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Original Article

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Formulation of a broad spectrum nanoemulsion from thymus vulgaris essential oil with enhanced antimicrobial activity against problematic gram negative bacteria and fungi

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Abstract

***Background & Aims*:** *Thymus vulgaris* L. belonging to the Lamiaceae family has been widely used for medicinal purposes. *T. vulgaris* essential oil (EO), which is derived from the aerial parts of the plant, has shown potent antimicrobial activities in previous studies. However, its hydrophobic nature limits its application as a natural antimicrobial agent. Focusing on this problem, the objective of this survey was to develop a nano-sized delivery system of the EO not only to enhance the water solubility but also protect it from degradation.

***Materials & Methods*:** In this study, *T. vulgaris* EO-loaded nanoemulsion was prepared using Tween 80 and Span 60 (surfactants) via high-pressure homogenization and physicochemical characteristics, long-term stability and antimicrobial activity on a broad range of microorganisms were evaluated.

***Results*:** The GC-MS of the EO showed that thymol was the primary compound with a 45.6% value. TEM and AFM images showed the spherical shape of nanoparticles with an average droplet size of 175.6 ± 0.96 nm. Interestingly, the final formulation had significantly lower MICs and MBCs in comparison with pure oil. Furthermore, it showed the lowest MIC and MBC values against *Ent. faecalis* and *B. subtilis*, respectively. Regarding the antifungal effects of the formulation, it was more effective on *C. albicans* than *A. niger*.

***Conclusion*:** The obtained data revealed that encapsulation of the EO as nanoemulsion significantly elaborates its antimicrobial properties, which can be considered as an stable and effective antimicrobial formulation for various purposes such as a food preservative.

***Keywords*:** Antimicrobial Activity, Essential Oil, Nanoemulsion, Physicochemical Characteristics, *Thymus Vulgaris* L

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Introduction

In recent years' microbial resistant has become a growing concern worldwide. Health organizations such as Centers for Disease Control and Prevention (CDC), European Centre for Disease Prevention and Control (ECDC), and World Health Organization (WHO) are consistently trying to explore new therapeutic strategies to combat Antimicrobial Resistant (AMR) and Multi Drug Resistant (MDR) bacteria (1). Therefore, to overcome the increased cost and side effects of prescribed drugs and AMR against various antimicrobial agents, scientists are enforced to develop new approaches. Usage of herbs and complementary medicines seems to be a potential solution to such unsettled problems since they have been used widely by the ancestors for their healing properties (2). Essential oils (EOs) are one of the 80000 plant-derived compounds which have been recognized as secondary metabolites and obtained by distillation by water or steam and cold-press techniques (3). EOs containing various substances are mainly composed of terpenoids and phenolic agents that exert antimicrobial and antiviral activity (4).

Thymus vulgaris L. is an annual woody sub-shrub specie of the *Thymus* genus belonging to the family Lamiaceae. Interestingly, in folk medicine, *Thymus* was famous for its antimicrobial and anthelmintic effects in the treatment of a variety of respiratory system diseases and skin disorders (5).

Several studies have been done to understand the exact action mechanism of antimicrobial properties of EOs, but at the end, there is just some hypothesis. One of the proposed mechanisms of action is that the phenolic component of EOs can penetrate through the microorganism membrane, disrupt the cell permeability, interrupt the function of the cellular energy (ATP) generation system, and cause leakage of cell content eventually microorganism death (6).

However, despite the considerable antimicrobial potential, the application of thyme EO might be limited due to its high hydrophobicity, instability of volatile components, intense aroma, and taste of thyme EO (7). One strategy to overcome these challenges is the application of a well-designed colloidal delivery system for antimicrobial, cosmetics, food, and pharmaceutical products known as nanoemulsions (8). Encapsulation of EO is a practical approach to enhance its efficacy along with physical stability and subsequently decrease the possibility of undesired interaction of EO with food ingredients when it is used as a food preservative (9).

Previous studies show that *T. vulgaris EO (*TyEO)-loaded nanoemulsion has been prepared by different methods, and antimicrobial as well as physicochemical properties were evaluated (10). Nevertheless, based on our knowledge, preparation method used in this study was not reported to date by other researchers for the encapsulation of TyEO. Furthermore, a broad range of Gram-positive and Gram-negative bacteria and fungi were selected despite previous studies that studied antimicrobial effects of TyEO-loaded nanoemulsion against a few numbers of microorganisms. Therefore, this study was designed to develop *T. vulgaris EO*-loaded nanoemulsion using high-pressure homogenization, characterizing the physicochemical properties of nanodispersions. Another aim of this work was to investigate antimicrobial activities against 14 microorganisms to provide an authenticated comparison between the efficiency of pure and nanocapsulated EO.

Materials & Methods

Gas chromatography/mass spectrometry (GC/MS) analysis:

TyEO was purchased (Zardband Pharmaceuticals, Tehran, Iran) and a Gas chromatography-mass spectrometry system (GC-MS) (Agilent Technologies, Palo Alto, USA, model: Agilent 7890A/5975C GC-MS) was used to determine the composition. The major constitutes of EO were investigated by the use of the GC-MS with an HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, film thickness of 0.25 µm). The oven temperature was kept at 40°C for 5 min initially. Following that the temperature raised at the rate of 10°C/min to 230°C followed by an increase to 30°C/min to reach 280°C. The injector and detector temperatures were set at 240°C and 250°C, respectively.

