using the hydrodistillation method and Clevenger's apparatus (KOL, Behr, Germany) at the temperature of 100 °C for three hours. Afterwards, the EO was collected and dried using anhydrous sodium sulfate, filtered through 0.22-μm filters, and stored in a sterile bottle made of dark glass at the temperature of 4 ºC for further analysis [11].

2.2. GC-MS Analysis

An equipped gas chromatograph (AGILENT 7890 B, Santa Clara, CA) with an HP-5MS column (length: 60 m, film thickness: 0.25 mm, I.D.: 0.25 mm), which was coupled to a mass spectrometer (AGILENT 5977A), was applied to determine the chemical composition of REO using the method described by Raeisi et al. (2016) with some modifications.

The flow rate of helium (He2) as the carrier gas was 1.5 ml/min. The initial temperature of the column was maintained at 50 °C for five minutes and programmed to rise gradually to 150 °C at the 5°C/min gradient (holding time: 5 min), while it increased to 300°C with the gradient of 20 °C/min (holding time: 5 min). The temperature of the injector and detector was set at 270 °C and 250 °C, respectively. The injected volume and split ratio were determined to be one microliters and 1:50, respectively. The obtained mass spectra were compared to those available in the database (Wiley-VCH 2001 data /NIST Ver 11, 2017) in order to identify the existing compounds [12].

2.3. Preparation of the Bacteria

The lyophilized bacteria cultures of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 15224), *Pseudomonas aeruginosa* (ATCC 15442), Shewanella sp. (ATCC 1711), *Listeria monocytogenes* (ATCC 13932), and *Salmonella enteritidis* (ATCC 14028) were purchased from the Iranian Research Organization for Science and Technology (IROST) in Tehran, Iran and prepared in accordance with the instructions of the IROST manual for each bacterium.

A loopful of the stock culture was transferred to the brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated at the temperature of 35 ºC for 18-24 hours so as to prepare fresh suspensions of the tested bacteria. Following that, the freshly prepared suspensions were set to the absorbance of 0.08-0.1 at 600 nanometers in order to obtain an enumeration equal to 108 CFU/ml of the bacteria using a spectrophotometer (Milton Roy Company, Warminster, USA). In addition, bacterial count was confirmed by the tenfold serial dilution and surface spreading of the bacteria on the plate count agar media and incubation at the temperature of 35 ºC for 24 hours [13].

2.4. Preparation of the REO Nanoemulsion

At this stage, the high-amplitude ultrasonic method was used for the preparation of the REO nanoemulsion. To this end, various concentrations of REO and Tween 80 with Span 80 as the surfactant were mixed (Table 1). The combination of REO and surfactant was mixed on a hot plate using a magnetic stirrer for 10 minutes. Afterwards, the solution was initially blended and homogenized using a high-speed homogenizer (model: Silent Crusher M, Heidolph, Germany) at 9,000 rpm for 20 minutes. Following that, sonication was performed for three minutes at the maximum rate.

Table 1 shows the REO nanoemulsion with various formulations. As can be seen, the prepared nanoemulsions had a transparent structure with no turbidity. All the prepared formulations were preserved for up to five months at room temperature (25 °C) and refrigerated temperature (4 °C). No changes were observed in the particle size and polydispersity index (PDI), confirming the long-term sustainability of the nanoemulsions and specified maintenance period at room and refrigerated temperatures.

Table 1: Various Formulation of REO for Preparation of Optimal Nanoemulsions

Formulation	REO (w/w %)	Tween 80 (w/w%)	Span 80 (w/w%)	PDI	Mean size (nm)
1	5	12.5	0	1.000 ± 0.00	74.91 ± 7.08
2	5	10	0	0.467 ± 0.194	39.006 ± 15.38
3	5	8.75	0	1.000 ± 0.00	68.17 ± 2.75
4	5	10	5	0.140 ± 0.014	124.76 ± 1.26
5	5	8.75	2.5	0.228 ± 0.023	116.23 ± 3.35
6	5	7.5	2.5	0.329 ± 0.014	92.18 ± 0.64
7	5	5	2.5	0.289 ± 0.003	121.23 ± 1.16
8	5	7.5	1.25	0.282 ± 0.031	33.84 ± 0.31
9	5	8.75	1.25	0.321 ± 0.043	62.28 ± 1.006
10	5	5	1.25	0.370 ± 0.021	59.9 ± 1.45
11	5	3.75	2.5	0.230 ± 0.009	61.73 ± 0.94
12	5	3.75	1.25	0.295 ± 0.016	78.27 ± 3.90
13	5	2.5	2.5	0.372 ± 0.012	119.32 ± 1.10

