Bulletin of Insectology ***73*** *(1): 153-160, 2020*
ISSN 1721-8861 eISSN 2283-0332

Nanoemulsions based on thymol-eugenol mixtures: characterization, stability and larvicidal activity against *Aedes aegypti*

Alejandro LUCIA[1,2], Ariel Ceferino TOLOZA[3], Micaela FANUCCE[4], Laura FERNÁNDEZ-PEÑA[5], Francisco ORTEGA[5,6], Ramón G. RUBIO[5,6], Carlos COVIELLA[1,7], Eduardo GUZMÁN[5,6]

[1]*Instituto de Ecología y Desarrollo Sustentable (INEDES, CONICET-UNLu), Luján, Buenos Aires, Argentina*
[2]*Centro de Investigación en Sanidad Vegetal (CISaV), Facultad de Ciencias Agrarias y Forestales, UNLP, La Plata, Argentina*
[3]*Centro de Investigaciones de Plagas e Insecticidas (UNIDEF/CONICET), Buenos Aires, Argentina*
[4]*Centro Nacional de Diagnóstico e Investigaciones Endemo-epidémicas (CeNDIE), Administración Nacional de Laboratorios e Institutos de Salud (ANLIS), Buenos Aires, Argentina*
[5]*Departamento de Química Física, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, Spain*
[6]*Instituto Pluridisciplinar, Universidad Complutense de Madrid, Spain*
[7]*Departamento de Ciencias Básicas, Universidad Nacional de Lujan, Argentina*

Abstract

Dengue, Zika, chikungunya and yellow fever are the most important vector-borne diseases worldwide transmitted to humans by *Aedes aegypti* (L.) (Diptera Culicidae). Thus, the control of this vector is of vital importance in order to avoid epidemics in tropical and neo-tropical areas. To find new and effective larvicidal formulations for control programs against mosquito populations, aqueous dispersions containing thymol and eugenol were characterized and tested against *Ae. aegypti* larvae. The dispersion and stabilization of thymol and eugenol in water was possible using a triblock copolymer with two lateral blocks of poly(ethylene oxide) and a central block of poly(propylene oxide), the so-called poloxamer 407, which allows obtaining oil in water (o/w) emulsion. Dynamic Light Scattering (DLS) points out that emulsions containing eugenol were in most cases monodisperse with an average apparent hydrodynamic diameter of the droplets in the 20-25 nm without destabilization after 28 months from their preparation, which plays a key role for the potential application of the studied formulations. On the other side, those emulsions containing only thymol as oil phase had higher polydispersity, suggesting a central role of eugenol in the dispersion of thymol in water. Furthermore, the combined effect of thymol and eugenol against *Ae. aegypti* larvae was evaluated. The nanoemulsion containing thymol as main component of the oil phase (100%) showed the lowest LC_{50} and the introduction of eugenol to the nanoemulsions facilitated the dispersion and stability of thymol in water, even though reducing the effectiveness of the emulsions. The findings on the larvicidal effects of the combined application of the monoterpenes tested could be considered a promising contribution to the development of botanical-derived larvicidal formulations against mosquitos.

Key words: yellow fever mosquito, essential oils, nanoemulsions, larvicidal activity, botanical products, thymol, eugenol.

Introduction

The mosquito *Aedes aegypti* (L.) (Diptera Culicidae) is among the main vectors of different diseases affecting humans, including dengue, chikungunya, Zika and yellow fever (Gubler and Clark, 1995; Weaver *et al*., 2016; Ferreira-de-Brito *et al*., 2016). Dengue fever and dengue hemorrhagic fever are the most important and serious mosquito-borne diseases in Argentina (Masuh, 2008). However, the absence of vaccines for treating dengue fever requires the control of its vectors as an essential tool for ensuring the interruption of the epidemics outbreaks in tropical and neo-tropical areas. Nevertheless, the U.S. Food and Drug Administration announced the first vaccine for dengue fever, but with major restrictions (Godói *et al*., 2017). Current approaches to control mosquitoes rely primarily on source reduction and the application of insecticides against either mosquito larvae or adults. This strategy has been employed for more than two decades, especially with the use of pyrethroids and organophosphates (Lucia *et al*., 2007). Concerning the latter, the application of the larvicide temephos as sand granules (Abate® 1% SG) has been demonstrated to be effective for weeks (Carvalho and Silva, 2000; Chen *et al*., 2009). However, it is highly rejected by residents due to its strong odour and slight turbidity of the drinking water containers where it is applied. The biological larvicides based on *Bacillus thuringiensis israelensis* (Bti) and insect growth regulators like methoprene and novaluron have been shown to be effective against several mosquito species (Boyce *et al*., 2013; Lau *et al*., 2015), but are expensive for large-term treatments (Ritchie *et al*., 2010).

In recent years, an extensive research has been focused on seeking new, low-risk, eco-sustainable formulations for insect pest control. Insecticidal formulations based on essential oils (EOs) and their relative compounds seems promising alternatives for this purpose (Regnault-Roger *et al*., 2012; Mossa, 2016; Pavoni *et al*., 2019; Samar, 2019). EOs are lipophilic secondary metabolites obtained from aromatic plants, with terpenoids being their main components (Tisserand and Balacs, 1995; Isman, 1999).

Despite the potential interest, the number of practical applications for botanical insecticides is rather limited, mainly due to the scarce knowledge about their effectiveness with respect to currently used synthetic insecticide formulations (Isman and Grieneisen, 2014). Moreover, the low solubility of EOs in water together with their chemical instability make their handling and transport

difficult, reducing the practical application of these new formulations.

EOs and their components can act either active ingredients or adjuvants in pesticide formulations. The latter use has attracted lately increasing interest because the existence of synergistic effects against various insect pests has been reported after the combined application of different EOs or its components with various synthetic insecticide (Tong and Bloomquist, 2013; Faraone *et al*., 2015; Norris *et al*., 2015; Gross *et al.*, 2017). These synergistic effects might be mainly explained on the basis of two different aspects: (i) terpenoids are good penetration enhancers for both lipophilic and hydrophilic compounds (Williams and Barry, 1991), and (ii) terpenoids inhibit metabolic enzymes such as cytochrome P_{450} monooxygenases and carboxylesterases (Tong and Bloomquist, 2013).

