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Essential oil-in-water microemulsions for topical application: structural study, cytotoxic effect and insect repelling activity

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Abstract

Essential oils (EOs) extracted from aromatic plants have been well recognized for their insect repellent properties. Although EOs are effective when freshly applied, their protective effect usually dissipates quickly due to low chemical stability and rapid volatilization of their ingredients. To address this issue, encapsulation in protective carrier materials at the nanoscale is proposed.

In this study, oil-in-water (O/W) microemulsions containing geraniol or Citrepel® 75 (citrepel) as the oil phase, a mixture of water and diethylene glycol monoethyl ether (DEGEE) as the aqueous phase and caprylocaproyl polyoxyl-8 glycerides as surfactant were formulated and structurally characterized. DLS analysis showed that the emulsified oil droplets had low polydispersity ($PdI < 0.3$) and average diameter ranging from 13.7 ± 0.7 to 35.2 ± 2.7 nm, depending on composition. Mean viscosity of all prepared microemulsions was from 15.7 ± 0.3 cP to 23.8 ± 0.2 cP indicating the suitability of all preparations for topical application. EPR spectroscopy using spin probes gave information about the rigidity of the surfactants' monolayer. In all cases an outer compact configuration of the interfacial layer and a more flexible inner one was observed. SAXS measurements revealed significant changes in the low q values related to the impact of the essential oils in microemulsion formulations. Looking at the high q region of the spectra, the curves were affected by the presence of the EOs in a concentration depended manner.

Cytotoxicity studies on normal human epidermal keratinocytes (NHKs) confirmed that the sub-toxic dosage for geraniol-in-water and citrepel-in-water microemulsions is $56.25 \mu\text{g}$ active ingredient per cm^2 of cell monolayer. Finally, diluted geraniol-in-water microemulsions were evaluated for repellence action against the Asian tiger mosquito (*Aedes albopictus*) showing activity for 3 hours at a dose of $190 \mu\text{g}/\text{cm}^2$ active ingredient.

Keywords

Microemulsions; geraniol; SAXS; EPR; repellent; mosquitoes

1. Introduction

Essential oils are complex volatile natural mixtures of compounds formed by aromatic plants as secondary metabolites. Generally, essential oils contain low molecular weight organic compounds at different concentrations. According to their chemical structure, the main components found in essential oils are terpenes, terpenoids and phenylpropenes. This rich chemical composition of essential oils confers a variety of biological activities including antioxidant, anti-inflammatory, antimicrobial, antiviral, insect repellent and insecticidal activities. As a result, essential oils are of great interest in food and cosmetic industries, human health, and sustainable agriculture [1,2]. However, direct application of essential oils results in a rapid volatilization of their ingredients, thus, reducing their effectiveness. To address this issue, nanoscale encapsulation has emerged as an alternative that

could provide protection, and controlled release that results in prolonged action. The most frequently used carriers are polymeric particles, liposomes, and solid lipid nanoparticles [3]. Current trends focusing on emulsion-based delivery systems have unlocked the potential of colloidal liquid-in-liquid nanodispersions, such as micro- and nanoemulsions as effective carriers of essential oils [4-8].

Microemulsions are isotropic, thermodynamically stable, liquid mixtures of oil, water, and surfactant with dispersed domain diameter varying approximately from 10 to 50 nm. They are structurally classified into three main categories: oil-in-water (O/W), water-in-oil (W/O) and bicontinuous microemulsions characterized by equal volume fractions of water and oil. They exhibit low viscosity and low interfacial tension combined with a high interfacial area between the immiscible liquids. Microemulsions are potentially excellent carriers of bioactive compounds offering the advantage of spontaneous formation, thermodynamic stability, optical clarity, ease of manufacture and scale-up. In parallel, microemulsions demonstrate the ability to carry both lipophilic, as well as hydrophilic compounds [9-12].

The aim of this work was to formulate, study and characterize natural products-based microemulsions for topical application. Therefore, geraniol was selected as the model oil phase, while microemulsion with citrepeel served as control.

Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is a naturally occurring monoterpenic alcohol found in essential oils extracted from aromatic plants, such as rose, lavender, and lemongrass. Researchers have shown geraniol to be an effective plant-based insect repellent also exhibiting many pharmacological activities including antifungal, antibacterial, anticancer and neuroprotective properties [13,14].