2.5. Determining the Size of the Nanoemulsion Particles

The particle size of the nanoemulsion was determined using the dynamic light dispersion device (ZEN 3600, Malvern, Investor Company, GB) at the temperature of 25 °C. To this end, one milliliter of each sample (diluted with deionized water 20 times) was poured into the cell of the target device [14].

2.6. Antibacterial Effects of the REO Nanoemulsion

In the current research, three methods were used to assess the antibacterial effects of REO and REO nanoemulsion.

2.6.1. Agar Diffusion Assay (Disk-Diffusion Method)

In the present study, the disk-diffusion method was applied to determine the antibacterial effects of REO and REO nanoemulsion. Briefly, 100 microliters of each of the mentioned bacterial suspensions containing 10^7 CFU/ml were spread on the Müller-Hinton agar culture media (Merck, Darmstadt, Germany), and sterile disks (diameters: 6-10 mm) were transferred onto the culture media containing the bacteria. Afterwards, 10 microliters of various concentrations of filtered REO and REO nanoemulsion (1%, 2%, 4%, and 8%) were transferred onto the disks, and the agar medium plates were incubated at the temperature of 37 °C for 24 hours. Finally, the antibacterial activity of REO and REO nanoemulsion was determined by measuring the growth inhibition zone (GIZ) of each bacterium. In this process, disks containing chloramphenicol (30 u/g) were used as the positive control [15].

2.6.2. *Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

To determine the minimum inhibitory concentration (MIC), the broth microdilution method was used as recommended by the CLSI with some modifications. Briefly, 160 microliters of the BHI broth was added to each well of a 96-well microplate. Following that, 20 microliters of the each fresh overnight bacterial inoculum (5×10^6 CFU/ml set by 0.5 McFarland standard tubes) and 20 microliters of each concentration of the REO and REO nanoemulsion solutions (0.5%, 1%, 2%, 4%, and 8%) were added. A well containing 20 microliters of the inoculum of each bacterium and 180 microliters of the BHI broth was considered as the positive control, and another well containing 180 microliters of the BHI broth and 20 microliters of filtered REO was considered as the negative control.

The microplates were incubated at the temperature of 37 °C for 24 hours. The minimum concentration of the EOs visibly showing bacterial inhibition growth was considered as the MIC. The wells without bacterial growth (pre-determined 99% reduction of bacterial population) were defined as the minimum bactericidal concentration (MBC) of REO and REO nanoemulsion [16].

2.6.3. Steam-phase Diffusion Method

The steam-phase diffusion method was applied in accordance with the method described by Masoomi et al. (2016). Initially, 100 microliters of the bacterial suspension with the inoculation dose of 107 CFU/ml was spread on the BHI agar culture medium. Following that, 0.5 milliliter of a semi-melted agar medium was placed at the center of the plate door. In addition, a sterile blank disk was placed on this section, and 20 microliters of the mentioned pure bioactive materials (REO and REO nanoemulsion) was added to the blank disk. The culture plates were incubated at the temperature of 37 °C for 24 hours, and the GIZ (mm) indicated the steam diffusion rate of the REO and REO nanoemulsion [17].

2.7. Statistical Analysis

All the experiments were conducted in triplicate. Data analysis was performed using the mean and standard deviation (SD) of the variable in Microsoft Excel software (2010).

3. Results and Discussion

3.1. Composition of the EO

The chemical composition of REO is shown in Table 2. The GC-MS analysis revealed 47 compounds, representing 100% of the total contents of the REO. The main compounds in REO included 3-carene (13.25%), -α-bornyl acetate (11.9%), verbenone (11.77%), eucalyptol (10.69%), (+)-2-bornanone (9.71%), camphene (5.40%), 3-octanone (4%), D-limonene (3.97%), and linalool (3.54%).

