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Antimicrobial and Antioxidant Effects of Emulsions and Nanoemulsions of *Salvia officinalis*, *Pimpinella anisum*, *Dracocephalum moldavica*, and *Syzygium aromaticum* Against Foodborne Bacteria

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Abstract

Due to their antimicrobial and antioxidant properties, essential oils are used as natural preservatives. The purpose of this study was to investigate the chemical composition, antioxidant properties, and antimicrobial activity of emulsion and nanoemulsion forms of *Salvia officinalis*, *Pimpinella anisum*, *Dracocephalum moldavica*, and *Syzygium aromaticum* essential oils. The Agar well-diffusion assay results obtained from the experiment suggested that nanoemulsion of *Dracocephalum moldavica* essential oil had the maximum antimicrobial activity against the pathogenic microorganisms drawn in the experiment. The inhibition zone diameters of the nanoemulsion of this essential oil against *Shigella dysenteriae*, *Salmonella* **Typhimurium**, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Bacillus cereus* were 11.03, 11.82, 13.02, 13.13, 13.13, 13.62, and 14.10 mm, respectively. In contrast, the inhibition zone diameters of the emulsion form of this essential oil against *S. dysenteriae*, *S.* **Typhimurium**, *P. aeruginosa*, *S. aureus*, *L. monocytogenes*, *E. coli*, and *B. cereus* were 9.66, 10.34, 10.84, 11.84, 11.34, 11.17, and 11.24 mm, respectively. The major components of *Dracocephalum moldavica* essential oil included geraniol (27.24%), geranial (10.75%), alpha-copaene (8.16%), alpha-pinene (7.37%), carvacrol (7.41%), limonene (6.86%), and nerol (6.45%). The nanoemulsion form of the essential oils investigated thus possessed a significantly greater antioxidant potential compared to their emulsion form. This study also demonstrated that the nanoemulsions exhibited significantly lower IC50 values compared to the emulsions. From the results, it was seen that the nanoemulsion form of *Dracocephalum moldavica* essential oil had the lowest IC50 and EC50 values of 22.17 µg/ml and 4.51 µg/ml, respectively.

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1. Introduction

Statistics published by WHO shows that every year a large number of people across the world die from alimentary diseases. Also, huge amounts of antibiotics have given rise to resistant microbes. However, food spoilage threatens consumer health and incurs a big economic loss from countries **(Burt, 2004).** Some of the methods to tackle pathogenic as well as spoilage microorganisms include introducing chemical preservatives. In the past few decades, the growing awareness of consumers about harmful effects of chemical preservatives, especially the carcinogenic properties, has therefore increased the demand of foods containing natural preservatives **(Skandamis *et al*., 2001).** Medicinal plants have been used since ancient times in medicine, in the preparation of aromatic cosmetics, in the control of spoilage and pathogenic microorganisms in food, and in enhancing the flavor of foods. Plant secondary metabolites such as essential oils, aromatic compounds and volatile compounds have extensive applications in traditional medicine, flavoring and food preservation. These substances have shown antibacterial, antifungal, antiviral and antiparasitic effects. The biological effects of medicinal plants and their essential oils or extracts are largely due to their chemical compounds, particularly to the phenolic compounds they contain **(Kelen and Tepe, 2008; Shahbazi *et al*., 2016).** The chemical compositions of essential oils in various plants may vary due to genetic and environmental factors such as geographical conditions, climatic and seasonal changes, and growth stages of the plant **(Ruiz-Navajas *et al*., 2012).** *Salvia officinalis*, *Pimpinella anisum*, *Eugenia caryophyllata*, and *Moldavian dragonhead* contain active substances, which can effectively inhibit the growth of pathogenic microorganisms and reduce food oxidation. These effects have been the subject of investigations in various studies. In one study to investigate the antibacterial activity of an essential oil mixture from some medicinal plants, including *Malva Sylvestris* and *Salvia officinalis*, against bacteria responsible for common oral infections, it was found that these oils exhibited significant growth inhibitory activity against both Gram-positive and Gram-negative bacteria and represent a natural substitute to chemical mouthwashes such as chlorhexidine (Eghbal *et al*., 2021). The extract of *Pimpinella anisum*, beyond exhibiting antimicrobial effects, has demonstrated a considerable degree of antioxidant activity in oil and emulsion systems by means of free radical-scavenging activity, which positively reflected the oxidative stability of oil during storage, showing promise for application in food preservation (Singh *et al*., 2008). *Eugenia caryophyllata* is another herbal plant extensively studied

for its antimicrobial and antioxidant properties. The study on the bioactive properties and composition of clove essential oil reported the presence of a plethora of phenolic and terpenoid compounds that possess potent antimicrobial activity against many bacteria, fungi, and yeasts. In addition, the essential oil from this herb is able to scavenge free radicals and serve as a natural antioxidant Kennouche *et al*., 2015). In another study, the chemical composition, antioxidant activity, and antimicrobial properties regarding the essential oil of Moldavian dragonhead (*Dracocephalum moldavica* L.) were evaluated. The results indicated that the main components of Moldavian dragonhead essential oil are terpenoids and phenolic compounds, thus giving it a very high potency against free radical scavenging, resulting in its importance as a natural antioxidant. Antimicrobial assays also proved that both essential oil and hydrolate of Moldavian dragonhead were successful against various microorganisms, mainly pathogenic bacteria (Acimovic *et al*., 2022).