Citrepeel®75 is a pale-yellow viscous liquid produced by aging of *Eucalyptus citriodora* oil. It contains around 75% p-menthane-3,8-diol (PMD), a highly volatile organic compound classified as a diol and a terpenoid which is a well-known repellent on human skin and clothing and has been registered as a biocide in European Union (Biocidal Products Regulation 528/2012). PMD has been found to be effective against mosquitoes, midges, ticks and flies [15,16]. Both geraniol and PMD are slightly soluble in water, lipophilic compounds. In Figure 1, the chemical structure of geraniol and p-menthane-3,8-diol is shown.

To formulate the microemulsions, labrasol® (caprylocaproyl polyoxyl- 8 glycerides), a self-emulsifying, water dispersible, non-ionic surfactant was used. Emulsions stabilized by non-ionic surfactants are preferably used in the agricultural industry as delivery vehicles for insecticides, fungicides and pesticides, due to their low foaming properties and lack of interaction with their loaded

active compounds [17]. Labrasol® is widely used in topical formulations due to its solubilizing power and has the ability to increase membrane lipid fluidity resulting in enhanced membrane permeability of various drugs [18]. To enhance solubilisation of the essential oils in the surfactant/water mixture, diethylene glycol monoethyl ether (DEGEE), a very good solvent for many compounds that are poorly soluble in either water or oil was added [19].

In this study, geraniol O/W microemulsions were formulated and structurally characterized using a wide range of methods such as viscometry, dynamic light scattering (DLS), electron paramagnetic resonance (EPR) spectroscopy, and small-angle X-ray scattering (SAXS). The effect of composition on physical properties like droplet shape and size, polydispersity index, stability, membrane dynamics and viscosity were determined for all microemulsions. To disclose the potential use of the formulated microemulsions based on geraniol for topical application as an insect repellent, their cytotoxicity over mammalian cells, including normal human epidermal keratinocytes, was evaluated. The natural-product-in-water microemulsions based on citrepele were also produced and served as controls both for structural characterization and toxicity evaluation studies. Finally, a diluted geraniol-in-water microemulsion was evaluated through *in vivo* repellence tests against the invasive mosquito species *Aedes albopictus*, also known as Asian tiger mosquito.

2. Experimental

2.1. Materials

Caprylocaproyl polyoxyl- 8 glycerides (labrasol®) HLB 12 was kindly donated by Gattefossé, France. Geraniol, 98%, diethylene glycol monoethyl ether (DEGEE), 99%, and 16-DOXYL stearic acid (16-DSA) were from Sigma-Aldrich (Chemie GmbH Munich, Germany). 5-DOXYL stearic acid (5-DSA), ammonium salt, >99% purity, was from Avanti Polar Lipids (Alabama, USA). Citrepele®75 (containing 75% *p*-menthane-3,8-diol) was kindly donated from Chemian Technology Limited, UK. Deionized water was used throughout the study.

2.2. Formulation of microemulsions

For the formulation of O/W microemulsions, water and DEGEE, at 1:1 weight ratio, were used as the aqueous phase, labrasol® as the emulsifier and essential oils as the oil phase. All microemulsions were prepared by mixing the aqueous phase and the emulsifier and titrating with either geraniol or citrepele under gentle stirring. The temperature was kept at 25° C. The final composition of the microemulsions is summarized in Table 1.

Diluted O/W microemulsions containing 2% w/w geraniol, 10% w/w labrasol[®] and 88% w/w aqueous phase were also prepared and used for in vivo repellence tests.

Table 1. Composition of the microemulsions.

Ingredients (% w/w)	Formulation code					
	MG1	MG2	MG3	MC1	MC2	MC3
Water	27.3	25.0	23.1	27.3	25.0	23.1
DEGEE	27.3	25.0	23.1	27.3	25.0	23.1
Labrasol [®]	36.4	33.3	30.7	36.4	33.3	30.7
Geraniol	9.0	16.7	23.1	-	-	-
Citrepel®75	-	-	-	9.0	16.7	23.1

2.3. Viscosity measurements

Viscosity measurements of the microemulsions were performed with a Brookfield DV-I Prime Digital Viscometer (Brookfield Engineering Laboratories, Middleboro, MA, USA) at given shear rate. The viscometer was equipped with cone spindle CPA-40Z (Brookfield Engineering Laboratories, Middleboro, MA, USA). The temperature was kept constant at 25°C using a water bath. Experimental results in units of centipoise (cP) are presented as mean value \pm standard deviation (S.D.). All measurements were performed in triplicate.

