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Essential Oils Nanoemulsions: Preparation, Characterization and Study of Antibacterial Activity against *Escherichia Coli*

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Abstract

This research studies the application of essential oil nanoemulsion as herbal medicine instead of using antibiotics and chemicals. Thyme, shirazi thyme and rosemary essential oils were selected as herbal drugs. Essential oil nanoemulsions with Tween 80 and/or Sodium dodecyl sulfate (SDS) surfactants were prepared and investigated. Physicochemical characterizations such as hydrodynamic diameter, pH, conductivity, optical clarity and antibacterial activity against gram negative bacteria, E.coli, have been studied. Morphology of the nanoemulsions was evaluated by transmission electron microscope (TEM). Nanoemulsions prepared with the mixture of SDS−Tween 80 had particle diameters significantly smaller than those prepared with Tween 80 (2-11.7 nm in comparison with 189-200 nm). Formulated nanoemulsions had long-term stability at ambient temperature; as there were little changes in droplet diameter after storage for 2 months. MTT assay showed non-toxicity of prepared nanoemulsions. Antibacterial activity against E.coli was also studied by counting the number of survival bacteria in a broth medium. The in vitro test indicated efficacy of all prepared emulsions on E.coli, especially those containing thyme essential oil. The results suggested that the formulated nanoemulsions might be used as potential carrier in food, pharmaceutical and drug delivery systems.

Keywords: *Nanoemulsion, Thyme oil, Rosemary oil, MTT assay, In vitro test.*

1. INRODUCTION

Essential oils are natural compounds containing a mixture of nonvolatile and volatile compounds produced by aromatic plants. Because of high antimicrobial, antioxidant and antifungal activity of essential oils; their application in various fields such as food, cosmetic and pharmaceutical industries have been raised recently [1]. Due to their volatile and aromatic components, essential oils have different applications as flavors and fragrances [2]. Thyme oil extracted from Thymus vulgaris has inhibitory activities against different bacteria and yeasts [3, 4]. Thymol, one of the main components of thyme oil [5], has antimicrobial activity against several bacteria and fungi [4, 6, 7]. Zataria multiflora with the vernacular name of Aavishan-e-shirazi in Iran is a thyme-like plant belonging to the Lamiaceae family which grows mainly in Iran, Pakistan and Afghanistan [8, 9]. It has several traditional uses such as antiseptic, carminative, stimulant, anesthetic, anti-spasmodic and analgesic [10, 11]. Rosmarinus officinalis, known as rosemary, is well-known for its powerful antioxidant [12], antibacterial and chemopreventive properties [13]. Today, rosemary essential oil is used in food flavors [14], cosmetic industry such as bathing essences and hair lotions [15] and medicinally formulation like skin care or pain relief creams [16].

Encapsulation of essential oils in nanoemulsions has been proved as a new method for enhancing their efficacy, stability, and utilization [17]. Nanoemulsions are categorized as nano-sized delivery systems of lipophilic

compounds [18] such as flavors [19], vitamins [20] and antimicrobials [21]. As nanoemulsions have small particle size (radius <100nm), they are transparent or translucent while macroemulsions (radius > 100nm) are usually turbid [19]. Owing to their smaller particles size; nanoemulsions are much more stable against flocculation, coalescence and creaming [22, 23] which make them an excellent candidate for food industries and as delivery agents in pharmaceutical and drug delivery.

Ziani et al. [24] have studied the effect of surfactant charge on antimicrobial activity of thyme oil nanoemulsions. In the absence of thymol, ionic surfactants such as Lauric alginate and SDS exhibited strong antifungal activity, but no antimicrobial activity in its presence. It was concluded that thymol droplets may reduce the effect of antimicrobial surfactants. The impact of ripening inhibitors on thyme oil nanoemulsion stability has also been investigated [4]. A ripening inhibitor like corn oil or medium chain triglycerides (MCT) was incorporated into the nanoemulsions to increase stability and inhibit particle growth. Addition of the ripening inhibitors decreased the antimicrobial activity of the thyme oil in the nanoemulsions which is depended on the oil type. Silva et al. [25] have evaluated the influence of the type of surfactant (Tween 20, SDS and DTAB) and processing conditions on the stability of oil-in-water nanoemulsions. Results showed that processing parameters such as homogenization pressure, surfactant concentrations and oil/water ratio significantly affected the values of hydrodynamic diameters and polydispersity of nanoemulsions. The impact of oil type on nanoemulsion formation and Ostwald ripening stability has been studied by Wooster et al. [26]. The physical properties of the oil phase and the nature of the surfactant layer were found to have a considerable impact on nanoemulsion formation and stabilization. Nirmala et al. [27] have worked on a cinnamon oil nanoemulsion drug delivery system for azithromycin using sonication technique. The results indicated that this system has reduced the surfactant concentration as compared to the microemulsion system which may have further influence in the reduction of gastrointestinal irritation.

The main purpose of this research was preparing physically stable nanoemulsions containing essential oils and mixture of non-ionic and/or anionic surfactants without using ripening inhibitors and studying their physical and biological properties. Two different surfactants, Tween 80 and SDS, were applied in this study. Tween 80 is a nonionic, biocompatible, and non-toxic surfactant [28, 29] and SDS is an anionic surfactant which reduces surface tension of aqueous solutions. It is used as fat emulsifier, wetting agent and detergent in cosmetics and pharmaceuticals [30] and has strong antimicrobial and antibacterial activity [31, 32]. Results of the present study have important applications in different fields such as food, pharmaceutical, and drug delivery systems

2. MATERIAL AND METHODS

2.1. Materials

Thymus vulgaris L., Zataria multiflora, and Rosmarinus officinalis L. were provided from grocery and dried under ambient temperature. Tween 80 and SDS were purchased from Merck Chemicals Ltd. and used without further purification.