The enhancement of the dispersion in water of poorly soluble compounds remains an important challenge, which has driven the research on the development of several methodologies, such as physical and chemical modifications of compounds, particle size reduction, crystal engineering, salt formation, solid dispersion, use of surfactant or complexation (Savjani *et al*., 2012; Khadka *et al*., 2014). An alternative for encapsulation of lipophilic active ingredients is the use of polymeric micelles consisting of amphiphilic block copolymers into which active ingredients (a.i.) can be physically incorporated (Kim and Park, 2010). The simplicity of the physical incorporation or solubilisation of active ingredients within block copolymer micelles has driven to a further development of this approach for encapsulation of active molecules (Batrakova *et al*., 2006; 2008). This allows one to take advantage of the versatility of polymer micelles for encapsulation, which makes it possible the use of amphiphilic block copolymer micelles as a good chemical environment for dispersing EO compounds in an aqueous environment. This colloidal dispersion is sometimes referred as oil-in-water emulsion (o/w), as result of the oil entrapment within the hydrophobic domains of the polymer micelles. This may be considered a promising alternative for preparing new formulations for pest control, enabling the facilitated distribution and bioavailability of the active ingredients (Moretti *et al*., 2002; Ragaei and Sabry, 2014; Campolo *et al.* 2017).

A previous study has evidenced considerable bioactivity of polymeric formulations containing low concentrations of EOs compounds (1.25 wt%) against permethrin-resistant head lice, *Pediculus humanus capitis* De Geer (Anoplura Pediculidae) (Lucia *et al*., 2017). Thus, the use of poloxamer 407 (P407), a triblock copolymer containing two lateral blocks of poly(ethylene oxide) and a central one of poly(propylene oxide), can be considered as a good alternative for obtaining a good EO encapsulation yield, with the copolymer acting as adjuvant in the formulation. At the best of our knowledge, there is no available data regarding bioactivity of polymeric nanoformulations based on a combination of monoterpenes against larvae of *Ae. aegypti* as alternative to synthetic insecticides. The goal of the present work was to develop binary nanoemulsions containing eugenol and thymol stabilized by a triblock copolymer to be applied against the larvae of *Ae. aegypti*. It is expected that the combination of monoterpenes can provide the basis for designing new, efficient formulations for insect-pest management.

Materials and methods

Chemicals

Eugenol (purity ≥ 99%), thymol (purity 99.5%) and P407, also known as Pluronic® F-127, were purchased from Sigma-Aldrich (Germany). P407 is a triblock copolymer formed for two lateral blocks of poly(ethylene oxide) (PEO) containing 101 monomers and a central block of poly(propylene oxide) (PPO) containing 56, leading to an average molecular formula $[PEO_{101}PPO_{56}PEO_{101}]$ and a molecular weight around 12.5 kDa. All chemicals were used as received without further purification. The molecular formulas for eugenol, thymol and P407 are shown in figure 1.

The water used for materials cleaning and samples preparation was ultrapure deionized water (Milli-Q water) obtained by a multicartridge purification system (Younglin 370 Series, South Korea). This water presents a resistivity higher than 18 MΩ cm and a total organic content lower than 6 ppm.

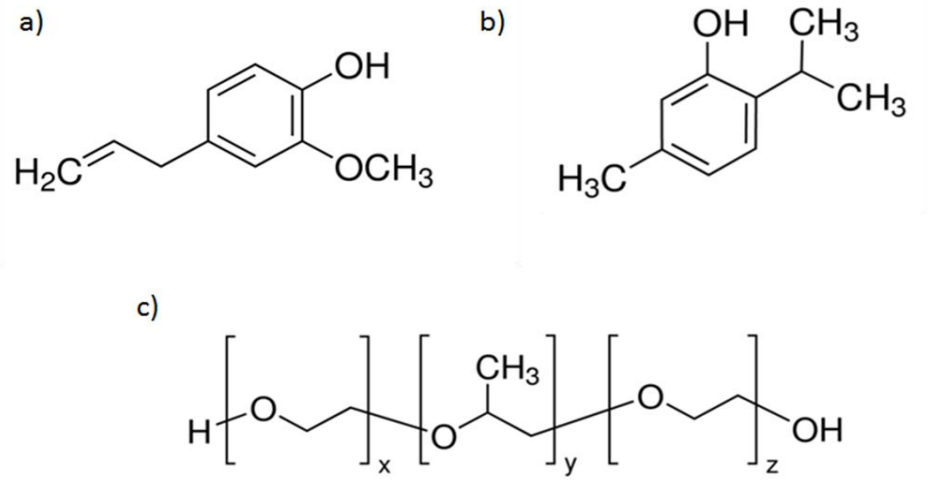

Figure 1. Molecular formulas of eugenol (a), thymol (b) and P407 (c).

Table 1. Composition of the different emulsions studied.

Nanoemulsion [a]	P407 (wt%)	Thymol (wt%)	Eugenol (wt%)	Water (wt%)
$E_{(100)}$	5	0 (0)	1.25 (1)	93.75
$T_{(25)}E_{(75)}$	5	0.3125 (0.25)	0.9375 (0.75)	93.75
$T_{(50)}E_{(50)}$	5	0.6250 (0.5)	0.6250 (0.5)	93.75
$T_{(75)}E_{(25)}$	5	0.9375 (0.75)	0.3125 (0.25)	93.75
$T_{(100)}$	5	1.25 (1)	0 (0)	93.75

[a] numbers in brackets indicate the weight fraction of each EO compounds in relation to the total composition of the oil phase (1.25 wt% of emulsion composition).

Samples preparation

Samples were prepared in tubular glass vials (10 mL) following a procedure adapted from our previous publication (Lucia *et al*., 2017). For this purpose, P407 aqueous solutions (concentration 10.25 wt%) were prepared by weight in a vial and then it were subjected to stirring at 500 rpm overnight to ensure copolymer solubilisation. Afterward, EO compounds, namely eugenol, thymol or their mixture, were poured into the vial to obtain a mixture with an EO concentration of 2.5 wt%. This process leads to the formation of opalescent dispersions, which are diluted by addition of Milli-Q water (1:1 ratio) to obtain the final transparent emulsion. The compositions of the different samples studied are summarized in table 1.