2.4. Dynamic Light Scattering (DLS) measurements

Droplet size and size distribution of the microemulsions were determined using a Zetasizer Nano ZS (ZEN3600) analyser (Malvern Instruments Ltd., Malvern, UK) equipped with a He-Ne laser (633 nm) and Non-Invasive Backscatter (NIBS) optics. Before each measurement, samples were filtered through 0.45 μ m hydrophilic PTFE filters (Millex-LCR from Millipore Merck KGaA, Darmstadt, Germany) and contained in dust free quartz cuvettes. Measurements were carried out in triplicate at 25 °C. The calculations were handled by Malvern Zetasizer Nano software, version 6.32

(Malvern Instruments Ltd., Malvern, UK) which fits a spherical model of diffusing particles with low polydispersity. Results are expressed as mean value \pm standard deviation (SD).

2.5. Electron paramagnetic resonance (EPR) spectroscopy measurements

Interfacial properties of the O/W microemulsions were investigated applying EPR spin-probing spectroscopy. EPR is a spectroscopic technique that measures the absorption of microwaves by paramagnetic centres with one or more unpaired electrons. In this study, 5-doxyl stearic acid, ammonium salt and 16-doxyl stearic acid, two fatty acid derivatives labelled at different positions of the aliphatic chain (C5 and C16), were used to probe dynamics of the surfactant's monolayer at different depths.

EPR measurements were performed with an EMX EPR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at the X-Band (9.8 GHz). Samples were contained in a quartz flat cell (Wilma-LabGlass, Cortecnet Europe, Voisins-Le-Bretonneux, France). Instrument settings were center field 0.348 T, scan range 0.01 T, receiver gain 5.64×10^4 , time constant 10.24 ms, conversion time 5 ms, modulation amplitude 0.4 mT. Sample preparation was as follows: Initially, stock solutions (10^{-2} M) of the spin probes in ethanol were prepared. Then, appropriate amounts of the stock solutions were inserted in eppendorf tubes. After ethanol evaporation, 1 ml of each microemulsion was added to obtain a final concentration of 10^{-4} M.

The Bruker WinEPR acquisition and processing program (Bruker BioSpin GmbH, Germany) was used for data collection and analysis. EPR spectra were analysed in terms of rotational correlation time (τ_R) and order parameter (S).

2.7. Small Angle X-ray Scattering (SAXS) measurements

Small angle X-ray Scattering (SAXS) experiments, for microemulsions in the presence and absence of the two essential oils (geraniol and citreol), were carried out on a XEUS 2.0 device (XENOCSS, Grenoble, France). Coupled to a FOX 3D single reflection optical mirror centered on the Cu K α radiation ($\lambda = 1.54 \text{ \AA}$), the GeniX 3D source delivers a beam of 8 keV, which is collimated and defined by a set of 2 motorized scatterless slits. The samples were put in thin glass capillaries (optical path 2mm) and exposed for 3 hours. The data were collected by a two-dimensional PILATUS-300k detector (DECTRIS, Switzerland) placed perpendicularly to the direct beam at a distance of 1634 mm, calibrated with a Silver behenate standard. Rectangular images with shape (619, 487) are obtained, giving access to a range of scattering wave vectors q from typically 0.007 \AA^{-1} to 0.24 \AA^{-1} as the vertically off-centered direct beam (actually blocked by a beam catcher

shadowing a circular region of radius about 14 pixels) would impact close to the lower limit of the detector.

2.8. Cultivation of cell lines and cytotoxicity assay

Normal human epidermal keratinocyte cell lines (DSMZ, Braunschweig, Germany) were cultured in keratinocyte SF medium supplemented with 0.2 ng/ml hEGF and 15 µg/ml pituitary extract (Gibco). Cytotoxicity was assayed, as described previously, by the neutral red uptake (NRU) assay [20]. Briefly, the cells were seeded into 96-well plates and allowed to adhere for 24 hours at 37°C. Subsequently the medium was replaced by fresh medium containing the respective microemulsions for dilutions ranging from 0-100% (v/v). The cells were incubated for 1 h with the test microemulsions, before neutral red (Sigma Aldrich) application (final concentration of 0.04 µg/mL) and incubated for a further 3 hours. Subsequently the medium was discarded, and the neutral red was extracted by living cells using destaining solution containing 50% ethanol and 1% acetic acid. Finally, absorbance at 540 nm was measured with an absorbance microplate reader (Infinite 50-Tecan, Switzerland).

