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Article

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Development of an environmentally friendly larvicidal formulation based on essential oil compounds blend to control *Aedes aegypti* larvae: Correlations between physico-chemical properties and insecticidal activity

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Abstract:

Oil in water (o/w) emulsions stabilized by an amphiphilic copolymer have been studied in relation to their potential insecticidal activity against *Aedes aegypti* mosquito larvae. These emulsions contain as oil phase different blends of two isomeric essential oil compounds, thymol and carvacrol. The results show that the addition of carvacrol facilitates the dispersion of the oil within the aqueous phase, with the stabilization and polydispersity of the emulsions being controlled by the change of the ratio between the copolymer concentration and that of the oil phase ($R_{cop/EOC}$). Emulsions containing pure essential oil compounds as oil phase do not present any significant difference on their larvicidal activity against mosquito larvae, with emulsions containing only thymol being slightly more effective than those containing only carvacrol as oil phase. Furthermore, the use of blends containing different weight fractions of thymol and carvacrol as oil phase results in formulations with an additive larvicidal activity in relation to those with the pure compounds. Despite the larvicidal activity of the emulsions, they do not provoke inhibition to the emergence of adult individuals in *Aedes aegypti* populations. The spreading and evaporation of the emulsions onto solid surface, which may be an important parameter for the performance of larvicidal formulations, was found to be dependent on the same parameters that govern the stability of the emulsions. This study helps on seeking new alternatives for the preparation of new eco-sustainable formulations against insect pest.

Keywords: emulsions; essential oils; insect pest control; isomers, thymol; carvacrol; larvicidal

Introduction

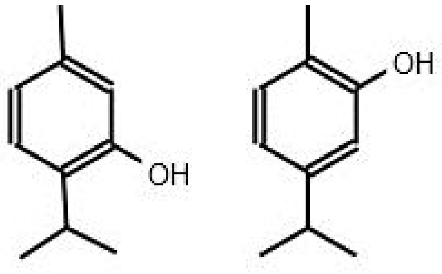
The biological properties of essential oils (EOs),[1-4] and their classification as GRAS (Generally Recognized As Safe) products with a reduced impact on environment and health,[5] have led to an increasing interest for their potential use in several fields, from cosmetics to food science and from pharmaceuticals to agriculture.[6-9] However, the inclusion of EOs in consumer products remain rather limited yet due to their volatility, thermal lability, poor water solubility, and stability, which are important drawbacks for the manipulation and handling of these compounds [10, 11]. Therefore, it is necessary to design appropriate strategies for taking advantage of the EOs biological activity on the manufacturing of new eco-sustainable products [12-14].

Seeking new strategies for the manipulation and use of essential oils in commercial formulations, the use of encapsulation procedures is a promising alternative to reduce the volatility of the EOs, improve their dispersion in water and enhance their availability [6, 15-22]. This can be easily done by using oil-in-water (o/w) emulsions, that is a widely extended method for the design of formulations in many industrial processes [23-25]. This route enabling an easier handling of essential oils can be exploited on the preparation of new formulations of botanical insecticides with low impact for environment and non-target organisms.[26-29]

Different studies have shown that the inclusion of EOs within the oil phase of o/w emulsions (alone or combined with an organic solvent) presents a significant impact against different insect pests. A dose dependent larvicidal activity against *Aedes aegypti* larvae was reported for nanoemulsions of *Rosmarinus officinalis L.* and *Ocimum basilicum L.* oils [30, 31], with the susceptibility of the larvae being stronger than essential oil incorporated within the oil phase the emulsion than the free form [32]. Similar results were found upon the exposure of *Culex quinquefasciatus* to eucalyptol, with the time required to obtain a 100% of mortality being reduced 6-fold when insects are exposed to emulsions in relation to the exposure to the same dose of the free essential oil [33]. Furthermore, the size of the oil droplets was found to be a critical parameter on the modulation of the bio-toxicity of the formulations, the smaller the droplets the higher the mortality is [34]. Similar impact of the droplet size on the biological activity of different nanoemulsions against *Rhopalosiphum padi* has been also reported recently by Pascual-Villalobos et al.[35]

It is worth mentioning that most of the studies related to emulsions containing EOs for insect pest control explore the impact of the whole EO on the insecticidal activity of the formulations [36, 37], and scarce data are available about the utility of individual essential oil compounds or their mixtures against insect pest. It is know that the combination of several EO compounds may result in an enhanced bio-toxicity of the formulations against specific insect pest, even though the mechanisms of this synergism are not clear yet [38-40]. Similar results have been found by adding a second essential oil compound (thymol) to o/w emulsions containing eugenol, with the larvicidal activity against *Aedes aegypti* larvae of such dispersion being significantly enhanced as the concentration of the second component is increased.[41]. These results opened new perspectives on the development of new botanical-derived larvicidal formulations against mosquitos. It is worth mentioning that even though the use of commercial insecticides, many of them as emulsifiable concentrates, including essential oils for pest management has undergone a moderate growth in recent years, especially in the USA and the EU,[26] none of these products have been tested as mosquito larvicidal, in spite of a great deal of research in this direction.[42] This may be associated with a potential toxicity against non-target aquatic organisms which is a barrier to regulatory approval of the use of these products. Thus, it is necessary to seek new alternatives for taking advantage of the toxicity of essential oils against insect larvae, minimizing the potential risks for environment and non-targeted organism, which is only possible by a careful examination of the stabilizing molecules and by the minimization of the use of organic solvents to vehicle the essential oil. These aspects are addressed in this work, where essential oils compounds are directly incorporated as the oil phase of the emulsions, without any additional organic solvent for their dispersion within the formulation. Furthermore, the use of an emulsifying molecule with low toxicity such as the amphiphilic triblock copolymer Pluronic® F127 favors the minimization of environmental risks.[43] Pluronic® F127 is a triblock copolymer with two lateral blocks of poly(ethylene-glycol) and a central one of poly(propylene-glycol), enabling the dispersion of essential oil compounds within the hydrophobic region of the supramolecular aggregates formed by such polymer in aqueous solution.

This work analyzes the effect of the nature of the oil phase, formed by different blends of carvacrol and thymol (positional isomers, see Scheme 1 for their molecular structures), on the larvicidal activity of o/w emulsions stabilized by Pluronic® F127 against Aedes aegypti larvae. It is worth mentioning that the similarity of thymol and carvacrol may not be correlated to their biological activity, as it was reported in previous studies for their respective activity against *Pediculus humanus capitis* [44] or their anti-microbial properties [45]. Therefore, it should be expected that the biological activity of their mixtures cannot be to be rationalized in terms of the behavior of the pure components.



Scheme 1. Molecular structures for thymol (left) and carvacrol (right).

It is expected that this work can be useful as a preliminary step towards the fabrication of new more eco-friendly formulations for insect pest control, that further have reduced health impact. In this work we will take advantage of the stabilization and ease of the dispersion process of essential oil compound drops in water provided by the eco-friendly surfactant character of the Pluronic® F127 copolymer[44]. This allows preparing the formulations *in situ* which may contribute to a significant reduction of the economic costs and pollutant emissions associated with the transport of large amounts of aqueous formulations from factory to the application place, helping on the minimization of the CO_2 footprint.