2.2. Preparation of Essential Oils

Microwave assisted hydro-distillation (MAHD) method has been selected for extracting of the essential oils. It has been proved that MAHD method has significant advantages in comparison with traditional hydro-distillation such as shorter extraction times, less environmental hazard and more energy-efficacy [33, 34]. MAHD also increases the quality of essential oil [33]. Extraction was performed by using a microwave oven (Tecnokit Chen, Italy,

Tek-2611) equipped with a Clevenger-type apparatus. It was a multimode microwave reactor 2450 MHz with a maximum power of 900W. Leaves of thyme, shirazi thyme and rosemary were heated for 20 minutes with addition of 300cc double-distillated water separately. The extraction process continued until no more essential oil obtained. Then the essential oils were collected from Clevenger apparatus and stored at laboratory condition (25°C). The oils obtained by this method were used without further purification or filtration.

2.3. Preparation of Nanoemulsion

2v/v% Tween 80 was dissolved in double-distillated water at room temperature. The mixture was shaken with a magnetic type stirrer for 10 minutes to get a homogeneous solution. The essential oil was added slowly and mixed with a direct driven stirrer (Fine TECH, SDS-41; South Korea) for 15 minutes. The resulting crude emulsion was sonicated using a 25 kHz ultrasonic Homogenizer (USH650, max power: 650 watt). The sonication time was fixed for 20 minutes in all cases. Ultrasonification generates intensive and disruptive forces in order to minimize nanoemulsion droplets [35].

In another experiment, 0.25 w/v% SDS was added to the solution containing Tween 80 and distillated water. Similarity, essential oils were added slowly and mixed with a direct driven stirrer for 15 minutes and sonicated using an ultrasonic homogenizer for 20 minutes. The resultant nanoemulsions were stored in the laboratory condition (25°C). Nanoemulsions compositions prepared in this study are listed in Table 1.

3. CHARACTERIZATION

3.1. Nanoemulsion Properties

Physicochemical characterization studies were carried out to characterize and control the physical stability of the formulated nanoemulsions. The pH values of the nanoemulsions were measured by immersing the pH electrode into the undiluted emulsion using a pH meter (744 pH Meter Metrohm). Conductivity of the nanoemulsion formulations was measured by using 712 Conductometer Metrohm. All the tests were determined at room temperature.

Table 1.*Initial delivery system composition*

Sample Name	Essential oil Type	Essential oil (%v/v)	Tween 80 (%v/v)	SDS (%w/v)
ZT12	*Zataria multiflora*	1	2	-
ZT22	*Zataria multiflora*	2	2	-
ZTS12	*Zataria multiflora*	1	2	0.25
ZTS22	*Zataria multiflora*	2	2	0.25
ZTS32	*Zataria multiflora*	3	2	0.25
TTS	*Thymus vulgaris*	1	2	0.25
RTS	*Rosmarinus officinalis*	1	2	0.25

3.2. Particle Size Measurements

Nanoemulsion droplets Diameters were measured using a dynamic light scattering instrument (DLS, MAL1008078, Malvern, UK). This instrument determines the particle size from intensity-time fluctuations of a laser beam (633 nm) scattered from a sample [36]. The mean particles size or Z-Average size reported by DLS is the intensity-weighted mean diameter derived from the cumulants analysis [37]. Before measurements, the samples were diluted with distilled water to an appropriate concentration (1:10) to avoid multiple scattering effects.

3.3. Turbidity

For turbidity indication, the absorbance of essential oil nanoemulsions was measured at 600 nm (Abs600) using a Lambda 25, PerkinElmer spectrophotometer. Measurements were repeated at least three times for each sample and the average values were reported.

3.4. Transition Electron Microscopy

Nanoemulsions were observed by negative-staining electron [38]. Nanoemulsions were diluted with distilled

water and filtered through filter paper. Then samples were adsorbed onto carbon-coated copper grids for 1 min. and negatively stained with phosphotungstic acid for 10 min at room temperature. The grids were observed in a transmission electron microscope (PHILIPS, model CM10) at an acceleration voltage of 100 kV.

3.5. MTT Assay

The cytotoxicity of the nanoemulsions was quantitatively assessed further by MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide] assay on L929 cells. MTT assay is a standard colorimetric assay for the quantification of living cells. After sterilization under UV exposure for 30 minutes, 50 µl of nanoemulsions was added to each 24-well plate containing 450 µl of cell suspension (104 cells/ml) in RPMI-1640 media supplemented with 10% FBS in triplicate. Negative control wells were considered as cell suspension without nanoemulsions. Plates were incubated for 24 and 48 hours in CO2 incubator at 37°C with 5% CO2 and 85% humidity. After each period of incubation, 100 µl MTT (5mg/ml) solutions were added to each of the wells and incubated for an extra 4 hours. Cells were washed with phosphate buffer saline (PBS). 0.5 ml DMSO was then added to each well and final optical densities were measured at 570 nm.

3.6. *In Vitro* Study

The antibacterial activity of the nanoemulsions against *Escherichia coli* (*E. coli*, ATCC# 25922), a Gram negative bacteria, was measured by the following method [39]. Briefly, *E. coli* was cultivated in a Tryptic Soy broth (17g Casein, 3g Soybean Peptone, 2.5g Dextrose, 5g Sodium Chloride and 2.5g Dipotassium Phosphate) medium to reach optical density of 0.2 (about 2×107 Bacteria). 100 µL of each essential oil nanoemulsion were mixed with 1 ml bacteria/TSB medium and inoculated for 24 hours in a 37◦C shaker. After incubation, 100 µL of the bacteria/TSB medium was added to 900 µL of sterilized broth solution to prepare several decimal dilutions and then 10 µL of the diluted bacteria/TSB solutions were gently spread on a plate containing TSA agar (15g Pancreatic Digest of Casein, 5g Papaic Digest of Soybean, 5g Sodium Chloride and 15g Agar). Plates were incubated at 37 ◦C for 24 hours and the number of bacteria was counted. A blank nanoemulsion without essential oil was used as the control.