Though essential oils possess their own biological properties, in their application within food products, they face certain challenges. For instance, high concentrations of these oils may lead to undesirable organoleptic properties in foods due to the inherent antimicrobial properties of these oils. Additionally, their hydrophobic character, low solubility in water, poor chemical stability, and volatile nature add tremendous challenges in the technology applicable for essential oils in food products **(Shavisi et al., 2017).** To solve these problems, encapsulation of essential oils into nanoparticles is considered a way to improve their applicability, stability, and efficacy. Of all systems of nanoparticles, nanoemulsions are found to be most advantageous in food, health, and cosmetic applications because of easy preparation and favorable functional properties. Nanoemulsions, whose particles have diameters in the range of 10-200 nm, are one of the most successful carrier systems for lipophilic compounds, including drugs, flavorings, antioxidants, and antimicrobial agents **(Rao and McClements, 2011; McClements and Rao, 2011).** A nanoemulsion is an oil phase dispersed in an aqueous phase, with fine droplets surrounded by a thin interfacial layer of surfactant and/or amphiphilic molecules, providing stability to the system **(Borrin *et al*. 2016).** Therefore, decreasing the size of the oil phase in the structure of the nanoemulsion while increasing its surface area increases the efficiency with which it improves interaction between active compounds and biological membranes for their transport **(Perugini Biasi-Garbin *et al*. 2015).** It was reported that the antimicrobial property of nanoemulsions is directly related to their formation methods **(Shavisi *et al*., 2017).** There are many methods for

nanoemulsion production, including many high-energy and low-energy approaches. One example of a high-energy method is ultrasonication, which produces quickly and effectively nanoemulsions with small particle sizes and uniform distribution **(McClements, 2012).** Iran is vastly rich in special resources, especially in plant cover and plant diversity. In terms of biodiversity, Iran ranks among the top eight countries in the world, with 8423 plant species, two-thirds of which are European plant species. Out of more than 2300 plant species in Iran, 1730 have specific medicinal properties, and all of these are unique to and endemic in this country where, naturally, they grow in about 8.84 million hectares of dense, semi-dense and sparse rangelands. Given that, this study aims to prepare essential oils and nanoemulsions of the plants Salvia officinalis, Pimpinella anisum, Eugenia caryophyllata and Moldavian dragonhead grown in various regions of Iran, and to evaluate and compare their activities with respect to antibacterial and antioxidant activity.

2.Material & Methods

2.1.Plant Materials

The plants used in this research, *Salvia officinalis*, *Pimpinella anisum*, *Syzygium aromaticum*, and *Dracocephalum moldavica*, were freshly harvested in the harvesting season and authenticated in the Botany Department of Gorgan University of Agricultural Sciences and Natural Resources. After the authentication, the plants were dried under shade in a well-ventilated place, avoiding direct sunlight.

2.2. Essential oil Extraction

Powdered plant samples were used for the extraction of essential oils using a Clevenger-type apparatus (Avijeh, Iran) according to the standard method. The powdered plant samples were put into distilled water, and the extraction began as soon as the water boiled; extraction continued for a total of 180 min. The oil was subsequently dried over sodium sulfate and purified by passing through filters of 0.22 µm pore size. Finally, the purified oils were kept in dark vials at a temperature of 4˚C to maintain their integrity until analysis **(Purkait *et al*., 2018).**

2.3. GC/MS Analysis

A GC/MS instrument (Agilent 7890B, USA) with a nonpolar capillary column - Agilent Hp5Ms (30 m length, 0.25 mm inner diameter, and 0.25 µm film thickness) was used. The GC injector was kept at 80 °C for 5 min and then its interface was kept at 270 °C. The

injection volume into the device was 1 µl. **The carrier gas was also Helium with a flow rate of 1 mL/min (Chamorro *et al.*, 2012). The analysis was conducted at Golestan University of Medical Sciences.**

2.4. Preparation and Characterization of the Emulsion and Nanoemulsion of Essential Oils

It has now become 0.5 percent w/v of essential oil in sterile distilled water, and with TWEEN 80 (0.2 g w/w EO) used as an emulsifier. This emulsion was stirred continuously for 10 min to form a clear, stable, and uniform emulsion. This has been prepared according to the method explained by Ghosh et al. (2013) with slight modifications. Thereafter, the emulsion was given 3 min treatment using Ultra Turrax homogenizer (OPTIMA, XL100K, Clausthal, Germany) at 3000 r/min and subsequently sonic emulsifier at 50 °C; frequency: 50 kHz; pulse: 45 s; rest: 15 s for a total of 6 min (probe diameter: 15 mm). The size of particles was analyzed using a dynamic light scattering (DLS) device (Nanophox Sympatec GmbH, Clausthal, Germany), which measures the size distribution of particles suspended under conditions of Brownian motion characterized by a low charge density and negligible hydrodynamic interactions. **The analysis was conducted at Mashhad University of Medical Sciences.**

2.5. Total Phenolic Contents

The Folin-Ciocalteu reagent assay was employed to measure the total phenolic content in the extracted essential oils. To begin, a solution of each essential oil dissolved in methanol at a concentration of 2 mg/mL was combined with 2.25 mL of distilled water and 250 µL of Folin-Ciocalteu reagent. was combined with 2.25 ml of distilled water and 250 µl of Folin-Ciocalteu reagent. The mixture was vortexed thoroughly and left to react for five min. Then, two ml of a 7.5% Na2CO3 solution was added. After allowing the mixture to sit at room temperature for 120 min, its absorbance was measured at 760 nm using a spectrophotometer (model: LKB Novaspec II; Pharmacia, Cambridge, England). The results were reported as mg of gallic acid equivalent per gram of essential oil, based on a standard curve generated from known concentrations of gallic acid **(Alizadeh *et al.*, 2013).**

2.6. Antibacterial Effects of the Essential Oils

Various essential oils were evaluated for their antibacterial activity against four Gram-negative bacteria (*Escherichia coli* PTCC 1399, *Salmonella* **Typhimurium** ATCC 13311, *Pseudomonas*

aeruginosa PTCC 1616, and *Shigella dysenteriae* PTCC 1188) and three Gram-positive bacteria (*Staphylococcus aureus* PTCC 1112, *Listeria monocytogenes* PTCC 1298 and *Bacillus cereus* PTCC 1154). These assays included agar well-diffusion and microdilution methods to determine antibacterial activity. The bacterial strains were obtained from the culture collection of the Food and Drug Deputy at Golestan University of Medical Sciences in Gorgan, Iran.

