Development of Mosquito Protective Textiles Using Nanoemulsion of *Eucalyptus Globulus* and *Syzygium Aromaticum* Essential Oils Against Malaria Vector, *Anopheles Stephensi* (Liston)

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Abstract

Background

The aim of current study was to determine the protection efficacy of *Eucalyptus globulus* and *Syzygium aromaticum* essential oils nanoemulsions-loaded textiles versus bulk essential oil- treated textiles against the malaria vector, *Anopheles stephensi*.

Methods

The components of *E. globulus* and *S. aromaticum* essential oils were determined using gas chromatography/mass spectrometry. Then, the nanoemulsions of both essential oils were prepared using a low energy emulsification method. Their stability and droplet sizes were determined, and the repellent efficacy against landings/bites of the starve mosquito females was examined using textile panels of polyester/cotton, impregnated with serial concentrations of the nano-emulsion.

Results

The main compositions of *E. globulus* essential oil were 1, 8-cineol (64.58%) and alpha-pinene (10.63%), whereas those of *S. aromaticum* essential oil were 2-methoxy-3-(2-propenyl) (77.04%) and trans-caryophyllene (11.99%). Transparent oil in water nanoemulsion system consisting of essential oils, Tween-20, Tween-80 and propylene glycol was developed. The median droplet size was 11.2-23.1nm depending on dilution ratio. Protection time of nanoemulsion-loaded textile (285 ± 30 min) was noticeably higher than that of bulk essential oils (< 5 min).

Conclusions

It was concluded that nanoemulsion of essential oils may be interesting options in control of mosquito-related diseases.

Introduction

Mosquitoes are of the most important insects, which are nuisance vectors of different diseases such as malaria, dengue and filariasis [1]. According to World Health Organization report, malaria cases were 229 million worldwide in 2019 [2]. Among malaria vectors, *Anopheles stephensi*, vector of urban malaria, is an important vector with wide distribution and spreading in a large parts of Arabian Peninsula and certain countries of south east Asia include India, Afghanistan and Iran [3].

One of the principal strategies to protect human from the mosquito bites, is using of repellent gents. These agents are mostly available as lotions, gels, solution, creams and sprays. Mosquito repellents have been widely used and consist of natural or synthetic chemical compounds [4] [5]. Natural repellents using herbal essential oils are now being considered as alternative human safe and eco-friendly compounds for repelling mosquitoes and other insects [6].

Research and development in herbal repellents has been focused on increasing the longevity of their repellency properties and decreasing the evaporation rate of volatile components [6]. For instance, in a comparative study of repellency, effect of 38 essential oils, include *Eucalyptus globulus* and *Syzygium aromaticum*, in three concentrations (10%, 50% and undiluted) was evaluated. 32 of them prevented mosquito bites in undiluted form [7]. It is interesting to note that some of essential oils (e.g. *E.globulus* and *S.aromaticum*) duration of repellency strongly depends on concentrations, experiment designs, and mosquito species [6].

The main ingredient of *E. globulus* essential oil is Eucalyptol. Eucalyptol is an aromatic component with a fresh camphor-like smell and a spicy, cooling taste. Generally, it is used as an insect repellent, insecticide, mosquito larvicide and ovipositional repellent with acetylcholinesterase inhibition activity [8].

Eugenol is the main extracted constituent (70–90%) of clove and is responsible for clove aroma. Eugenol is a volatile phenolic constituent of clove essential oil obtained from *S.aromatium* buds and leaves, mainly harvested in Indonesia, India and Madagascar [9]. Eugenol is recognized as a relatively strong and moderately durable mosquito repellent [6].

In a study to achieve formulations of the plant-based mosquito repellents, using some plants including *E. globulus* and *S. aromaticum* (both of extract and essential oil) concentrations of 10% (V/V%) were determined to be effective mosquito repellents [10]. While it was reported that the minimum effective concentration of soaked clove leaves (*S. aromaticum*) for repellency of *Anopheles sp*., was 1% [11]. Furthermore, it was shown that the hexane extract of clove (*S. aromaticum*) was more effective for repellency of malaria vector, *An. stephensi*, at 2.5, 5 and 10% concentrations [12].

Among the mosquito protection methods and mosquito repellent applications, a common method is use of textile-based mosquito protection in the form of nets, clothes, etc. [13]. According to Anitha et al., a mosquito repellent textile is a product which protects the human beings from the bite of mosquitoes [14]. Application of textiles treated with mosquito repellents to protect from the mosquito bites and mosquito borne disease such as malaria, is an innovate method (5).