3.7. Statistical Analysis

Microsoft Excel software was used to determine P values by t test and analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

4. RESULTS AND DISCUSSION

4.1. Physicochemical Properties

All nanoemulsions were characterized by measuring their physicochemical properties; pH and conductivity (Table 2.). Conductivity is the ability to conduct electrical current between two points. Electrical conductivity of the nanoemulsions was determined to check the stability and nature of the formulation [40]. Conductivity measurement provides information about continuous phase of nanoemulsions (oil or water continuous) and phase inversion phenomenon [41]. Higher value of nanoemulsion conductivity points out more water content which provides more spaces for ions movements. If the conductivity stays stable after storage at room temperature, it can be assigned as a sign of stability without phase inversion [40]. When phase inversion occurs, the conductivity decreases noticeably [42]. As presented in Table 2, conductivity of the nanoemulsions increased by increasing essential oil concentration which demonstrated that water is the continuous phase ($P<0.05$). Comparison between results of nanoemulsions prepared with Tween 80 and blend of two surfactants showed that

addition of SDS increased conductivity of the nanoemulsions (P<0.05). This is due to this fact that conductivity of the solutions is directly proportional to the amount of ions and increases by increasing the ions.

The pH value is important for determining emulsions stability; because pH fluctuation is an indication of chemical reactions occurrence that can affect the quality of the final product [43]. The prepared emulsions had stable pH for almost all conditions (As it is shown in Table 2.). Because pH values were fixed through various conditions, so it can be concluded that the prepared nanoemulsions are stable. These results are in agreement with results of other literatures [44].

Table 2*. Physicochemical Properties of Nanoemulsions.*

Sample Name	ZT12	ZT22	ZTS12	ZTS22	ZTS32	TTS	RTS
Conductivity (μs/cm)	140±0.5	157±0.7	632.1±0.3	707.6±0.4	715.7±0.4	681.2±0.3	777.7±0.5
pH (After Preparation)	7.1±0.03	7.4±0.02	7.88±0.04	7.93±0.03	7.3±0.02	7.6±0.02	6.75±0.01
pH (After 2 months storage)	7	7	8	8	7	7.8	7

4.2. Hydrodynamic Diameter of Emulsions

Mean particle diameters of essential oils nanoemulsion were evaluated by DLS method. The results of experiments are compiled in Table 3. Mean droplet sizes of nanoemulsions prepared with 2 v/v% Tween 80 were about 200 nm. Addition of SDS decreased emulsions diameters significantly (P<0.05). In comparison with 1v/v% shirazi thyme oil emulsified by 2v/v% Tween 80 (d=189 nm), the equivalent nanoemulsion prepared with the mixture of Tween 80−SDS had much smaller droplets with a mean diameter of 2.03 nm. This can be explained according to the molecular weight of the surfactants (Mw SDS < Mw Tween 80). Generally small molecule emulsifiers like SDS can form, under the same processing situations, smaller particle diameters [45, 25]. This has been assigned to differences in adsorption rates and interfacial properties such as thickness, charge, permeability, and environmental responsiveness [25]. An emulsifier with faster adsorption rate and smaller interfacial forces (which leads to smaller interfacial tension) produces smaller particle sizes [46, 47].

Because of higher droplet collisions and then coalescence during emulsification, increasing essential oil concentration led to increase of mean droplets sizes (P>0.05). Comparison between mean particle diameters of three kinds of essential oils nanoemulsion showed no meaningful difference and they were all in the range of 2 nm.

The values of polydispersity index (PDI) are also presented in Table 3. PDI represents the uniformity of droplet size in nanoemulsion. The higher value of polydispersity indicates the lower uniformity of droplet size of nanoemulsion [48]. PDI can be defined as the ratio of standard deviation to the mean particle diameter [48]. The PDI is dimensionless and scaled such that values smaller than 0.07 are seldom observed other than highly monodisperse systems [49]. Samples with a very broad size distribution have PDI values greater than 0.7 and probably are not suitable for DLS analysis [49]. Polydispersity values near 1.0 are indication of a polydisperse system [51]. All the nanoemulsions had low polydispersity index values which indicate the overall stability and uniformity of the formulation

4.3. Turbidity of Emulsions

Photographs of nanoemulsions prepared with various concentrations of essential oil are shown in Figures 1-2. As it can be observed from the photos, emulsions prepared with Tween 80 are turbid, while

those prepared with both surfactants are transparent. This is because of their lower particle diameters leading to the more transparent solutions.