2.6.1. Agar Well-Diffusion Assay

To begin, 100 ml of nutrient agar (Merck, Darmstadt, Germany) was seeded with 1 ml of **actively growing** 18 h bacterial broth cultures (1.5×10^6 CFU/ml). The combined mixture, which was cooled to 45°C, was mixed well for 2 min, transferred to sterile plates, and allowed to solidify. 4 wells were then aseptically prepared in the agar with a sterile cork-borer, and 10 µl of the essential oils were placed in each well. DMSO served as the negative control. The plates were incubated for 72 h at 37°C, then the inhibition zone around the wells was measured in mm using a manual caliper (Vernier, Mitutoyo, Japan) **(Mirtaghi *et al.*, 2016).**

2.6.2. Determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Using the Broth Microdilution Assay

The assay was conducted as per **Turgis et al. (2012),** with changes made to the methodology. Prior to the assay, the essential oils were dissolved in 10% DMSO so that the maximum concentration could reach 10,000 µg/ml, then serially diluted twofold, from 10 to 10,000 µg/ml. A total of 125 µl of each of the above essential oils solutions was introduced into wells 1-11 of a 96-well microplate (Sarstedt, Montreal, QC, Canada). A total volume of 140 µl was achieved from each well by adding 15 µl of mueller hinton broth (Merck, Germany) containing 10^6 CFU/ml of the **actively growing cultures of the target microorganisms** into all wells. Three rows of the microplate were used for each bacterium. The negative controls (two rows) contained 15 µl of saline instead of the bacteria, while the positive controls contain 125 µl of growth media and 15 µl of the bacterial cultures. The samples were incubated for 24 h at 37°C, after which absorbance was determined using a Biotech ELX800O microplate reader (Biotek Instruments Inc., Winooski, VT, USA) at 595 nm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the antimicrobial agent that completely inhibited visible bacterial growth. The minimum bactericidal concentration (MBC) is the lowest concentration required to kill at least

99.9% of the initial number of bacteria, determined by subculturing 10 µl from each well showing no visible growth onto nutrient agar plates, and incubating for 72 h at 37°C.

2.7. Antioxidant Activity of the Essential Oils

The antioxidant activity of essential oils was evaluated using two methods, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and reducing power assay.

2.7.1. DPPH Assay

The free radical scavenging activity of the emulsion and nanoemulsion forms essential oils was evaluated using the DPPH assay (Sigma-Aldrich, Steinheim, Germany), following the protocol established by **Erkan *et al*. (2008)** with minor changes. Initially, 50 µl of each essential oil at various concentrations (10-10,000 µg/ml) and the reference antioxidant butylated hydroxytoluene (BHT) were mixed with 2 ml of a 0.2 mM methanolic DPPH solution. After shaking well, they were kept at room temperature in the dark place for 60 min. Subsequently, the absorbance of the mixture was measured at 517 nm using a spectrophotometer (model: LKB Novaspec II, Pharmacia, Cambridge, England). The blank sample contains methanol solvent with DPPH. The radical scavenging activity was calculated using the following formula:

$$\% I = [A_{blank} - A_{sample}/A_{blank}] \times 100$$

In the above relation, I represent the percentage of free radical DPPH scavenging, A_{blank} is the absorbance of the control, and A_S is the absorbance of the sample, all measured in nm.

The IC50 value was derived from the scavenging activity curve plotted against the concentrations of the essential oils, indicating the total antioxidant activity required to achieve a 50% reduction in the initial DPPH radical concentration.

2.7.2. Reducing Power Assay

The reducing power of the essential oils was evaluated using the method established **by Oyaizu (1986).** In this procedure, the essential oils were prepared by different concentrations of essential oils (10- 1000 µg/ml) and 2.5 ml 0.2 M phosphate buffer pH=6.6 were added with potassium ferricyanide ($K_3Fe[CN]$) were added as 1% potassium ferricyanide ($K_3Fe[CN]_6$). The mixtures were incubated for 20 min at 50 °C The samples were added with 2.5 ml of the 10% trichloroacetic acid after incubation, which was then centrifuged for 10 min at 1,036 rpm. The upper layer was subsequently transferred to 2.5 ml Distilled water and combined with 2.5 ml ferric chloride (1%).

The absorbance at 700 nm was measured by a double-test UV-Vis spectrophotometer (model: LKB Novaspec II) (Pharmacia, Cambridge, England), the blank contained all reagents except the essential oils. Increased absorbance is shown to have more reducing power Butylated hydroxytoluene (BHT) as a positive control; Additionally, this contained the absorbance of each essential oil fraction which provided an EC50 (absorbance value of 0.5), against the standard antioxidant (BHT).

2.8. Statistical Analysis

Data analysis was performed in SPSS version 16 (SPSS Inc., Chicago, IL, USA), and all the assays were performed in triplicate. Tukey's test was used to compare the differences between the mean values obtained from the experiments at the significance level of $P < 0.05$.