Experimental

Chemicals. Thymol (2-Isopropyl-5-methylphenol) and carvacrol **(5-Isopropyl-2-methylphenol)** with purities ≥ 99% were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Pluronic® F127 was also purchased from Sigma-Aldrich (Saint Louis, MO, USA). Pluronic® F127 is a triblock copolymer which presents a molecular weight of 12.600 kDa (4.4 kDa for each poly(ethylene-oxide) and 3.8 kDa for the central poly(propylene-oxide), i.e. 101 and 56 monomers, respectively). All the chemicals were used as received without further purification.

Ultrapure deionized water used for cleaning and preparation of the dispersions was obtained by a multicartridge purification system AquaMAX™-Ultra 370 Series. (Young Lin Instrument Co., Ltd., Gyeonggi-do, South Korea), with a resistivity higher than 18 MΩ·cm, and a total organic content lower than 6 ppm.

Preparation of emulsions stabilized by Pluronic® F127. The samples were prepared in tubular glass vials (10 mL) following a procedure adapted from our previous publication.[44] Briefly, it consists in the initial addition of weighted amounts of a stock aqueous solution of Pluronic® F127 (concentration 10 wt%) in the vial, followed by the pouring of weighted amounts of thymol and carvacrol to obtain final mixtures with a total concentration of oil phase (essential oil compounds blend) within the 1-5 wt% range and different thymol:carvacrol weight ratios. Afterwards the mixtures are then diluted with ultrapure water to obtain the final mixtures, with a Pluronic® F127 concentration (c_{cop}) composition in the 1-7.5 wt% range. Once all the components have been added, the mixtures are homogenized using a magnetic stirrer (1000 rpm) at 70 ºC during 5 hours. This heating is important because thymol is a solid compound (melting point around 50ºC), and their incorporation within the oil phase of the emulsions is facilitated by the increase of the temperature. Once the final liquid mixtures are obtained, they are cool down and left to age during 1 week before using them to perform any physico-chemical or biological test.

Characterization of the dispersions. The determination of the compositional ranges, in which emulsions remain stable during at least six month (microemulsions or nanoemulsions), was performed at a temperature of 25 °C for mixtures containing different thymol:carvacrol weight ratios. This type of studies allows mapping the compositional regions in which pseudo-single phase mixtures with potential utility in the fabrication of new botanical insecticides may be prepared.

Dynamic Light Scattering. Dynamic Light Scattering (DLS) experiments were carried out using a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom) in quasi-backscattering configuration (scattering angle, $\theta = 173º$) using the radiation from the red line of a He-Ne laser (wavelength, $\lambda = 632$ nm). DLS experiments provides information of the characteristic diffusion time of Brownian scatters τ at fixed temperature (25ºC in our studies) which is related to the apparent diffusion coefficient D_{app} of such scatters [46, 47]

$$\frac{1}{\tau} = D_{app} q^2 , \qquad (1)$$

where $q = \left(4\pi n / \lambda\right)\sin(\theta / 2)$ is the wavevector, n the refractive index of the continuous phase. The values of D_{app} allow one to estimate the apparent hydrodynamic diameter of the scatters, d_h^{app}, through the Stokes-Einstein equation

$$d_h^{app} = \frac{k_B T}{3\pi\eta D_{app}}, \qquad (2)$$

where k_B and T are the Boltzmann constant and the absolute temperature, respectively, and η is the viscosity of the solvent. It is worth mentioning that the aforementioned DLS technique can be only used on the analysis of transparent dispersions.

Spreading and evaporation of emulsion microdroplets. Measurements of the contact angle of emulsion microdroplets (2-4 μL) deposited onto silicon wafers (Siltronix, Archamps, France) using a Hamilton microliter syringe (SN 701, 10 μL) were performed using a set-up consisting on a cylindrical steel chamber (13 cm of diameter, 10 cm of depth) fitted with flat glass windows on the side and at the bottom positioned in such a way that allow one to follow the time evolution of the droplet contour [48, 49]. The images of the drop shape were captured using a CCD camera (KODAK IT CCD KAI340), at a maximum rate of 60 frames per second with a 640 x 480 pixels resolution. The temperature of the measuring chamber was controlled at 25.0± 0.2°C.

The images of the sessile droplets were analyzed according to the axisymmetric drop shape analysis profile (ADSA-P) method [50] allowing one to obtain the contact diameter, 2L, and its height h. This allows estimating the contact angle θ and the volume V of the sessile droplet assuming that it has a spherical-cap shape

$$\frac{\theta}{2} = \tan^{-1}\left(\frac{h}{L}\right), \qquad (3)$$

and

$$V = \frac{\left[\frac{\pi}{3}(L)^3\right](1-\cos\theta)^2(2+\cos\theta)}{\sin^3\theta}. \qquad (4)$$

Larvicidal bioassays. A colony of *Aedes aegypti* (Rockefeller Strain, Venezuela), maintained in a bioterium of PIET-INEDES (CONICET-UNLu) from 2018 at 25±1°C, 65-80 % of Relative Humidity (RH), and a L12/D12 photoperiod (exposure to alternate 12 hours periods of light and darkness) and free of exposure to pathogens, insecticides, or repellents, was used for larvicidal bioassays [51]. These involves the addition of different volumes (0.05

to 2 mL) of the tested emulsions to drinking water up to a final volume of 225 mL (final emulsion concentration within 1-250 ppm range), followed by the addition of 25 mL of water containing 20 late third-instar or early four-instar larvae of *Aedes aegypti* into the container. Control experiments were performed following the same procedure described above, replacing the emulsions by a Pluronic® F127 aqueous solutions (concentration 10 wt%). Food supply was offered to larvae and the larval mortality was recorded after 24 h of exposure [41].

The mortality was assumed for those insects which failed to move, were unable to rise to the surface within a 1 min or does not evidence the characteristic diving reaction when the water is disturbed [52]. The percentage of larvae affected or the degree of mortality was determined, and a Probit analysis was performed to estimate lethal concentration affecting to the 50% of the individuals (LC_{50}) [53]. Lethal concentrations (LCs) correspond to the concentrations of thymol, eugenol or their mixtures dispersed in the emulsions and are expressed as parts per million in the final mixture (ppm). Four replicates were performed of each experiment, and the percentage of mortality (%) was obtained as the average value. Furthermore, the effective concentration required for the reduction of the emergence of adult individuals by 50% , i.e. the median *emergence inhibition* (EI_{50}), was evaluated as the last specimen of the experimental group that emerged or died. This was done in experiments in which 5 mg of larval food were added to the container with the diluted emulsion after 24 hours of exposure. The food supply was maintained until more than 90% of the control mosquitoes emerge or die, with the latter being evaluated. Mortality data were corrected using Abbott's formula when the mortality ranged between 10-20% of the insects tested in control groups.[54] Note that values of mortality below 10% of the insects tested were assumed within the natural variability.