The volatile components of essential oils are responsible for their repellent effects against mosquitoes. However, problems related to poor solubility and short-term stability of aromatic compounds of essential oils, hinder the development of effective essential oils formulations. Therefore, use of novel preparations such as nanoemulsions have been sugested [7] [15].

Nanoemulsions are colloidal dispersion systems composed of two immiscible liquids, mixed along with emulsifying agents (surfactants and co-surfactants) to form a single phase, with droplet sizes on the order of 100nm. Their small size leads to useful properties such as high surface area, robust stability, optically transparent appearance, and tunable rheology [16] [17].

The present study, focused on preparation of a nanoemulsion from *E.globulus* and *S.aromaticum* essential oils. Repellency efficiency of the treated fabrics with nanoemulsions was tested against *An. stephensi*, using mosquito repellency cage test.

Matherials And Methods

Essential oils

E. globulus essential oil was purchased from Kesht-o-Sanate Golkaran Kashan Co, (Iran). The buds of clove (*S. aromaticum)* were grained by home grinder and hydro-distilled for 6h in a Clevenger type apparatus in August 2019. The oil was dried over anhydrous sodium sulphate. The essential oils were kept in dark glass at 4-5°C in a refrigerator until analysis.

Water dilutions of 1%, 3% and 5% of *E. gobulus* as well as 0.5%, 1% and 3% of *S. aromaticum* essential oils were prepared. To find the mixing effects, three different combinations ratios of 1:1, 1:2 and 2:1 (v/v) according to the results of repellency tests of essential oil-impregnated textiles, were prepared.

Chemicals

Polyoxyethylene Sorbitan Monooleate (Tween-80) and Polyoxyethylene Sorbitan Monolaurate (Tween-20) were purchased from SigmaAldrich, (Germany). Propylene Glycol was purchased form Kimyagaran Emrooz Co, (Iran).

Analysis of essential oils

The chemical composition of the essential oils was analyzed by gas chromatography–mass spectrometry (GC-MS). A gas chromatograph 7890B (Agilent Technologies, USA) equipped with a 30 m DB-5 capillary column (0.25 mm inner diameter, 0.25 μm film thickness) was used in combination with a mass spectrometer 5977A (Agilent Technologies, electron ionization detector). All injections were performed in split mode with auto sampler. Ten-microliter syringes were used to inject 1μl of samples. The injector temperature was set at 280°C and the flow rate was maintained at 1.0 ml/min using helium as the carrier gas. The initial oven temperature was set at 50°C for 5 min and ramped at 10°C /min to 280°C. The ion source and interface temperature were 230°C and 300°C, respectively. The MSD was used in the electron impact (EI) full scan monitoring mode with a solvent delay time of 5 min.

Impregnating textile with the essential oil

The textile fabric made of cotton: polyester (30:70) weighing 190 grams per square meter (Boroujerd Textile Co.) was used in the current study. The fabrics (25cm × 25cm) were separately impregnated with different concentrations of essential oils and /or combination of both essential oils according to WHO protocol for impregnating the bed nets [18]. The repellency tests were separately performed for each concentration in four replications. Nanoemulsions were loaded on fabrics based on spray dying protocol [19]. The best ratio of essential oils combination with higher repellency efficiency was chosen to prepare the nanoemulsion. Deionized Water-treated fabrics were used as controls.

Mosquitoes

Adult females of *An. stephensi*, used in this study, were from the laboratory colonies reared and maintained in the Anopheles insectary of faculty of public health of Tehran University of Medical sciences (28 ± 2°C, 60 ± 10% relative humidity (RH) and 12:12 (L: D) hours photoperiod). The mosquitoes were fed with 10% sugar solution and offered guinea pig blood twice in a week. In the present study, non-blood fed 2–5 day old females, starved for 4–6 hours prior to the bioassays, were used for repelling tests.

Repellency bioassay

In present study, repellency bioassays were conducted as previously described [20] [5]. A transparent Plexiglas box (30 × 30 × 30 cm dimension) was used for accurate observation of the mosquito landing on the fabrics in the cage. Thirty mosquitoes were used for each repellency bioassay. The mosquitoes were simultaneously exposed to hand of a volunteer, which was covered with 25 × 25 cm treated textile. The controls were essential oil free textiles. The exposed time was 5 minutes, each repellency test was performed in four replicates and protection time was considered as the time between the beginnings of test to the mosquito first landing.

