Quantification through TLC-Densitometric analysis, repellency and anticholinesterase activity of the homemade extract of Indian cloves

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ABSTRACT: The rise of mosquito's transmitted diseases, like dengue, zika and chikungunya in Brazil in the last years has increased the concerns on protection against mosquito's bites. However, the prohibitive prices of the commercially available repellents for the majority of the Brazilian population, has provoked a search for cheaper solutions, like the use of the homemade ethanolic extract of Indian clove (*Syzygium aromaticum L.*) as repellent, which has been reported as quite efficient by the local press. In order to verify this, we performed here, the quantification of the main components of this extract through high-performance thin-layer chromatography (HPTLC)-densitometry and evaluated its efficiency as repellent, as well as the acetylcholinesterase (AChE) inhibition capacity. Our results have proved HPTLC-densitometry as an efficient and appropriate method for this quantification and confirmed the repellency activity as well as its capacity of AChE inhibition.

Keywords: Syzygium aromaticum L., insect repellent, eugenol, anticholinesterase, Aedes aegypti

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INTRODUCTION

Indian clove (*Syzygium aromaticum L.*) is a plant from the *Myrtaceae* family which dried flower buds are quite appreciated in the world culinary, as an aromatic condiment. In the popular medicine these buds are used, after decoction, as diuretics and to treat asthma, allergies, stomachache and toothache (Kim et al., 1998).

In Brazil the flower buds, acquired in popular markets, are also used in the preparation of homemade repellents. The buds are usually macerated in ethanol for 4 to 10 days and the filtrate applied over the skin to protect against mosquitos bytes, mainly the *Aedes aegypti (Affonso et al., 2012).*

The ethanolic extract presents an intense smell and corresponds to 14-21% of the chemical content of the flower buds (Evans, 2002). According to the extraction method and the origin of the flower bud, the percentage of each component can change and, consequently, the intensity of the biological response. Its main components (Fig. 1) are eugenol (major component), eugenyl acetate and β-caryophyllene (Alma *et al*., 2007). Several biological activities have been observed for this extract such as antioxidant (Viuda-Martos *et al*., 2010), antineoplasic (Dwivedil *et al*., 2011), antiviral (Khan *et al*., 2009), anesthesic (Alma *et al*., 2007) and antimicrobial (Fu *et al*., 2007; Chaieb *et al*., 2009), as well as cytotoxicity to human skin cells (Prashar *et al*., 2006). Many of these activities are attributed to eugenol, which pharmacological effects have been intensively studied (Kong *et al*., 2014). Also, there are reports of antioxidant (Lee & Shibamoto, 2001), insecticide (Kafle & Shih, 2013), antiviral (Musthafa & Voravuthikunchai, 2016) and antibacterial (Musthafa & Voravuthikunchai, 2016) activities of eugenyl acetate.

The classic methods of extracts quantification are based on non-aqueous titration, gas chromatography and high efficiency liquid chromatography (Ghani & Khan, 2015), which present a high operational cost. Here we report the simultaneous quantification of eugenol

and eugenyl acetate (Fig. 1) in the ethanolic extract of flower buds of *Syzygium aromaticum* (*S. aromaticum*) through high-performance thin-layer chromatography (HPTLC)-densitometry, an efficient analytical tool, with low operational cost and simple protocol for sample preparation, requesting minimal amounts of sample (Minz, Kaurav, Sahu, Mandal, & Pandey, 2015). This method is, also, able to run several samples simultaneously with a small amount of mobile phase, reducing the time and cost of analysis (El-Kafrawy & Belal, 2016). As far as we know, no other TLC-densitometric methods have been reported for simultaneous quantification of eugenol and eugenyl acetate. TLC has been reported in literature only for quantification of eugenyl acetate (Rana et al, 2011; Srivastava, 1991; Annegowda et al., 2013).

The ethanolic extract was prepared according to its popular use in Brazil for repellency purposes. Also, to check the efficacy of this preparation, we evaluated the anticholinesterase and repellence activities of the extract quantified through densitometry.

FIGURE 1. Molecular structures of Eugenol (E), Eugenyl Acetate (EA) and β-caryophyllene (BC).

EXPERIMENTAL

Chemicals

Flower buds were acquired from a local marked in Rio de Janeiro, characterized and quantified as described by Affonso *et al*., (2014). The standards for chromatography of eugenol (purity > 99%) and eugenyl acetate (purity > 98,5%) were acquired from Sigma-Aldrich (Rio de Janeiro, Brazil). The solvents used (ethanol and toluene) were of spectroscopic grade.