The data were processed using the PoloPlus v.2.0 software (LeOra Software, Berkley, California, United States of America) and the values were considered statistically significant when the 95% confidence limits did not overlap. Drinking water quality was analyzed by Química MATCO (Luján, Buenos Aires, Argentina) and the results were the following:

Drinking water composition (mg/mL): alkalinity 399, chloride 35, calcium 66.7, magnesium 51, total hardness ($CaCO_3$) 37, dissolved oxygen < 0.20, nitrate < 5, sulfate 41.5, nitrite <0.01 and residual chlorine 0. Conductivity (μS/cm): 1058. pH: 7.84.

Biological interactions between thymol and carvacrol. The interaction between thymol and carvacrol in the blends containing different thymol:carvacrol ratios (1:0; 0.75:0.25; 0.50:0.50; 0.25:0.75 and 0:1) was evaluated by the comparison of the expected value of LC_{50} and that obtained experimentally: R=LC_{50} expected/LC_{50} experimental. The expected values were determined based on Wadley's calculation as [55]

$$\text{expected LC}_{50} = \frac{x_t + x_c}{\frac{x_t}{LC^t_{50}} + \frac{x_c}{LC^c_{50}}}, \tag{5}$$

where x_t and x_c represent the weight fractions of thymol and carvacrol in the mixture, respectively, and LC^t_{50} and LC^c_{50} are the values of LC_{50} for pure thymol and pure carvacrol, respectively. Thus, according to Wadley [55] it is possible to assume that $R > 1.5$ indicates synergistic interaction; $1.5 \geq R > 0.5$ appears for additive interactions and $R \leq 0.5$ evidences an antagonistic interaction.

Results and Discussion

Determination of the stability regions for essential oil in water emulsions. An important preliminary step on the design of formulations of o/w emulsions with potential application in pest control is the evaluation of the stability regions for the studied pseudo-ternary mixtures (water/essential oil compounds/copolymer), i.e. the definition of the compositional regions within emulsions are obtained. Figure 1 shows the compositional maps for the two pseudo-ternary mixtures in which the oil phases are formed for the pure EOs (thymol or carvacrol) and for a mixture with an oil phase formed by a equimolar blend of both essential oil compounds (thymol:carvacrol ratio 0.5:0.5).

The analysis of the compositional maps obtained for the pseudo-ternary system evidences the existence of at least three different types of mixtures within the explored composition ranges: (i) phase separated mixtures, (ii) opalescent emulsions and (iii) transparent emulsions. The transition from phase separated mixtures to transparent emulsions, passing through the compositional range corresponding to opalescent emulsions, appears with the increase of the ratio between the concentration of the copolymer (Pluronic® F127) and the concentration of the essential oil compound in the pseudo-ternary mixtures, $R_{cop/EOC}$. This suggests the existence of a minimum concentration of copolymer below which it is not possible to accomodate all the essential oil compound within the hydrophobic environment of the emulsion, resulting in the phase separation of the mixtures. Similar conclusions are obtained from the evaluation of the emulsions containing 0.75:0.25 and 0.25:0.75 thymol:cavacrol ratios.

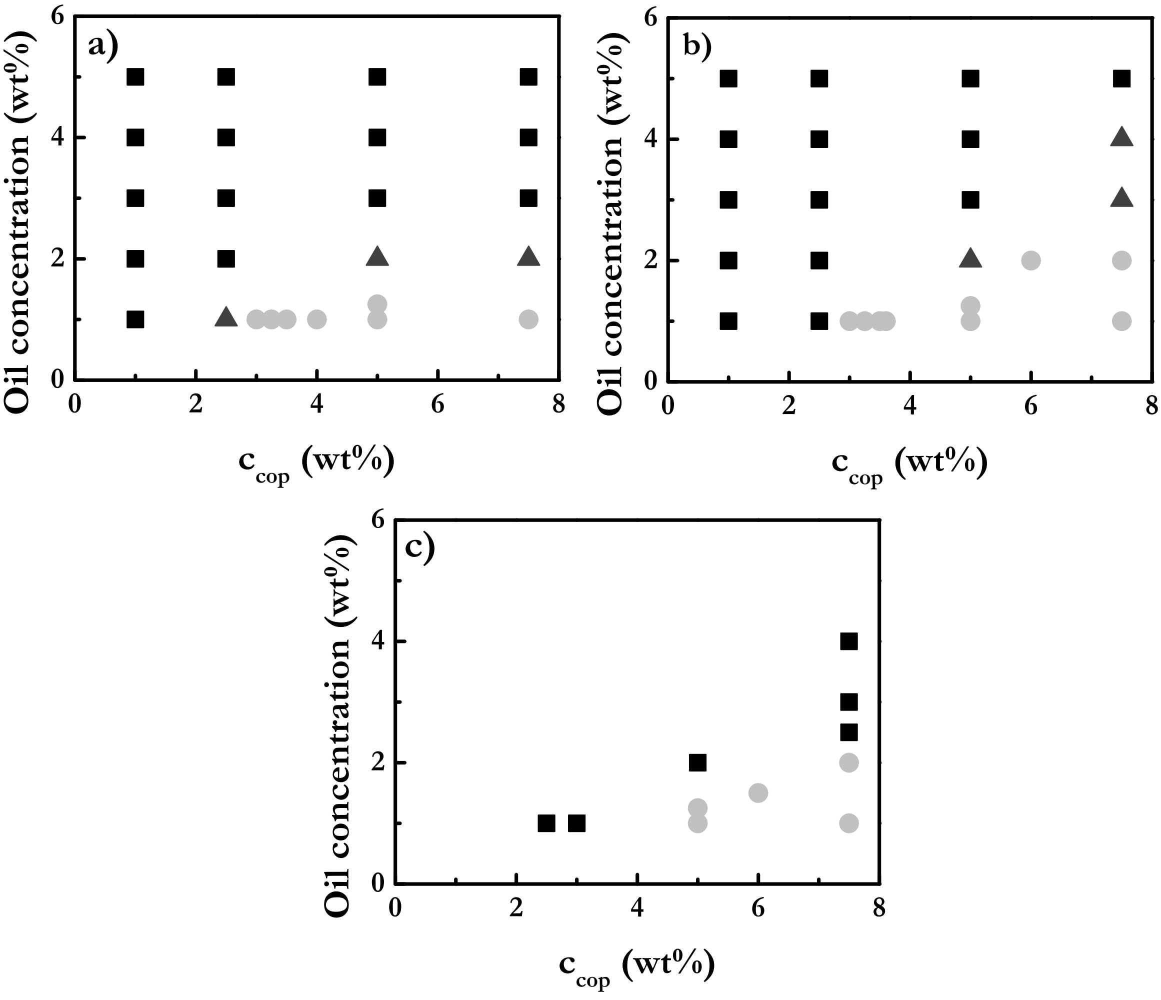


Figure 1. Compositional maps for the different pseudo-ternary mixtures (water/essential oil compound/copolymer) represented as the dependence of the oil phase concentration on the Pluronic® F127 concentration (c_{cop}): (a) Mixtures with thymol as oil phase. (b) Mixtures with carvacrol as oil phase (c) Mixtures with an equimolar thymol:carvacrol blend as oil phase

(thymol:carvacrol ratio 0.5:0.5). In the three panels: (■) Phase-separated mixtures. (▲) Opalescent emulsions. (●) Transparent emulsions.