The carrier gas was helium with a linear velocity of 1 ml/min (11).

Preparation of Nanoemulsion:

TyEO nanoemulsion was prepared as previously described by Moghimi *et al.* (12). To prepare the nanoemulsion, Tween 80 and Span 60 (both from MERK, Gernsheim, Germany) were used as non-ionic surfactants. The prepared nanoemulsion included 2% w/w surfactant, 96% w/w distilled water, and 2% w/w TyEO. Briefly, distilled water heated up to 50 ℃ and Span 60 then dissolved in it. The solution homogenized at 8000 rpm for 3 minutes with a high-speed homogenizer (Heidolph, Schwabach, Germany). The mixture of Tween 80 and TyEO added drop-wise to the solution, and the speed of homogenization was elevated up to 12000 rpm for 3 minutes (13).

Characterization of Nanoemulsion:

A Malvern Nanosizer (Malvern Instruments, Worcestershire, UK) was used to measure droplet size and the size distribution of nanoemulsion. The morphology of the synthesized nanoemulsion was shown by atomic force microscopy (JPK, Berlin, Germany, model: Nano Wizard 2) and transmission electron microscopy (Philips, Eindhoven, Netherlands, model: Philips EM 208S-200kV).

***In virto* Release of Essential Oil:**

The release of TyEO was determined by using the dialysis method. Two ml of the TyEO-loaded nanoemulsions were placed in the bags and immersed in 15 ml of phosphate buffer solution (PBS), containing 0.5% Tween 80 at pH of 7.4. All sets were incubated at 37 ºC. Sampling has been done at the times 1, 2, 4, 6, 8, 18, and 24 h after immersion. The absorbance was measured at 276 nm using a UV–VIS spectrophotometer (Jenway, Barcelona, Spain). The same procedure was performed on pure TyEO dissolved in the phosphate buffer (14).

Antimicrobial Activity Assay:

Microbroth dilution assay was performed to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of TyEO and nanoemulisified TyEO (NEO) against standard microbes strains from Persian Type Culture Collection (PTCC) which were included: *Escherichia coli* 1330, *Pseudomonas aeruginosa* 1310, *Staphylococcus aureus* 1337, *Enterococcus faecalis* 13294, *Klebsiella pneumonia* 1035, *Bacillus subtilis* 1023, *Proteus vulgaris* 1312, *S. epidermidis* 1435, *Listeria monocytogenes* 1298, *B. cereus* 1015, *Micrococcus luteus* 1408, *Salmonella typhi* 1709, *Candida albicans* 5027, and *Aspergillus niger* 5012 (15).

Each well contained a mixture of 100 µl media (Tryptic Soy Broth for bacteria and *A. niger* and Yeast Mold Broth for *C. albicans*) and different concentrations of TyEO or NEO, respectively. In the next step, 0.5 McFarland adjusted microbial suspension was inoculated into each well. The final concentration of NEO and TyEO were 0.5 to 9.7×10^{-4} mg/ml and 2.5×10^{-1} to 4.8×10^{-4} mg/ml in each well, respectively. Microbial free media and microbial suspension inoculated media were considered as negative and positive controls, respectively. Additionally, the probable antimicrobial activities of Tween 80 and Span 60 were studied as other controls. Then, 96-well plates were incubated at 37 ℃ for at least 24 h (48 h for *A. niger*). MIC was defined as the lowest concentration of TyEO or NEO that inhibited bacterial growth following incubation. MBC values were determined by plated 5 µl of wells with no visible growth onto agar media. Then, plates were incubated at 37 ℃ for further 24 h, and the lowest concentration of the samples with no microbial growth (killed 99.9% of microorganisms) was considered as MBC (16). Furthermore, The MICs and MBCs of amikacin, teicoplanin, and amphotericin B were determined as positive controls in parallel experiments.

P. aeruginosa was not affected by TyEO or TyNE in microbroth dilution method. 5 ml of NE and Broth culture with TyEO equivalent to NE were prepared with 5 concentrations (undiluted, 0.25, 0.1, 0.001, and 0.0001) in falcons. A falcon containing 5 ml of NE components except EO used as control. 100 µl of microbial inoculum of the microorganism was added to falcons. Then, all of them were incubated for 72 h in shaker incubator with the speed of 180 rpm, and

sampling was done at 0, 4, 8, 12, 18, 24, 48, and 72 h. After 24 h of incubation in 37 ℃, agar plates were investigated for the existence of colonies.

Statistical analysis:

All of the experiments were performed in triplicates except microbroth dilution assay, which was carried out in duplicate. The obtained data are expressed as the mean ± standard deviation (SD).