Table 3. *Nanoemulsions Particle Diameters and Polydispersity Index*

Sample	ZT12	ZT22	ZTS12	ZTS22	ZTS32	TTS	RTS
Dia.(nm)	189±20	200±25	2±0.6	7±0.8	11.7±1.5	2.9±0.7	2.9±0.6
PDI	0.21±0.01	0.07±0.002	0.28±0.04	0.36±0.03	0.22±0.04	0.51±0.02	0.52±0.01

Ultraviolet−visible spectroscopy (UV-vis) is an absorption spectroscopy that uses the ultraviolet and visible region of the electromagnetic spectrum for qualitative and quantitative analyses of samples [52]. UV-vis spectroscopy evaluates the amount of light absorbed by chemical substances by measuring the intensity of light passing through the sample [53]. The results of UV−vis spectrophotometer are presented in Table 4. According to the Abs600 results of shirazi thyme oil emulsions, addition of more essential oil increased absorption rate and consequently turbidity of the nanoemulsions ($P>0.05$). Nanoparticles have optical properties that are very sensitive on size, shape, agglomeration and concentration changes [54]. Increasing absorption rate indicated that the amount of nanoparticles is increased which led to the increase of nanoemulsion turbidity. Adding SDS decreased absorption rate and increased transparency of nanoemulsion ($P>0.05$). It can be explained by this fact that SDS decreased the particle size of nanoemulsion and absorption rate is directly proportional to the size of particles. Comparison between three kinds of essential oils absorption rates revealed no significant difference among them. All above results are consistent with the visual appearance shown in the Figures 1-2.

Figure 1. *Appearance of essential oil nanoemulsions prepared with 2 v/v% Tween 80, shirazi thyme oil concentration is 1 and 2 v/v% from left to right.*

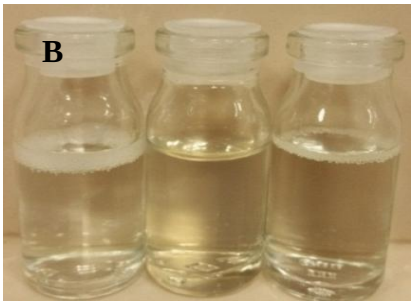

Figure 2. *Appearance of essential oil nanoemulsions prepared with 2 v/v% Tween 80 and 0.25 w/v% SDS. A: shirazi thyme oil concentration is 1, 2 and 3 v/v% from left to right and B: Essential oil type is shirazi thyme oil, thyme oil and rosemary from left to right.*

4.4. Nanoemulsion Particles Morphology

Transmission electron microscopes (TEM) are electron optical instruments similar to light microscopes. However, an electron beam rather than light is transmitted through the sample [55]. Morphology of the nanoemulsions containing 1 & 2 v/v% shirazi thyme oil prepared with 2 v/v% Tween 80 and 0.25 w/v% SDS were visualized by TEM

(Figure 3.). It can be inferred from the figures that most of the droplets of nanoemulsions were almost spherical in shape. The droplets had uniform shape and size. Emulsions with 2v/v% essential oil showed bigger particles which is in agreement with the results of DLS test

Table 4. *Absorbance at 600 nm*

Sample Name	ZT12	ZT22	ZTS12	ZTS22	ZTS32	TTS	RTS
Abs 600	2.08 ± 10^{-4}	4.42±0.01	0.09±0.003	1.31±0.005	4±0.01	0.11±0.01	0.13±0.003

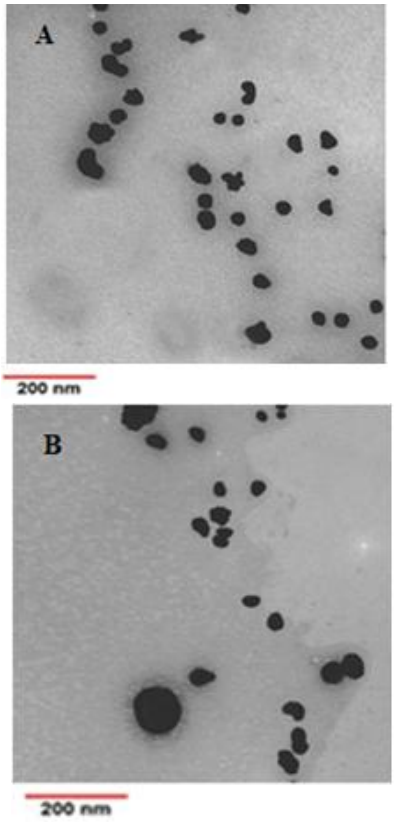

Figure 3. *TEM images of shirazi thyme oil nanoemulsion with 2v/v% Tween 80 and 0.25 gr SDS (A) essential oil 1 v/v%, (B) essential oil 2v/v%*

4.5. Cytotoxicity Test

Quantitative assessment of cytotoxicity of the nanoemulsions was done by MTT assay. Two different time intervals were investigated in this assay; 24 and 48 hours. The results of MTT assay are shown in Figure 4. After 24 hours of contact, both nanoemulsions (thyme and rosemary essential oils) showed 79-84% metabolically active cells compared to cells without the drugs (negative controls). After 48 hours of contact, cell viability reached to 88-90% for nanoemulsion containing thyme and rosemary oil respectively which showed that prepared nanoemulsions are safe and non-toxic.

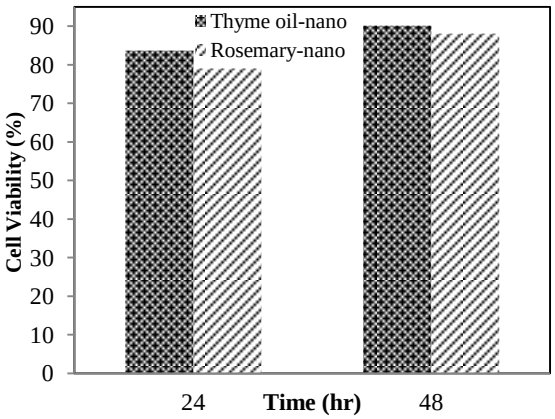

Figure 4. *Cell viability of the prepared nanoemulsions.*

4.6. *In Vitro* Study

The antibacterial activities of nanoemulsions with good physical stability were determined against E.coli by using the viable cell-counting method. Two different concentrations of 1 & 2 v/v% were used for evaluating the antibacterial activity of rosemary and thyme oil. The antibacterial activity of thyme and rosemary essential oil against E.coli has been investigated previously [56, 57, 58]. Burt [59] reported the antibacterial activity of essential oils against different bacteria. In this review, the minimum inhibitory concentrations (MIC) of thyme and rosemary essential oil against E.coli were reported 0.45-1.25 and 4.5–>10 µl/ml respectively. The MIC is cited by the most researchers as a measure of the antibacterial performance of essential oils

and is the lowest concentration of a chemical, usually a drug, preventing visible growth of bacterium. In a study performed by Teixeira et al. [60], the zones of inhibition for thyme and rosemary essential oil were calculated 47 and 6 mm respectively. All these results confirm higher antibacterial activity of thyme essential oil in comparison with that of rosemary.