Preparation of the sample solution

The sample was obtained by static maceration (96h) of 3.02g of Indian clove and 15 mL of ethanol, as described by Affonso *et al*. (2014). An aliquot of 0.1mL was transferred to a volumetric flask which had its volume completed to 10.00mL with ethanol, for densitometric analysis.

Standard mix solution preparation

A stock solution of eugenol and eugenyl acetate standards at 900 and 100 µg.mL^{-1} respectively was prepared through dissolution of 22.5 mg of eugenol and 2.5 mg of eugenyl acetate in ethanol in a volumetric flask of 25 mL. Aliquots (0.2, 0.4, 0.6, 0.8 and 1.0 mL) from this stock solution were transferred to volumetric flasks of 1 mL, which had the volume completed to 1 mL with ethanol, for each aliquot, in order to obtain solutions at the following concentrations: 180, 360, 540, 720 and 900 µg.mL^{-1} for eugenol and 20, 40, 60, 80, and 100 µg.mL^{-1} for eugenyl acetate. The proportions eugenol:eugenyl acetate in each final solution were, therefore: 18:2; 36:4; 54:6; 72:8; 90:10 µg.mL^{-1}, respectively.

Calibration curve for eugenol and eugenyl acetate

For evaluation of the calibration curve, 10µL of each concentration of the mix standard solution was applied in replicate (n = 3, band width: 5 mm, distance between the bands: 5 mm) on a pre-coated silica gel 60 F_{254} 0.2 mm (E. Merck) using ATS4 CAMAG applicator. The plate was developed in a solvent system of toluene:methanol (9:1, v/v) in a CAMAG glass chamber (20x10 cm) up to a distance of 4 cm. After development the plate was dried at room temperature (25ºC) and scanned at 278nm using a CAMAG TLC Scanner 3 and WinCats 1.4.4® software. The peak areas were recorded. Calibration curves of eugenol and eugenyl acetate were obtained by plotting peak areas vs applied concentrations of standard mix solutions.

Method validation

The validation of the method was performed by following the parameters of instrumental precision, stability, linearity range, specificity, accuracy, robustness and sensibility, in accordance with the ICH guideline (ICH, 2005), the AOAC international-Eurachem guide (AOAC, 2017) and the literature (Shabir, 2003; Fraley, 2011).

Instrumental precision

The instrument precision was checked by repeated scanning of the same spot of standard mix solutions six times and was expressed as coefficient of variance (% CV) (ICH, 2005; AOAC, 2017; Shabir, 2003).

Stability

The stability of the method was evaluated based on the application of standard and sample (extracts) in the chromatographic plate, eluting and analyzing at 190 nm for 0, 6, 12, 18, 24, 36 and 48 hours (ICH, 2005; AOAC, 2017; Shabir, 2003).

Linearity range

The standard stock solution was applied in five concentrations (eugenol:eugenyl acetate): 18:2; 36:4; 54:6; 72:8; 90:10 $\mu g.mL^{-1}$. Each of the five standards was applied in triplicate and analyzed three times. Calibration curves were constructed by plotting the absorbance against the concentration using linear regression calibration curve (ICH, 2005; AOAC, 2017; Shabir, 2003).

Specificity

The specificity of the method was verified through the calculation of the spectral purity of the signal between the analyte and its respective standard (ICH, 2005; AOAC, 2017; Shabir, 2003), using the software WinCats 1.4.4®.

Accuracy

To assess the method's accuracy, the reading was held in replicates (n = 6) with the same application in 6 different times (0, 6, 12, 24, 36 and 48h). The results were evaluated by analysis of variance (ICH, 2005; AOAC, 2017; Shabir, 2003).

Robustness

Robustness was determined during the method development based on a few changes in mobile phase (± 5% of methanol volume). The plates were activated in stove (± 100°C) for 30 minutes before analysis; the development distance was of ± 50 mm and the duration of the saturation was of ± 10min. The values obtained were statistically evaluated by t-student test (Fraley, 2011; ICH, 2005; AOAC, 2017; Shabir, 2003).