Nanoemulsion preparation

Nanoemulsion of essential oils was formulated using a low energy emulsification method by magnetic stirrer at laboratory temperature (55 min, 1200 rpm). Initially, Tween 80 and Tween-20 were added to essential oil of *E. globulus* during stirring, then propylene glycol (co-emulsifier) was slowly added to the solution. Similar procedure was performed to prepare nanoemulsion of *S. aromaticum* (clove) essential oil using only Tween 80 as an emulsifier.

Thermodynamic stability study

The stability of prepared nanoemulsions was measured through visual observation. Freeze-thaw cycles were performed by keeping the emulsions at cycles of − 23°C and 25°C for 48h at each temperature. Also the nanoemulsions were subjected to the thermodynamic stability tests by heating-cooling cycles (4°C and 40°C with storage of 48h at each temperature). The cycles were repeated four times.

Investigation of particle size and morphology

The measurement of droplet size of nanoemulsion formulations was determined using Dynamic Light Scatteroscope I (K-One LTD., Korea). The morphological study of nanoemulsion was carried using Transmission Electron Microscopy (Philips EM208S, 100KV, Netherland).

Statistical Analysis

Data generated in repellency activity or protection time experiments using repellency bioassays were expressed as mean of repellency (min) ± SD (Standard deviation). The data were then subjected to analysis of variance (ANOVA) followed by Post Hoc LSD test of multiple comparisons. All statistical analyses were performed by means of statistical software SPSS ver. 21.0 and the significance level was accepted when P-value < 0.05.

Results

Chemical analysis of essential oils

According to GC-MS analysis, 12 compounds from *E. globulus* essential oil and 8 compounds from *S. aromaticum* essential oil were identified. The main components of the *E. globulus* essential oil were 1,8-cineole (64.58%), alpha pinene (10.63%), benzene (8.31%), (+) spathulenol (3.28%) and (-)-globulol (2.60%), which showed the retention times of 8.306, 7.118, 8.221, 11.925 and 11.963 min respectively (Table 1). The main components of *S. aromaticum* essential oil were phenol, 2-methoxy-3-(2-propenyl) (77.04%), trans-caryophyllene (11.99%), phenol, 2-methoxy-4-(2-propenyl) (6.83%), alpha-caryophyllene (1.40%) and beta-cadinene (1.21%) with retention times of 10.721, 11.113, 11.559, 11,290 and 11.599 min, respectively (Table 2).

Table 1
Components of *Eucalyptus globulus* essential oil from GC/MS analysis

No.	Compounds	Rt* (min)	Compositions (%)
1	Alpha pinene	7.118	10.63
2	Benzene	8.221	8.31
3	1,8-Cineole	8.306	64.58
4	Gamma.-Terpinene	8.552	1.46
5	Bicyclo[3.1.1]heptan-3-ol	9.274	1.91
6	3-Cyclohexen-1-ol	9.546	1.10
7	Alpha terpinolene	9.637	1.30
8	(+)-Aromadendrene	11.208	1.78
9	1H-Cycloprop[e]azulene	11.328	1.73
10	(+) Spathulenol	11.925	3.28
11	(-)-Globulol	11.963	2.60
12	1-Propyl-3-(propen-1-yl)adamantane	12.681	1.31
	Total		99.99
*Retention Time			

Table 2
Components of *Syzygium aromaticum* essential oil from GC/MS analysis

No.	Compounds	Rt* (min)	Compositions (%)
1	Phenol, 2-methoxy-3-(2-propenyl)	10.721	77.04
2	Alpha.-Copaene	10.836	0.81
3	Trans-Caryophyllene	11.113	11.99
4	Alpha.-Caryophyllene	11.290	1.40
5	Delta.-Cadinene	11.365	0.29
6	Phenol, 2-methoxy-4-(2-propenyl)	11.559	6.83
7	Beta-Cadinene	11.599	1.21
8	Caryophyllene oxide	11.971	0.43
	Total		100
*Retention Time			

Physiochemical characterization of nanoemulsion

The results of thermodynamic stability study on prepared formulations are shown in Table 3. After thermodynamic stress and based on visual appearance, 8 proportions were transparent and among them, formulations F5 (5% *E. globulus* essential oil + 10%Tween-80 + 8% Tween-20 + 10% propylene glycol + 67% DW), F8 (5% *S. aromaticum* essential oil + 22% Tween-80 + 73% DW) and F15 (3% *E. globulus* essential oil + 2% *S. aromaticum* essential oil + 7%Tween-80 + 7% Tween-20 + 16% propylene glycol + 65% DW) as A, B and C formulations were chosen for further studies due to their emulsifier content. Figure 1 shows the DLS results of the samples. The effect of dilution on particle size of the samples are indicated in Table 4. Comparison of the dilution effect on the median particle size (nm) of A, B and C formulations is represented in Fig. 2. From the details, dilution has minimum effects on particle size of C formulation. Therefore, C formulation was considered as the final preparation and investigated using TEM (see Fig. 3). The TEM image shows spherical shape for the particles with particle size of 8.5 ± 3.0 nm.