The photos of the effects of essential oils on the growth of E. coli are shown in Figure 5. In all emulsions, the number of bacteria decreased in comparison with the blank sample ($P>0.05$). Figure 6 also shows quantitative amount of survival bacteria for prepared samples. The in vitro results obtained in this study indicated that the thyme oil nanoemulsion inhibited the growth of bacteria more than rosemary did ($P>0.05$). By increasing the concentration of thyme oil, the number of bacteria decreased more showing the dependency of antibacterial activity of nanoemulsion on thyme oil concentration. This is also in agreement with other findings [44, 61].

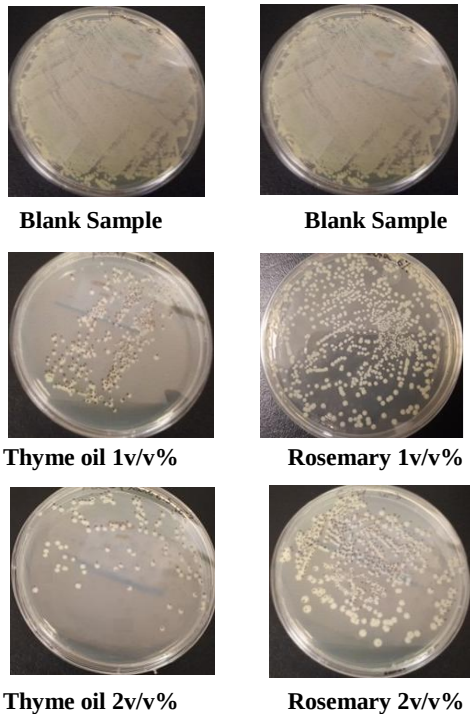

Figure 5. *Schematic of the antibacterial activity of rosemary and thyme oil against E. coli (Blank sample is a nanoemulsion without essential oil).*

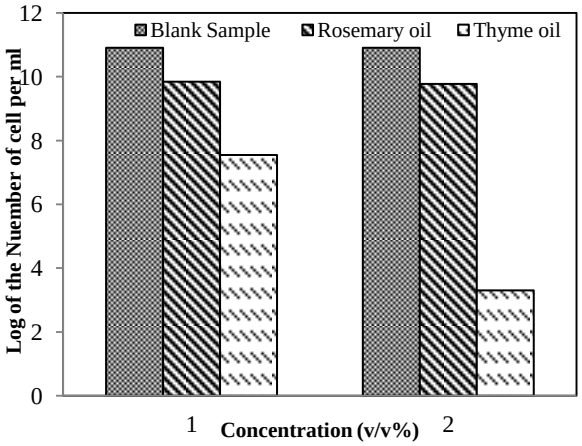

Figure 6. *Quantitative Antibacterial activities of nanoemulsions against E.coli (Blank sample is a nanoemulsion without essential oil.*

4.7. Storage Stability of Nanoemulsions

One of the main aspects of preparation of essential oil nanoemulsions is their stability against Ostwald ripening. Ostwald ripening is the growth of large oil droplets at the expense of smaller oil droplets due to diffusion of oil molecules through the intervening aqueous phase [24]. For using nanoemulsions as antimicrobial or antibacterial agents in delivery systems, having good long-term stability is essential. The particle size changes in thyme oil emulsions were evaluated by DLS method after storage at room temperature (25°C) for 60 days. When particle size distributions of emulsions prepared with Tween 80-SDS blend were compared before and after 2 months storage at room temperature (Figure 7), several trends were observed. The growth of particle size was observed for all the emulsions. However, the growth of average hydrodynamic diameters (Table 5) was not significant indicating the overall stability of nanoemulsions.

The TEM images of nanoemulsions after 2 months storage are also shown in Figure 8. The formulated nanoemulsions exhibited good stability against phase separation and creaming with little changes in particle diameter, although some diameter growth was observed which was in agreement with the DLS data.

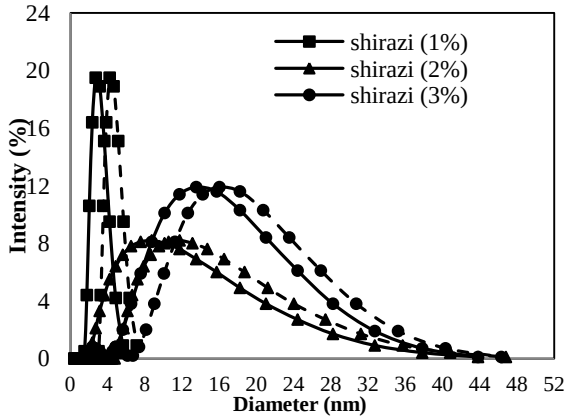

Figure 7. *Particle size distributions of nanoemulsions with thyme oil emulsified by Tween 80-SDS blend before (solid curves) and after (dashed curves) storage at room temperature for 2 months.*