Results

Volatile Composition: Table 1. represents the chemical composition of TyEO. Twenty-two components amounting to 97.36% were identified by GC-Mass analysis. The major volatile components were thymol (45.62%), p-cymene (25.97%), γ -terpinene (4.76%), carvacrol (4.75%), and linalool (4.05%).

Physicochemical Characterization*:* The average size of prepared nanoemulsion was 120.60 ± 0.96 nm, with a polydispersity index of 0.32 ± 0.038 (Figure 1). Figure 2. represents a typical profile of the negative zeta potential of particles, which was –29.66 ± 0.44 mV.

Table 1. The essential oil composition of *T. vulgaris*

Component	Retention time (min)	Composition (%)
Tricyclene	11.455	0.173
Alpha-pinene	12.352	1.080
Camphene	13.310	1.996
Beta pinene	14.988	0.301
Beta myrcene	14.482	1.313
P-cymene	19.615	25.967
(Z)-b-ocimene	20.648	0.092
Gamma-terpinene	21.577	4.762
Alpha terpinolene	23.166	0.337
Linalool	25.004	4.047
Terpinen-4-ol	29.422	0.151
Alpha-terpineol	34.254	0.788
Citronellol	37.607	0.154
Thymol	40.341	45.621
Carvacrol	40.501	4.746
Thymol acetate	42.871	0.181
Geranyl acetate	44.149	0.908
Z-caryophyllene	45.686	2.937
Bromoacetic acid, 2-octyl ester	46.246	0.152
(E)-Caryophyllene	47.460	0.378
Delta-cadinene	51.615	0.061
Caryophyllene oxide	55.101	1.217

Table 2. The MIC and MBC of antibiotics, *Thymus vulgaris* essential oil and essential oil-loaded nanoemulsion against selected microorganisms, the data shown are multiplied in 10^4.

Tested Microorganisms/PTCC	AB[a]	MIC AB* (mg/ml)	MBC AB (mg/ml)	MIC TyEO[e] (mg/ml)	MBC TyEO (mg/ml)	MIC TyNEO[f] (mg/ml)	MBC TyNEO (mg/ml)
P. aeruginosa (1310)	Amik[b].	39	78	_	_	_	_
E. coli (1330)	Amik.	156	625	4590	4590	1560	1560
K. pneumonia (1035)	Amik	156	312	4590	4590	3120	3120
S. typhi (1709)	Amik.	156	625	4590	4590	1560	1560
P. vulgaris (1312)	Amik.	156	625	4590	4590	1560	6250
B. cereus (1015)	Amik.	156	625	36720	293750	6250	12500
S. aureus (1337)	Tei[c]	4	4	9180	9180	6250	25000
S. epidermidis (1435)	Tei	7.8	31	4590	18360	3120	50000
M. luteus (1408)	Tei	156	31	4590	9180	780	1560
B. subtilis (1023)	Tei	4	7.8	4590	4590	390	390
E. faecalis (13294)	Tei	0.2	1	4590	18360	90	1560
E. hirae (12598)	Tei	0.2	6	4590	9180	1560	1560
C. albicans (5027)	Amph[d].B	40	40	4590	4590	90	390
A. niger (5012)	Amph.B	40	80	4590	4590	780	780

[a] Antibiotic, [b] Amikacin, [c] Teicoplanin [d] Amphotricin B, [e] *Thymus vulgaris* essential oil, [f] *Thymus vulgaris* essential oil-loaded nanoemulsions,

Table 3. Macrobroth dilution method results

Antimicrobial agent	Conc (v/v)	Time (h)							
		0	4	8	12	18	24	48	72
Nanoemulsion/ Control	UnD/C	3+/-	3+/-	3+/-	-/-	-/-	-/-	-/-	-/-
	0.2/C	3+/-	3+/-	3+/-	-/-	-/-	-/-	-/-	-/-
	0.1/ C	3+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	0.01/C	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Essential oil	0.2	3+	-	-	-	-	-	-	-
	0.04	3+	-	-	-	-	-	-	-
	0.004	-	-	-	-	-	-	-	-
	0.0004	-	-	-	-	-	-	-	-

UnD: undiluted C: control

Presence of so many colonies is reported as – (no bactericidal activity), presence of 5-12 colonies is reported as + (slight microbicidal activity), presence of 1-5 colonies is reported as 2+ (moderate microbicidal activity) and no colony presence is reported as 3+ (high microbicidal activity). TyNE has antibacterial activity as fast as EO, but with more duration