According to the information in Table 2, monoterpene and monoterpenoid were the main components of REO, which is inconsistent with the results of the previous studies in this regard (Table 3). The discrepancy in the chemical constituents of EOs could be attributed to several

factors, including the differences in climate, geographical conditions, plant parts, cultivation conditions, cultivar type, planting season, methodology and duration of extraction, and duration and condition of EO storage [12].

3.2. Nanoemulsion and Particle Size Characteristics

Table 1 shows various formulations of the REO nanoemulsion. Based on the mean size and PDI, the optimal prepared nanoemulsion was formulation No. 11, which contained 5% (w/w), 3.75% (w/w), and 2.5% (w/w) of REO, Tween 80 and Span 80, respectively, with the mean particle size of 61.73 ± 0.94 nanometers and mean PDI of 0.230 ± 0.009.

The most important stage in the present study was the accurate formulation and proper production of oil in water nanoemulsion. If the formulation was prepared improperly, the emulsion system would collapse [22]. The foremost parameters that influenced the chemical characteristics and stability of the nanoemulsion included the homogenization pressure, rotation rate, and concentration of the surfactants [23]. The mean particle size largely depended on the surfactant-to-oil ratio (SOR), which decreased with increased SOR. In a study in this regard, Martin Piñero et al. (2018) reported that obtaining smaller particle sizes than 200 nanometers is possible with the 30% increase of the surfactant and reduction of the EO concentration [24].

Similarly, Restrepo et al. (2018) reported the mean particles size of rosemary nanoemulsion to range from 164 ± 9 to 676 ± 26 nanometers [25].

Table 2: Chemical Composition of *Rosmarinus officinalis* Essential Oil

No	Compound Label:	Area Sum	RT	Kovats index
1	3-Carene	13.25	8.78	948 iu
2	Camphene	5.4	9.177	943 iu
3	Bicyclo[3.1.0]]hex-2-ene, 4- methylene-1-(1-methylethyl)-	0.72	9.246	879 iu
4	1-Octen-3-ol	0.38	9.49	969 iu
5	3-Octanone	4	9.648	952 iu
6	beta.-Myrcene	2.2	9.744	958 iu
7	3-Octano	0.45	9.781	979 iu
8	alpha.-Phellandrene	0.24	10.104	969 iu
9	(+)-4-Carene	0.53	10.316	919 iu
10	o-Cymene	2.01	10.453	1042 iu
11	D-Limonene	3.97	10.548	1018 iu
12	Eucalyptol	10.69	10.644	1059 iu
13	gamma.-Terpinene	0.34	11.03	998 iu
14	Cyclohexene,1-methyl-4-(1-methylethylidene)-	0.82	11.565	1052 iu
15	Linalool	3.54	11.655	1082 iu
16	2,7-Octadiene-1,6-diol, 2,6-dimethyl-	0.18	11.724	1325 iu
17	2,6,6-Trimethylbicyclo [3.2.0] hept-2-en-7-one	0.6	11.851	1119 iu
18	Fenchol	0.13	12.057	1138 iu
19	1,7,7-Trimethylbicyclo [2.2.1]hept-5- en-2-one	0.2	12.232	1103 iu
20	trans-Verbenas	0.3	12.576	1136 iu
21	(+)-2-Bornanone	9.71	12.661	1121 iu
22	Ketone,2,2-dimethylcyclohexyl methyl	0.14	12.714	1151 iu
23	3-Cyclopentene-1-ethanol, 2,2,4-trimethyl-	0.19	12.809	1209 iu
24	Bicyclo[3.1.1]heptan-3-one, 2,6,6- trimethyl-, (1.alpha.,2.beta.,5.alpha.)-	2.22	12.873	1109 iu
25	Bicyclo[2.2.1]heptan-2-ol,1,7,7- trimethyl-, (1S-endo)- (-α-bornyl acetate)	6.61	12.973	1138 iu
26	trans-3(10)-Caren-2-ol	0.21	13.037	1131 iu
27	Terpinen-4-ol	2.3	13.095	1137 iu
28	Benzenemethanol, .alpha.,.alpha.,4-trimethyl-	0.18	13.164	1197 iu
29	alpha.-Terpineol	2.67	13.28	1143 iu
30	(-)-Myrtenol	0.41	13.407	1191 iu
31	3-Cyclopentene-1-ethanol, 2,2,4-trimethyl-	1.5	13.455	1209 iu
32	Bicyclo[3.1.1]hept-3-en-2-one,4,6,6-trimethyl-, (1S)- (Verbenone)	10.55	13.683	1119 iu
33	Ethanol, 2-(3,3-dimethylcyclohexylidene)-, (Z)-	1.81	14.053	1281 iu
34	D-Carvone	0.2	14.106	1190 iu
35	(1S,3S,4S,5R)-1-Isopropyl-4- methylbicyclo [3.1.0]hexan-3-ol	1.36	14.159	1079 iu
36	3-Methyl-2-(2-methyl-2-butenyl)- furan	0.19	14.54	1115 iu
37	Bicyclo[2.2.1]heptan-2-ol, 1,7,7- trimethyl-, acetate, (1S-endo)- (-α-bornyl acetate)	5.29	14.742	1277 iu
38	Phenol, 2-methyl-5-(1methylethyl)-	0.18	14.821	1262 iu
39	Bicyclo[2.2.1]hept-2-ene, 1,7,7-trimethyl-	0.24	15.398	932 iu
40	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)-	2.01	15.71	1223 iu
41	Cyclopentane, 1-acetoxymethyl-3-isopropenyl-2-methyl-	0.27	15.996	1315 iu
42	trans-Farnesol	0.15	16.049	1710 iu
43	Cyclopentane, 1-acetoxymethyl-3-isopropenyl-2-methyl-	0.47	16.123	1315 iu
44	Bicyclo[3.1.1]hept-3-en-2-one,4,6,6- trimethyl-, (1S)- (Verbenone)	0.22	16.356	1119 iu
45	Caryophyllene	0.52	17.087	1494 iu
46	5,9-Undecadien-2-one, 6,10-dimethyl-,(E)-	0.17	17.193	1420 iu
47	Isoaromadendrene epoxide	0.3	19.925	1281 iu
			SUM =100.02	