Sensibility

For evaluation of the sensibility the values of limit of detection (LD) and limit of quantification (LQ) were calculated as described in the ICH guide (ICH, 2005) and shown in equations 1 and 2.

$$LD = \frac{(Standard deviation of the intercept \times 3)}{curve slope} \quad (1)$$

$$LQ = \frac{(Standard deviation of the intercept \times 10)}{curve slope} \quad (2)$$

Biological activities

Repellency

Larvae and mosquitos selection

The repellency tests followed the guidelines of the world health organization (WHO) (World Health Organization, 2009) and the study was approved by the ethical committee of the Anhaguera Educational (CEP/AESA), report number: 37413514.0.0000.5372. The Larvae and pupae from mosquito *Aedes Aegypti* were kindly given by the Faculty of medicine of the University of Brasilia (UnB).

In the first weeks of adult life the mosquitoes were kept in the insectary of the UnB inside an acrylic cage measuring 35 x 35 x 40 cm at T = 25 ±2°C and relative humidity of 70 ± 5%. They were submitted to a photoperiod of 12/12 hours (clarity/darkness) and fed with aqueous solution of sucrose at 10% (p/v) and blood from living mice. The place was free of pathogens, insecticides or repellents.

Validation of the mosquitoes lots

Adult females were identified and selected for the repellence tests in lots of 50 mosquitoes that were kept under starvation for 72 hours before each test. The validation of each lot was done according to a protocol proposed by the World Health Organization (WHO, 2009) in which a volunteer introduce one forearm, with the hand protected by latex gloves, in the cage containing the lot of mosquitoes (Fig. 2). The lot is considered validated only when at least 10 landings in the forearm is observed in a period of 30 s.

FIGURE 2. Validation test for the mosquitoes lots.

Repellency tests

Volunteers for the repellence tests were healthy people from both genders (2 men aged 25 and 24 years old, and 2 women aged 25 and 22 years old), non-smokers and had no lesion or any skin disease. They were advised to not use any type of fragrance (from soap, creams, perfumes, etc…) in the 12 hours preceding the tests. Just before each test they had the forearms washed with neutral soap, dried with a towel and the hands protected by rubber gloves. All volunteers also signed the consent and free clarification terms (CFCT) authorizing the tests and the use of the data obtained.

The repellence test was performed according to a protocol of the World Health Organization (WHO, 2009) by spreading out 1.0 mL of the solution (pure ethanol, ethanolic extract or the commercially used repellent N,N-Diethyl-3-methylbenzamide, also known as DEET, over the whole internal extension of the volunteer forearm, followed by the introduction of the forearm inside the cage (as shown in Fig. 2), keeping it there for 1 minute for the counting of the number of mosquitos landings.

Each volunteer first performed tests with ethanol in one forearm and DEET 20% in the other. After, the volunteer had the forearms washed with neutral soap and sterilized with ethanol and waited 30 minutes before performing the tests with ethanol and the ethanolic extract of *S. aromaticum.* These 2 tests were repeated after one hour. Each volunteer repeated the test 3 times in different cages in consecutive weeks in order to generate enough data for statistic treatment.

Statistical treatment of the repellence data

The statistic treatment was developed through the software R (R Core Team, 2015). The necessary premises to run this test were found through the normality test Shapiro-Wilk and the Bartlett test of variance homogeneity (Fraley, 2011). A Two-Way-Anova was used to

evaluate the data and, when necessary, the differences among groups were evaluated through the Tukey-HSD test (Quinn & Keough, 2002). The difference in the landing frequency among the groups analyzed were evaluated through factorial variance, correlating all groups among themselves. The analysis of variance was also used to evaluate the variation on the landing frequency according to the gender. The differences were considered significant when *p < 0.05 (Quinn & Keough, 2002).

The landing percentage was calculated based on equation 3:

$$\%\ of\ landings = \frac{L}{50} \times 100 \qquad (3)$$

Where: L = number of landings. The percentage of repellency was calculated according to equation 4:

$$\%\ of\ repellency = \lfloor \frac{(C-T)}{C} \rfloor \times 100 \qquad (4)$$

Where: C = negative control (ethanol) and T = number of landings of the group analyzed. Both formulas in equations 3 and 4 are preconized by the WHO (World Health Organization, 2009).