2.9. Rearing of *Aedes albopictus* in the laboratory

Mosquito adults were obtained from a laboratory colony of *Ae. albopictus* which was maintained in the Benaki Phytopathological Institute (Kifissia, Attiki, Greece) at 25±2°C, 80% relative humidity, and photoperiod of 16:8-h light/dark (L/D) [5,21]. Females were fed with fresh bovine blood using the Hemotek® blood-feeding (Hemotek, UK) system.

2.10. Repellent activity bioassay

For the repellent activity of geraniol-in-water microemulsion the assessment was based on the human landing counts. A plastic glove with an opening measuring of 5×5 cm was employed for all the bioassays [21]. The study conducted into a cage (33×33×33 cm) with a 32×32 mesh and each cage contained 100 adult mosquitoes (sex ratio, 1:1), 5- to 10-day old, starved for 12 hours at 25±2 °C, and 70-80 % relative humidity. Control treatments included geraniol, citrepiel and empty microemulsion (without geraniol) were also included in the repellency tests as standards. The diluted microemulsion with 2% w/w geraniol was also evaluated in terms of duration of action (up to 3 hours). All the tested materials were applied at 190 µg/cm² (0.2 µl/cm²) active ingredient and each treatment was repeated eight times and four human volunteers were used.

2.11. Determination of repellency index

The landing numbers were converted to repellency index (RI±SE) using the following equation

$$RI = 1 - (T / C) = (C - T) / C,$$

where C is the number of landings in the control and T the number of landings in the compound treatment.

2.12. Statistical analysis

The effects of tested compounds on the *Ae. albopictus* female landings were estimated using the Kruskal–Wallis test. When significant differences were detected, Mann–Whitney U tests with Bonferroni correction were carried out for pair-wise among treatment [22,23].

2. Results and discussion

3.1. Formulation of microemulsions

A series of O/W microemulsions containing oil phase from 9.0 to 23.1 % w/w were produced with the phase titration method. The oil phase consisted either of geraniol or citrepele. The aqueous phase was a 1:1 mixture of water and DEGEE. According to preliminary studies (data not shown), DEGEE concentrations less than 50% of the aqueous phases were not applicable when encapsulation of such high concentration of essential oil oils in the microemulsions was required. To emulsify the two immiscible liquid phases, labrasol® was used at a 2:3 weight ratio to the aqueous phase (Table 1). Labrasol® is a non-ionic, water dispersible surfactant composed of well-characterized polyethylene glycol (PEG) esters, a small glyceride fraction and free PEG. All microemulsions were thermodynamically stable and once formed, remained optically clear without any precipitate or phase separation. In a recent study by Grice and coworkers, the development of labrasol® and DEGEE containing O/W microemulsions as effective delivery vehicles for enhanced targeted skin delivery of antitumor agents has been reported [24]. In an older study, O/W microemulsions stabilized by non-ionic surfactants (labrasol®, Tween 20, Span 20) and DEGEE as co surfactant, were formulated for dermal delivery of silymarin, a plant extract known for its skin benefits [25]. In the present study, 4 component O/W microemulsions containing water, labrasol®, DEGEE and essential oil, were formulated by a low-energy emulsification procedure in contrast to other essential oil-based microemulsions where high-energy techniques were required [26]. Follow-

ing, all essential-oil-in-water microemulsions were characterized using analytical techniques based on viscometry, scattering and spectroscopy to elucidate their structural details in relation to their composition.

3.2. Viscosity

Viscosity is an important factor in controlling the flow of microemulsions fabricated to serve as carriers of bioactive compounds. In general, emulsion viscosity depends on the initial viscosity of the continuous phase, the viscosity of the dispersed phase, the ratio of continuous to dispersed phase, the size of the droplets and also the type and concentration of surfactants. This macroscopically observable parameter is important for a variety of applications of microemulsions [9].