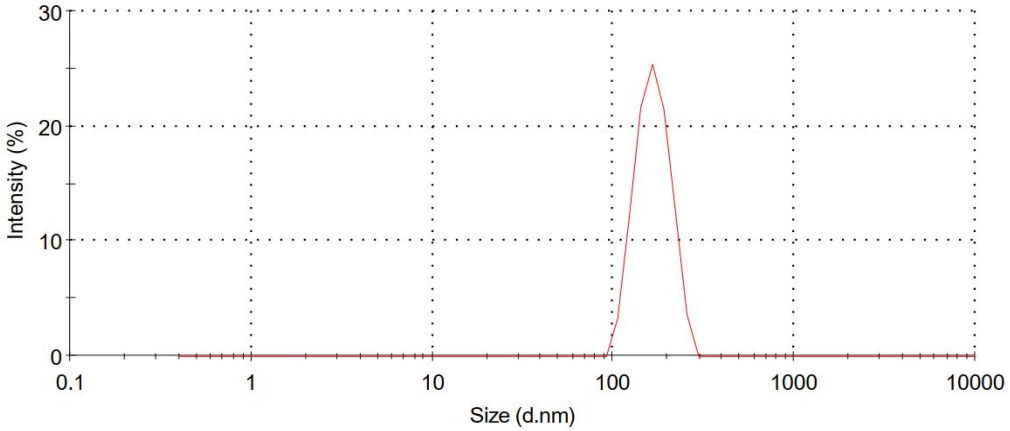

Fig. 1. The particle size distribution of *T. vulgaris* essential oil-loaded nanoemulsion

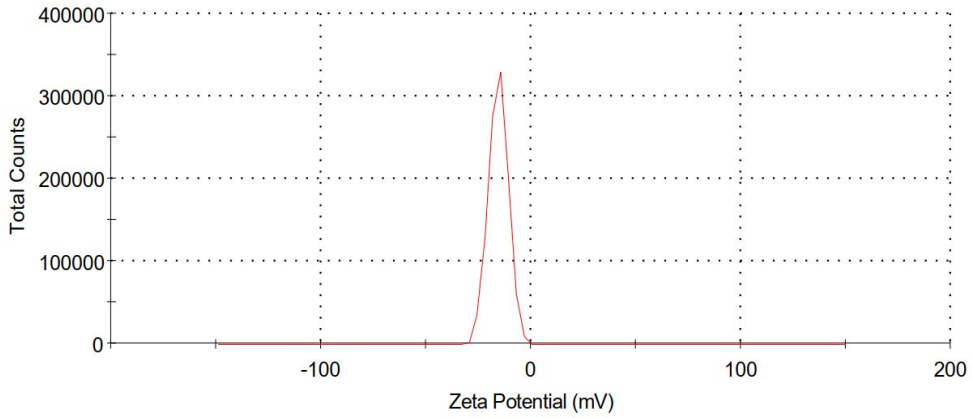

Fig. 2. Zeta potential of *T. vulgaris* essential oil-loaded nanoemulsion

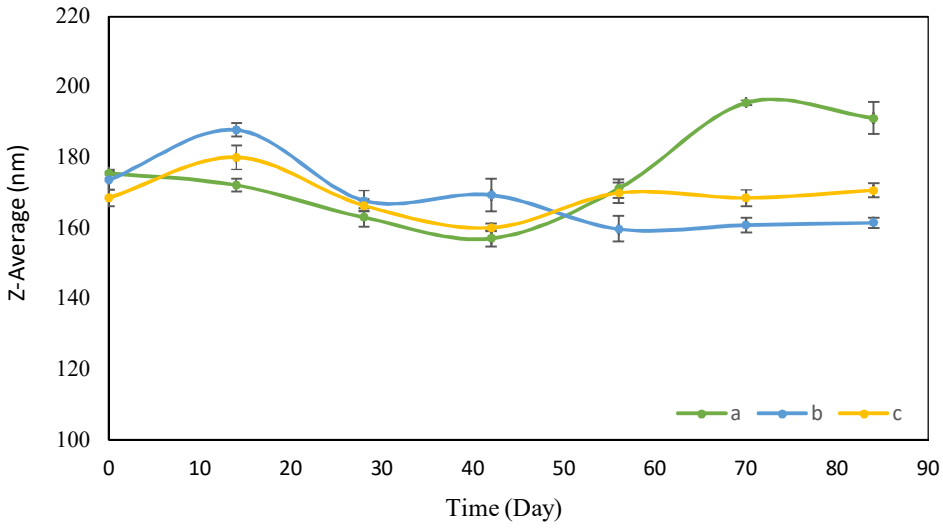

Fig. 3. Stability of *T. vulgaris* essential oil-loaded nanoemulsion at 25 °C (a), 4 °C (b) and 40 ± 1 °C (c) (data shown are the mean ± SD)