Table 3: Comparison of Main Components of REO Based on Previous Studies

Main components	Part of plant (Extract or EO)	part of Plant	Origins of plant	References
3-Carene(12.25%), Verbenone (11.77%), Eucalyptol (10.69 %),, (+)-2-Bornanone (9.71%) , Camphene(5.40%), 3-Octanone(4%) , D-Limonene (3.97% , Linalool (3.54%)	EO	Seed	Zanjan Iran	Present study
Camphor (24.12%) ،1R- alpha- Pinene(11.04%), Eucalyptol(16.87%) ،Beta-Pinene(5.51%) ،Camphene(5.16%)	EO	Seed	Ho chi Minh City, Vietnam	[18]
1,8-cineole (31.50%), a-pinene (18.33%), camphor (9.72%), a-Terpineol (9.42%) and Borneol (5.05%)	EO	Seed	Guelma, Tebessa (East Algeria)	[19]
α-pinene (48.58%), 1,8-cineole (33.4%) camphene (8.69%) , Camphor (2.58)	EO	Seed	Morocco	[20]
Camphor (23.17%) ,α-Pinene (18.56%) , Verbenone (11.32%) , 1,8-Cineole (11.89%), Borneol(8.89%)	EO	Seed	Tehran, Iran	[21]
α-Pinene (32.44%), 1,8-Cineole (25.04%), Verbenone (4/15%), limonene (3.97%) , camphene (3.93%)	EO	Seed	Kerman, Iran	[21]

3.3. Antimicrobial Properties of the REO and REO Nanoemulsion

Table 4 shows the growth inhibitory effects of the REO and REO nanoemulsion against *E. coli, S. enteritidis*, *Shewanella* sp., *L. monocytogenes*, *S. aureus*, and *P. aeruginosa* based on the agar disk-diffusion and steam-phase diffusion methods. Table 5 shows the MIC and MBC of the REO and REO nanoemulsion against these pathogens based on the broth microdilution method.

The findings of the current research indicated no significant antibacterial effects of the nanoemulsion on the studied bacteria compared to the REO using the disk-diffusion and steam-phase diffusion methods (Tables 4 & 5). In the nanoemulsion particles, the EO droplets were covered by a liquid phase containing the surfactant. Therefore, the antibacterial effects of the EO were not observed in the nanoemulsion escape phase as was found in pure EO (Table 5) [17].