Table 3
Effect of ingredients' concentration on visual appearance of the preparations after thermodynamic stability cycles

Tube Lable	*E. globulus* Essential oil (%)	*S. aromaticum* Essential oil (%)	Tween-80 (%)	Tween-20 (%)	Propylene glycol (%)	Water (%)	visual appearance
F1	5	0	5	5	10	75	Turbid
F2	5	0	6	6	15	68	Turbid
F3	5	0	8	8	15	64	Turbid
F4	5	0	10	12	15	68	Transparent
F5	5	0	10	8	10	68	Transparent
F6	5	0	10	10	10	65	Transparent
F7	0	5	20	0	0	75	Turbid
F8	0	5	22	0	0	73	Transparent
F9	0	5	27	0	0	68	Transparent
F10	0	5	30	0	0	65	Transparent
F11	4	1	15	15	15	55	Turbid
F12	1	4	12	15	12	61	Turbid
F13	3	2	10	10	10	65	Turbid
F14	2	3	12	12	10	68	Transparent
F15	3	2	7	7	16	65	Transparent

Table 4
Particle size of the selected nanoemulsions of *E. globulus* and *S. aromaticum* before and after dilution with water

Formulation	Dilution	Particle Size (nm)
F5	**No dilution**	**23.1**
	1:5	12.6
	1:10	12.3
	1:20	11.2
F8	**No dilution**	**21.3**
	1:5	14.8
	1:10	11.5
	1:20	13.5
F15	**No dilution**	**17.7**
	1:5	14.4
	1:10	11.4
	1:20	11.2

Repellency bioassays

The protection time (min) ± SD of the impregnated textures with different concentrations and ratios of *E. globulus* and *S. aromaticum* essential oils as well as nanoemulsion-impegrated textiles are represented in Table 5.

Table 5
Protection times of the textures impregnated with different bulk or nanoemulsion of essential oils against *An. Stephensi* mosquitoes

Textile treatments	Concentrations or ratios of EOs (v/v)	Mean protection time (min) ±SD	Standard Error	P-Value
E. globulus EO*	1%	0.85 ± 0.09[b]	0.05	0.9640
	3%	3.00 ± 0.23	0.12	0.0004
	5%	3.82 ± 0.20	0.10	0.0002
S. aromaticum EO	0.5%	1.25 ± 0.50[b]	0.25	0.0750
	1%	3.00 ± 0.82	0.41	0.0001
	3%	3.25 ± 0.50	0.25	0.00006
3% v/v *E.globulus* EO + 1% v/v *S.aromaticum* EO	1:1	2. 75 ± 0.50	0.25	0.00001
6% v/v *E.globulus* EO + 1% v/v *S.aromaticum* EO	2:1	4.00 ± 0.82	0.41	0.000001
3% v/v *E.globulus* EO + 2% v/v *S.aromaticum* EO	1:2	4.500 ± 0.58	0.29	0
(Nanoemulsion) 3% *E. globulus* EO + 2% *S. aromaticum* EO + 7%Tween-80 + 7% Tween-20 + 16% propylene glycol + 65% DW	1:2	285 ± 30[a]	15	0
Deionized Water (control)	-	0.08 ± 0.07[b]	0.04	-

*Essential Oil

a: significant deference with all groups

b: no significant deference according to Post Hoc (LSD) test (p-value = 0.05)

According to Table 5, the protection times of impregnated textures, treated with *E. globulus* 1% and *S. aromaticum* 0.5% were less than 2 min and significantly lower than others (p-value < 0.05). Furthermore, the protection time of textiles treated with *E. globulus* 1% and *S. aromaticum* 0.5% were not significantly different from water treated textiles as controls (p-value > 0.05). The protection times of impregnated textures, treated with *E. globulus* 3% and *E. globulus* 5% were not significantly different, as well as for the textiles treated with *S. aromaticum* 1% and *S. aromaticum* 3% (p-value > 0.05). Among the different combinations of essential oils (1:1, 2:1 and 1:2 ratios) based on the minimum effective concentrations of essential oils, the most effective combination was considered to formulate the nanoemulsions. Accordingly, variety of formulations regarding to the fixed proportion of essential oils (5%) were prepared and optimized, as already shown in Table 3. In total, nanoemulsion-impegrated textiles with *E. globulus* and *S. aromaticum* essential oils (3%:2%) showed the highest protection time (285 ± 30 min) compared with others (< 5 min) (Table 5).