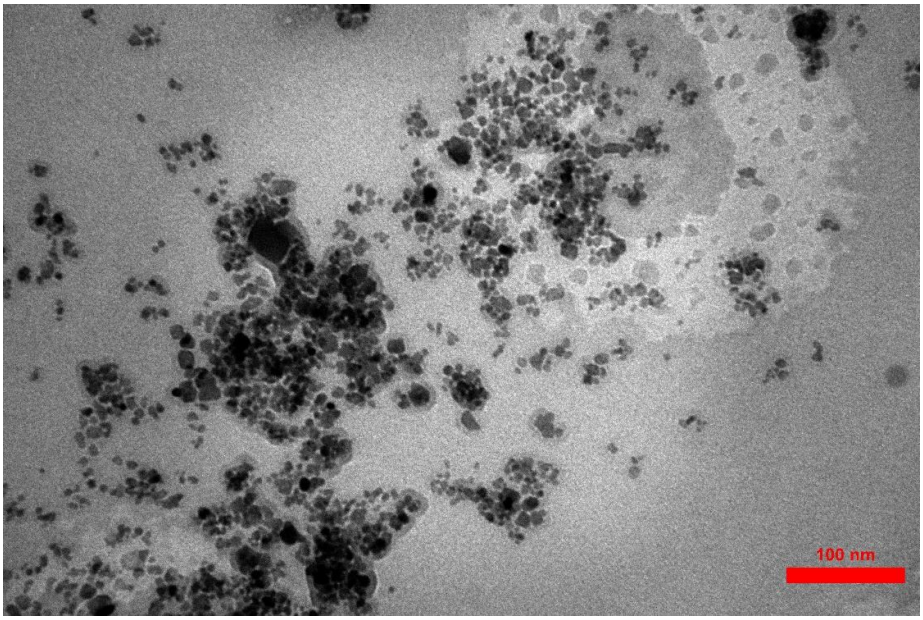

Fig. 4. *TEM images T. vulgaris* essential oil-loaded nanoparticles

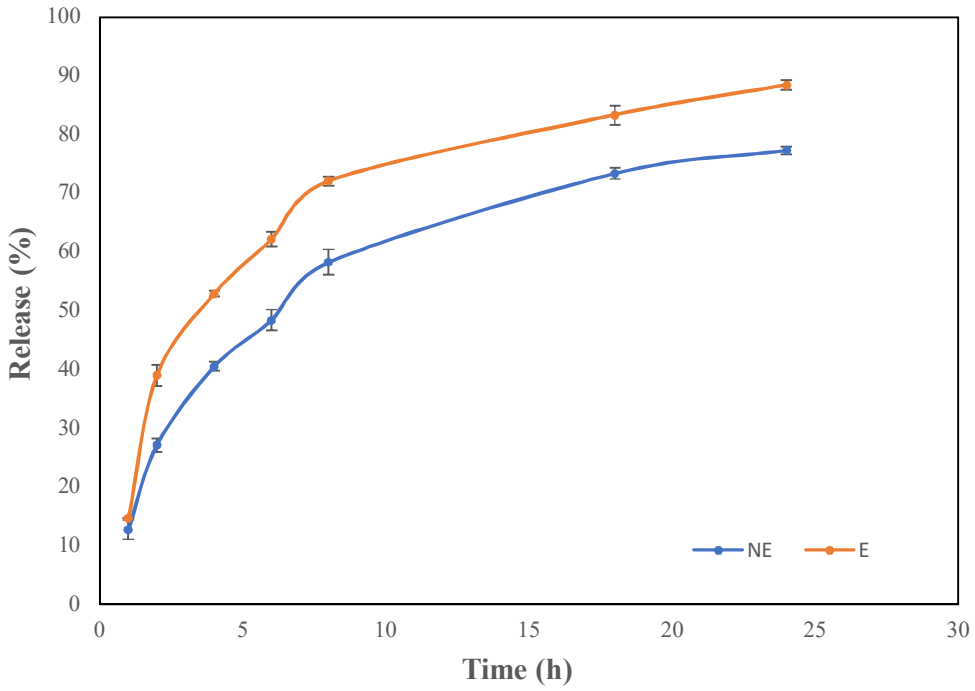

Fig. 5. *In vitro* release profile of *T. vulgaris* essential oil from nanoparticles (data shown are the mean ± SD)

To evaluate the long-term stability, nanoemulsion was incubated at 4℃, room temperature (25 ℃), and 40±1℃ up to three months. After the incubation time, the mean diameter size (Z-averages) and size distribution (PDI) of samples were measured by DLS analysis. Particle size and distribution were followed

every week during the storage period at three different temperatures. As shown in Figure 3. the formulation was physically stable, and the particle size changed slightly over 90 days. Also, comparisons between particle sizes at different temperatures did not show significant changes.

TEM image confirmed the nanometric size of oil in water nanoemulsion. Figure 4 illustrates the nanoemulsion semi-spherical shape within the size range of 10 to 25 nm.

***In Vitro* Release Study**: The *in vitro* release profile of TyEO-loaded nanoemulsion in phosphate buffer solution was obtained and represented as a cumulative percentage (Figure 5). The release behavior indicated an initial burst followed by a slow release.

Determination of MIC and MBC: The antimicrobial activities of TyEO and NEO were evaluated using microbroth dilution assay (Table 2). The lowest MIC values of TyEO were against *E. coli, K. pneumonia, Sal. typhi, P. vulgaris, S. epidermidis, M. luteus, B. subtilis, Ent. faecalis* and *Ent. hirae* (0.459 mg/ml). Considering MBCs, TyEO showed highest bactericidal activity against *E. coli, K. pneumonia, Sal. typhi, P. vulgaris, and B. subtilis* (0.459 mg/ml).