3. Results and Discussion

3.1.Identification of Essential Oil Components

Using mass spectrum similarity and the mass library of the gas chromatography-mass spectrometry (GC-MS) device, 34, 9, 24, and 13 compounds were identified in the essential oils of *Salvia officinalis* (Table 1), *Pimpinella anisum* (Table 2), *Dracocephalum moldavica* (Table 3), and *Syzygium aromaticum* (Table 4), respectively. According to the results in Table 1, the main compounds of *Salvia officinalis* essential oil include Beta Thujene (13.74%), 1,8-Cineole (12.13%), Alpha Thujene (9.52%), Alpha Fenchyl Acetate (6.12%), Camphor Bicyclo Heptan (7.36%), Caryophyllene (4.52%), Viridifloral (3.15%), α-Pinene (3.11%) and β-Pinene (2.15%). These compounds together made up 61.8% of the identified compounds.

Salvia officinalis is one of the most important medicinal and aromatic plants and has antioxidant, antimicrobial, spasmolytic, astringent, antihydrotic, and sensory properties. The essential oil of the plant, which is mainly formed in the very young leaves, is partly responsible for these activities. This essential oil is mainly composed of monoterpenes such as 1,8-Cineole, α- and β-Thujene, and Camphor, and it is produced and stored in epidermal glands **(Grausgruber-Gröger *et al*., 2012).** Other researchers in Morocco studied the chemical properties of *Salvia officinalis* and found that 36 compounds are present in the chemical structure of this plant. Among them, 1,8-Cineole, camphor, borneol, α-pinene, β-pinene, α-humulene, α-caryophyllene, β-caryophyllene, and viridiflorol were the dominant compounds **(Delamare *et al*., 2007).** In another study, **Couladis *et***

248 ***al.* (2002)** examined the chemical properties of *Salvia officinalis* medicinal plant samples in Serbia
249 and Montenegro and reported that α-thujone, β-thujone, 1,8-cineol, camphor, borneol, and bornyl
250 acetate were the dominant compounds in the leaves of this medicinal plant, while in the flowers,
251 the percentages of camphor and thujone were much lower. Different parts of medicinal plants vary
252 in their chemical composition, but the diversity and quantity of compounds are much higher in the
253 reproductive organs of plants than in other parts. A comparison of the chemical analysis results of
254 *Salvia officinalis* leaf essential oil showed many similarities between these researchers' analyses
255 and the results of the present study, confirming the aforementioned points.

256 **Table 1.** Identified Compounds in *Salvia officinalis* Essential Oil.

Number	Compound	RT (min)	% of Total
1	Cis salvene	3.12	0.48
2	Tricyclene	4.17	0.53
3	α-Thujene	4.28	0.17
4	α-Pinene	4.72	3.11
5	β-Pinene	5.18	2.15
6	β-Myrcene	5.42	1.18
7	L Phellanderene	5.53	1.84
8	α-Terpinene	6.12	0.70
9	Benzene 1 Methyl 3 (1-Methylether)	6.28	0.56
10	1,8 Cineole	6.41	12.13
11	Gamma Terpinene	6.72	2.17
12	α-Terpinolene	7.12	0.10
13	β-Thujene	7.33	13.74
14	α-Thujene	7.84	9.52
15	Camphor Bicyclo Heptan	8.17	7.36
16	Isoborneol	8.34	1.14
17	Borneol L	8.89	1.52
18	α-Terpineol	9.11	0.30
19	Trans Carveol	9.29	0.56
20	1,3,6 Octatriene, 3, 7 Dimethyl	10.18	2.17
21	Cis 3 Hexenyl Isovalerate	10.74	2.52
22	α-Fenchyl Acetate	12.49	6.12
23	Sabinyl Acetate	13.09	1.50
24	Myrtenyl Acetate	14.17	0.35
25	Eugenol	15.82	2.17
26	α-Copaene	16.13	1.15
27	Caryophyllene	17.21	4.52
28	Aromadendrene	17.38	1.15
29	α-Caryophyllene	19.12	1.19
30	Spathulenol	21.17	0.10
31	Caryophyllene Oxide	22.33	1.78
32	viridifloral	22.84	3.15
33	Camphene	23.14	2.72
34	β-Clovene	25.54	0.92
Total			90.77

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In another part of this study, using gas chromatography-mass spectrometry, nine compounds were identified in the essential oil of Pimpinella anisum (Table 2). Trans-anethole (60.17%) with a retention time of 12.15 min was the most abundant compound identified in this essential oil. Additionally, γ-himachalene (14.19%) and Trans-ocimenone (5.85%) were ranked next. The identified compounds in Pimpinella anisum essential oil have been reported in various forms in previous studies. **Orav *et al*. (2008)** reported that the main compound in all essential oil samples of Pimpinella anisum collected from different European countries was Trans-anethole, ranging from 76.9% to 93.7%, and other major compounds included γ-himachalene, trans-pseudoisoeugenyl 2-methylbutyrate, p-anisaldehyde, and methylchavicol. Furthermore, **Ullah *et al.* (2014)** reported Trans-anethole at 82.1% and γ-himachalene at 7% as the main compounds in the essential oil of this plant. Abdel-Reheem and Oraby (2015) also identified the main constituents of Pimpinella anisum essential oil as trans-anethole (82.1%), cis-anethole (5.8%), methylchavicol (2.5%), linalool (2.3%), α-terpineol (1.5%), and methyl eugenol (1.3%). It could say there are differences not great among major constituents of the essential oil of this plant, but in general, any difference in the composition may arise from variation in climate among different eco-regions. Also, the parts of a plant may widely differ in their chemical composition at a particular time, and it is essential to pick up the part when it has the highest concentration of the active ingredient. Other important considerations for the level of active compounds during harvesting are the appropriate harvest time depending on the plant's genetics, type of cultivation, and times of cultivation, location and soil used, irrigation level, light exposure, altitude, and many others **(Hendawy et al., 2018, Ali-Shtayeh et al., 2018).**

Table 2. Identified Compounds in *Pimpinella anisum* Essential Oil.