Several reports have denoted the antibacterial effects of REO. In a study conducted by Raeisi et al. (2016) regarding the antimicrobial effects of REO against foodborne pathogens, the mean diameters of the GIZ formed by pure REO on *E. coli*, *L. monocytogenes*, *S. typhimurium*, and *S. aureus* were estimated at 23.2 ± 0.4, 31.3 ± 0.3, 21.4 ± 0.2, and 30.4 ± 0.3 millimeters, respectively [12]. In another study in this regard, Fu et al. (2007) reported that the mean GIZ and antibacterial effects of REO against *S. aureus*, *E. coli*, *P. aeruginosa* were 18.5 ± 1.3, 10.0 ± 0.8, and 6.0 ± 0 millimeters, respectively [26]. The results of the aforementioned studies are inconsistent with the current research.

In another study by Evangelista Martínez et al. (2018), the mean antibacterial effects of REO against *E. coli*, S. aureus, and *S. typhimurium* were reported to be 11.7 ± 0.1, 11.8 ± 1.7, and 8.2 ± 0.7 millimeters, respectively [27]; these values are in line with the results of the present study. On the other hand, Jawad et al. (2018) reported that the mean GIZ of REO against three types of bacteria (*E. coli*, *Pseudomonas*, and *Bacillus cereus*) at two REO concentrations of 64 and 512 µl/ml was 19, 18, and 19 millimeters and 26, 25, and 26 millimeters, respectively [28].

To the best of our knowledge and based on the literature review, no similar studies have been focused on the effects of REO nanoemulsion against foodborne pathogens. Comparison of the results of the present study with the previous studies regarding the nanoemulsion of EOs indicated similar outcomes. For instance, the mean GIZ for thyme EO nanoemulsion against *E. coli* O_{157}:H_7 has been reported to be 9.72 ± 0.3 millimeters and zero based on the disk-diffusion and steam-phase diffusion methods, respectively [17]. On the other hand, the EO surrounded by the surfactant layer in the nanoemulsion particles could not penetrate into the vapor phase, and the antibacterial effects of the REO became subsequently weaker compared to the pure EO [17].

In the present study, the MIC and MBC analysis of REO and the prepared REO nanoemulsion indicated that REO and its nanoemulsion inhibited all the studied bacteria, while REO exhibited more significant inhibitory effects (Table 4). This is inconsistent with the results obtained by Moghimi et al. (2016), which demonstrated the four-fold antibacterial activity of EO nanoemulsion compared to the bulk oil against *E. coli* and *S. typhi* based on the MIC and MBC measurements [28]. The difference in this regard could be due to the active binding sites of the bioactive ingredients of EO with the applied surfactant, as well as the inhibition of bringing the EO into the proximity of the bacterial cell membrane [29]. On the other hand, the results of the present study demonstrated that *P. aeruginosa* had lower sensitivity to REO, which is in line with the findings of Jawad et al. (2018) and Thanh et al. (2017) [18,30].

According to the findings of the current research, the REO and REO nanoemulsion most significantly affected *Shewanella* sp., *L. monocytogenes*, *S. aureus*, *S. enteritidis*, *E. coli*, and *P. aeruginosa*, respectively. In addition, the results of all the antimicrobial experiments indicated that the REO and REO nanoemulsion had more significant antibacterial effects against gram-positive bacteria (*Shewanella* sp., *L. monocytogenes*, and *S. aureus*) compared to the gram-negative bacteria (*S. enteritidis*, *E. coli*, and *P. aeruginosa*). In gram-positive bacteria, ion permeability enhanced the cell membrane due to the direct contact of the lipophilic components of the EO with the phospholipid layer of the cell membrane, and the absence of an outer phospholipid membrane facilitated intracellular excretion [15].

Table 4: Growth Inhibition Zone (mm) of REO and REO Nanoemulsion against Foodborne Pathogens Based on Disk-diffusion Method (Mean ± SD)