Discussion

The percentage of 1,8 cineole in chemical profile of *E. globulus* essential oil (64.58%) was different from the 1.8 cinelole of the essential oil of *E. globulus* in previous reports collected from other countries such as Spain (63.8%), India (68.8%), Algeria (78.45%), Italy (84.9%), Montenegro (85.5%) and China (94.30%) [21] [22] [23].

Furthermore, the proportion of Eugenol and its derivatives in the essential oil of *S.aromaticum* (77.043) was different from those collected from other countries. Major components which were identified in the essential oil of *S.aromaticum* from Indonesia were m-Eugenol (69.44%) and Eugenol acetate (10.79%) [24]. While the main compositions of *S.aromaticum* from India were Eugenol (70%), beta-caryophyllene (19.5%) and Eugenol acetate (2.1%). Those from Madagsacar were Eugenol (82.6%), beta-caryophyllene (7.2%) and Eugenol acetate (6%) [25]. Previous studies indicated that the environmental factors including temperature, rainfall, humidity and solar radiation as well as the amount of soil macro- and micronutrients are able to influence the production of metabolites. Thus, plants under conditions of stress induced by climate factors may show changes in the production of different metabolites [26].

Previous researches have shown that attractiveness of human/ animal hosts to mosquitoes depends on the sensory cues (e.g. CO_2, temperature and the quantity of components) which emit from the host's skin [27] [28] [29]. It is already known that at least 277 compounds [30], including ammonia, lactic acid and 1-octen-3-ol (octenol), which are produced by human, are detected by the olfactory receptor neurons of mosquitoes [29]. The olfactory system of mosquitoes contains various protein receptors, connected to olfactory neurons, articulated in different parts of the mosquito body, particularly on the antennae and plays a key role in attraction towards human hosts [31]. However, some plant act as repellent or deterrent for mosquitoes as a protection strategy against the herbivores, pathogenic organisms, insects and pests [32] [26] [33]. The olfactory receptor neurons are responsible for the recognition of the repellent agents from these plants [29] [34]. Some volatile compounds which are detected by olfactory receptor neurons of mosquitoes, have the potential to be applied as repellents to decrease mosquito-human contact [7] [6] [35].

Essential oils, as a main class of herbal repellants [36] [31], are only effective when are freshly applied. They lose their repellency effects after a short time [7, 37]. This issue has been resolved through preparing the formulations which are able to maintain the active ingredients on the skin for longer time periods. Formulations based on nano/microparticles not only increase the stability of the essential oils, but also facilitate their absorption into the skin/cloths, so, increase their repellency efficacy [36] [15].

Depending on the type of application, various methods have been used to determine the repellency properties of essential oils against mosquitoes including their dermal application [20] [38] [39] [10] [40]. The repellency activity of microemulsion of Eucalyptus (*E. globulus*) essential oil against field mosquitoes has been reported. The results indicated that microemulsions of 5, 10 and 15% eucalyptus essential oil showed repellency activity for 82, 135 and 170 min, respectively [41]. The repellency activity of nanoemulsified *Mentha piperita* and *E. globulus* essential oils against *An. stephensi* showed longer protection time compared with bulk essential oils of *E. globulus* and *M. piperita* [42]. There two studies agree with our findings in increasing the duration of effect of essential oil when formulated in a nanoemulsion. Other studies evaluate the repellency activity of impregnated textiles with essential oils. A study on textiles treated with microencapsulated citronella oil indicated higher and longer lasting protection compared to the textiles treated with an ethanol solution of the essential oil [43], similar to our results. This may be due to the lower rate evaporation of volatile components of essential oils in the nanemulsion formulation. In nanoemulsion particles, the inner phase (i.e. essential oil) is preserved in a layer of emulsifier molecules, therefore, evaporation of the essential oils molecules becomes less noticeable to form a slow and continuous release [44] [15]. In addition, the small particle size of the produced nanoemulsion (d50 < 20 nm) plays an important role in the increasing protection time, since nano-size particles probably have improved penetration into different materials including textile fibers [45] [15]. Moreover, it is well-known that the small size of the droplets allows them to be sprayed or deposited uniformly on target areas [44].