Number	Compound	RT (min)	% of Total
1	D limonene	11.72	2.86
2	Meta anisaldehyde	11.96	3.58
3	Stragole	12.06	1.96
4	Trans anethole	12.15	60.17
5	Trans ocimenone	12.26	5.85
6	Muurolene	13.50	3.31
7	Curcumene	14.11	2.37
8	γ –himachalene	14.53	14.19
9	Beta biabolene	14.92	2.75
Total			97.04

In another part of this study, the identification of active compounds in the essential oil of *Dracocephalum moldavica* (Table 3) showed that 74.23% of this essential oil consisted of Geraniol

(27.24%), Geranial (10.75%), Alpha Copaene (8.16%), Alpha Pinene (7.37%), Carvacrol (7.41%), Limonene (6.86%), and Nerol (6.45%). Yousefzdeh *et al*. (2013) and Kakasy *et al*. (2006) reported that the three compounds Geranyl acetate, Geranial, and Geraniol constituted the major components of the essential oil in *Dracocephalum moldavica*. According to the findings of Holm *et al*. (1988), the main components of the essential oil in *Dracocephalum moldavica* were oxygenated cyclic monoterpenes, including Geraniol, Geranial, Geranyl acetate, and Nerol. **Sonboli *et al*. (2008)** reported that Nerol, Geranial, Geranyl acetate, and Geraniol, with 32.1%, 21.6%, 19.9%, and 17.6%, respectively, were the major components of *Dracocephalum moldavica* essential oil.

Table 3. Identified Compounds in *Dracocephalum moldavica* Essential Oil.

Number	Compound	RT (min)	% of Total
1	α- Thujene	9.20	1.18
2	α-Pinene	9.27	7.37
3	Camphene	9.36	2.58
4	β-Pinnene	9.54	1.17
5	α-Phellandrene	9.72	0.59
6	Limonene	9.81	6.86
7	Linalool oxide	9.93	0.94
8	Linalool	10.52	1.15
9	Terpinen 4 ol	11.02	3.14
10	α-Terpineol	11.15	1.12
11	Nerol	11.43	6.54
12	Neral	11.55	1.90
13	Geraniol	11.62	27.24
14	Geranial	11.71	10.75
15	Thymol	12.12	3.15
16	Carvacrol	12.31	7.41
17	Geranyl acetate	12.54	1.25
18	α-Copaene	12.67	8.16
19	β-Bourbonene	12.75	0.78
20	β-Caryophyllene	13.14	1.25
21	γ –Muurolene	13.36	0.50
22	γ –Cadinene	13.95	0.88
23	Caryophyllene oxide	14.30	0.33
24	Viridifloral	14.78	0.56
Total			96.8

Syzygium aromaticum was another essential oil studied in this research. Based on the results in Table 4, the compounds identified in this essential oil with their respective retention times were Eugenol (28.13%) with a retention time of 13.26 min, Caryophyllene (22.17%) with a retention time of 14.03 min, Eugenol acetate (17.75%) with a retention time of 14.86 min, and Caryophyllene oxide (9.51%) with a retention time of 15.19 min.

Such was the state of affairs with respect to the highest identified compounds found in the essential oil of *Syzygium aromaticum*. A lot of researchers have worked on *Syzygium aromaticum* essential

oil components. For instance, Myint *et al.* (2007) reported that Eugenol, Caryophyllene, and Eugenol acetate were the main components in the ethanolic extract of *Malaysian Eugenia caryophyllata*. Another study conducted by Nassar *et al.* (2007) revealed that Eugenol, Eugenol acetate, and Caryophyllene are the major constituents among 16 volatile compounds isolated from hexane extract of *Syzygium aromaticum*. Fichi *et al.* (2007) reported similar results for chemical analysis of clove essential oil, where Eugenol (59.3%) and Caryophyllene (24.9%) were the major compounds.

Table 4. Identified Compounds in *Syzygium aromaticum* Essential Oil.

Number	Compound	RT (min)	% of Total
1	Carvacrol	1270	1.18
2	Alpha Cubebin	1313	2.14
3	Eugenol	1326	28.13
4	M Eugenol	1348	3.72
5	Trans Caryophyllene	1382	0.70
6	Caryophyllene	1403	22.17
7	Trans Isoeugenol	1417	0.38
8	Isoeugenol	1426	3.17
9	Alpha Humulene	1441	1.15
10	Eugenol acetate	1486	17.75
11	Caryophyllene oxide	1519	9.51
12	Hexadecanoic acid	1718	0.80
13	Eicosane	1829	0.56
Total			91.36

3.2. Particle size of emulsion and nanoemulsion of essential oils

The diameter of emulsion and nanoemulsion particles of essential oils is shown in Fig 1. Based on the results, the size of the emulsion and nanoemulsion particles was 874-973 and 50.4-64.9 nm, respectively.