The examination of the three compositional maps shows that the replacement of thymol for carvacrol in the oil phase of the pseudo-ternary mixtures results in an enhanced dispersion of the oil within the aqueous continuous phase, i.e. higher amounts of essential oil compounds may be dispersed for a fixed amount of copolymer without any signature of phase separation. This suggests that carvacrol plays an important role for improving the solubilization of the solid thymol (melting point around 50 ºC) within the oil phase, enhancing its dispersion within the aqueous medium. The worst capacity of the copolymer for dispersing thymol than carvacrol is clear from the threshold values of $R_{cop/EOC}$ above which the phase separation occurs in each system. This value is above 0.5 and 0.6 for emulsions with thymol and carvacrol as oil, respectively. This may present a critical impact in the further development of these type of systems for the solubilization and distribution of other bioactive molecules.

Determination of the sizes of the droplets for essential oil compounds in water emulsions. The size of the emulsions belonging to the compositional region in which transparent emulsions are formed may be estimated from DLS measurements in terms of the apparent hydrodynamic diameter d_H^{app}. The evaluation of the size of the droplets presents a key importance when systems for pest control are considered, mainly because an enhanced penetration of the formulation through the insect cuticle and consequently a higher toxicity of the formulations may be expected as the average size of the droplets is reduced [34, 35]. Figure 2a shows, for the sake of example, the intensity auto-correlation functions obtained for transparent emulsions containing a fixed amount of carvacrol as oil-phase and increasing copolymer concentrations.

The intensity auto-correlation functions shift to smaller relaxation times with the increase of the copolymer concentration, which is ascribed to a decrease of the average size of the droplets. However, the clear bimodal character of the intensity auto-correlation functions found for most of the explored mixtures (only emulsions with the highest values of $R_{cop/EOC}$ appear as mono-modal) makes it difficult to obtain an evaluation of the size of the droplets from the time-scale within the auto-correlation function decay. Therefore, the bimodal character of the intensity auto-correlation functions is a signature of polydispersity. It is worth mentioning that the decrease of the copolymer concentration makes the intensity autocorrelation more clearly bimodal. This is explained assuming that the dispersion of essential oils droplets in water requires a minimum amount of copolymer to ensure the formation of a shell with a hydrophobic pool, thus enabling the distribution of the essential oil, and giving stability to the droplets [56, 57]. However, the decrease of the copolymer concentration leads to a situation in which the number of molecules is not enough to stabilize monodisperse emulsions with small droplets, thus the droplets start to coalesce which results in an increase of the average size of the droplets and, hence, the polydispersity of the emulsions. The increase of the average size of the droplets allows a minimization of the area of the interface between the droplets and the continuous phase, and matter of fact reduces the amount of copolymer required for coating the droplets. The limit case of this phenomena is the onset of the compositional region in which opalescent emulsions are formed, and after a further decrease of the copolymer concentration the emergence of the phase separation region (see Figure 1). It is worth noting that DLS does not allow investigating the two latter systems. Even though, the above discussion has been focused in emulsions containing only carvacrol on the oil phase, similar results were found independently of the composition of the essential oil compounds blend forming the oil phase (see insets of Figure 2 for the case of emulsions containing an equimolar blend of thymol and carvacrol as oil phase).

The bimodal character of the auto-correlation function results is also evidenced from the distributions of the apparent hydrodynamic diameters shown in Figure 2b. Such distributions show, for most of the copolymer concentrations, two separated distributions, the first one corresponding to droplets with an average diameter in the 25-75 nm range and the second containing droplets with an average apparent hydrodynamic diameter more than one order of magnitude larger. It is worth mentioning that the droplets with the smallest size correspond to the most important contribution, which is explained considering that the scattered intensity increases by a 10^6 factor with the size of the scatters, thus the population having the most significant contribution in the distribution may not correspond to that involving the highest number of scatters [46, 47].

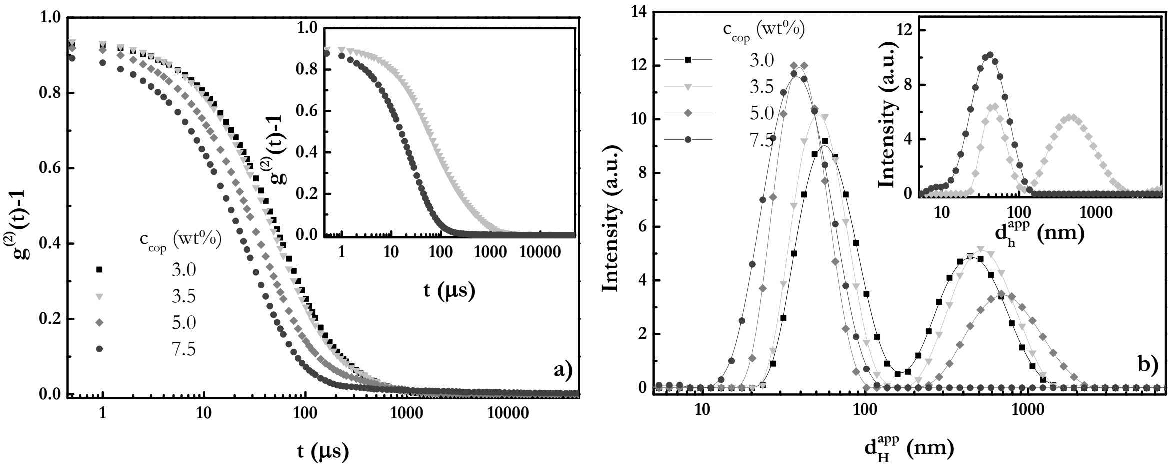


Figure 2. Intensity auto-correlation functions (a) and d_h^{app} distributions (b) for emulsions with a fixed carvacrol concentration (1 wt%) and different Pluronic® F127 concentrations (c_{cop}). The data corresponds to emulsions in which the oil phase is pure carvacrol. The insets corresponds to the same results shown in the main panels for emulsions in which the oil phase is formed by equimolar blend of thymol and carvacrol (thymol:carvacrol ratio 0.5:0.5).

A detailed analysis of the distributions points out that the smallest droplets decrease their size as c_{cop} increases, whereas the opposite is true for the droplets with the highest sizes. Furthermore, the contribution associated with the smallest droplets becomes more important with the increase of c_{cop}, whereas the second contribution decrease its importance until its complete disappearance for the highest copolymer concentrations. This confirms again the aforementioned importance of a minimal copolymer concentration for preventing the destabilization processes which result in an increase of the sizes of the droplet and polydispersity. Thus, the increase of the size of the biggest droplets until its disappearance for the highest copolymer concentration can be considered as a signature of a destabilization by Ostwald ripening. This is rationalized assuming that the decrease of the size of the smallest droplets with the increase of the copolymer concentration results from the availability of higher amounts of copolymer to coat larger interfacial areas, providing the bases for the reduction of the average size of the droplets. Furthermore, the decrease of the size of the smallest droplets leads to an increase of the internal pressure differences between big and small droplets which drives to the Ostwald ripening, and the growing of the biggest droplets following process. However, the contribution associated with the biggest droplets reduces its importance with the increase of the polymer concentration, disappearing for the highest copolymer concentrations. This is explained considering that the increase of the polymer availability leads to an increase of the stabilization of the droplets, i.e. the coating is strong enough to reduce the impact of the Ostwald ripening. Notice that the increase of importance of the low-size contribution indicates that the ratio between the number of small and large droplets increases steeply, which may affect to the bioactivity of the formulations.