Conclusion

A high protection time from nano-formulation of *E. globulus* essential oil and *S. aromaticum* (clove) essential oil was reported. This indicates the effect of nano-formulation on fixing the volatile components as active ingredients of essential oils and reduction of evaporation rate from impregnated textiles. However, more investigations on nano-formulations of other essential oils with mosquito repellent properties, the synergistic effect of active components of mixed essential oils as well as their droplet size, thermodynamic stability and repellency effect after dilutions are required.

Declarations

Acknowledgment

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Ethics approval

This research was approved by ethics committee of Tehran University of Medical Sciences (ir.tums.sph.rec.1397.244)

Consent for publication

Not applicable

Availability of data

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

The study came out from the corresponding author's graduate fieldwork. ZSh carried out the laboratory bioassays and field tests. AA supervised nano-synthetizing of essential oils, HRB as a main investigator has designed the project, supervised all procedure, SMK supervised mosquito bioassays, KA performed statistical analyze, YY advised nano-synthetized of essential olis, MA and FA extracted the essential oils. All authors read and approved the final manuscript.

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Figures

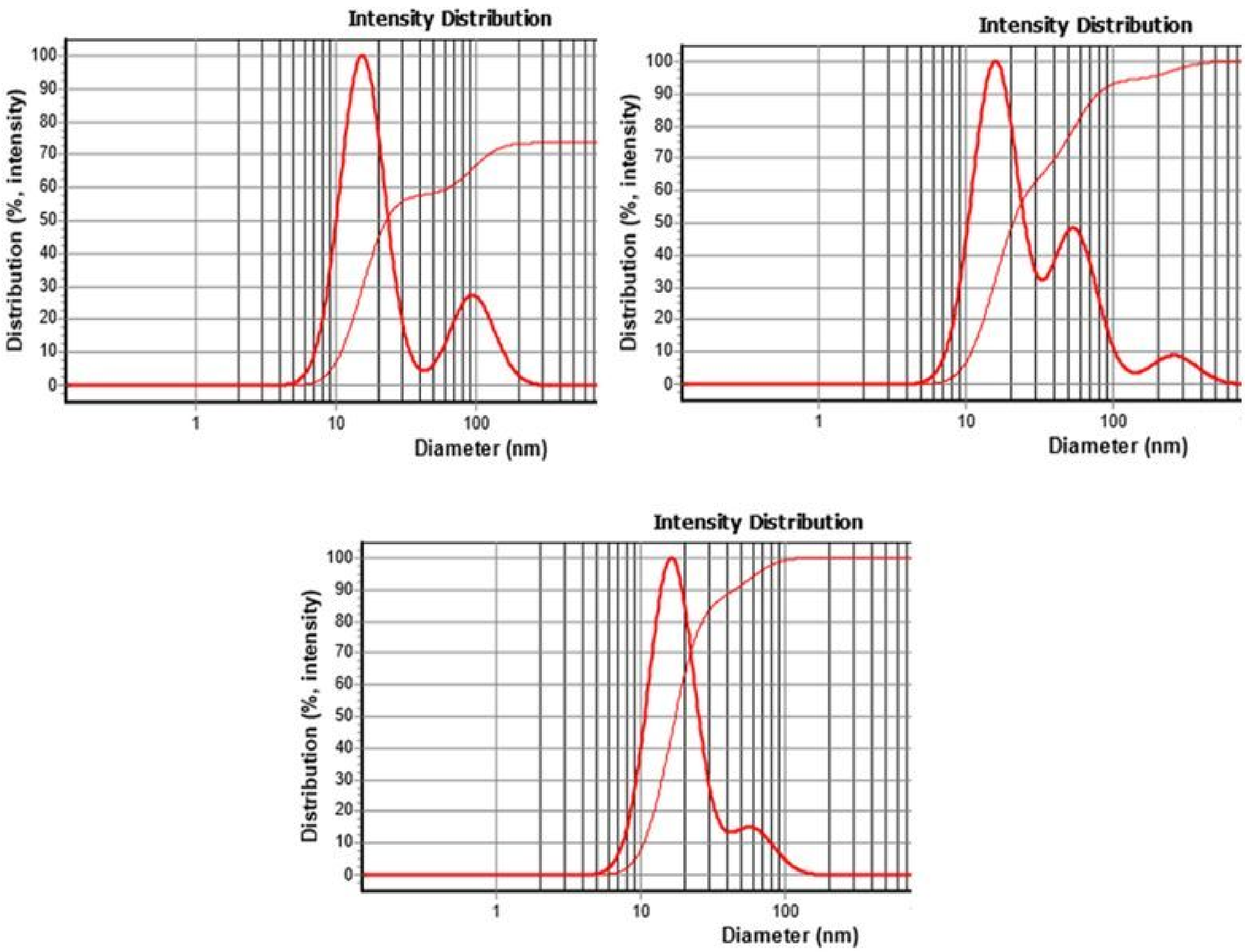

Figure 1

Particle size distributions for three formulations (A: up-left, B: up-right and C: down), using Dynamic Light Scattering

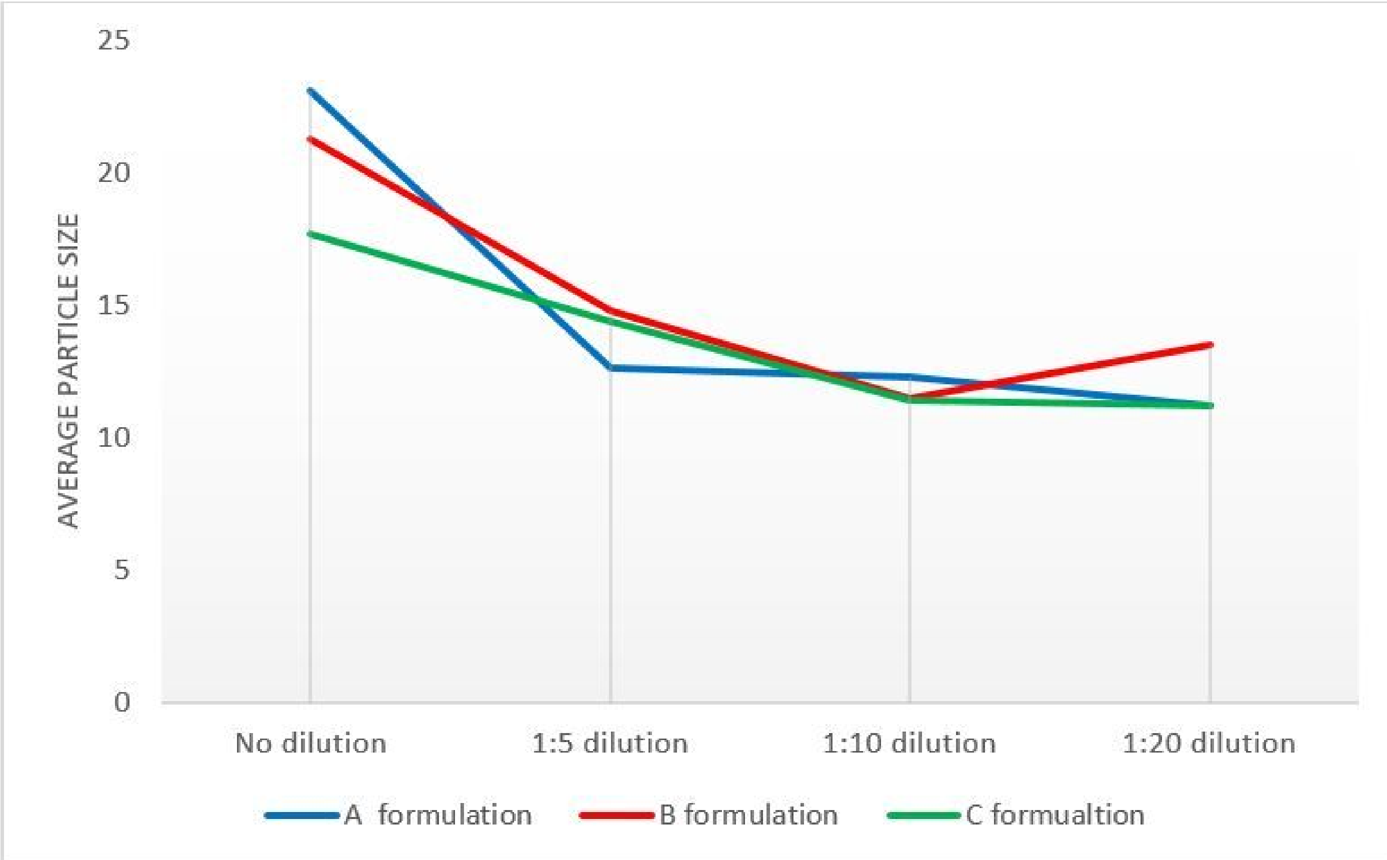

Figure 2

Comparison of the dilution effect on the average particle size (nm) of three formulated nanoemulsions