Size of the nanoemulsions droplets

The average particle size and the distribution of the particle size of each sample was determined using the dynamic light scattering (DLS), that estimates fluctuations in the intensity of light scattering following a Brownian motion of the particles (Berne and Pecora, 2013). Furthermore, DLS was also used for evaluating the stability of after 1 week and 28 months of aging.

Insects

A colony of *Ae. aegypti* (Rockefeller Strain, Venezuela) was used for bioassays. The colony was maintained since 2018, free of exposure to pathogens, insecticides, or repellents, at 25 ± 1 °C, 65-80% of relative humidity (RH), and a L12/D12 photoperiod in the bioterium of PIET-INEDES (CONICET-UNLu). All larval instars were fed on a mixture of rabbit pellets and yeast in a 3:1 proportion. Adult mosquitoes were fed on raisins, and a human arm was offered three times a week for females to produce their eggs.

Larvicidal bioassays

The larvicidal bioassays were performed following a method adapted from our previous study (Lucia *et al*., 2007). For this purpose, tested emulsions were added directly to drinking water. Different volumes (0.5 to 4 mL) of each emulsion were incorporated into drinking water to obtain a final volume of 225 mL in a 500-mL plastic jar. Then, 25 mL of water containing 20 late third-instar or early four-instar larvae of *Ae. aegypti* were added into the jar. As control, P407 aqueous solutions (concentration 10.25 wt%) were prepared by weight in a vial and mixed with drinking water. Food supply was offered *ad libitum* to larvae. The emulsions of EOs were tested at different final concentrations in a range of 1-250 ppm. All bioassays were performed in a laboratory under controlled conditions (25 ± 1 °C, 70 ± 5% RH and a L12/D12 photoperiod). Mortality was recorded after 24 hours exposure. The dead larvae in four tests were combined and expressed as a percentage of larval mortality for each concentration. Mortality criteria of larvae consisted of insects that failed to move or were unable to rise to the surface within a 1 minute or not showing the characteristic diving reaction when the water was disturbed (Lucia *et al*., 2008).

Statistical analysis

The percentage of larvae affected, and the percentage of mortality were determined and a Probit analysis was calculated to estimate lethal concentrations affecting 50% and 90% of the individuals (LC_{50} and LC_{90}). Lethal concentrations (LCs) correspond to the concentration of thymol, eugenol or their mixtures dispersed in the aqueous nanoemulsion and were expressed as parts per million in the final concentration (ppm). These values were considered statistically significant when the 95% confidence limits did not overlap. Data were processed using the PoloPlus v.2.0 software (LeOra Software, Berkley, CA).

Binary interaction of the nanoemulsions

For this purpose, a comparison of between the observed and theoretical LC_{50} values was performed. This latter was obtained following the description given by Wadley (1967):

$$\text{Theoretical } LC_{50} = \frac{a+b+c+....+n}{\frac{a}{LC_{50}(a)}+\frac{b}{LC_{50}(b)}+\frac{c}{LC_{50}(c)}+....+\frac{n}{LC_{50}(n)}}, \quad (1)$$

where the letters (from a to n) indicate the components involved in the mixture. The ratio between the theoretical and the experimental value of the LC_{50} [R = LC_{50} (theoretical) / LC_{50} (experimental)] was determined. According to Wadley (1967), R values > 1.5 indicate a synergistic interaction between the components of the mixtures, 1.5 ≥ R > 0.5 additive interaction and R values ≤ 0.5 indicate an antagonistic interaction.

Results and discussion

Characterization and stability of the nanoemulsions

The transparent character of the obtained dispersions enables their characterization using DLS, which allows one to estimate the size of the droplets as the apparent hydrodynamic diameter d_H^{app}. The corresponding d_H^{app}

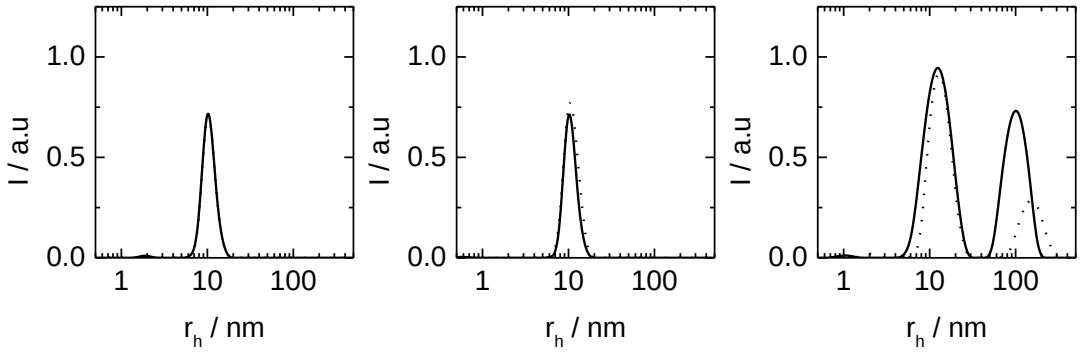

Figure 2. d_H^{app} Distributions for selected emulsions after 1 week (continuous lines) and 28 months (dotted lines) of their preparation: $E_{(100)}$ (left panel), $T_{(50)}E_{(50)}$ (central panel) and $T_{(100)}$ (right panel).

distributions obtained using DLS measurements are shown in figure 2. It is worth mentioning that the initial characterization of the obtained nanoemulsions was performed one week after their preparation in order to ensure that this emulsion had good stability and were not affected by any potential destabilization of the systems.

The results evidenced the monodisperse character (polydispersity index, PDI ~ 0.1) of emulsions with eugenol concentration > 0.5. On the other hand, the absence of eugenol (emulsion 100 wt% thymol) led to the appearance of polydisperse emulsions, in which droplets with two well separated d_H^{app}distributions, centred on 25 nm and 200 nm were found. This suggested that eugenol plays an important role for improving the dispersion of thymol in an aqueous medium, thus resulting in monodisperse droplets providing during the preparation process of emulsions an accurate environment for the solubilisation of the solid thymol (melting point around 50 °C) within the oil phase.