The above discussion corresponds to the impact of the copolymer on the stabilization of the emulsions. However, another important parameter is the amount of oil incorporated within the emulsion. This requires an analysis of the effect of the oil concentration on the size of the droplets for a fixed copolymer concentration. Figure 3 shows the intensity auto-correlation function for emulsions with a fixed concentration of copolymer and different carvacrol concentrations. The results show again the importance of the $R_{cop/EOC}$ value on the stabilization of the emulsion droplets, with the polydispersity of the emulsions, evidenced from the intensity auto-correlation function and the size distribution, being reduced as the amount of available copolymer increases.

This work is focused on the study of the larvicidal activity of emulsions containing as oil phase different essential oil compounds blends, this makes it interesting to evaluate the impact of the composition of the oil phase on the dispersion of the oil. Figure 4a and 4b shows the intensity auto-correlation functions and the d_h^{app} distributions, respectively, for emulsions containing different essential oil blends as oil phase in which the concentrations of copolymer and essential oil are maintained constant. The intensity-autocorrelation functions does not evidence any significant difference on the character of the obtained dispersions, appearing independently of the nature of the oil phase a bimodal decay. However, the differences as function of the composition of the essential oil blend are more evident from the d_h^{app}

distributions (see Figure 4b). It is true that it is not easy to obtain an evaluation of the average sizes corresponding to small and big droplets because they start to be coupled as the thymol concentration is increased (lower values of x_c). However, the clearer separation of the populations corresponding to the droplets having different sizes as carvacrol content increases suggests a better dispersion of the thymol within the hydrophobic shell formed for the copolymer and, consequently, it would possible possible to assume that the higher the concentration of carvacrol in the oil phase the better the dispersion of the essential oil within the aqueous phase is. This confirms the picture obtained from the compositional maps shown in Figure 1, with the increase of the carvacrol concentration leading to an increase of the amount of oil that can be distributed in the aqueous phase without the aqueous continuous phase. This agrees with previous results for o/w emulsions stabilized with Pluronic® F127 using blends of thymol and eugenol as oil phase [41].

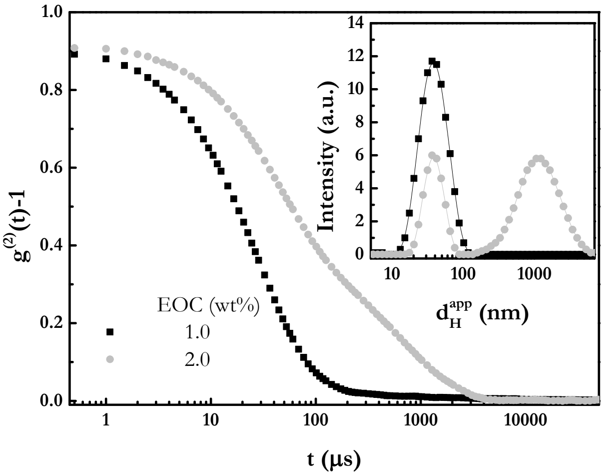


Figure 3. Intensity auto-correlation functions for emulsions, in which the oil phase is carvacrol, with a fixed Pluronic® F127 concentration (7.5 wt%) and two different carvacrol concentrations. The inset shows the d_h^{app} distributions corresponding to the samples on the main panel.

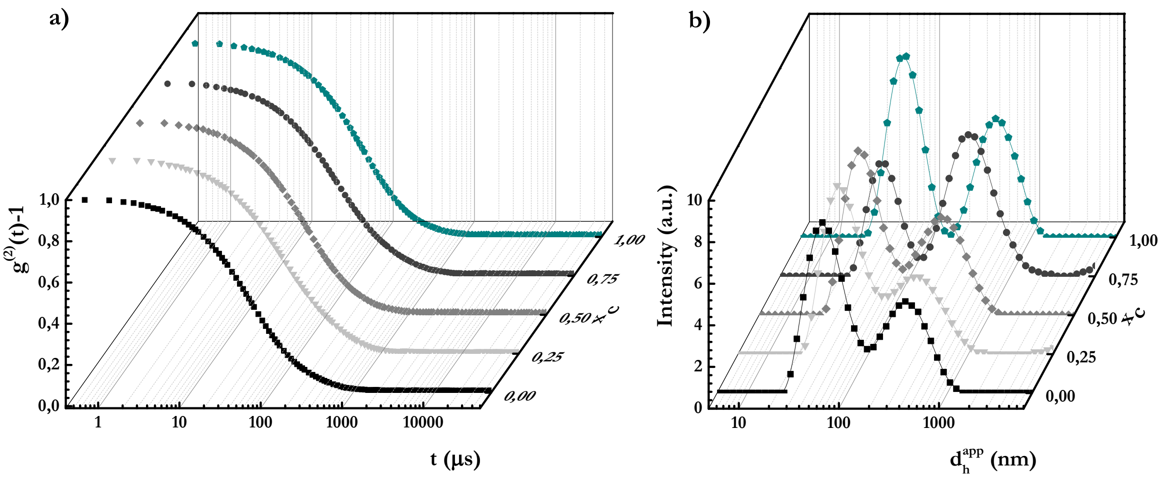


Figure 4. (a) Intensity auto-correlation functions for emulsions with different essential oil blends as oil phase (indicated by the weight fraction of carvacrol x_c), and fixed Pluronic® F127 (3.25 wt%) and essential oil compounds (1 wt%) concentration. (b) d_h^{app} distributions for selected emulsions with different essential oil blends as oil phase (indicated by the weight fraction of carvacrol x_c), and fixed Pluronic® F127 (3.25 wt%) and essential oil compounds (1 wt%) concentrations.

Larvicidal activity of essential oil in water emulsions against *Aedes aegypti* larvae. The larvicidal activity of different transparent emulsions presenting thymol:carvacrol blends with different ratios between the essential oil compounds forming the oil phase, and a fixed copolymer (5 wt%) and total essential oil compounds (1.25 wt%) concentrations was evaluated in terms of the lethal concentration affecting 50% of the exposed larvae (LC_{50}) and the effective concentration to reduce adult emergence by 50% (EI_{50}), with both being calculated by Probit analysis with an overlapping of the confidence intervals of 95%. Figure 5 shows the values of the values of LC_{50} and EI_{50} as function of the fraction of carvacrol in the oil phase, x_c.

The tested emulsions results, in all the cases, in acute toxicity in mosquito larvae, although significant differences exist on their toxicity depending on the composition of the oil phase. Emulsions containing only thymol in the oil phase present the highest larvicidal activity against *Aedes aegypti larvae*, which is worsened as carvacrol is introduced within the oil phase, i.e. emulsions containing thymol:carvacrol blends as oil phase presents a worst larvicidal activity than when thymol is the only component of the oil phase. Nevertheless, the larvicidal activity of emulsions containing only carvacrol is not significant different to those containing only thymol. On the other hand, the use of thymol:carvacrol blends to substitute the thymol from the oil phase results in a reduction on the larvicidal activity of the emulsions. It is worth noting that even though there are no significant differences between the emulsions of the pure compounds, those containing only thymol as oil phase present significant differences in their activity with respect to emulsions containing thymol:carvacrol blends as oil phase. However, the differences on the activity of emulsions containing only carvacrol and those containing essential oil compound blends as oil phase are not significant.