Discussion

Volatile Composition: Table 1. shows twenty-two components of TyEO. As it is obvious, the major volatile components were thymol (45.62%). Based on the literature data, the amount of thymol as the primary component of thyme oil can differ from 6.8% to 64.5%. Variation of thyme oil components can be due to the plant genotype, nature of the soil, climate, season of collecting, and extraction method (17). The results are similar to those previously reported in other studies (18). A study conducted by Jianu showed that p-cymene (8.41%), γ-terpinene (30.90%), and thymol (47.59%) were significant components of thyme plant obtained from Romania. In another similar research, the data revealed that the major constituent of *T. vulgaris* was thymol (40.02%) (19). The GC-Mass analysis of TyEO which was collected from the local market in Saudi Arabia showed that thymol was the major compound followed by camphene, caryophyllene, humulene, α-terpeniol and para-cymene, respectively (20). However, Bouguerra reported that linalool (82.88%), thymol (4.92%), linalyl acetate (2.43%), ascabin (1.74%), camphor (0.80%), α-terpineol (0.34%), eucalyptol (0.22%), and borneol (0.18%) were the primary components of TyEO which was obtained from Blida (21).

Physicochemical Characterization*:* As Figure 1. and Figure 2. represents the average size of nanoemulsion, polydispersity index and zeta potential of particles were 120.60 ± 0.96 nm, 0.32 ± 0.038 and –29.66 ± 0.44 mV, respectively. Difference in the electrical charge between the interfacial double layer at the location of particles and the charge of bulk fluid surrounding this particle is defined as zeta potential (22). Despite the nature of tween 80 which can provide a small negative charge with a value of almost close to –5 mV (23), the prepared nanoemulsion exhibited highly negative zeta potential. To hypothesize further based on the literature, the dissociation degree and ionizable functional groups of TyEO might be responsible for the strong negative zeta potential. Besides, chemical compositions of TyEO might be broken up during mechanical stresses, including ultrasonication and high-shear homogenization. Consequently, the released hydroxyl and carboxyl groups of TyEO adsorb on the oil-water interface, strengthening the negative zeta potential (23).

To evaluate the long-term stability, nanoemulsion was incubated at 4℃, room temperature and 40 ± 1℃ up to three months. As it is obvious from Figure 3. the formulation was physically stable and the particle size changed slightly over 90 days. The slight increase in the droplet diameter of nanoemulsions which were incubated at 4℃ and 25℃ might be due to many physicochemical mechanisms such as flocculation, coalescence, and Ostwald ripening which is known as the major reason for the instability of nanoemulsions. Poor water solubility of the EO might inhibit this process by a compositional ripening effect (24).

TEM image confirmed the nanometric size of semi-spherical shape of nanoemulsion. The results obtained

by TEM showed that the droplet's size was smaller than that of DLS analysis. This difference is due to the DLS feature that measures the hydrodynamic size (25).

***In Vitro* Release Study**: The release behavior of TyEO-loaded nanoemulsion in phosphate buffer solution indicated an initial burst followed by a slow release (Figure 5). In addition, the release of pure TyEO from bag dialysis was investigated to compare the results. According to Figure 3, encapsulation of TyEO enables a slower release over time which approached 50% after 6h and indicated plateaux. It can thus be suggested that the biphasic release of TyEO ascribed to the high affinity of TyEO with the lipophilic matrix of nanoparticles (14).

Determination of MIC and MBC: Based on the Table 2, the lowest MIC values of TyEO were against *E. coli, K. pneumonia, Sal. typhi, P. vulgaris, S. epidermidis, M. luteus, B. subtilis, Ent. faecalis* and *Ent. hirae*. Considering MBCs, TyEO showed highest bactericidal activity against *E. coli, K. pneumonia, Sal. typhi, P. vulgaris and B. subtilis*. Sienkiewicz and co-workers reported that TyEO had the lowest MIC value (6.25×10^{-2} µl/ml) against vancomycin-resistant standard strain—*Ent. faecalis*. Based on the data, this microorganism was the most susceptible bacteria to the TyEO between the clinical strains of *E. coli, Staphylococcus*, *Enterococcus*, and *Pseudomonas* genera (26). Also, MIC and MBC of TyEO against *C. albicans* and *A. niger* were the lowest value (0.459 mg/ml). Considering antifungal effects, the reason for high anti-candida activity of the TyEO could be ascribed to presence of thymol and carvacrol among ingredients based on the literature (27). Previous studies showed that the major components of the oil are thymol, p-cymene, carvacrol and γ –terpinene; whilst, other constitutes such as ketones (camphor, verbenone), alcohols (linalool, (Z)-verbenol, terpinen-4-ol, a(alpha)-terpineol, geraniol, spathulenol), and oxygenated thymol derivates (thymol methyl ether) are present in a negligible amount (28).

The proposed mechanism of bioactivity of TyEO is the hydrophobic TyEO passes through the lipid layer of the cell membrane, deteriorating the structure of polysaccharides, fatty acids and phospholipids, and increases the permeability of the membrane (29). The antimicrobial activity of TyEO is mostly dependent on terpenoids, such as thymol and carvacrol. These phenolic constitutes diffuse in the lipid phase of the cytoplasmic membrane, suppressing the calcium and potassium transport (29). It is assumed that their hydroxyl group's interaction with porins in cell membrane affects its permeability, changing the integrity of the lipid bilayer, leading to an increase of passive proton flux across the cell membrane which leads to leakage of nucleic acids, proteins, potassium, and finally microorganism's death. Additionally, thymol, carvacrol and p-cymene directly interact with fungal cytoplasmic membrane ergosterol, resulting in disruption of the cell membrane and release of cell contents (30).