Rosemary essential oil (REO)						
Concentration	***S.entritidis***	***L.monocytogenes***	***Shewanella sp.***	***S. aureus***	***E.coli***	***P.aeroginosa***
1	7.16 ± 0.28	8.16 ± 0.76	9.16 ± 0.76	8.26 ± 0.68	7.83 ± 0.76	9.16 ± 0.76
2	10.5 ± 0.86	10.5 ± 0.5	10.1 ± 0.79	9.13 ± 0.32	10 ± 1	10.5 ± 0.86
4	12.16 ± 0.28	11.45 ± 0.18	11.25 ± 0.66	11 ± 0.5	11.83 ± 0.28	11.75 ± 0.25
8	13.5 ± 0.5	12.13 ± 0.32	13.01 ± 0.9	12.36 ± 0.51	12.66 ± 0.57	11.83 ± 0.76
Pure	0.5 ± 15	14 ± 0.5	16.5 ± 0.5	14.58 ± 0.38	14.41 ± 0.80	13.02 ± 0.55
Control	0.54 ± 25.35	18.8 ± 0.61	26.16 ± 1.04	24.16 ± 0.76	19.33 ± 1.52	22.36 ± 1.30
REO Nanoemulsion						
1	6.66 ± 0.28	6.76 ± 0.25	7 ± 0.5	6.9 ± 0.1	6.73 ± 0.25	7.1 ± 0.36
2	7.16 ± 0.56	7.06 ± 0.73	7.63 ± 0.55	7.43 ± 0.40	7.78 ± 0.40	7.5 ± 0.5
4	7.83 ± 0.28	7.76 ± 0.25	8.33 ± 0.28	8.25 ± 0.25	8.46 ± 0.45	7.5 ± 0.5
8	8.76 ± 0.25	9 ± 0.5	9.16 ± 0.28	9.43 ± 0.43	9.66 ± 0.28	8.4 ± 0.36
Pure	10 ± 0.5	10.1 ± 0.36	10.51 ± 0.21	10.03 ± 0.35	10.43 ± 0.60	9.83 ± 0.15
Control	25.03 ± 0.55	19.16 ± 0.76	25.43 ± 1.40	25.1 ± 0.79	18.83 ± 0.76	22.43 ± 0.92

Table 5: Growth Inhibition Zone (mm) Based on Steam-phase Diffusion Method (Mean ± SD) and MIC and MBC (%) of REO and REO Nanoemulsion against Foodborne Pathogens

		S.entritidis	***L.monositojenes***	***Shewanella sp***	***S. aureus***	***E.coli***	***P.aeruginosa***
REO	24 ± 3.605	21.66 ± 3.78	24.33 ± 0.57	24.33 ± 0.57	17.33 ± 3.21	16 ± 2	13.66 ± 1.52
REO nanoemulsion	Zero	Zero	Zero	Zero	Zero	Zero	Zero
REO	2	1	1	1	1	2	4
	4	4	2	2	4	8	8
REO nanoemulsion	2	2	1	1	2	4	4
	8	8	4	4	8	8	8

4. Conclusion

According to the results of the microdilution and disk-diffusion assays, both the REO and REO nanoemulsion exerted more significant antibacterial effects against gram-positive bacteria compared to the gram-negative bacteria. However, no significant differences were observed in the antibacterial effects of REO nanoemulsion and REO based on the disk-diffusion and steam-phase diffusion methods. In conclusion, it could be stated that REO and REO nanoemulsion are proper candidates for the growth inhibition of foodborne pathogens and could be used as effective antimicrobial agents in food industries for the improvement of food safety and to extend the shelf life of food products.

Authors' Contributions

H.H.A., and S.Y., designed the manuscript; A.G.H., and M. A., managed the analysis of the literature search; S.Y., and M.F., performed data acquisition; H.H.A., performed the statistical analysis; H.H.A., and S.Y., drafted the manuscript.

Conflict of Interest

None declared.

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References

1. Severino R, Ferrari G, Dang Vu kh, Donsi F, Salmieri S, Lacroix M. Antimicrobial Effects of Modified Chitosan Based Coating Containing Nanoemulsion of Essential Oils, Modified Atmosphere Packaging and Gamma Irradiation against *Escherichia coli* O_{157}:H_7 and *Salmonella Typhimurium* on Green Beans. *Food Control.* 2015; 50: 215-22.

2. Alizadeh Sani M, Ehsani A, Hashemi M. Whey Protein Isolate/Cellulose Nanofiber /TiO_2 Nanoparticle/ Rosemary Essential Oil Nanocomposite Film: Its Effect on Microbial and Sensory Quality of Lamb Meat andGrowth of Common Foodborne Pathogenic Bacteria during Refrigeration. *Int J Food Microbiol.* 2017; 251: 8-14.