Table 5. *Hydrodynamic Diameter of Emulsions After 2 months*

Sample Name	ZTS12	ZTS22	ZTS32
Dia.(nm)	5±0.6	12±0.8	20±1.5
PDI	0.188±0.02	0.202±0.03	0.26±0.06

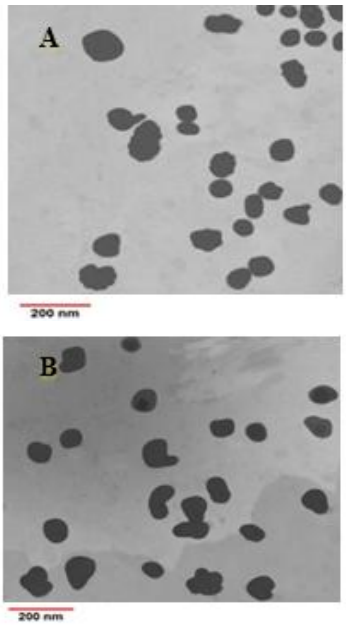

Figure 8. *TEM images of shirazi thyme oil nanoemulsion prepared with 2v/v% Tween 80 and 0.25 gr SDS after 2 months storage (A) essential oil 1v/v%, (B) essential oil 2v/v%*

The pH values of formulations were determined by pH indicator (pH-indicator strips pH 0 – 14, Universal indicator, Merck Millipore) and they were all in the range of 7-8 (As shown in Table 2) which confirmed the stability of nanoemulsions even after storage of two months at room temperature.

5. CONCLUSION

Nanoemulsions have obtained great attentions as pharmaceutical, drug delivery, food products, and cosmetics formulation. Nanoemulsions with different essential oil and surfactant concentrations were prepared and investigated in this study. Tween 80 and SDS were chosen as emulsifier and thyme, shirazi thyme and rosemary as herbal drugs. Various tests such as DLS, TEM, UV-vis and in vitro were performed and physiochemical properties such as pH and conductivity were measured. Particle sizes of nanoemulsions prepared by Tween 80−SDS blend were much smaller than those prepared with individual surfactant and were in the range of 2-11.7 nm. The droplet size increased with increasing the level of essential oils which was also verified by TEM test. Absorption content was measured by UV-vis test and showed that increasing the amount of essential oils increased nanoemulsion turbidity. Cytotoxicity test proved that prepared nanoemulsions are safe and non-toxic. The results of in vitro test also showed the antibacterial activity of all prepared nanoemulsions against E.coli including rosemary and thyme, but thyme oil had a higher antibacterial ability. The formulated nanoemulsions were physically stable over 2-months storage at ambient temperature which makes them interesting for practical applications as mentioned previously.