In the present study, mean viscosity of all prepared microemulsions was from 15.7 ± 0.3 cP to 23.8 ± 0.2 cP (Table 2). This finding is consistent with one of the basic characteristics of microemulsions, which is low viscosity. The highest viscosity belongs to a microemulsion consisted of 1:1 water/DEGEE, labrasol® 30.7% w/w and citrepele 23.1 % w/w (MC3) and the lowest (15.7 ± 0.3 cP) belongs to the same system containing geraniol instead of citrepele (MG3). Since both systems have the same composition except for the nature of the oil, we conclude that the observed difference in viscosities is due in part to the different viscosity of the oils, namely 56.1 cP for citrepele and 8.2 cP for geraniol. The same observation was also made when MC1, MG1 and MC2, MG2 microemulsions were compared (Table 2).

In summary, essential oil-in-water microemulsions were prepared that are more viscous than water or DEGEE and far less viscous than vegetable oils. Based on these viscosity properties, all microemulsions are suitable for several ways of topical application, including spraying or spreading.

3.3. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is one of the most applied techniques to study structural properties of liquid colloid systems. In the present study DLS measurements were carried out to evaluate the size and size distribution of the dispersed oil droplets in the essential oil-in-water microemulsions.

Table 2 shows the mean droplet diameter and the polydispersity index of the microemulsions (MG1-MC3). As can be observed, both geraniol (MG1, MG2, MG3) and citrepele (MC1, MC2, MC3) containing microemulsions showed similar droplet sizes and polydispersity when all other

components were the same and in the same proportions. In other words, droplet size and PDI of MG1 and MC1, MG2 and MC2, MG3 and MC3 were not considerably varied. On the other hand, mean droplet diameter was increased in all cases as the percentage of essential oil was increased from 9.0% w/w to 23.1% w/w and the surfactant content was decreased from 36.4% w/w to 30.7% w/w, indicating the formulation of larger oil droplets. Interestingly, the polydispersity was decreased from 0.26 ± 0.02 to 0.16 ± 0.05 (geraniol-in-water microemulsions) and from 0.27 ± 0.01 to 0.11 ± 0.01 (citrepel-in-water microemulsions) when the oil content was increased.

In conclusion, monodispersed ($PDI < 0.3$) oil droplets of low hydrodynamic diameter ($D < 40$ nm) were obtained using geraniol, citrepel and components generally regarded as safe for human use. As well established in the literature, particle size and particle size distribution are important indicators for the stability, uniformity and dispensibility of microemulsions especially when applied for the solubilization and protection of bioactive compounds [5,27,28]. In this sense, all prepared formulations can be regarded as appropriate carriers of geraniol and citrepel.

Table 2. Mean droplet diameter, PDI and viscosity of the O/W microemulsions measured immediately after preparation.

Formulation	Diameter (nm)	PDI	Viscosity (cP)
MG1	13.7 ± 0.7	0.26 ± 0.02	17.8 ± 0.6
MG2	19.9 ± 0.0	0.21 ± 0.04	16.5 ± 0.3
MG3	27.8 ± 4.8	0.16 ± 0.05	15.7 ± 0.3
MC1	15.3 ± 0.3	0.27 ± 0.01	20.1 ± 0.8
MC2	20.3 ± 0.4	0.20 ± 0.01	22.2 ± 0.5
MC3	35.2 ± 2.7	0.11 ± 0.01	23.8 ± 0.2

3.4. Electron paramagnetic resonance (EPR) spectroscopy

The degree of flexibility and local viscosity of the surfactant film in the essential oil-in-water microemulsions was investigated using EPR and two spin probes, namely 5-DSA ammonium salt and 16-DSA. The paramagnetic doxyl moiety of the nitroxide spin probes was covalently attached to the 5th and 16th carbons of the fatty acid aliphatic chain, respectively. The doxyl group of 5-DSA is localized in the part of the surfactant monolayer that is closer to the polar head groups and consequently closer to the continuous aqueous phase. In 16-DSA, the doxyl group is anchored in a deeper part of the same monolayer closer to the dispersed oil. As a result, these interface-located spin

probes give EPR spectra reflecting the influence of the various constituents on the fluidity and structure of the membrane [4].

Figures 2a and 2b show experimental EPR spectra of 5-DSA ammonium salt and 16-DSA in essential oil-in-water microemulsions containing 9.0% w/w geraniol (MG1) or citrepele (MC1). Both spectra were characterized by the presence of three lines of unequal heights and widths. The two doxyl stearate spin probes differ in the attachment of the nitroxide free radical to the fatty acid and consequently their location among the oriented surfactant molecules is not the same. The observed EPR spectra anisotropy reflects changes in the mobility and order of the surfactants' monolayer. In other words, the width and intensity of spectral lines depend on interactions near molecules containing an unpaired electron (spin probes) and therefore their analysis can provide data on molecular motion and organization near them.