3. Falcó I, Verdeguer M, Aznar R, Sánchez G, Randazzo W. Sanitizing Food Contact Surfaces by the Use of Essential Oils. *Innov Food Sci Emerg Technol.* 2019; 51: 220-8.

4. Jemaa MB, Falleh H, Serairi R, Neves MA, Snoussi M, Isoda H, et al. Nanoencapsulated Thymus Capitatus Essential Oil as Natural Preservative. *Innov Food Sci Emerg Technol.* 2018; 45: 92-7.

5. Lorenzo Leal AC, Palou E, López Malo A. Evaluation of the Efficiency of Allspice, Thyme and Rosemary Essential Oils on Two Foodborne Pathogens in In-Vvitro and on Alfalfa Seeds, and Their Effect on Sensory Characteristics of the Sprouts. *Int J Food Microbiol.* 2019; 295: 19-24.

6. Elbanna K, Assiri AM. Tadros M, Khider M, Mohdaly A, Ramadan MF. Rosemary (Rosmarinus officinalis) Oil: Composition and Functionality of the Cold Pressed Extract. *J Food Meas Charact.* 2018; 12(3): 1601-9.

7. Acevedo Fani A, Salvia Trujillo L, Rojas Graü MA, Martín Belloso O. Edible Films from Essential-Oil-Loaded Nanoemulsions: Physicochemical Characterization and Antimicrobial Properties. *Food Hydrocoll.* 2015; 47: 168-77.

8. Salvia Trujillo L, Rojas Graü MA, Soliva Fortuny R, Martín Belloso O. Effect of Processing Parameters on Physicochemical Characteristics of Microfluidized Lemongrass Essential Oil-Alginate Nanoemulsions. *Food Hydrocoll.* 2013; 30(1): 401-7.

9. Prakash B, Kujur A, Yadav A, Kumar A, Singh PP, Dubey NK. Nanoencapsulation: An Efficient Technology to Boost the Antimicrobial Potential of Plant Essential Oils in Food System. *Food Control.* 2018; 89: 1-11.

10. Roy A, Guha P. Formulation and Characterization of Betel Leaf (Piper Betle L) Essential Oil Based Nanoemulsion and Its InVitro Antibacterial Efficacy against Selected Food Pathogens. *J Food Process Preserv.* 2018; 42(6): e13617.

11. Ahmadyasbchin S, Mostafapor Rami M, Rajaee Maleki S. The InVitro Inhibitory Effects of the Rosemary Essential Oil on Some Gram Positive and Negative Bacteria. *J Ilam Uni Med Sci.* 2015; 24 (2): 80-9.

12. Raeisi M, Tajik H, Aminzare M, Sangin Abadi S, Yarahmadi A, Yarahmadi E, et al. The Role of Nisin, Monolaurin, and EDTA in Antibacterial Effect of Rosmarinus officinalis L. and Cinnamomum Zeylanicum Blume Essential Oils on Foodborne Pathogens. *J Essent Oil Bear Plants.* 2016; 19(7): 709-20.

13. Abdollahzadeh E, Rezaei M, Hosseini H. Antibacterial Activity of Plant Essential Oils and Extracts: The Role of Thyme Essential Oil, Nisin, and Their Combination to Control *Listeria monocytogenes* Inoculated in Minced Fish Meat. *Food Control*. 2014; 35(1): 177-83.

14. Samani SS, Soleimanian Zad S, Sheikh Zeinoddin M, Fathi M. Evaluation of *Zataria multiflora* Boiss. and *Carumcopticum L.* Essential Oil Based Nanoemulsions in Inhibition of Byssochlamys fulva Growth in Apple Juice. *J Agric Sci Technol*. 2019; 21(2): 357-68.

15. Aminzare M, Hashemi M, Hassanzadazar H, Amiri E, Abbasi Z. Antibacterial Activity of Corn Starch Films persicum Essential Oils. *Annu Res Rev Biol*. 2017; 19(1): 1-9.

16. Mihajilov Krstev T, Radnović D, Kitić D, Stojanović Radić Z, Zlatković B. Antimicrobial Activity of Satureja *hortensis L.* Essential Oil against Pathogenic Microbial Strains. *Arch Biol Sci*. 2010; 62(1): 159-66.