REFERNCES

1. Davidson, P., (2005). "Food antimicrobials: Back to nature", in *First International Symposium on Natural Preservatives in Food Systems*.
2. Bauer, K., Garbe, D., Surburg, H., (2008). "*Common fragrance and flavor materials: preparation, properties and uses*", John Wiley & Sons.
3. Gaysinsky, S., Davidson, P., McClements, D., Weiss, J., (2008)."Formulation and Characterization of Phyto-Phenol-Carrying Antimicrobial Micro emulsions", *Food Bio. physics.*, 3(1): 54-65.
4. Chang, Y., McLandsborough, L., McClements, D. J., (2012). "Physical Properties and Antimicrobial Efficacy of Thyme Oil Nanoemulsions: Influence of Ripening Inhibitors", *J. Agric. Food Chem.*, 60: 12056-12063.
5. Gaysinsky, S., (2007). "*Emulsions and micro emulsions as antimicrobial delivery systems*", University of Massachusetts, PhD Dissertation, Amherst.
6. Tawakkal, I. A., Cran, M. J., Bigger, S. W., (2016). "Release of thymol from poly(lactic acid)-based antimicrobial films containing kenaf fibres as natural filler", *LWT - Food Sci. Technol.*, 66: 629-637.
7. Galotto, M. J., de Dicastillo, C. L., Torres, A., Guarda, A., (2016). "Thymol: Use in Antimicrobial Packaging", *Antimicrobial Food Packaging*, Academic Press, 553-562.
8. Manikandan, R., Chandrasekar, K., Srivastava, S. K., (2012). "Life form analysis of the family Lamiaceae in Jammu & Kashmir", *Phytotaxonomy.*, 12: 7-19.
9. "World Checklist of Selected Plant Families," *Kew Sceience*, (2018). [Online]. Available: http://wcsp.science.kew.org/namedetail.do?name_id=215611.
10. Sajed, H., Sahebkar, A. H., Iranshahi, M., (2013). "Zataria multiflora Boiss. (Shirazi thyme)—An ancient condiment with modern pharmaceutical uses", *J. Ethnopharmacol.*, 145(3): 686-698.
11. Taherpour, A. , Hashemi, A. , Fallah, F., Erfani, Manesh S., Taki, E., (2014). "Evaluation of Zenian and Avishan-e Shirazi Antibacterial Activity against Vibrio cholerae Strains", *J. Med. Bacteriol.*, 2(1): 17-21.
12. Visentin, V., Rodríguez-Rojo, S., Navarrete, A., Maestri, D., Cocero, M. J., (2012). "Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process", *J. Food Eng.*, 109(1): 9-15.
13. Oluwatuyi, M., Kaatz, G. W., Gibbons, S., (2004). "Antibacterial and resistance modifying activity of Rosmarinus officinalis", *Phyto Chemistry.*, 65: 3249-54.
14. Shokri, A., Saeedi, M., Fakhar, M., Morteza-Semnani, K., Keighobadi, M., Hosseini, Teshnizi S., Kelidari, H., Sajadi, S., (2017). "Antileishmanial Activity of Lavandula angustifolia and Rosmarinus Officinalis Essential Oils and Nano-emulsions on Leishmania major (MRHO/IR/75/ER)", *Iran J. Parasitol.*, 12(4): 622-631.
15. Hernández, M. D., Sotomayor, J. A., Hernández, A., Jordán, M. J., (2016). "Rosemary (Rosmarinus officinalis L.", in *Essential Oils in food preservasion flavor and safety*, 677-688.
16. "11 Amazing Benefits of Rosemary Oil," (2018). [Online]. Available: https://www.organicfacts.net/health-benefits/essential-oils/health-benefits-of-rosemary-oil.html.
17. Moghimi, R., Ghaderi, L., Rafati, H., Aliahmadi, A., McClements, D. J., (2016). "Superior antibacterial activity of nanoemulsion of Thymus daenensis essential oil against E. coli", *Food Chem.*, 194: 410-415.
18. Xue, J., Zhong, Q., (2014). "Thyme Oil Nanoemulsions Coemulsified by Sodium Caseinate and Lecithin", *J.Agric. Food Chem.*,62: 9900-9907.
19. Rao, J., McClements, D. J., (2011). "Food-grade micro emulsions, nanoemulsions and emulsions: Fabrication from sucrose mono palmitate & lemon oil", *Food Hydrocoll.*, 25: 1413-1423.
20. Relkin, P., Jung, J. M., Ollivon, M., (2009). "Factors affecting vitamine degradation in oil-in-water nano emulsions", *J. Therm. Anal. Calorim.*, 98: 13-18.
21. Donsì, F., Annunziata, M., Sessa, M., Ferrari, G., (2011). "Nano encapsulation of essential oils to enhance their antimicrobial activity in foods", *LWT - Food Sci. Technol.*, 44: 1908-1914.
22. Weiss, J., Gaysinsky, S., Davidson, M., McClements, J., (2009). "Nanostructured encapsulation systems: food antimicrobials", *Global issues in food science and technology*, 425-479.
23. Xuem J., (2015). "*Essential Oil Nanoemulsions Prepared with Natural Emulsifiers for Improved Food Safety*", University of Tennessee, PhD Dissertation, Knoxville.
24. Ziani, K. H., Chang, Y., McLandsborough, L., McClements, D. J., (2011). "Influence of Surfactant Charge on Antimicrobial Efficacy of Surfactant-Stabilized Thyme Oil Nanoemulsions", *J. Agric. Food Chem.*, 59: 6247-6255.
25. Silva, H. D., Cerqueira, M. A., Vicente, A. A. , (2015). "Influence of surfactant and processing conditions in the stability of oil-in-water nanoemulsions", *J. Food Eng.*,167: 89-98.
26. Wooster, T. J., Golding, M., Sanguansri, P., (2008). "Impact of Oil Type on Nanoemulsion Formation and Ostwald Ripening Stability", *Langmuir.*, 24: 12758-12765.
27. Nirmala, M. J., Allanki, S., Mukherjee, A. , Chandrasekaran, N., (2013). "Azithromycin: Essential Oil Based Nanoemulsion Drug Delivery Systems", *Int. J. Pharm .Sci.*, 5(4): 236-238.