Anticholinesterase activity

In order to determine the anticholinesterase activity we used the Ellman method (Ellman *et al*., 1961) modified for a 96-wells plate (Lima *et al*., 2009). The velocities of substrate hydrolysis by the enzyme acetylcholinesterase (AChE) as function of sample concentration were evaluated for the AChE from *Electrophorus electricus* (*Ee*AChE). AChE and Ellmann reagent (DTNB) were prepared in phosphate buffer (100 mM, pH 7.4). Acetylthiocholine iodide (ATCI) was prepared in distilled water. Stock Samples (100 mg/mL) were prepared in MeOH and appropriately diluted in phosphate buffer to the desired concentrations

immediately before use. All solutions were kept on ice during the experiment. All experiments were performed at 37 ± 1 °C.

All experimental wells received AChE (0.01 U/mL), DTNB (0.25 mM), and phosphate buffer (control – enzyme activity) or sample solutions (0.01 to 10 mg/mL). The mixture was incubated for 10 min. Then, ATCI (0.5 mM) was added to all wells and the plate was read immediately for 5 min in a spectrophotometer (Spectramax 340PC, Molecular Device®). The spontaneous hydrolysis of the substrate was evaluated by replacing enzyme for buffer. The solvent (methanol or ethanol) was evaluated at the highest concentration (5%) used in the experiment. All concentrations refer to final concentrations. The samples were tested in at least 5 concentrations. The enzyme activity (absorbance/min) in sample presence was determined by comparison with the control (mixture without sample) and expressed as the change in the optic deviation at 412 nm. The values of absorbance/min were calculated by the software Softmax Pro 6.4®. Inhibition values were calculated through non-linear regression with the software Graph Pad Prism 5®. For each sample, results correspond to average ± standard deviation of two experiments, being each one performed in triplicate.

RESULTS AND DISCUSSION

Calibration curve for eugenol and eugenyl acetate

The mobile phase chosen to build the calibration curve was toluene:metanol 9:1 (v/v), the same reported by Pathak, et al (2004). This solvent system provided an excellent resolution for eugenol [Retention factor (R_f) = 0.25] and eugenyl acetate (R_f = 0.31) (Fig. 3).

FIGURE 3. R_f for eugenol and eugenyl acetate in toluene:metanol 9:1 (v/v).

The identities of the bands of eugenol and eugenyl acetate in the sample extract were confirmed by overlaying their UV absorption spectra with those of their respective reference standards (Fig. 4). The purity of each of these bands in the sample extract was confirmed by comparing the absorption spectra recorded at start, middle and end positions of the bands. The profiles of both spectra (Fig. 4) points to a high degree of purity of the sample (black lines), compared to the standards of eugenol (green line) and eugenyl acetate (red line).

FIGURE 4. Overlaying of the UV spectra of the sample (black lines) and standards of eugenol (green line) and eugenyl acetate (red line).

The method was evaluated in terms of instrumental precision, repeatability and accuracy (Tab. 1). The range of linearity for eugenol and eugenyl acetate was of 720-3600 $mg.mL^{-1}$ and 250-1230 $mg.mL^{-1}$, with correlation coefficients of 0.983 and 0.998 respectively (Tab. 1). The instrumental precision and the repeatability, expressed as % CV, suggest that the proposed method is quite precise and reproductive. The precision average (% of recovery) of eugenol and eugenyl acetate obtained after maceration in ethanol for 96 hours, was of 99.84 % and 99.21 %, respectively. The contents of eugenol and eugenyl acetate estimated in the clove samples by this method were found to be in the ranges of 87-92% and 13-8% respectively. These results confirm the (HPTLC)-densitometry as an efficient method for the simultaneous quantification of eugenol and eugenyl acetate in ethanolic extracts.

TABLE 1. Validation parameters and results.

Biological activities

Repellence

The ethanolic extract of *S. aromaticum* after 96 hours of static maceration presented a statistically significant repellence activity ($F_{2.24}$ = 18.450, P<0.0005) (Table 2), calculated according with equations 3 and 4 and using the Tukey test-HSD (Quinn & Keough, 2002), when compared to the blank (pure ethanol) as shown in Fig. 5. During the time of evaluation (1 minute) the blank presented 15.5 ± 2.1 landings, corresponding to 32 % of the total landings (TL) and 0% of repellence (R), while the extract presented 5.8 ± 2.0 landings, corresponding to 12 % of TL and 63.16% of R. The positive control (DEET 20%) presented 100 % of repellence. The tukey-HSD test evidenced that the 96 h extraction resulted in less landings than the ethanol considering a value of P<0,05.