Analysis of EPR spectra in terms of rotational correlation time (τ_R) and order parameter (S) are presented in Table 3. The detailed equations and the physical meaning of those parameters have been reported in previous studies [28,29]. Increased τ_R and S values are related to increased local viscosity and membrane rigidity, respectively. When geraniol-in-water microemulsions (MG1, MG2, MG3) were studied by EPR using 5- and 16-DSA as spin probes, higher values for both τ_R and S were obtained in the case of 5-DSA as compared to the corresponding values of 16-DSA. This finding indicates that the 5-DSA salt is located in a region, where the surfactant molecules of the monolayer are organized in a tighter way, in contrast to the more flexible environment near the oil phase. The same behavior regarding local viscosity and flexibility was observed when citrepele-in-water microemulsions (MC1, MC2, MC3) were studied with the same spin probes.

Furthermore, by comparing rotational correlation times (τ_R) of the two microemulsions, it must be underlined that the outer part of the surfactant monolayer is more viscous in the case of citrepele-in-water microemulsions than in the corresponding geraniol based microemulsions. Regarding flexibility as expressed by order parameter (S), citrepele-in-water microemulsions seem to have a less flexible outer part as compared to the ones containing geraniol in the dispersed oil droplets. However, this was not encountered in the case of 16-DSA indicating that the inner part of the monolayer and close to the oil phase is equally flexible. Similar results were obtained in an older study where O/W microemulsions based on water as the continuous phase, limonene as the dispersed phase and a mixture of pharmaceutically acceptable surfactants were developed as carriers of ibuprofen. EPR spectroscopy applying the same spin probes revealed that the outer part of the surfactants' monolayer was more compact in correlation to the inner part. This finding was related to the ability of the nanocarriers to effectively release the drug [28].

Table 3. Rotational correlation time (τ_R) and order parameter (S) of 5-DOXYL stearic acid ammonium salt (5-DSA) and 16-DOXYL stearic acid (16-DSA) in essential oil-in-water microemulsions.

Formula- tion	5-DSA		16-DSA	
	τ_R (ns)	S	τ_R (ns)	S
MG1	1.49±0.05	0.06±0.00	0.34±0.03	0.03±0.01
MG2	1.29±0.03	0.06±0.00	0.73±0.43	0.06±0.03
MG3	1.60±0.27	0.10±0.04	0.49±0.33	0.04±0.01
MC1	1.93±0.02	0.10±0.01	0.44±0.02	0.03±0.00
MC2	2.09±0.04	0.12±0.01	0.57±0.15	0.03±0.01
MC3	2.0±0.06	0.13±0.02	0.76±0.32	0.04±0.02

3.5. Small Angle X-ray Scattering (SAXS)

SAXS is a fundamental analytical and non-destructive technique providing structural and morphological information of solids, gels and liquid dispersions [30]. The regime covered by SAXS typically covers the range from 0.5 to 100 nm, which turns out to be a very useful range for the examination of nanomaterials [31,32]. In the present study, SAXS experiments were carried out to clarify possible alterations provoked in the structure of the microemulsions after the incorporation of each essential oil in the dispersed phase

Figures 3 and 4 show the (background-subtracted) intensity profile of the microemulsions in the absence and presence of citrepele and geraniol, respectively. The addition of the essential oils was investigated aiming to detect whether the gradual addition of each oil could affect the structure of the final microemulsion, and secondly whether there could be differences between the two types of essential oils. For the comparison of the microemulsions containing 23.1% w/w essential oil (MG3, MC3), apart from intensity variations, the two systems (\blacktriangle red, \blacktriangledown red), have apparently the same effect on the structure of the system as revealed by the SAXS spectra. The two systems were compared with the mixture without essential oil (\bullet black) and it can be observed that with the addition of essential oil an alteration in the characteristic size between the empty and essential oil loaded system occurred. These results complete the results obtained from DLS and EPR, where differences upon the addition of the essential oils were observed both on the size and rigidity of the micellar

membrane. Looking at the high q region of the spectra, the curves appear to be barely affected by the increase in the concentration of essential oils (geraniol or citrepe). At smaller scattering wave vector q , a clear effect is an enhancement of the "microemulsion peak" (becoming both sharper and more intense) upon the increase of each essential oil. Moving towards smaller angles, this latter effect was associated with an increase in the structural size. This result indicates that the observed alteration in the low q values is significant and related to the impact of the essential oils on the formulation of the microemulsion. Other authors have reported similar findings where the addition of lapachol [33] or *Cinnamomum zeylanicum* essential oil [34] promotes the increase of droplet size since a shift toward lower q -values is observed.