28. Kerwin, B. A., (2008). "Polysorbates 20 and 80 used in the formulation of protein bio therapeutics: structure and degradation pathways", *J. Pharm. Sci.*, 97: 2924-2935.
29. Merk Milipore Co., (2018). "*Tween 80 MSDS*", [Online]. Available: http://www.emdmillipore.com/US/en/product/Tween-80,MDA_CHEM-822187.
30. U.S. National Library of Medicine, "*National Center for Biotechnology Information* ", OPEN CHEMISTRY DATABASE," [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/compound/Sodium_dodecyl_sulfate #section=Top
31. Askerm D., Weissm J., McClementsm D. J., (2011). "Formation and stabilization of antimicrobial delivery systems based on electrostatic complexes of cationic-non-ionic mixed micelles and anionic polysaccharides", *J. Agric. Food Chem.*, 59(3): 1041-1049.
32. Yousry, M. I., Sabrein, H. M. , Abd-El Baset, M., (2016). "Chemically modified carbon paste and membrane sensors for the determination of benzethonium chloride and some anionic surfactants (SLES, SDS, and LABSA): Characterization using SEM and AFM", *Talanta.*, 155: 158-167.
33. Moradi, S., Fazlali, A., Hamedi, H., (2018). "Microwave-Assisted Hydro-Distillation of Essential Oil from Rosemary: Comparison with Traditional Distillation", *Avicenna J. Med. Biotechnol.*, 10(1): 22-28.
34. Mohammadi, S. Z., Khorasani-Motlagh, M., (2012). "Synthesis and Characterization of α-Fe2O3 Nanoparticles by Microwave Method", *Int. J. Nanosci. Nanotechnol.*, 8(2): 87-92.
35. Ghosh, V., Mukherjeem, A., Chandrasekaran, N., (2013). "Ultrasonic emulsification of food-grade nanoemulsion formulation and evaluation of its bactericidal activity", *Ultrason Sonochem.*, 20(1): 338-344.
36. Saberi, A., Fang, Y., McClements, D. J., (2014). "Stabilization of Vitamin E-Enriched Nanoemulsions: Influence of Post-Homogenization Cosurfactant Addition", *J. Agric. Food Chem..*, 62: 1625-1633.
37. Kaszuba, M. , (2015). "*Malvern Dynamic Light Scattering Manual*", Malvern Instruments.
38. Shakeel, F., Ramadan, W., Ahmed, M. A., (2019). "Investigation of true nanoemulsions for transdermal potential of indomethacin: characterization, rheological characteristics, and ex vivo skin permeation studies", *J. Drug Target.*, 17(6): 435-441.
39. Abdelgawada, A. M., Hudsona, S. M., Rojasb, O. J. , (2014). "Antimicrobial wound dressing nanofiber mats from multicomponent (chitosan/silver-NPs/polyvinyl alcohol) systems", *Carbohydr. Polym.*, 100: 166-178.
40. Talegaonkar, S., Tariq, M., Alabood, R. M. , (2011). "Design and Developement of O/W Nanoemulsion for the Transdermal Delivery of Ondansetron", *Bull. Pharm. Res.*,1(3): 18-30.
41. Grumezescu, A. M. , (2016). "Nanoemulsions-based delivery systems", in *Emulsions*, 304.
42. Junyaprasert, V. B., Teeranachaideekul, V., Souto, V., Boonme, P., Müller, R. H. , (2009). "Q10-loaded NLC versus nanoemulsions: Stability, rheology and in vitro skin permeation", *Int. J. Pharm.*, 377: 207-214.
43. Bernardi, D. S., Daniela, Pereira, T. A., Maciel, N. R., Bortoloto, J., Viera, G. S., Oliveira, G. C., Rocha-Filho, P. A. , (2011). "Formation and stability of oil-in-water nanoemulsions containing rice bran oil: in vitro and in vivo assessments", *J. Nanobiotechnology.*, 9: 44.
44. Heydari, M. , Bagheri, M. , Rabieh, S., (2014). "High Energy Ultrasonification Preparation of Thyme Oil Nanoemulsion", in *5th International Congress on Nanoscience & Nanotechnology*, Tehran, Iran.
45. Donsì, F., Sessa, M., Ferrari, G., (2011). "Effect of emulsifier type and disruption chamber geometry on the fabrication of food nanoemulsions by high pressure homogenization", *Ind. Eng. Chem. Res.*, 51(22): 7606-7618.
46. Qian, C., McClements, D. J. , (2011). "Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: Factors affecting particle size", *Food Hydrocoll.*, 25: 1000-1008.
47. McClements, D. J., (2010). "Emulsion Design to Improve the Delivery of Functional Lipophilic Components", *Annu. Rev. Food Sci. T.*, 1: 241-269.
48. Jaiswal, M., Dudhe, R., Sharma, P. K., (2015). "Nanoemulsion: an advanced mode of drug delivery system", *Biotech.*, 5: 123-127.
49. Hughes, J. M., Budd, P. M., Grieve, A., Dutta, P., Tiede, K., Lewis, J., (2015). "Highly monodisperse, lanthanide-containing polystyrene nanoparticles as potential standard reference materials for environmental “nano” fate analysis" *J. Appl. Polym. Sci.*, 132: 42061.
50. Malvern Co., (2011). "*Dynamic Light Scattering Common terms defined*," Malvern Instruments Limited.
51. Otoni, C. G., Pontes, S. S. F., Medeiros, E. A. A., Soares, N. F. F., (2014). "Edible Films from Methylcellulose and Nanoemulsions of Clove Bud (Syzygium aromaticum) and Oregano (Origanum vulgare) Essential Oils as Shelf Life Extenders for Sliced Bread", *J. Agric. Food Chem.*, 62: 5214-5219.
52. Abdelhalim, M. K., Mady, M. M., Ghannam, M. M., (2012). "Physical Properties of Different Gold Nanoparticles: Ultraviolet-Visible and Fluorescence Measurements" *Nanomed. Nanotechnol.*, 3(3): 1-5.
53. Sanda, F. M., Victor, M. E., Monica, T. A., Alina, C., (2012). "*Spectrophotometric Measurements techniques for Fermentation Process*", Hungary-Romania Cross-BorderCo-Operation Programme 2007-2013.

54. Tomaszewsk, E., Soliwod, K., Kadziol, K., Tkacz-Szczesn, B., Celichowski, G., Cichomski, M., Szmaj, W., Grobelny, J., (2013). "Detection Limits of DLS and UV-Vis Spectroscopy in Characterization of Polydisperse Nanoparticles Colloids", *Nanomaterials.*, 2013: 1-10.
55. Zinin, P., "*Transmission Electron Microscope*," University of Hawai, Lecture 14, Honolulu, USA.
56. Farag, R. S. , Daw, Z. Y. , Hewwdi, F. M., EL-Baroty, G. S. A., (1989). "Antimicrobial Activity of Some Egyptian Spice Essential Oils", *J. Food Protect.*, 52(9): 665-667.
57. Gutierrez, J., Barry-Ryan, C., Bourke, P., (2008). "The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients", *Int. J. Food Microbiol*, 124: 91-97.
58. Schelz, Z., Molnar, J., Hohmann, J., (2006). "Antimicrobial and antiplasmid activities of essential oils", *Fitoterapia.*, 77: 279-285.
59. Burt, S. , (2004). "Essential oils: their antibacterial properties and potential applications in foods—a review", *Int. J. Food Microbiol.*, 94: 223-253.
60. Teixeira, B., Marques, A., Ramos, C., Neng, N. R., Nogueira, J. M. F., Saraiva, J.A., Nunes, M. L., (2013). "Chemical composition and antibacterial and antioxidant properties of commercial essential oils", *Ind. Crop Prod.*, 43: 587-595.
61. Chang, Y. , McLandsborough, L., McClements, D. J. , (2015). "Fabrication, stability and efficacy of dual-component antimicrobial nanoemulsions: Essential oil (thyme oil) and cationic surfactant (lauric arginate)", *Food Chem.*, 172: 298-304.