FIGURE 5. Number of landings observed in volunteers exposed to *Aedes aegypti for* 1minute after *treatments*. Values correspond to average ± *standard error (n*=6).

Repellent activity between genders

We also evaluated the capacity of repellency of the ethanolic extract of *S. aromaticum* between genders. As can be seen in Fig. 6, the male gender presented more landings (10.42 ± 1.25) than the female (6.17 ± 2.02), corresponding to 35% more landings calculated according to equation 3 ($F_{1.24}$ = 10.793, P<0.005) (Table 2). The same landing behavior was also observed in the control group (Fig. 6).

As mentioned before, the differences between genders were evaluated by the Tukey test-HSD (Quinn & Keough, 2002) and the variance analysis was used to evaluate the variation in frequency of landings according to the gender, with differences being considered statistically

significant when *p<0.05. The results, observed in the bars for each gender in Fig. 6, show that the differences between genders were statistically significant.

FIGURE 6. Number of landings according to the gender of each volunteer. Values correspond to the average ± standard error (n=6).

TABLE 2. Anova table presenting the statistical differences between the treatments and between genders.

It has been demonstrated (Takken, 1991) that the capacity of repellence of DEET in women is higher and longer than in men. This is more related to the gender than to DEET, once women present lower levels of lactic acid in the blood even after intense physical activity. The lactic acid is one of the constituents of the human sweat and an important attractive to mosquitoes (Affonso *et al*., 2013). Our results, reported in Fig. 6, therefore, corroborate with the literature reports.

Anticholinesterase activity

The toxicity mechanism of insecticides involves the cholinergic neurotransmission in the central nervous system (CNS) and has been investigated by Abd-Ella *et al*. (2015) and Corbel *et al*. (2009) who has showed that DEET is a reversible inhibitor of both human AChE (*Hss*AChE) and butyril cholinesterase (*Hss*BChE). DEET binds to the catalytic sites of *Hss*AChE and *Hss*BChE, strongly inhibiting them at concentrations of 4.67 and 1.08 mM, respectively, but does not penetrate in the active site of AChE from *Drosofila melanogaster*

(*Dm*AChE) (Corbel *et al*., 2009), probably because the active site gorge of *Dm*AChE is about 50% narrower than in *Hss*AChE (Harel *et al*., 2000).

Our anti AChE activity results (Fig. 7) show that eugenol and the ethanolic extract of *S. aromaticum* are able to inhibit *Ee*AChE. Eugenol was about three times more potent to inhibit *Ee*AChE (IC_{50} = 0.90 ± 0.01 mg/mL) than the ethanolic extract (IC_{50} = 2.69 ± 0.08 mg/mL).

Figure 7. Inhibition of *Ee*AChE by eugenol and the ethanolic extract of *S. aromaticum*. The values are the average ± standard deviation of two independent assays, each one performed in triplicate.

Eugenol presented AChE inhibitory potency (IC_{50} = 0.90 ± 0.01 mg/mL / IC_{50} = 5.50 ± 0.18 mM) similar to that of DEET (4.67 mM) while the *S. aromaticum* extract was less potent (IC_{50} = 2.69 ± 0.08 mg/mL). As the catalytic triad of *Ee*AChE is composed by the same amino acids as in *Hss*AChE (Wiesner *et al*., 2007), the results observed for *Ee*AChE can be extrapolated to *Hss*AChE, despite the structural and functional differences between these two enzymes could result in differences in affinity and potency of the inhibitors.

The toxicity of DEET to humans has been attributed to its action on the CNS (Lipscomb *et al*., 1992; Schaefer, 1992; Clem *et al*., 1993; Koren *et al*., 2003). If combined with pesticides, DEET can cause severe seizures and lethality (Abdel-Rahman *et al*., 2004; Abou-Donia *et al*., 2004), probably due to its cholinesterase inhibitory action. It is possible that the extract of *S. aromaticum* obtained according to the methodology of this work, presents low toxicity levels to humans.

CONCLUSION

We reported for the first time the HPTLC-densitometry as an efficient and appropriate method for simultaneous quantification of eugenol and eugenyl acetate in ethanolic extracts of *S. aromaticum*. Also, the ethanolic extract of the flower buds of *S. aromaticum*, prepared according to its popular use (96 hours of static maceration) was efficient as repellent, with 63 % of repellency when compared to the positive control DEET, present in commercial repellents. Regarding the antiacetylcholinesterase activity, eugenol and DEET presented similar inhibitory potencies, while the ethanolic extract was able to inhibit the enzyme only at higher concentrations.