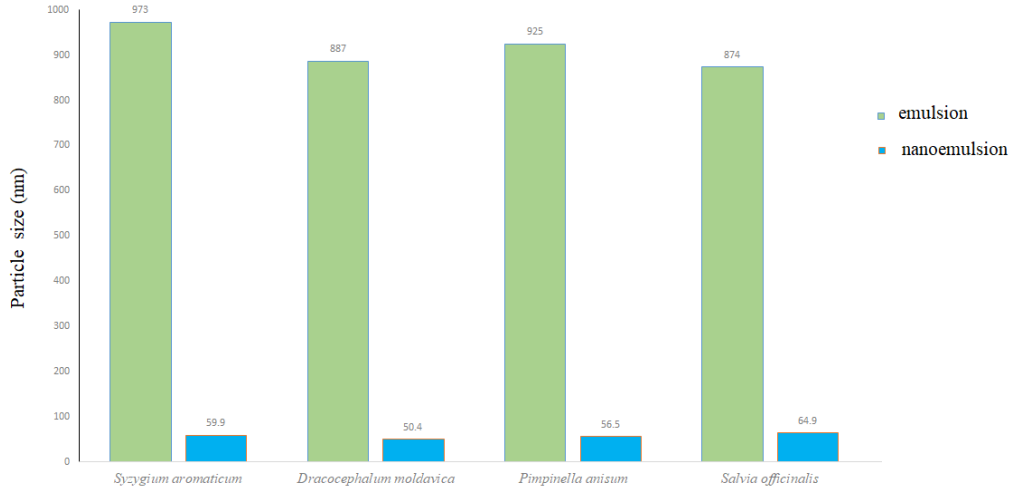

Fig 1. The particle diameter of emulsion and nanoemulsion of different essential oils.

3.3. Antimicrobial Activity of Emulsion and Nanoemulsion Forms Essential Oils

In order to compare the antimicrobial activity of the emulsion and nanoemulsion of plant essential oils studied in this research, the well diffusion and microdilution methods were used. The results of the antibacterial activity assessment of these essential oils are shown in Tables 5 and 6, respectively.

Table 5. Inhibitory zone diameter (mm) of emulsion and nanoemulsion forms of essential oils against bacterial pathogens using the well diffusion method.

Bacteria		*Salvia officinalis*	*Pimpinella anisum*	*Dracocephalum moldavica*	*Syzygium aromaticum*
S. Typhimurium	emulsion	9.28±0.12	10.11±0.05	10.34±0.2	9.82±0.17
	nanoemulsion	10.13±0.05^{*Ce}	10.94±0.22^{*Bd}	11.82±0.12^{*d}	10.78±0.2^{*Bc}
S. aureus	emulsion	10.12±0.07	11.61±0.11	11.84±0.22	10.74±0.31
	nanoemulsion	11.34±0.15^{*Dd}	12.86±0.15^{*Bb}	13.13±0.10^{*Ac}	11.89±0.23^{*Cb}
S. dysenteriae	emulsion	8.62±0.17	9.24±0.09	9.66±0.13	8.76±0.22
	nanoemulsion	9.92±0.31^{*Cf}	10.86±0.27^{*Ad}	11.03±0.09^{*Ae}	10.3±0.17^{*Bd}
L. monocytogenes	emulsion	10.12±0.04	10.92±0.17	11.34±0.12	10.73±0.25
	nanoemulsion	11.78±0.26^{*Cc}	12.36±0.2^{*Bc}	13.13±0.13^{*Ac}	12.32±0.27^{*Bb}
P.aeruginosa	emulsion	10.26±0.15	10.92±0.27	10.84±0.12	10.13±0.09
	nanoemulsion	11.82±0.26^{*Cc}	12.38±0.15^{*Bc}	13.02±0.12^{*Ac}	12.9±0.18^{*Aa}
B. cereus	emulsion	10.63±0.26	11.36±0.02	11.24±0.10	10.82±0.16
	nanoemulsion	12.14±0.1^{*Cb}	13.76±0.28^{*Ba}	14.10±0.13^{*Aa}	12.33±0.20^{*Cb}
E. coli	emulsion	10.54±0.22	10.8±0.34	11.17±0.10	10.36±0.26
	nanoemulsion	13.12±0.15^{*Ba}	13.81±0.23^{*Aa}	13.62±0.27^{*Ab}	12.14±0.19^{*Cb}

- The asterisk (*) in each column for each bacterium separately indicates a significant difference between the inhibitory effect of the emulsion and nanoemulsion forms of the studied essential oils ($P<0.05$).
- Similar uppercase letters in each row and similar lowercase letters in each column indicate no significant difference between the mean antibacterial effects of the nanoemulsion forms of the studied essential oils ($P<0.05$).

Tale 6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (µg/mL) of emulsion and nanoemulsion forms of essential oils against pathogenic bacteria.

Bacteria		*Salvia officinalis*		*Pimpinella anisum*		*Dracocephalum moldavica*		*Syzygium aromaticum*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. Typhimurium	emulsion	10000	10000	2500	5000	2500	5000	5000	10000
	nanoemulsion	5000	5000	1250	2500	1250	2500	2500	5000
S. aureus	emulsion	2500	5000	1250	2500	1250	2500	2500	5000
	nanoemulsion	1250	2500	625	1250	312.5	625	625	1250
S. dysenteriae	emulsion	5000	10000	2500	5000	2500	5000	2500	5000
	nanoemulsion	2500	2500	625	1250	625	625	625	1250
L. monocytogenes	emulsion	2500	5000	1250	2500	1250	2500	2500	5000
	nanoemulsion	1250	1250	625	1250	312.5	625	625	1250
P.aeruginosa	emulsion	2500	5000	2500	5000	1250	2500	2500	5000
	nanoemulsion	1250	2500	2500	5000	1250	2500	1250	2500
B. cereus	emulsion	1250	2500	1250	2500	625	1250	2500	5000
	nanoemulsion	625	1250	312.5	625	312.5	625	1250	2500
E. coli	emulsion	5000	10000	2500	5000	2500	5000	5000	5000
	nanoemulsion	1250	2500	625	1250	625	1250	1250	2500