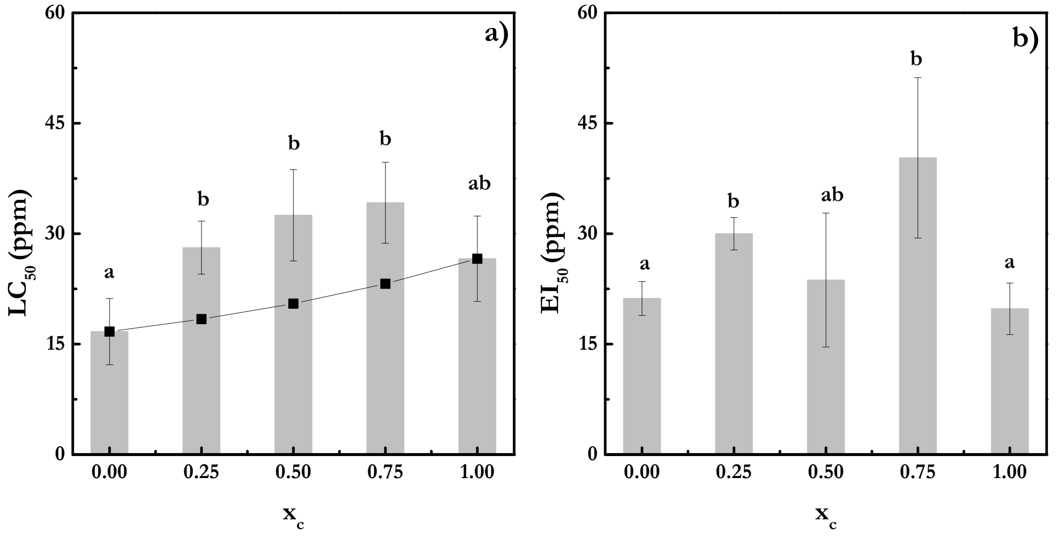


Figure 5. Larvicidal activity of emulsions of thymol, carvacrol and their mixtures against larvae of *Aedes aegypti*. Each bar represents the LC_{50} (a) and the EI_{50} (b) values obtained by probit analysis on the carvacrol fraction contained in the oil phase (all the emulsions present a copolymer and total essential oil compounds concentrations of 5 wt% and 1.25 wt%, respectively). The error bars indicate the variability of the data, with the average values of LC_{50} and EI_{50} being obtained by Probit analysis with an overlapping of the confidence interval of 95%. The symbols (■) in the panel a correspond to the values obtained for the expected LC_{50} calculated according the Wadley's calculation represented by equation (5) and the line is a guide for the eyes. The lowercase letters above each bar evidence the significance in the difference between the results obtained for the different formulations, with the same

letters indicating the absence of significant differences between treatments. The number larvae tested for each formulations was 480.

It is true that the toxicity of the essential oil compounds dispersed in the emulsions against *Aedes aegypti* larvae is around five orders of magnitude lower than that of the synthetic insecticide pyriproxyfen or one thousand times lower than that corresponding to the spinosad [58]. However, the dispersion of essential oil compounds as the oil phase of o/w emulsions leads to a larvicidal activity several times higher than that reported for free essential oils [59]. Therefore, even though emulsions containing essential oils are less effective than most of the commonly used synthetic insecticides, they may be considered as promising tools in the design of new formulations for eco-sustainable botanical insecticides [26]. It must be stressed that it is always possible to include pyriproxyfen, or another synthetic insecticide, in the oil droplets to increase its activity, but containing a smaller amount of insecticide than that corresponding to the normal application dose of the insecticide, thus making the formulation less eco-aggressive. Furthermore, the substitution of the flammable organic solvents (xylene, toluene or gas-oil) commonly used in pesticide manufacturing by oily derivatives of essential oils would make it possible to reduce the risks associated with the manufacturing and use of insecticidal products.

The above results allow one to evaluate the interactions occurring between thymol and carvacrol in their blends, which was found additive (R values in the 0.6-0.7 range for all the studied emulsions) in agreement with the results by Youssefi et al.[60] for the activity of thymol:carvacrol mixtures against eggs of *Culex pipiens*. However, this additive effect contrasts with the synergetic activity of thymol and carvacrol against *Culex pipiens* larvae [60], *Dermanyssus gallinae* [61], *Dermacentor nitens* [62], *Amblyomma sculptum* [62], *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus sanguineus* [40], and the antagonistic interaction found against *Drosophila melanogaster* [63]. Furthermore, the comparison of thymol:carvacrol and thymol:eugenol[41] mixtures show significant differences, with the latter evidencing an antagonistic interaction between the components. Thus, the results suggest that chemical similarity between thymol and carvacrol presents a certain role on the interactions occurring between the components.

The IE_{50} values do not show any significant difference with those obtained for the LC_{50}. Therefore, it is possible to assume the absence of any significant impact of the emulsions on the growth of the remaining *Aedes aegypti* larvae, and consequently no inhibition of adult emergence in addition to larvae mortality was found after 24 hours of exposure. Thus, emulsions present an initial acute toxicity of larvae, without any subsequent sublethal effect on emergence of adult individuals. Therefore, these results suggest that within the concentration range studied, the emulsions do not present any pupicidal activity, which may be explained considering the higher resistance of pupae than larvae (LC_{50} has been reported to be between 10 and 100 fold higher for pupae than for larvae [64]). It is worth mentioning that the LC_{50} of Pluronic® F127 required for producing mortality on pupae was found around a value of 570 ppm,[44] which is a value one order of magnitude higher than the corresponding to the LC_{50} reported here for the emulsions.

The above discussion suggests that the organization of the essential oil within the emulsions or the size of the droplets do not play a significant role on the toxicity of the here studied thymol:carvacrol blends in water emulsions, with the chemical nature of the oil phase being probably the most critical role. This can be explained considering that even though the polydispersity of the different tested formulations may be very different, the average size of the smaller droplets is very similar independently of the essential oil compound blend included within the emulsion. Therefore, the results suggest that the specific nature of the oil phase may be a parameter with a stronger impact on the biological activity of the emulsions than the differences on the droplets concentration.