The results for the average d_H^{app} obtained from the analysis of the DLS experiments showed that in all the cases in which monodisperse emulsions were obtained, droplets with average sizes < 25 nm were found, with a slight increase with the thymol concentration. On the other hand, emulsions containing only thymol present an average d_H^{app} value for the smallest population above 25 nm. The increase of the width of the distribution (error bars) showed that the increase of the weight fraction of thymol increase the polydispersity of the droplets, confirming the worsening of the dispersibility of thymol when it becomes the main compound of the formulation. In addition to the simple characterization of the size of the obtained formulations, the development of systems with potential technological application also requires the existence of long-term stability. For this purpose, the stability of the emulsions after an aging of 28 months was evaluated for visual inspection and quantified by DLS. The visual inspection of emulsions did not show any evidence of macroscopic phase separation in the studied samples. However, we were unable to exclude that the formulations may undergo some destabilization process. In order to elucidate the role of the storage time on the formulation stability, DLS experiments of samples after 28 months of aging were performed. Figure 2 shows selected d_H^{app} distributions.

The analysis of the dependence of the average d_H^{app} on the weight fraction of the EO emulsions before and after aging showed that samples containing eugenol as main component did not present any significant change on their homogeneity after 28 months of aging, maintaining their monodisperse character and a constant droplets size (figure 3). Furthermore, for emulsions containing a weight fraction of 0.25 of eugenol in the oil phase, it was found the disappearance of the d_H^{app} distribution related to the biggest droplets, and the narrowing of the size distribution with aging by a factor of 2. This might be a signature of the homogenization of the size of the emulsion drops to reach the steady state. On the other hand, the d_H^{app} values for emulsions containing only thymol as oil phase evidenced a poorer stability in comparison to those containing eugenol, with the increase of the average d_H^{app} of the droplets size (and more significant on droplets of the biggest size). This may be associated with the instability of these emulsions that favoured the droplet coalescence and the increase of its average size, and that may be considered as a further evidence of the important role of eugenol as a facilitating agent for the dispersion of thymol in aqueous medium.

Larvicidal activity against *Ae. aegypti*

The toxicity of the emulsions on *Ae. aegypti* larvae is shown in table 2. The lethal concentration affecting 50% and 90% of the exposed larvae (LC_{50} and LC_{90}) ranged approximately from 11 to 90 ppm and from 28 to 118 ppm, respectively. All the tested emulsions produced acute toxicity in mosquito larvae. However, significant differences were found in their performance.

The results showed that emulsions with thymol as main component of the oil phase had higher larvicidal activity than those containing eugenol as main component. For emulsions with an oil phase formed exclusively by thymol (T_{100}), LC_{50} was estimated to be 11.1 ppm, which decreased by a factor close to 3 (27.5 ppm) when eugenol was included at 25% ($T_{75}E_{25}$). On the other side, when eugenol was incorporated into the nanoemulsions at a concentration of 50% ($T_{50}E_{50}$) and 75% ($T_{25}E_{75}$) of the oil mixture, the LC_{50} decrease by a factor of 4 and 5 respectively, in comparison to the emulsion containing only thymol, without any significant difference in their activity with the eugenol content. Thus, the larvicidal activity is stronger in nanoemulsions containing only thymol, while the complete substitution of thymol for eugenol drive to a worsening of the effectivity of the formulations against *Ae. aegypti* larvae.

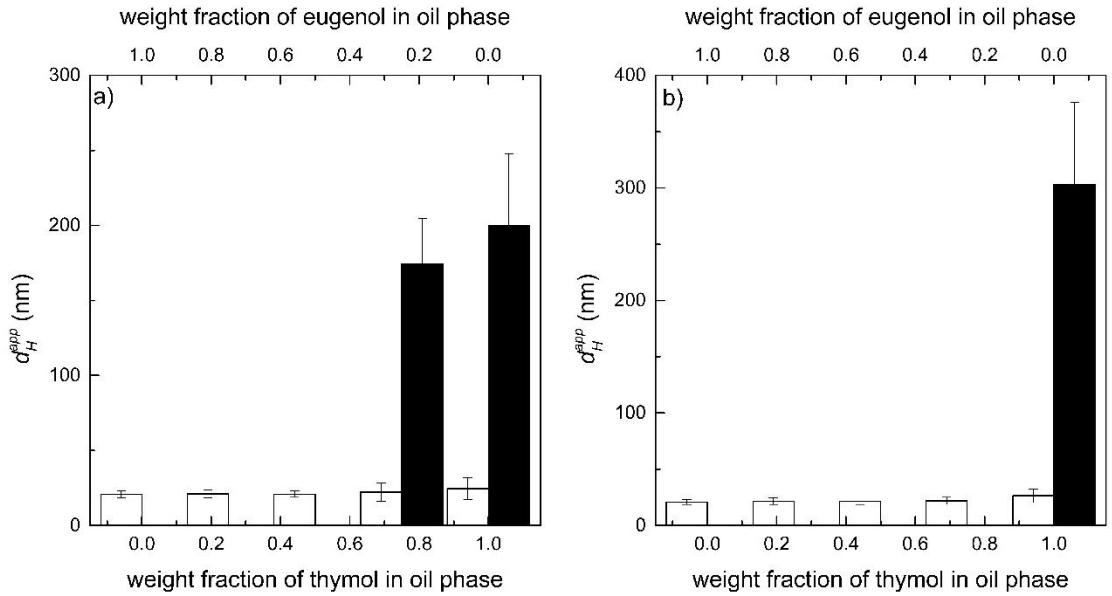

Figure 3. Dependence of the average d_H^{app} for the different distributions obtained from the analysis of DLS experiments on the weight fraction of each EO compound, namely eugenol or thymol, in the oil phase for the studied emulsions after 1 week (a) and 28 months (b) of their preparation. In both panels, the white bars correspond to the distribution with the smallest droplets size and the black ones correspond to the distribution with the highest droplet size in those case in which such second distribution was found. Notice that the error bars in this case provides information about the width of the d_H^{app} distributions.

Table 2. Larvicidal activity of emulsions of thymol, eugenol and their mixtures against larvae of *Aedes aegypti*.