The results from the well diffusion method (Table 5) and the microdilution method (Table 6) indicate that both emulsion and nanoemulsion forms exhibited significant antimicrobial effects against all tested microorganisms. Moreover, in all the studied essential oils, the antimicrobial properties significantly increased ($P<0.05$) when the particle size of the essential oil emulsion was reduced and converted into the nanoemulsion form. Antibacterial activity is most likely to be improved upon finely converting various essential oils into nano-sized particles. This is likely since, because of their low solubility in water, they cannot interact with the cell membrane easily. Nanoemulsions through their smaller size can easily approach the surfaces of the cell membranes (Moghimi *et al*., 2016). Generally, nanoemulsions could cause damage or kill bacteria by different mechanisms. Essential oils' mode of action is cited to involve the disruption and destabilization of the phospholipid bilayer structure, leading to the impairment of the cell membrane, interaction with membrane enzymes and proteins as proton carriers, and pH reduction across the membrane (Burt, 2004; Nazzaro *et al*., 2016). For instance, the liberation of ions by a nanoemulsion may react with thiol groups of proteins located on bacterial cell surfaces. Following this, these proteins become inactivated; lessening membrane permeability, which eventually results in death for the bacteria. Electrostatic interaction possible between the positively charged nanoparticle and the negatively charged cell membrane can result in disruption of the membrane through nanoparticle

364 binding. The accumulation of nanoparticles in both the cytoplasm and outer membrane can also
365 interfere with bacterial growth and their survival. Nanoemulsions formed through high-pressure
366 homogenization are even more potent than pure essential oils because of their size reduction
367 (Topuz *et al*., 2016). Moghimi *et al*. (2016) also reported that the nanoemulsion of Thymus
368 daenensis essential oil was ten times more antibacterial than the pure essential oil. Results depend
369 on many variables, including type and physical properties of essential oil, method of preparation,
370 and type of tested microorganism. As cited by Shahabi *et al*. (2017), the nanoemulsion of essential
371 oil extracted from *Zataria multiflora* had an enhanced antimicrobial potency toward *L.*
372 *monocytogenes* compared to that of *S.* Typhimurium when compared to the same essential oil. The
373 antimicrobial activity of the nanoemulsion, however, depends on the type of bacteria,
374 concentration of the nanoemulsion, and the exposure period to bacteria. In 2016, Moghimi *et al*.
375 (2016) evaluated and validated the antibacterial activity of thyme essential oil in its pure form and
376 in nanoemulsion form against *E. coli* by stating that for the nanoemulsion, the antibacterial activity
377 of the essential oil becomes increased as it gains faster access to the bacterial cells.
378 The nanoemulsion of *Dracocephalum Moldavica* indicated the highest action upon the pathogenic
379 microorganisms, followed by the nanoemulsion of *Pimpinella anisum*. The inhibition diameters
380 for the said nanoemulsion against *S. dysenteriae*, *S.* Typhimurium, *P. aeruginosa*, *S. aureus*, *L.*
381 *monocytogenes*, *E. coli*, and *B. cereus* were 11.03, 11.82, 13.02, 13.13, 13.13, 13.62, and 14.1 mm,
382 respectively (table 5). The findings indicate that this essential oil had the highest inhibitory effect
383 against *B. cereus* and the least effect against *S. dysenteriae* and *S.* Typhimurium ($P<0.05$). The
384 nanoemulsion of *Pimpinella anisum* has also shown a significantly lower inhibitory effect against
385 both *S.* Typhimurium and *S. dysenteriae* ($P<0.05$).
386 Microdilution method was also used to compare the antimicrobial activity of emulsified and
387 nanoemulsified essential oils studied in this work. As results presented in Table 6 indicate, the
388 antimicrobial activity of all essential oils was enhanced as a result of the decreasing minimum
389 inhibitory concentration (MIC) of the nanoemulsion compared to the emulsified form against all
390 studied microorganisms. In fact, in many cases, the MIC of nanoemulsions was half of those of
391 the emulsified essential oils. Moreover, as shown in Table 5, the highest antibacterial effects were
392 observed of the nanoemulsion forms of *Dracocephalum Moldavica* and *Pimpinella anisum* against
393 gram-positive bacteria.

In this study, antibacterial effects were evaluated by liquid dilution and well diffusion methods. Tables 5 and 6 show that among the aforementioned essential oils, *Dracocephalum moldavica* showed a relatively stronger antibacterial activity on the Gram positive than on the Gram negative organisms. Antimicrobial activity of essential oils from plants is dependent on their chemical constituents. Analysis of essential oils originating from different plants using gas chromatography revealed that these oils contained many compounds such as monoterpenes, sesquiterpenes and other oxygenated compounds such as alcohols, aldehydes, esters, ethers, ketones and phenols (Nazemisalman *et al*., 2024). The main constituents of essential oil of *Dracocephalum moldavica* included terpenoids; citral accounted for a substantial part of them, as investigated by Maham *et al*. (2013). Citral is well known for its prominent antimicrobial activity against Gram-positive and Gram-negative bacteria, the results of this research agree with those analyzed (Wójtowicz *et al*. 2017). El-Baky and El-Baroty (2008) evaluated the antibacterial activity of *Dracocephalum moldavica* essential oil through bioautography and reported that, among its components, geraniol, neral, geranyl acetate, geranial, nerol, neryl acetate, and methyl nerolate exhibited antibacterial activity. They also showed that the essential oil of *Dracocephalum moldavica* possesses a significant antibacterial activity against *S. aureus*, *Micrococcus luteus*, and *Serratia marcescens*; the present study further showed that 74.23% of the essential oil of *Dracocephalum moldavica* is composed of geraniol. Geraniol is an aliphatic monoterpene structure mainly related to the functional alcohol group in its organic composition. The possible mechanism of the antimicrobial action of geraniol via its lipophilicity would be its ability to attach to cell membrane lipids of the microorganism, and then increase membrane permeability while binding to sites that are essential in cells, which eventually disrupts their structures (Lira *et al*., 2020).