Spreading and evaporation of essential oil in water emulsions onto solid surface. The evaluation of the spreading and evaporation of microdroplets upon their deposition onto solid surfaces present interest on the design of formulations for pest control because these phenomena may contribute to the distribution on the formulations within the environment and even on their interactions with the insect. This is especially important when the toxicity by contact is considered, which is especially important when the control of terrestrial or aerial insect pest are considered and hence the evaluation of the spreading and evaporation may present a limited impact on the interaction of the studied emulsions against larvae as result of the aquatic life cycle of Aedes aegypti larvae occurs. However, considering that larvae can be found in two possible situations: larvae at the air/wáter interface or below the surface, it is expected that the formulation will increase its larvicidal activity when the EO droplets wet completely the chitin exoskeleton, thus displacing the water layer. Despite this is an obvious conclusion, it is not easy to stablish straightforward methods for the design of the appropriate oil characteristics and the correct surfactant to be used in the formulation. This is because the chemical composition and the topography of the exoskeleton is not uniform. The irregularities of the surface can lead to the pinning of the three-phase contact line as well as to a wetting behavior intermediate between the Cassie-Baxter and the Wenzel scenarios.[65] Therefore, no clear conclusions about the wetting ability of the EO droplets stabilized by the surfactant can be extracted in the absence of a good determination of the topography of the exoskeleton and the building of a model surface with the appropriate chemical and topological characteristics. Therefore, even though the analyzed situation is far from the real situation appearing during the interaction of larvicidal formulations with larvae and the surface used is a very simple hydrophilic surface (silicon wafers with a silicon oxide layer of around 2-3 nm on their surface as was evaluated independently by ellipsometry and neutron reflectometry [66]) which is far from the composition of the cuticle surface of the insects, [67, 68]. the study of the spreading and evaporation processes of microdroplets of the formulations may present a big interest for the optimization of larvicidal formulations. Thus, the evaluation of the time dependences of the droplet contact angles, volumes and radius may help on different aspects related to the optimization of botanical insecticides based on essential oil compound in water emulsions [69]. Figure 6 shows the time dependences of the contact angle, volume and radius for a set of droplets belonging to different emulsions containing only thymol as oil phase onto the silicon oxide surface.

The decrease of the contact angle and volume of the droplets and the increase of their radius with the time may be explained assuming the coupling between the spreading of the droplets on the surface and the evaporation of the water (main component of the studied emulsions) [70-72]. The results show similar time dependences for the geometrical parameters, independently of the considered emulsions, only variations of the time scales involved in the spreading/evaporation process and the values of contact angle, volume and radius on the composition of the emulsions are observed from the experimental rate. This may be explained considering that the spreading rate is mainly governed by the oil phase and the copolymer, with the water evaporation, for such diluted emulsions, proceeding with a similar rate than in pure water droplets [72]. Thus, water evaporation pushes the oil droplets to the proximity of the surface and force their aggregation, with the coalescence and Ostwald ripening rates controlling the spreading process.

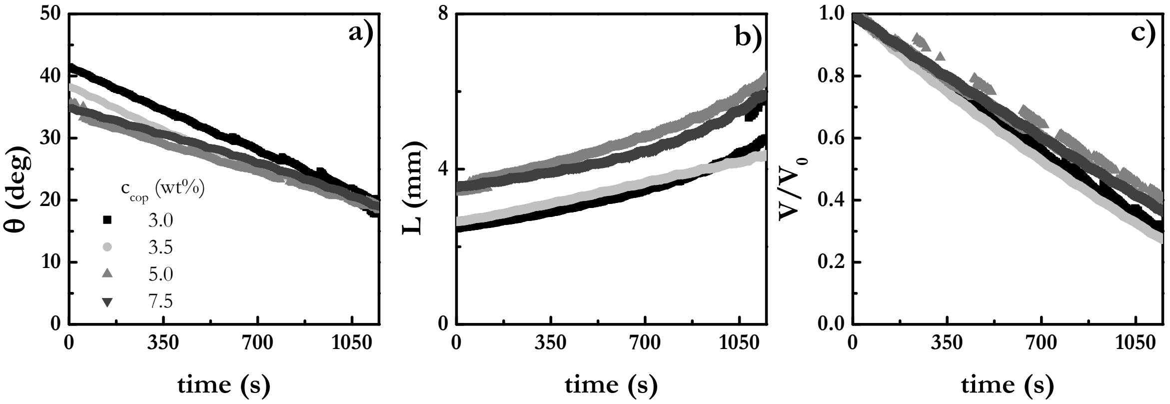


Figure 6. Time dependences for droplet contact angle θ (a), radius L (b) and reduced volume V/V_0 (with V_0 is the volume after deposition of the droplet onto the surface) (c) for emulsions with an oil phase formed by 1 wt% of thymol and different concentrations Pluronic® F127.

The effect of the copolymer concentration is clear from the results in Figure 6, with the emulsions stabilized with the lowest copolymer concentrations having a contact angle closer to that corresponding to the advancing contact angle of a droplet of pure water (55 ± 3º). This contact angle decreases with the concentration of the copolymer. Thus, considering the diluted character of the emulsions, it may be expected that the evaporation rate of water may control the process, and only when the oil droplets remains entrapped within a small aqueous droplet, the oil starts to spread onto the wafer surface driving the formation of an oil film upon the complete evaporation of the water. This spreading of the essential oil is possible due to an adhesion mediated by the copolymer and consequently it should be reduced as the concentration of the copolymer is decreased. This justifies the enhanced spreading found as the copolymer concentration is increased (faster decrease of contact angle). This picture is also supported on the stronger increase of the droplet radius with the copolymer concentration which is an indication of a copolymer mediated adhesion of the oil phase on the surface. Furthermore, the evaporation of the water is slightly reduced with the increase of the copolymer concentration, which can be explained considering an adsorption of part of the copolymer at the water/vapor interface preventing partially the evaporation.

Although one of the most important aspects for the optimization of a formulation based on an emulsions is the control of the stabilization process, and their impact on the properties of the formulations, this work tries to analyze the impact of the composition of the oil phase on the toxicity of the formulations. Therefore, it is required analyze the spreading/evaporation for emulsions containing oil phases with different composition because it may impact on their insecticidal activity upon contact with the targeted organism. Figure 7 shows the time dependences of the geometrical parameters of emulsion droplets, with different essential oil blends forming their oil phase (evidenced by the values of x_c), deposited onto a silicon wafers. It is worth mentioning that the time dependences of the geometrical parameters follows the general trend discussed above.

The results show that the spreading of the oil phase and the evaporation of the water is enhanced with the introduction of carvacrol in the oil phase. This may be rationalized considering the different nature of thymol and carvacrol. Thus, it may be expected that the presence of thymol within the oil phase lead to the formation of ordered domains of essential oil compound which may limit the diffusion of the molecules contained in the oil phase and consequently slows down the spreading process. This ordered essential oil domains can probably modify also the dynamic of the water within the emulsion droplets, altering the evaporation rate.

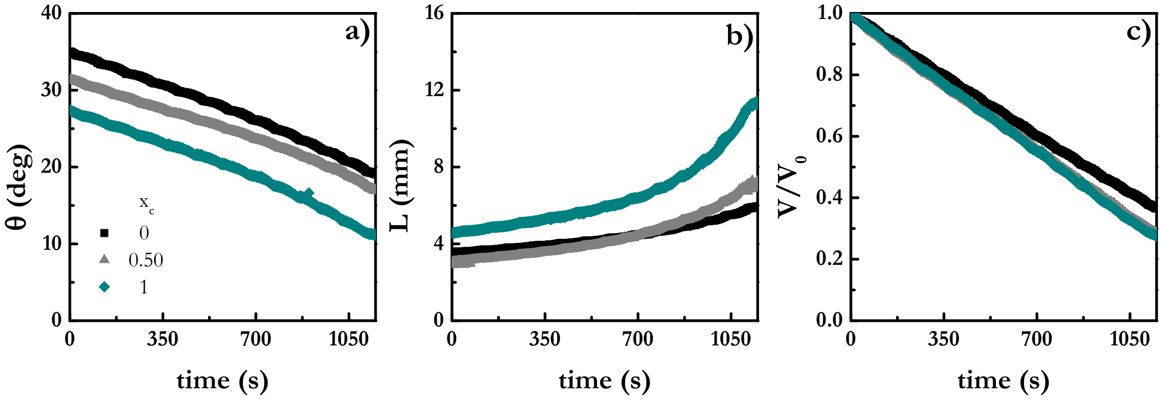


Figure 7. Time dependences for droplet contact angle θ (a), radius L (b) and reduced volume V/V_0 (with V_0 is the volume after deposition of the droplet onto the surface) (c) for emulsions with oil phases containing different essential oil compounds blends and a concentration of Pluronic® F127 of 7.5 wt%.