Emulsion [a]	Observed LC [b]		Slope (± SE)	χ^2	df	n	Theoretical LC_{50} [c] (ppm)	R [d]
	LC_{50}, ppm (95% CI)	LC_{90}, ppm (95% CI)						
$E_{(100)}$	89.4 (85.5-93.6)	117.8 (109.9-131.2)	10 ± 2	30.2	22	480	89.4	1
$T_{(25)}E_{(75)}$	51.3 (46.1-56.2)	81.8 (73.3-95.7)	6.3 ± 0.8	29.9	18	400	29.2	0.6
$T_{(50)}E_{(50)}$	45.1 (40.9-50.5)	67.9 (58.7-88.0)	7.0 ± 1	41.1	18	400	15.9	0.4
$T_{(75)}E_{(25)}$	27.5 (24.1-31.0)	49.3 (42.3-61.6)	5.1 ± 0.7	19.3	14	320	12.4	0.4
$T_{(100)}$	11.1 (9.3-13)	28.4 (23.6-35.8)	4.5 ± 0.6	34.2	22	480	11.1	1

[a] Numbers in brackets indicate the weight fraction of each EO compound (T: Thymol and E: Eugenol) in relation to the total composition of the oil phase (1.25 wt% of emulsion composition concentration); [b] LC_{50} and LC_{90} obtained by probit analysis with a confidence interval (CI) at 95%); [c] Wadley's calculation of expected LC_{50} calculated according equation (3); [d] Synergy ratio from Wadley's calculation: $R > 1.5$ indicates synergistic interaction, $1.5 \geq R > 0.5$, additive interaction and $R \leq 0.5$, indicates an antagonistic interaction; n: Number of larvae tested for each emulsion (mixture), each assay was independently repeated 4 times in group of 20 larvae but the tested concentrations differ according to the mixture.

On the basis of the obtained results, it was possible to determine the type of interactions between thymol and eugenol in their mixtures. The results showed than in most of the cases, the mutual interaction between eugenol and thymol had an antagonist effect in relation to the pure components, and presented only a significant additive effect in terms of larvicidal activity, when eugenol was the main component ($T_{25}E_{75}$).

According to Pavela (2015), EOs with $LC_{90} < 100$ ppm should be considered as highly promising alternatives for developing plant-borne larvicides against mosquitoes. Thus, most of studied combinations evaluated here can be considered as highly effective larvicides, as their LC_{90} values were in most cases below 100 ppm. It is worth mentioning that the studied emulsions do not evidence any pupicidal activity, which may be explained by the fact that pupae are more resistant than larvae, LC_{50} being between 10 and 100 fold higher than for larvae (Andrade-Ochoa *et al.*, 2018). It is important to mention that for the solution of P407 alone the LC_{50} value for pupae was 570 ppm.

In summary, this work focused on the preparation of emulsions of eugenol and thymol in water using as stabilizing agent poloxamer P407. DLS experiments showed that the presence of eugenol facilitated the dispersion of thymol in water as monodisperse emulsion with good long-term stability (28 months). This lead to new possibilities for engineering this type of system in the design of new ready-to-use formulations for insect pest control, with long-term stability that can be stored for more than 2 years without any significant modification. This combination with their aqueous based character provides the bases for the application of such systems. Furthermore, high water content facilitates the availability of the formulation in the environment by reducing the risks and hazard for either human or animals and plants. On the other side, the simplicity of the methodology used for the preparation of the formulations provides further advantages for the development of these formulations, limiting the use of complex machinery, e.g. high pressure homogenizer, and facilitating the *in situ* preparation of the formulations which reduces the costs associated with their preparation and transport. Emulsions containing thymol were found to be effective against *Ae. aegypti* larvae, with a LC_{90} lower than 100 ppm. This makes them promising alternatives for the development of botanical-derived larvicides. It is worth mentioning that the introduction of eugenol in emulsions facilitate the dispersion of thymol in water, even though it reduces the effectiveness of the emulsions. It is therefore necessary to optimize the formulations in order to ensure the good dispersion of thymol, with the highest insecticidal activity, without compromising the activity of the obtained emulsions.

As mentioned by Isman (2008), developing countries impose less restrictive regulatory constraints so hazard for human pesticide poisonings are most prevalent. However, the relative high costs of conventional mosquitocidals (larvicides and adulticides) and the supplying problems of these products led to interruption of vector control programs. Because of it, there is a great need for the development of new larvicides easily available in developing countries and less toxic to both humans and non-target organisms (Benelli, 2016; Benelli and Mehlhorn, 2016; Masetti, 2016). Most of the research on the impact of EOs on non-target animals were performed on fishes (Govindarajan *et al*., 2016; Pavela and Govindarajan, 2016; Baskar *et al*., 2018), and very little on arthropods. This latter might be due to the mode of action of EOs and their compounds targeting several sites of the nervous system like acetylcholinesterase (AChE), GABA and octopamine receptors (Enan, 2001; Picollo *et al*., 2008; Regnault-Roger *et al*., 2012). Since these receptors are present in all arthropods, there are few chances to find high level of selectivity of EO in relation to non-targeted arthropods (Masetti, 2016).

The toxicity of *Origanum scabrum* Boiss. et Heldr. (Lamiaceae) whose main constituents were carvacrol and thymol were tested on vector mosquitoes, two mosquito predators and a fish (Govindarajan *et al*., 2016). The authors found that the EO was less harmful to the non-target organism in comparison to mosquito species. In addition, the swimming behaviour and survival of the *Dyplonichus indicus* Venkatesan et Rao (Hemiptera Belostomatidae), *Anisops bouvieri* Kirkaldy (Hemiptera Notonectidae) and *Gambusia affinis* (Baird et Girard) (Cyprinodontiformes Poeciliidae) were not altered after the exposure at LC_{50} and LC_{90} values of the EO. Another study analyzed the toxicity of the EO *Atalantia monophillia* (L.) (Rutaceae), whose main constituents were eugenol and sabinene, on three mosquito vectors and non-target zebrafish *Danio rerio* (Hamilton) (Cypriniformes Cyprinidae) (Baskar *et al*., 2017). The EO showed a high larvicidal and repellent activity against the mosquitoes and a limited impact on zebrafish.