Considering that DEET does not interact with *Dm*AChE but interacts with *Hss*AChE, the toxicity in humans observed for DEET should not be detected in the extract due to its low inhibitory potency with AChE.

Our results permit to propose that the ethanolic extract of *S. aromaticum*, prepared according to the popular tradition, is efficient in the combat to mosquitos, once it is cheap and of easy preparation, facilitating its use by low income populations as a homemade repellent.

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Conflicts of interests

The authors declare no conflict of interest.

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TABLES

TABLE 1.

Validation parameters and results.

Parameters	Results	
	E	EA
Instrumental precision (% CV, n=6)	0.82	0.92
Repeatability (% CV, n=6)	0.46	0.51
Precision (% Recovery)	99.74	99.21
LQ ($mg.mL^{-1}$)	18.55	22.40
LD ($mg.mL^{-1}$)	6.12	7.39
Specificity	Specific	Specific
Linearity (R^2)	0.983	0.998
Linear range ($mg.mL^{-1}$)	720 – 3600	250 – 1230
Equation	y = 2569.2x + 2570.9	y = 2604.1x + 184.81

TABLE 2.

Anova table presenting the statistical differences between the treatments and between genders.

	Degrees of freedom	**Sum of squares**	**Mean Square**	**F-value**	**Probability**
Treatment	3	555.79	185.264	18.450	7.444×10^{-6}
Gender	1	108.38	108.375	10.793	0.003893
Residuals	1	190.79	10.042	---	---

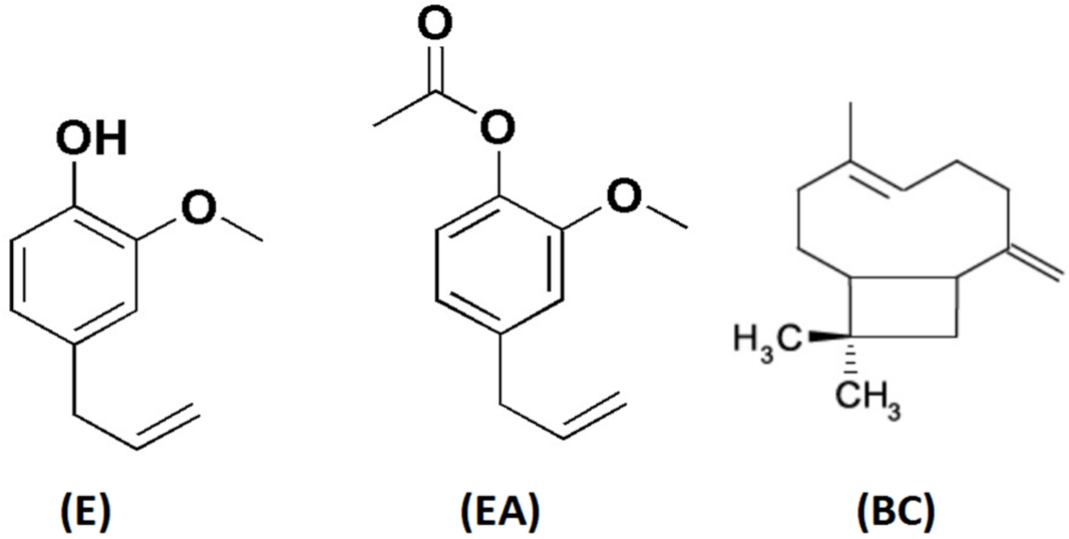

FIGURE 1. Molecular structures of Eugenol (E), Eugenyl Acetate (EA) and β-caryophyllene (BC).