However, antimicrobial properties of the oil could not be simply accredited to thymol and carvacrol since there might be a synergistic action among all the components. Interestingly, Rota *et al.* reported that an increased concentration of thymol might not cause in a better antimicrobial activity. In this study, *T. hyemalis* (43% thymol) and *T. vulgaris* (58% thymol) showed higher bactericidal effects against *Shigella sonnei* as well as *S. aureus* than *T. zygis* (68% thymol) (30). Also, Marianecci and Rango showed that thymol had higher MIC and MBC values comparing to those of TyEO. The data illustrated that other constitutes of TyEO might act synergistically and antimicrobial activity cannot be attributed only to one specific component (10). P-cymene acts as the precursor of carvacrol, demonstrating a synergism. Ultee showed that p-cymene facilitates the transportation of carvacrol into the cell by swelling the membrane of a microbial cell (31). In another study, it was found that the combination of thymol and carvacrol had additive antibacterial activities against *S. aureus*, *B. cereus*, *Sal. infantis*, and *E. coli*. Hence, the results of synergy testing revealed lower MIC values of the combination than thymol or carvacrol alone (32). Other studies have shown synergism between p-cymene and thymol against *B. cereus* (33). Generally, p-cymene and EOs containing

cymene as major component are more active against Gram-positive bacteria and yeasts like *C. lusitaniae* (34), but less active or inactive against Gram-negative bacteria (33, 35). Also, other terpenes in addition to monoterpenes like thymol also have more activity against Gram-positive bacteria compared to Gram-negatives (36). Caryophyllene, a sesquiterpene, has more effect on Gram-positive bacteria and fungi (37). In contrast, linalool is more active against Gram-negative bacteria rather than Gram-positives (36, 38). NEO exerted the highest bacteriostatic effect against *Ent. faecalis* with an MIC value of 9×10^{-3} mg/ml; while the best MBC values were against *B. subtilis* (39×10^{-3} mg/ml). The NEO formulation had higher antifungal activity against *C. albicans* (MIC and MBC of 9×10^{-3} and 39×10^{-3} mg/ml, respectively) than *A. niger (*MIC and MBC of 78×10^{-3} mg/ml).

As can be seen in Table 2, both pure TyEO and TyEO-loaded NEO were inactive against *P. aeruginosa*, which is well correlated with the result of the previous studies. Posing a high level of resistance to essential oils have been reported by studies. Al-Bayati reported that TyEO was effective against all pathogenic bacteria except *P. aeruginosa* (38). In Kacaniova's research, the only resistant bacteria to oregano and TyEO was *P. aeruginosa* (39). Furthermore, in a research conducted by Hammer and co-workers, only *Cymbopogon citratus*, *Origanum vulgare,* and *Pimenta racemosa* among fifty-two tested EOs, including *T. vulgaris* were effective against *P. aeruginosa* (40). This could be ascribed to the presence of impermeable outer membrane and robust mechanism of efflux in *P. aeruginosa* (40). However, the findings of the current study do not support the previous research of Prata's study in which *P. aeruginosa* was the most sensitive microorganisms to the encapsulated thyme among all the tested microorganisms named *Sal. typhimurium*, *Ent. faecium*, *Ent. hirae*, *Sal. choleraesuis*, *E. coli*, *S. aureus* and *C. albicans* (17).

MICs of TyEO against *B. cereus* and *S. aureus* were the highest values 3.918 and 0.918 mg/ml, respectively (Table 2). At the same time, TyEO exhibited the highest value of MBC against *B. cereus* (29.375 mg/ml), expressing that the lowest bactericidal activity of TyEO was against this bacterium. The results were in agreement with previous researches. In a study, *B. cereus* showed the least sensitivity to TyEO tested on three microorganisms with the highest MIC value (2.5 mg/ml) (41). Another research showed that thymol had the highest MIC value of 0.45 µL/mL for *B. cereus* (42).

B. cereus and *S. aureus* were the least sensitive microorganisms to TyNEO with an MIC value of 6.2×10^{-5} v/v. These results agree with data found in previous study. Interestingly, *S. aureus* showed great resistance to free and TyNEO in comparison to *E. coli* and *Sal. Typhi* (43) .

As it is evident in Table 2, MIC and MBC values of antibiotics were obtained as a control to TyEO as well as TyNEO. All microorganisms showed lower antibacterial susceptibility to TyEO in comparison to antibiotics.