In 2013, a vegetation spray containing 1% eugenol in a sugar solution was introduced for the control of mosquitoes in the USA as Terminix AllClear®, an attractive targeted sugar bait (ATSB) (Entwistle, 2014). Qualls *et al.* (2014) demonstrated that a single application of the product on vegetation around a golf course centre in Florida reduced the density of the most abundant mosquito specie, *Anopheles crucians* Wiedemann, over a 4-week period and had no significantly effect on non-target organisms.

Concerning studies of the toxicity of eugenol and thymol against non-target arthropods, thymol seemed to be relatively safe to adult bees when compared with synthetic pesticides (Gashout and Guzmán-Novoa, 2009). For example, Albo *et al*. (2003) reported that thymol was 50 times less toxic to workers bees than the organophosphate dimethoate, and Iwasa *et al.* (2004) demonstrated LC_{50} values < 15 µg/bee for neonicotinoid insecticides. These results suggest that the potential use of this compound in bee colonies would not result in higher toxicity levels to bees and larvae than those from the most employed commercial synthetic varroacides worldwide (Gashout and Guzmán-Novoa, 2009).The high effectivity of thymol controlling *Varroa jacobsoni* Oudemans (Acari Varroidae) has resulted in the development of several commercial varroacidal formulations (Imdorf *et al*., 1999). In addition, clove oil and its major constituent eugenol have shown high effective acaricidal properties against *Varroa destructor* Anderson et Trueman (Acari Varroidae) mites that infest honey bees (*Apis mellifera* L.) colonies (Maggi *et al*., 2010).

The tested emulsions herein were highly toxic to *Ae. aegypti* larvae, but both thymol and eugenol are considered safe and are exempted from the requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA-EPA) regulations. Moreover, both constituents have a 'Food and Agriculture Organization Generally Recognized as Safe (for human consumption) (FAO-GRAS)' status at a concentration up to 50 mg/kg. According to EU regulation No. 2377/90, eugenol is within group II of the non-toxic veterinary drugs, which do not require a maximum residue limit (MRL) (Girisgin *et al*., 2014). However, if promising larvicidal effectiveness is demonstrated for an EO or its constituents for which health hazards were not established, specific ecotoxicological assays should be carried out before starting any field trials (Masetti, 2016).

Therefore, the study and development of new EOs formulations with enhanced half-life and solubilisation should be further carried out. The EOs applications against mosquito larvae could be a valid alternative to conventional insecticides treatments hazardous to the environment and humans.

Acknowledgements

Alejandro Lucia is especially grateful to the Research Institute of Theoretical and Applied Physical Chemistry for the use of their services (Dynamic Light Scattering (DLS) experiments) (INIFTA/UNLP-CONICET), La Plata, Argentina. We also are grateful to C.A.I. de Espectroscopía y Correlación of the Universidad Complutense de Madrid, for the use of their facilities. Francisco Ortega, Ramón G. Rubio and Eduardo Guzmán carried out this research with the financial support of Programa Estatal de Investigación, desarrollo e innovación orientada a los retos de la sociedad with the Project title "Propiedades de nuevos sistemas nanoestructurados de importancia tecnológica" (CTQ2016-78895-R), and Alejandro Lucia and Ariel Toloza were funded by Agencia Nacional de Promoción Científica y Técnica (ANPCyT) under grant PICT 2016-1111 entitled "Nuevas formulaciones biocompatibles de origen botánico para el control de piojos de la cabeza resistentes a permetrina: un enfoque integral".

References

ALBO G. N., HENNING C., RINGUELET J., REYNALDI F. J., DE GIUSTI M. R., ALIPPI A. M., 2003.- Evaluation of some essential oils for the control and prevention of American foulbrood disease in honey bees.- *Apidologie*, 34: 417-427.

ANDRADE-OCHOA S., SÁNCHEZ-ALDANA D., CHACÓN-VARGAS K. F., RIVERA-CHAVIRA B. E., SÁNCHEZ-TORRES L. E., CAMACHO A. D., NOGUEDA-TORRES B., NEVÁREZ-MOORILLÓN G. V., 2018.- Oviposition deterrent and larvicidal and pupaecidal activity of seven essential oils and their major components against *Culex quinquefasciatus* Say (Diptera: Culicidae): synergism-antagonism effects.- *Insects*, 9: 25.

BASKAR K., SUDHA V., NATTUDURAI G., IGNACIMUTHU S., DURAIPANDIYAN V., JAYAKUMAR M., AL DHABI N. A., BENELLI G., 2018.- Larvicidal and repellent activity of the essential oil from *Atalantia monophylla* on three mosquito vectors of public health importance, with limited impact on non-target zebra fish.- *Physiological and Molecular Plant Pathology*, 101: 197-201.

BATRAKOVA E. V., BRONICH T. K., VETRO J. A., KABANOV A. V., 2006.- Polymer micelles as drug carriers, pp. 5-13. In: *Nanoparticulates as drug carriers* (TORCHILIN V. P., Ed.).- Imperial College, London, UK.

BATRAKOVA E. V., KABANOV A. V., 2008.- Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers.- *Journal of Controlled Release*, 130: 98-106.

BENELLI G., 2016.- Plant-mediated synthesis of nanoparticles: a newer and safer tool against mosquito-borne diseases?- *Asian Pacific Journal of Tropical Biomedicine*, 6: 353-354.

BENELLI G., MEHLHORN H., 2016.- Declining malaria, rising dengue and Zika virus: insights for mosquito vector control.- *Parasitology Research*, 115:1747-1754.

BERNE B. J., PECORA R., 2013.- *Dynamic light scattering: with applications to chemistry, biology, and physics*.- Dover Publications Inc., Mineola, USA.

BOYCE R., LENHART A., KROEGER A., VELAYUDHAN R., ROBERTS B., HORSTICK .O., 2013.- *Bacillus thuringiensis israelensis* (BTI) for the control of dengue vector: systematic literature review.- *Tropical Medicine & International Health*, 18: 564-577.