3.4. Antioxidant Activity of the Esential Oils

The measured phenol content of the studied essential oils and their antioxidant activity are shown in Table 7. According to the results in Table 7, no significant difference was observed in the total phenol content between the emulsion and nanoemulsion forms of each essential oil ($P<0.05$). The results indicated that the essential oils of *Dracocephalum moldavica* and *Pimpinella anisum* had the highest, and the essential oil of *Salvia officinalis* had the lowest total phenol content, respectively.

425 **Table 7.** Comparison of total phenolic content and antioxidant activity of various essential oils in
426 two forms: emulsion and nanoemulsion.

		Salvia officinalis	Pimpinella anisum	Dracocephalum moldavica	Syzygium aromaticum	BHT
DPPH (IC50)	emulsion	43.78±0.09^{b}	36.23±0.11^{c}	29.76±0.18^{d}	56.25±0.13^{a}	28.92±0.16^{d}
	nanoemulsion	31.82±0.18^{*b}	29.56±0.21^{*c}	22.17±0.1^{*e}	47.16±0.12^{*a}	28.22±0.16^{d}
Reducing power (EC50)	emulsion	6.27±0.17^{a}	5.36±0.13^{b}	5.82±0.10^{c}	6.11±0.08^{a}	1.31±0.12^{d}
	nanoemulsion	5.13±0.14^{*a}	4.62±0.28^{*b}	4.51±0.20^{*b}	5.15±0.10^{*a}	1.31±0.12^{c}
Total phenol content (mg of gallic acid per g of essential oil)	emulsion	259.14±0.52^{d}	317.12±0.38^{b}	392.51±0.41^{a}	289.53±0.12^{c}	
	nanoemulsion	260.21±0.36^{d}	319.28±0.45^{b}	397.82±0.28^{a}	292.39±0.40^{c}	

427 - The asterisk (*) in each column of the table, for each parameter separately, indicates a significant difference between
428 the emulsion and nanoemulsion forms ($P<0.05$).
429 - Similar lowercase letters in each row indicate no significant difference($P<0.05$).
430 -

431 In this study, the antioxidant effect of the essential oil emulsions and nanoemulsions was also
432 evaluated using two methods: DPPH and reducing power. The results from both methods are
433 presented in Table 7. As observed, although the antioxidant activity of both emulsions and
434 nanoemulsions is clearly lower than the standard group (BHT) in both methods, the overall
435 antioxidant activity of the nanoemulsions is higher than that of the emulsions, and this difference
436 is statistically significant ($P<0.05$). The study also demonstrated that the IC50 of the essential oil
437 nanoemulsions was significantly lower than that of the emulsions. According to the results, the
438 lowest IC50 and EC50 values were found in the emulsion form of *Dracocephalum moldavica*,
439 with values of 22.17 µg/ml and 4.51 µg/ml, respectively.
440 This is because of the phenolic content of the essential oils that are responsible for high-radical
441 scavenging activity. Generally, increasing the concentration of phenolic compounds directly
442 enhances the activity of different essential oils for inhibiting free radicals. It is inferred that at high
443 concentrations of phenolic compounds, there is an increased number of hydroxyl groups present
444 in the reaction environments, thus increasing the probability of hydrogen donation to free radicals
445 and leading to an increased scavenging capability of the extract. Many studies have shown the
446 relationship between the electron-donating ability of bioactive compounds and their scavenging
447 activity against free radicals. Results indicate that the highest phenolic essential oils exhibit the
448 strongest antioxidant (Baliyan *et* al., 2022). In fact, the radical scavenging power of different

essential oils depends to a large extent on the number and position of hydroxyl groups as well as the molecular weight of phenolic compounds. The more hydroxyl groups a phenolic compound has, the more readily accessible it becomes when dealing with other compounds in reactions and, therefore, is considered to be of lower molecular weights. Reducing properties in essential oils are also related to the presence of electron-donating compounds. In other words, as the amount of phenolic compounds in the extract increases, its reducing power also increases (Kumar and Goel, 2019; Liu and Yao, 2007).

4. Conclusions

The findings of this study highlighted the potent antimicrobial activity of *Dracocephalum moldavica* essential oil against major Gram-positive and Gram-negative foodborne pathogens. This effectiveness is attributed to its rich composition of bioactive compounds such as geraniol, geranial, alpha-copaene, and alpha-pinene. Considering the demonstrated antimicrobial and antioxidant properties of the nanoemulsion form of this essential oil, it holds great potential as a natural preservative and antioxidant in the food industry. Its application could effectively control the proliferation of key foodborne pathogens while enhancing the shelf life of food products. Furthermore, the high concentration of geraniol makes this essential oil valuable for use in other industries, including pharmaceuticals, cosmetics, and chemical manufacturing.

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