3.6. Cytotoxic activity to normal human epidermal keratinocytes

The normal human epidermal keratinocyte cell lines (NHKs) were exposed to geraniol-in-water (MG1, MG2, MG3) and citrepe-in-water (MC1, MC2, MC3) microemulsions at concentrations ranging from 0 to 100% (v/v) and their cytotoxic effect was determined using the NRU uptake assay [20].

Cells were susceptible to 100% (v/v) MG1, MG2, MG3 and MC1, MC2, MC3, corresponding to a dose of 56.25 mg/cm^2 active ingredient as shown in Figure 5A. Both MG1, MG2, MG3 and MC1, MC2, MC3 microemulsions exerted an equally toxic effect and they reduced cell viability below 60% compared to untreated cells at concentrations ranging from 0.0625 to 10% (v/v), yet cell viability was statistically significantly increased compared to undiluted microemulsions (Figure 5B). Further dilution of MG1 and MC1 (1/1000 v/v) resulted in no cytotoxic effect to NHKs (Figure 5C). Therefore, the safety of the MG1 and MC1 can be advocated for the following concentrations: 0.03% v/v DEGEE, 0.04% v/v labrasol®, 0.009% v/v geraniol for MG1 and 0.03% v/v DEGEE, 0.04% v/v labrasol®, 0.009% v/v citrepe for MC1 corresponding to $56.25 \text{ } \mu\text{g/cm}^2$ active ingredient.

3.7. Repellent activity bioassay

Geraniol and the diluted geraniol-in-water microemulsion were evaluated through *in vivo* repellence tests. Initially, the empty microemulsion has been examined to verify the absence of repellent action. Results revealed that the tested geraniol-in-water microemulsion had similar repellency index compared with geraniol and citrepe ($X^2=30,175$, $df=3$, $P<0.0001$, Figure 6) and its activity can last up to 3 hours at a dose of $190 \text{ } \mu\text{g/cm}^2$ ($X^2=30,184$, $df=3$, $P<0.0001$, Figure 7).

To summarize, in this work we proposed encapsulation of geraniol in micro-emulsions as a mean of decrease the dose to sub-toxic levels and achieve a satisfactory duration of protection. Geraniol is widely used as a fragrance in commercial cosmetic and household products [35]. It is also used as a biocide and as an ingredient of spatial insect repellents in diffusers and candles [36,37]. However, its use as a topical repellent on human skin is restricted because of its known allergenic and irritation potential in humans [38,39]. According to Notification of the 49th Amendment to the IFRA Code of Practice (IFRA49), geraniol concentration is restricted to 1.2% in leave-on body lotion products. Furthermore, geraniol similar to other plant repellents displays very short repellent duration due to its high volatility. Increase of geraniol concentration can prolong its action but can induce undesirable toxicity and allergic reactions. The repellency studies of geraniol against mosquitoes are limited. A study by Bernad and Xue showed that 25% geraniol applied topically repels mosquitoes for 3.2 to 4.8 h [40]. However, use of sub-toxic/sub-allergic geraniol dosages e.g. 2%, was found to be ineffective as topical repellents in animals [41].

In the present study, a microemulsion of 2% w/w geraniol was evaluated for repellency action against Asian tiger mosquito and the results revealed a protection time up to 3 hours, similar to that of 25% free geraniol reported by Barnard & Xue, [40]. Furthermore we showed that the prepared microemulsions of geraniol displays the same safety profile as the registered repellent citrepeel and it lacks toxicity at a dose of $56.25 \mu\text{g}/\text{cm}^2$ geraniol per cm^2 of NHK cell monolayer. Given that the concentrations ratio at which toxicity is observed is significantly lower in 2D cultures compared with the 3D cultures, and consequently to human skin [42,43] our formulation could be used for the development of efficient and safe plant-based insect repellents products for topical use.