CAMPOLO O., CHERIF A., RICUPERO M., SISCARO G., GRISSA-LEBDI K., RUSSO A., CUCCI L. M., DI PIETRO P., SATRIANO C., DESNEUX N., BIONDI A., ZAPPALÀ L., PALMERI V., 2017.- Citrus peel essential oil nanoformulations to control the tomato borer, *Tuta absoluta*: chemical properties and biological activity.- *Scientific Reports*, 7: 13036.

CARVALHO L. A. D. F., SILVA I. G. D., 2000.- Longitudinal evaluation of the activity of 1% temephos on *Aedes aegypti* (Lin., 1762).- *Entomologia y Vectores*, 7 (2): 191-201.

CHEN C. D., LEE H. L., CHAN C. K., ANG C. L., AZAHARI A. H., LAU K. W., 2009.- Laboratory bioefficacy of nine commercial formulations of temephos against larvae of *Aedes aegypti* (L.), *Aedes albopictus* Skuse and *Culex quinquefasciatus* Say.- *Tropical Biomedicine*, 26 (3): 360-365.

ENAN P., 2001.- Insecticidal activity of essential oils: octopaminergic sites of action.- *Comparative Biochemistry and Physiology, C*, 130: 325-337.

ENTWISTLE J., 2014.- Emerging technologies for urban mosquito management, pp. 23-42. In: *Urban insect pests: sustainable management strategies* (DHANG P., Ed.).- CABI, Wallingford, UK.

FARAONE N., HILLIER N. K., CUTLER G. C., 2015.- Plant essential oils synergize and antagonize toxicity of different conventional insecticides against *Myzus persicae* (Hemiptera: Aphididae).- *PLoS ONE*, 10 (5): e0127774.

FERREIRA-DE-BRITO A., RIBEIRO I. P., MIRANDA R. M., FERNANDES R. S., CAMPOS S. S., SILVA K. A. B., CASTRO M. G., BONALDO M. C., BRASIL P., LOURENÇO-DE-OLIVEIRA R., 2016.- First detection of natural infection of *Aedes aegypti* with Zika virus in Brazil and throughout South America.- *Memorias do Instituto Oswaldo Cruz*, 111: 655-658.

GASHOUT H. A., GUZMÁN-NOVOA E., 2009.- Acute toxicity of essential oils and other natural compounds to the parasitic mite, *Varroa destructor*, and to larval and adult worker honey bees (*Apis mellifera* L.).- *Journal of Apicultural Research*, 48 (4): 263-269.

GIRISGIN A. O., BAREL S., ZILBERMAN BARZILAI D., GIRISGIN O., 2014.- Determining the stability of clove oil (Eugenol) for use as an acaricide in beeswax.- *Israel Journal of Veterinary Medicine*, 69 (4): 192-196.

GODÓI I. P., LEMOS L. L., ARAÚJO V. E., BONOTO B. C., GODMAN B., GUERRA JÚNIOR A. A., 2017.- CYD-TDV dengue vaccine: systematic review and meta-analysis of efficacy, immunogenicity and safety.- *Journal of Comparative Effectiveness Research*, 6 (2): 165-80.

GOVINDARAJAN M., KADAIKUNNAN S., ALHARBI N. S., BENELLI G., 2016.- Acute toxicity and repellent activity of the *Origanum scabrum* Boiss. & Heldr. (Lamiaceae) essential oil against four mosquito vectors of public health importance and its biosafety on non-target aquatic organisms.- *Environmental Science and Pollution Research International*, 23: 23228-23238.

GROSS A. D., NORRIS E. J., KIMBER M. J., DUNPHY B., BARTHOLOMAY L. C., COATS J. R., 2017.- Essential oils enhance the toxicity of permethrin against *Aedes aegypti* and *Anopheles gambiae*.- *Medical and Veterinary Entomology*, 31: 55-62.

GUBLER D. J., CLARK G. G., 1995.- Dengue/dengue hemorrhagic fever. The emergence of a global health problem.- *Emerging Infectious Diseases*, 1: 55-57.

IMDORF A., BOGDANOV S., IBÁÑEZ OCHOA R., CALDERONE N. W., 1999.- Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies.- *Apidologie*, 30 (2-3): 209-228.

ISMAN M. B., 1999.- Pesticides based on plant essential oils.- *Pesticide Outlook*, 10: 68-72.

ISMAN M. B., 2008.- Botanical insecticides: for richer, for poorer.- *Pest Management Science*, 64: 8-11.

ISMAN M. B., GRIENEISEN M. L., 2014.- Botanical insecticide research: many publications, limited useful data.- *Trends in Plant Science*, 19: 140-145.

IWASA T., MOTOYMA N., AMBROSE. J. T., ROE R. M., 2004.- Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*.- *Crop Protection*, 23: 371-378.

KHADKA P., RO J., KIM H., KIM I., KIM J., KIM H., CHO J., YUN G., LEE J., 2014.-Pharmaceutical particle technologies: an approach to improve drug solubility, dissolution and bioavailability.- *Asian Journal of Pharmaceutical Sciences*, 9: 304-316.

KIM S., PARK K., 2010.- Polymer micelles for drug delivery, pp. 513-551. In: *Targeted delivery of small and macromolecular drugs* (NARANG A. S., MAHATO R. I., Eds).- CRC Press, Boca Ratón, USA.

LAU K. W., CHEN C. D., LEE H. L., NORMA-RASHID Y., SOFIAN-AZIRUN M., 2015.- Evaluation of insect growth regulators against field-collected *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) from Malaysia.- *Journal of Medical Entomology*, 52: 199-206.

LUCIA A., GONZALEZ AUDINO P., SECCACINI E., LICASTRO S., ZERBA E., MASUH H., 2007.- Larvicidal effect of *Eucalyptus grandis* essential oil and turpentine and their major components on *Aedes aegypti* larvae.- *Journal of the American Mosquito Control Association*, 23: 299-303.

LUCIA A., LICASTRO S., ZERBA E., MASUH H., 2008.- Yield, chemical composition andbioactivity of essential oils from twelve species of *Eucalyptus* on *Aedes aegypti* (L.) larvae (Diptera: Culicidae).- *Entomologia Experimentalis et Applicata*, 129: 107-114.

LUCIA A., TOLOZA A. C., GUZMÁN E., ORTEGA F., RUBIO R. G., 2017.- Novel polymeric micelles for insect pest control: encapsulation of essential oil monoterpenes inside a triblock copolymer shell for head lice control.- *Peer J*, 5: e3171.