It is worth recalling that the results concerning to the spreading and evaporation of emulsions droplets are related to their interaction with a hydrophilic surface, which is the opposite situation occurring when the interaction of an insecticide formulation with insects occurs by contact. Thus, it may be expected that the picture found in the application of the here studied emulsions as insecticide may be reversed in relation to the reported above. However, it is clear that the adhesiveness of the polymeric shell impact decisively on the interaction between the formulation and any surface, with the nature of the oil phase playing also a certain role.

Conclusions

This work has studied the preparation of essential oil compounds in water emulsions stabilized by an amphiphilic copolymer, Pluronic® F127, in which the oil phase is composed by blends of thymol and carvacrol, and their insecticidal activity against *Aedes aegypti* mosquito. The stabilization of the emulsions is strongly dependent on the composition of the pseudo-ternary mixture (water/essential oil/copolymer), with the increase of the compositional ratio between the copolymer concentration and that of the essential enabling the reduction of the average size of the droplets and polydispersity of the emulsions, which results in an enhancement of the stability of the emulsions. Furthermore, the composition of the essential oil blend also plays a very important role on the distribution of the hydrophobic phase within the copolymer shell, with the progressive substitution of thymol for carvacrol resulting in an increase of the amount of essential oil which can be dispersed within the aqueous phase. However, neither the size of the droplets nor the organization of the essential oils within the emulsions present any significant impact on the larvicidal activity of the emulsions against *Aedes aegypti larvae*, with the chemical nature of active ingredient contained within the oil phase, i.e. whether pure essential oil compounds or their blends are used as oil phase, being probably the most critical parameter on the evaluation of the toxicity of this type of formulations. The most effective emulsions against mosquito larvae were found those containing thymol as oil phase, with the introduction of carvacrol resulting in an additive effect on the larvicidal activity on the formulations. Even though the essential oil compound in water emulsions present a strong effect against mosquito larvae, their activity as growth regulators is limited. One of the most critical aspects related to the activity of any insecticidal formulation is their interaction with the insect cuticle, which acts as a barrier for the penetration of the active molecules. This is evaluated in a semi-quantitative way in terms of the spreading of the formulations onto a solid surface, with the results suggesting that the concentration plays an essential role on the control of the interaction of the active compounds (essential oils compounds) and the surface. Furthermore, the results have also evidenced an important role of the chemical nature of the essential oil compounds on the interactions of the formulations with the surface. However, a more appropriate discussion of the correlations between the spreading and evaporation of the formulations and the toxicity will require a careful examination of the characteristic tipping times associated with the interaction of the formulations and mosquito.

On the basis of the insecticidal activity of this formulations in relation to synthetic insecticide, and considering the simplicity of the methodology used for their preparation, essential oil in water emulsions should be considered as a promising alternative of the preparation of readyto-use formulations against insect pest. Furthermore, the high water content of the formulations may facilitate the bioavailability of the active compounds (essential oil), and limit their risks and hazards for human health and non-target organism (plants or animals). Therefore, even though a careful examination of the dose-response of the formulations against *Aedes aegypti* mosquito and of their long-term stability are required for obtaining a formulation with real field application, o/w emulsions containing essential oils are expected to be a promising safe and viable alternative for an eco-sustainable chemical control of insect pest.

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Contributor roles

Alejandro Lucia: conceptualization; methodology; software; validation; formal analysis; investigation; data curation; writing—original draft preparation; investigation; writing—review and editing; visualization; funding acquisition; resources. **Clemence Girard:** methodology; investigation; writing—review and editing. **Micaela Fanucce:** methodology; investigation; data curation; formal analysis; validation; writing—review and editing. **Carlos Coviella:** investigation; writing—review and editing; resources. **Francisco Ortega:** investigation; writing—review and editing; supervision; funding acquisition; resources. **Ramón G. Rubio:** validation; investigation; writing—review and editing; supervision; project administration; funding acquisition; resources. **Eduardo Guzmán:** conceptualization; methodology; software; validation; writing—original draft preparation; investigation; writing—review and editing; visualization; funding acquisition.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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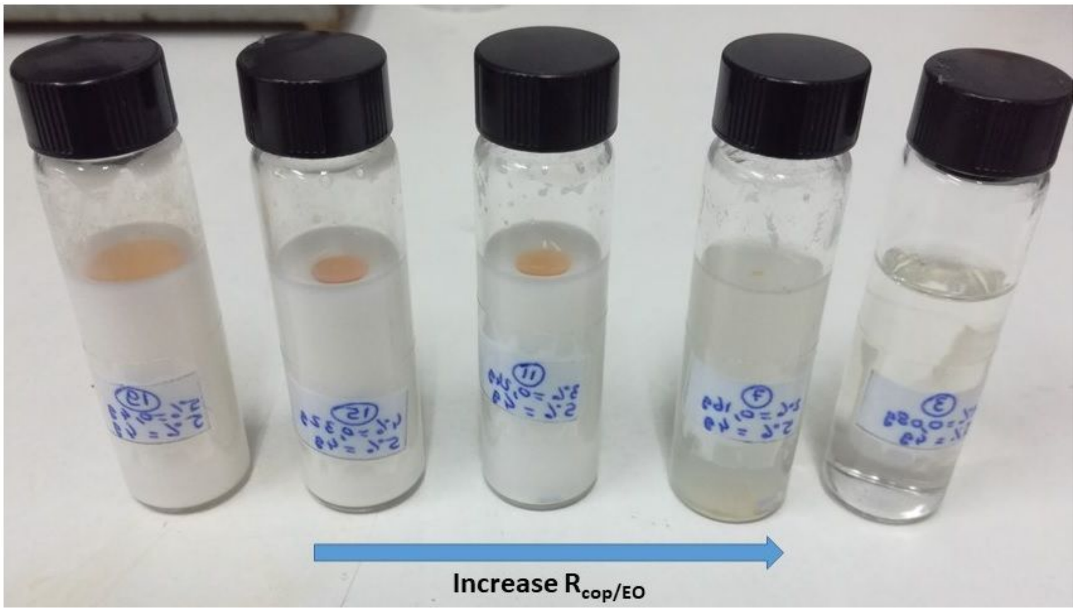

Essential oil compound in water emulsions are promising alternatives for designing eco-sustainable formulations for controlling *Aedes aegypti* larvae