17. Masoomi V, Tajik H, Moradi M, Forough M, Shahabi N. Antimicrobial Effects of *Zataria multiflora* Boiss. Essential Oil Nanoemulsion against *Escherichia coli* O_{157}: H_7. *Urmia Med J*. 2016; 27(7): 608-17.

18. Thanh TT, Lan LX, Thu H, Tam NK. Isolation by Different Processes and in Vitro Bioactivities of Rosemary (*Rosmarinus officinalis L.*) Essential Oil. *AIP Conf Proc*. 2017; 1878 (1): 020040.

19. Ksouri S, Djebir S, Bentorki AA, Gouri A, Hadef Y, Benakhla A. Antifungal Activity of Essential Oils Extract from Origanum Floribundum Munby, *Rosmarinus officinalis L.* and Thymus Ciliatus Desf. against Candida Albicans Isolated from Bovine Clinical Mastitis. *J Mycol Med*. 2017; 27(2): 245-9.

20. Fadil M, Fikri Benbrahim K, Rachiq S, Ihssane B, Lebrazi S, Chraibi M, et al. Combined Treatment of Thymus Vulgaris L., Rosmarinus officinalis L. and Myrtus communis L. Essential Oils against *Salmonella typhimurium*: Optimization of Antibacterial Activity by Mixture Design Methodology. *Eur J Pharm Biopharm*. 2018; 126: 211-20.

21. Raeisi M, Ebrahimi M, Hashemi M, Aminzare M, Khoshbakht R, Sadeghi A, et al. Comparison of Chemical Components and Antibacterial Activity of Rosemary Essential Oil Grown in Various Regions of Iran against Foodborne Pathogenic Bacteria. *Int J Pharm Sci Res*. 2017; 9(10): 1725-30.

22. Benjemaa M, Neves MA, Falleh H, Isoda H, Ksouri R, Nakajima M. Nanoencapsulation of Thymus Capitatus Essential Oil: Formulation Process, Physical Stability Characterization and Antibacterial Efficiency Monitoring. *Ind Crops Prod*. 2018; 113: 414-21.

23. Swathy JS, Mishra P, Thomas J, Mukherjee A, Chandrasekaran N. Antimicrobial Potency of High-Energy Emulsified Black Pepper Oil Nanoemulsion against Aquaculture Pathogen. *Aquaculture*. 2018; 491: 210-20.

24. Martin Piñero MJ, Ramirez P, Muñoz J, Alfaro MC. Development of Rosemary Essential Oil Nanoemulsions Using a Wheat Biomass-Derived Surfactant. *Colloids Surf B Biointerfaces*. 2019; 173: 486-92.

25. Restrepo AE, Rojas JD, Garcia OR, Sanchez LT, Pinzon MI, Villa CC. Mechanical, Barrier, and Color Properties of Banana Starch Edible Films Incorporated with Nanoemulsions of Lemongrass (Cymbopogon citratus) and Rosemary (Rosmarinus officinalis) Essential Oils. *Food Sci Technol Int*. 2018; 24(8): 705-12.

26. Fu Y, Zu Y, Chen L, Shi X, Wang Z, Sun S, et al. Antimicrobial Activity of Clove and Rosemary Essential Oils Alone and in Combination. *Phytother Res*. 2007; 21(10): 989-94.

27. Evangelista Martínez Z, Reyes Vázquez N, Rodríguez Buenfil I. Antimicrobial Evaluation of Plant Essential Oils against Pathogenic Microorganisms: In Vitro Study of Oregano Oil Combined with Conventional Food Preservatives. *Acta Univ*. 2018; 28(4): 10-18.

28. Moghimi R, Aliahmadi A, McClements DJ, Rafati H. Investigations of the Effectiveness of Nanoemulsions from Sage oil as Antibacterial agents on Some Food Borne Pathogens. *LWT Food Sci Technol*. 2016; 71:69-76.

29. Moghimi R, Ghaderi L, Rafati H, Aliahmadi A, McClements DJ. Superior Antibacterial Activity of Nanoemulsion of Thymus Daenensis Essential Oil against *E. coli*. *Food Chem*. 2016; 194: 410–15.

30. Jawad AM, Allawi AK, Ewadh HM. Essential oils of Rosemary as Antimicrobial Agent against Three Types of Bacteria. *Med j Babylon*. 2018; 15(1): 53-6.