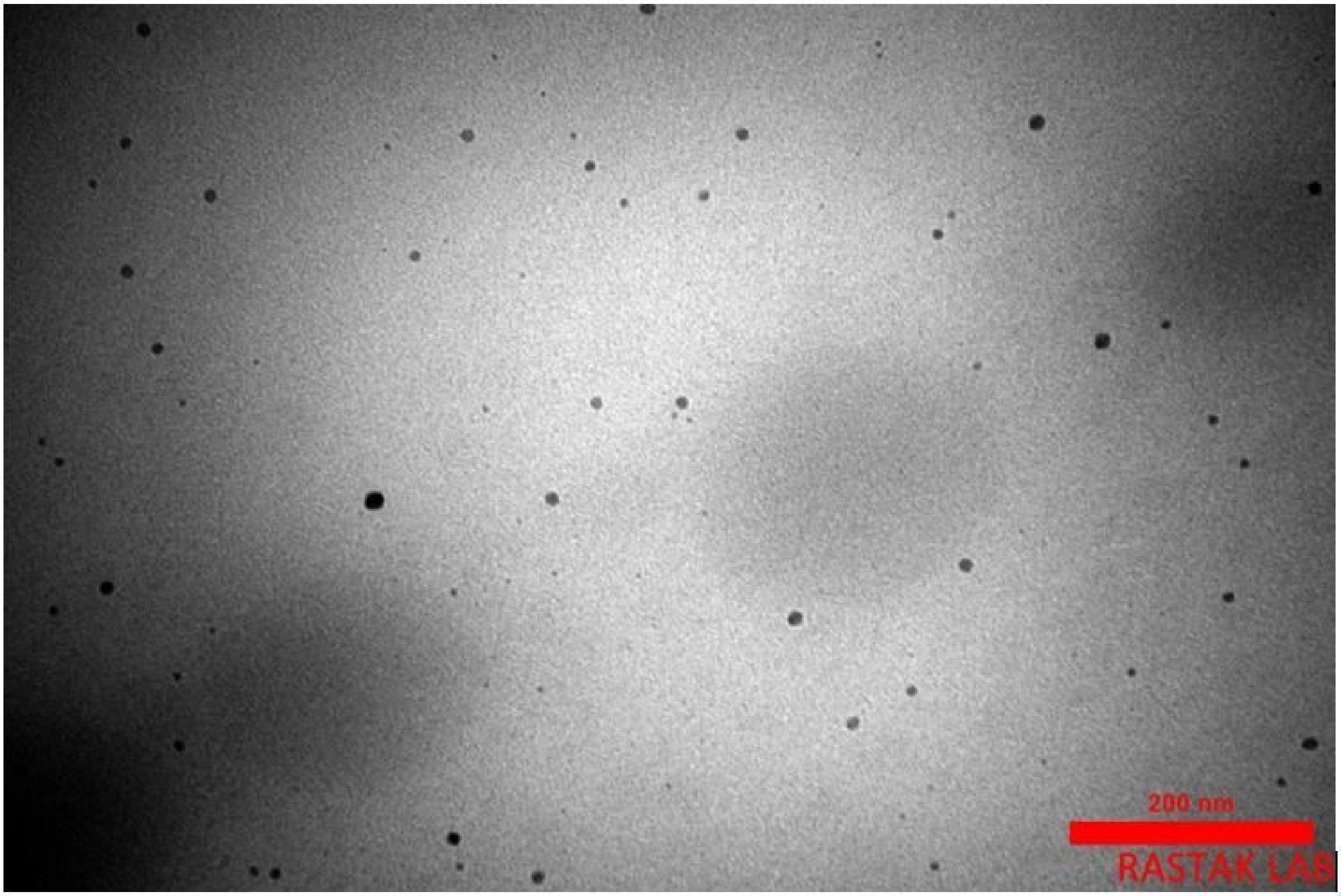

Figure 3

Morphology of nanoparticles of formulation C results from Transmission Electron Microscopy