Conclusions

In the present study, oil-in-water microemulsions based on geraniol or citrepeel as the dispersed oil phase, caprylocaproyl polyoxyl- 8 glycerides as surfactant, diethylene glycol monoethyl ether as co solvent and water as aqueous continuous phase were formulated and structurally characterized by means of viscometry, DLS, EPR and SAXS. In all cases, formulations of low viscosity containing monodispersed oil droplets of low hydrodynamic diameter were obtained indicating their suitability as delivery systems of essential oils. EPR spin probing spectroscopy gave information about the rigidity of the surfactants' monolayer. In all cases an outer compact configuration and a more flexible inner one was observed. SAXS results complete the results from the DLS and EPR techniques, where it was found that differences upon the addition of the essential oils were observed

both on the size and rigidity of the micellar membrane. Cytotoxicity studies of both geraniol- and citrepelel-in-water microemulsions showed a broad cytotoxic effect when directly administrated on a monolayer culture of cells *in vitro*. However, their dilution increased cell viability by more than 40%. Importantly, this study confirms that MG1 and MC1 microemulsions are safe for use at a dose of 56.25 $\mu\text{g}/\text{cm}^2$ geraniol or citrepelel as active ingredients for MG1 and MC1, respectively. Finally, a diluted microemulsion of 2% w/w geraniol was evaluated for repellency action against Asian tiger mosquito and the results revealed a protection time up to 3 hours.

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Figures Caption

Figure 1. Chemical structures of (a) geraniol and (b) p-menthane-3,8-diol.

Figure 2. EPR spectra of 5-DSA ammonium salt and 16-DSA in MG1 (a) and MC1 (b) microemulsions.

Figure 3. Raw data of SAXS spectra for empty microemulsion (black ●) and all the loaded systems with the essential oils: 9.0% citrepele (▼ blue), 16.7% citrepele (▼ green), 23.1% citrepele (▼ red), 9.0% geraniol (▲ blue), 16.7% geraniol (▲ green), 23.1% geraniol (▲ red).

Figure 4. Background-subtracted data of SAXS spectra for empty microemulsion (black ●) and all the loaded systems with the essential oils: 9.0% citrepele (▼ blue), 16.7% citrepele (▼ green), 23.1% citrepele (▼ red), 9.0% geraniol (Δ blue), 16.7% geraniol (Δ green), 23.1% geraniol (Δ red).

Figure 5. Cytotoxicity of geraniol (MG1, MG2, MG3) and citrepele (MC1, MC2, MC3) containing microemulsions to normal human epidermal keratinocyte cell lines (NHKs) is determined by the NRU uptake assay. A, Cytotoxicity effect of undiluted MG1, MG2, MG3 and MC1, MC2, MC3 to NHKs upon treatment for 1 hour, tested concentration of microemulsions: 100% (v/v) B, Effect of MG1, MG2, MG3 and MC1, MC2, MC3 microemulsions on NHKs viability, tested concentration of microemulsions: 0.0625-10% (v/v) C, Effect of MC1 and MG1 on NHKs at a ratio of 1/1000 in KSF medium (v/v). Data are expressed as % viability of untreated cells and are presented as median ± SEM from two independent experiments. All reactions were set up in triplicates in each experiment.

Figure 6. Repellent activities of tested materials. Box plots depicting landings distributions of *Ae. albopictus* mosquitoes after exposure to a dose of 190 µg/cm² (0.2 µl/cm²) active ingredient of tested material for five minutes. Different letters, a-b, indicate significant differences among treatments ($P < 0.05$, Mann–Whitney U test with Bonferroni correction). Geraniol, citrepele, and empty microemulsions were served as controls.

Figure 7. Repellent activities of 2% w/w geraniol-in-water microemulsion (geraniol microemulsion). Box plots depicting landings distributions of *Ae. albopictus* mosquitoes after exposure to a dose of 190 µg/cm² (0.2 µl/cm²) active ingredient of tested material for five minutes. Different letters, a-b, indicate significant differences among treatments ($P < 0.05$, Mann-Whitney U test with Bonferroni correction). The geraniol-in-water microemulsion (geraniol microemulsion) was evaluated immediately (geraniol microemulsion, 0 hours), then 1, 2 and 3 hours after the first application (geraniol microemulsion 0, 1, 2, and 3 hours, respectively).