Surprisingly, TyNEO showed the most potent antimicrobial effect against *C. albicans*. At first glance, it might appear that free TyEO has higher antimicrobial efficacy; however, considering the amount of loaded-TyEO in nanoemulsion (2 % v/v) entrapment of TyEO in nanoemulsion delivery system reduced MIC and MBC values against some microorganisms significantly and effectively. Considering MIC values, antimicrobial potency of nanoemulsion against all microorganisms increased in comparison with pure oil. This MIC reduction was 1.47 times against *K. pneumonia, S. epidermidis* and *S. aureus,* 2.94 times against *E. coli, Sal. typhi, P. vulgaris* and *Ent. hirae,* 5.88 times for *M. luteus* and *A. niger*, 11.76 times against *B. cereus* and *B. subtilis* and 51 times against *Ent. faecalis* and *C. albicans*. The MBC values showed that bactericidal and fungicidal activities of nanoemulsion improved 2.94 times against *E. coli* and *Sal. typhi*, 5.88 times against *M. luteus*, *Ent. hirae*, *A. niger*, 11.77 times against *Ent. Faecalis, B. subtilis* and *C. albicans* and 23.5 times against *B. cereus*.

The data are well correlated with recent studies which showed that encapsulation of EOs such as D-limonene and oregano oil into the nanoemulsion delivery system significantly enhanced their

antibacterial effects (44, 45). Moghimi and co-workers reported that *T. daenensis* essential oil-loaded nanoemulsion showed ten times higher antibacterial effects against *E. coli* than the bulk oil (46). In Jemaa's study, nanoemulsification process significantly improved antibacterial properties of *T. capitatus* essential oil (47). The ameliorated antibacterial effects might be due to the small particle size of nanoemulsions, causing an easier fusion with microbial cells, killing microorganisms. In Jimenez-Munguia's study, decreased MIC of 27-60% was observed in nanoemulsion in comparison with non-capsulated TyEO. Using ultrasonication as preparation method might have reduced the particle's size, facilitating their diffusion into the media. In fact, the low water solubility of EOs limits antimicrobial activities since only dissolved molecules can interact with the microbial cell. Thus, encapsulation of EOs increases their solubility, simplifying transportation of EOs molecules through the microbial cell membrane (48, 49). The antimicrobial activity enhancement as a result of nanoemulsification was significantly higher against fungi and Gram-positive bacteria rather than Gram-negatives. This is probably because of increased terpenes (especially p-cymene) solubilization and dissolution, which have low solubility due to their highly hydrophobic structure. As previously mentioned, p-cymene almost only affects Gram-positive bacteria and yeast and so they would be more influenced by increase in EO dissolution and solubilization in comparison to Gram-negative bacteria. However, increased antimicrobial activity against *A. niger* may not be completely through this mechanism, as it has very low susceptibility to p-cymene (50). Difference in susceptibility to EO or NE among various Gram-positive or Gram-negative bacteria may be attributed to difference in the cell wall structure and components. For example, the lipids in the cell wall may facilitate EO activity (51, 52). Another factor that may influence antimicrobial effect of NE is Tween 80, which in low concentrations may increase some microorganisms growth or decrease the growth of others (53). The results of determining antibacterial activities of TyEO and TyNE by microbroth dilution method are demonstrated in Table 3.

Both TyEO and TyNE in sufficient concentrations immediately inhibited growth. TyEO in concentrations of 0.2 and 0.04 completely inhibited growth at the moment, but it did not show any antibacterial activity in the first hour. Undiluted NE and its 0.2 concentrations indicated a complete bacteriostatic activity until the 8th hour, but concentration of 0.1 only indicated in 0 h. So both EO and NE had complete but temporary bacteriostatic activity, with the difference that NE prolongs antibacterial activity at least for 8 hours.

Conclusions

In recent years, the virulence of antimicrobial resistance has become a major health concern for societies globally, demanding novel approaches to combat microbial resistance. This study has indicated the potent antimicrobial properties of pure and NTyEO against 14 microorganisms. By applying GC-MS analysis, twenty-two components were identified; while, thymol was shown to be the primary constituent. Spherical nanoparticles with optimum droplet size were produced by high-pressure homogenization, being stable over three months at three different temperatures. For antimicrobial efficacy test, microbroth dilution method was used and the results demonstrated that nanoemulsification has significantly increased antimicrobial activities of the EO. Most antimicrobial activity increase was against the most of the Gram-positive bacteria and *C. albians* most likely because of increased dissolution of p-cymene, a major component of EO. Similar activity was observed against *A. niger* but probably not all of this is because of p-cymene dissolution. In microbroth dilution method NE enhanced duration of action against *P. aeruginosa*. Moreover, the nanostructured lipid system enhanced the physical stability of the volatile EO and minimized adverse effects including low water solubility which will turn it into a suitable ingredient that can be applied as a preservative for food, pharmaceutical and cosmetic products. Conclusively, nanoencapsulation can be considered as a suitable method which improves the

efficacy of lipophilic molecules. It is also worthy of elucidating the mechanism of action of nanoemulsion with microbial membranes in further studies.

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Ethical Statement

The thesis was approved and funded by Ethical Committee of Zanjan University of Medical Sciences with the code of IR.ZUMS.REC.1396.286 and the thesis code of A-11-673-13.

Data Availability

The raw data supporting the survey are available from the authors upon reasonable request.

Conflict of interest

The authors have no conflict of interest in this study.

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