MAGGI M. D., RUFFNENGO S. R., GENDE L. B., SARLO E. G., EGUARAS M. J., BAILAC P. N., PONZI M. I., 2010.- Laboratory evaluations of *Syzygium aromaticum* (L.) Merr. et Perry essential oil against *Varroa destructor*.- *Journal of Essential Oil Research*, 22: 119-122.

MASETTI A., 2016.- The potential use of essential oils against mosquito larvae: a short review.- *Bulletin of Insectology*, 69 (2): 307-310.

MASUH H., 2008.- Re-emergence of dengue in Argentina: historical development and future challenges.- *Dengue Bulletin*, 32: 44-54.

MORETTI M. D. L., SANNA-PASSINO G., DEMONTIS S., BAZZONI E., 2002.- Essential oil formulations useful as a new tool for insect pest control.- *AAPS PharmSciTech*, 3: 13.

MOSSA A. T. H., 2016.- Green pesticides: essential oils as biopesticides in insect-pest management.- *Journal of Environmental Science and Technology*, 9 (5): 354-378.

NORRIS E. J., GROSS A. D., DUNPHY B., BARTHOLOMAY L. C., COATS J. R., 2015.- Comparison of the insecticidal characteristics of commercially available plant essential oils against *Aedes aegypti* and *Anopheles gambiae*.- *Journal of Medical Entomology*, 52: 993-1002.

PAVELA R., 2015.- Essential oils for the development of ecofriendly mosquito larvicides: a review.- *Industrial Crops and Products*, 76: 174-187.

PAVELA R., GOVINDARAJAN M., 2016.- The essential oil from *Zanthoxylum monophyllum* a potential mosquito larvicide with low toxicity to the non-target fish *Gambusia affinis*.- *Journal of Pest Science*, 90: 369-378.

PAVONI L., PAVELA R., CESPI M., BONACUCINA G., MAGGI F., ZENI V., CANALE A., LUCCHI A., BRUSCHI F., BENELLI G., 2019.- Green micro- and nanoemulsions for managing parasites, vectors and pests.- *Nanomaterials*, 9: 1285.

PICOLLO M. I., TOLOZA A. C., MOUGABURE CUETO G., ZYGADLO J., ZERBA E., 2008.- Anticholinesterase and pediculicidal activities of monoterpenoids.- *Fitoterapia*, 79: 271-278.

QUALLS W. A., MÜLLER G. C., REVAY E. E., ALLAN S. S., ARHEART K. L, BEIER J. C., SMITH M. L., SCOTT J. M., KRAVCHENKO V. D., HAUSMANN A., YEFREMOVA Z. A., XUE R-D., 2014.- Evaluation of attractive toxic sugar bait (ATSB)-barrier for control of vector and nuisance mosquitoes and its effect on nontarget organisms in sub-tropical environments in Florida.- *Acta Tropica*, 131: 104-110.

RAGAEI M., SABRY A. K. H., 2014.- Nanotechnology for insect pest control.- *International Journal of Science, Environment and Technology*, 3: 528-545.

REGNAULT-ROGER C., VINCENT C., ARNASON J. T., 2012.- Essential oils in insect control: low-risk products in a high-stakes world.- *Annual Review of Entomology*, 57: 405-24.

RITCHIE S. A., RAPLEY L. P., BENJAMIN S., 2010.- *Bacillus thuringiensis* var. *israelensis* (Bti) provides residual control of *Aedes aegypti* in small containers.- *The American Journal of Tropical Medicine and Hygiene*, 82 (6):1053-1059.

SAMAR S. I., 2019.- Essential oil nanoformulations as a novel method for insect pest control in horticulture, In: *Horticultural crops* (BAIMEY H. K., HAMAMOUCH N., KOLOMBIA Y. A., Eds).- IntechOpen, London UK.

SAVJANI K. T., GAJJAR A. K., SAVJANI J. L., 2012.- Drug solubility: importance and enhancement techniques.- *ISRN Pharmaceutics*, 2012: 195727.

TISSERAND R., BALACS T., 1995.- *Essential oils safety: a guide for health care professionals*.- Churchill Livingstone, London, UK.

TONG F., BLOOMQUIST J. R., 2013.- Plant essential oils affect the toxicities of carbaryl and permethrin against *Aedes aegypti* (Diptera:Culicidae).- *Journal of Medical Entomology*, 50: 826-832.

WADLEY F. M., 1967.- *Experimental statistics in entomology*.- Graduate School Press, U.S.D.A Washington, USA.

WEAVER S. C., COSTA M. F., GARCIA-BLANCO A., KO A. I., RIBEIRO G. S., SAADE G., SHI P. Y., VASILAKIS N., 2016.- Zika virus: history, emergence, biology, and prospects for control.- *Antiviral Research*, 130: 69-80.

WILLIAMS A. C., BARRY B. W., 1991.- Terpenes and the lipid-protein-partitioning theory of skin penetration enhancement.- *Pharmaceutical Research*, 8: 17-24.

Authors' addresses: Alejandro LUCIA (corresponding author: luciaalejandro@yahoo.com.ar), Carlos COVIELLA, Instituto de Ecología y Desarrollo Sustentable (INEDES, CONICET-UNLu), ruta 5 y avenida Constitución, 6700 Luján, Buenos Aires, Argentina; Ariel Ceferino TOLOZA, Centro de Investigaciones de Plagas e Insecticidas (UNIDEF/CONICET), San Juan Bautista de La Salle 4397, B1603ALO Villa Martelli, Buenos Aires, Argentina; Micaela FANUCCE, Centro Nacional de Diagnóstico e Investigaciones Endemo-epidémicas (CeNDIE), Administración Nacional de Laboratorios e Institutos de Salud (ANLIS), Buenos Aires, Argentina; Laura FERNÁNDEZ-PEÑA, Francisco ORTEGA, Ramón G. RUBIO, Eduardo GUZMÁN, Departamento de Química Física, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, Ciudad Universitaria s/n., 28040 Madrid, Spain.

Received December 2, 2019. Accepted April 3, 2020.