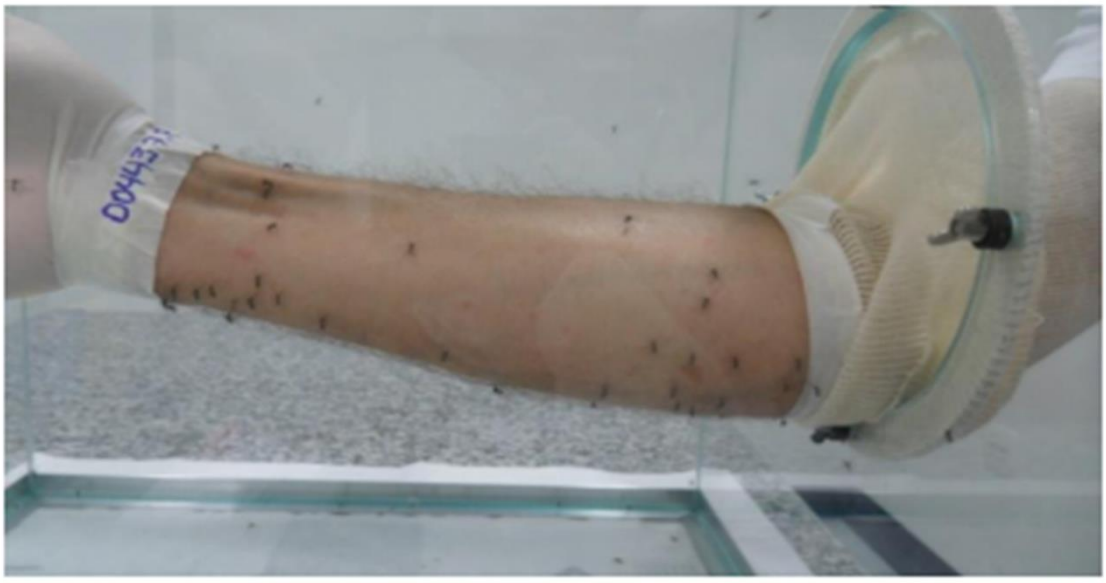

FIGURE 2. Validation test for the mosquitoes lots.

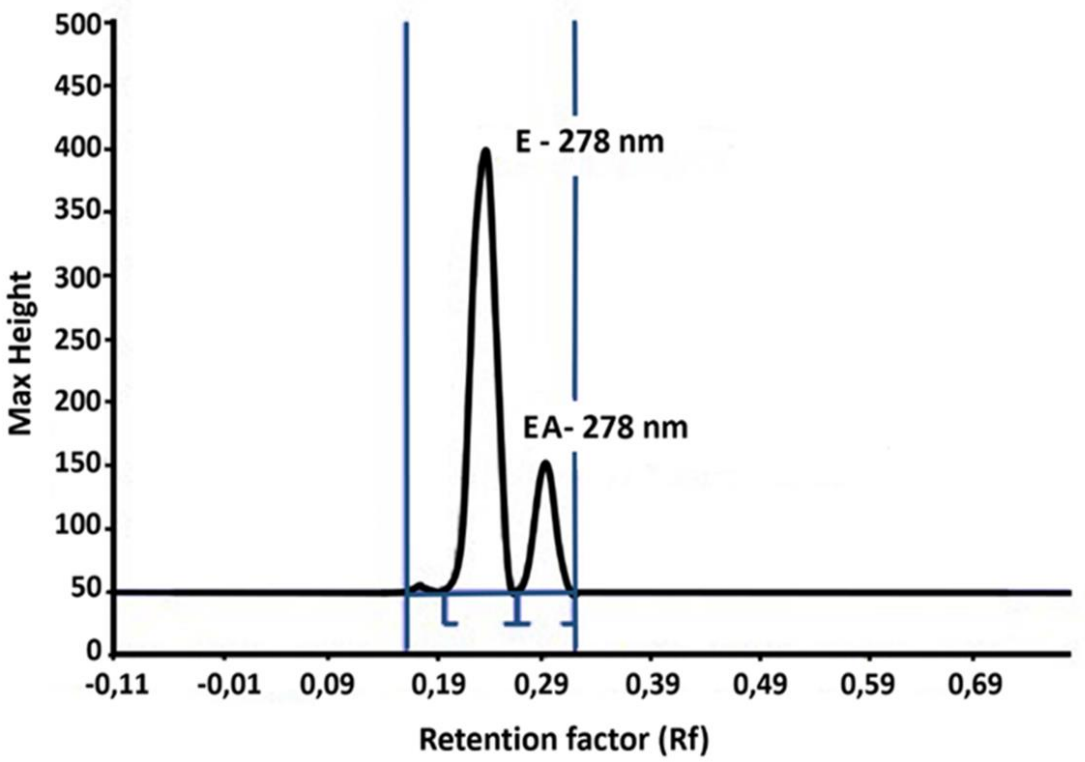

FIGURE 3. R_f for eugenol and eugenyl acetate in toluene:metanol 9:1 (v/v).

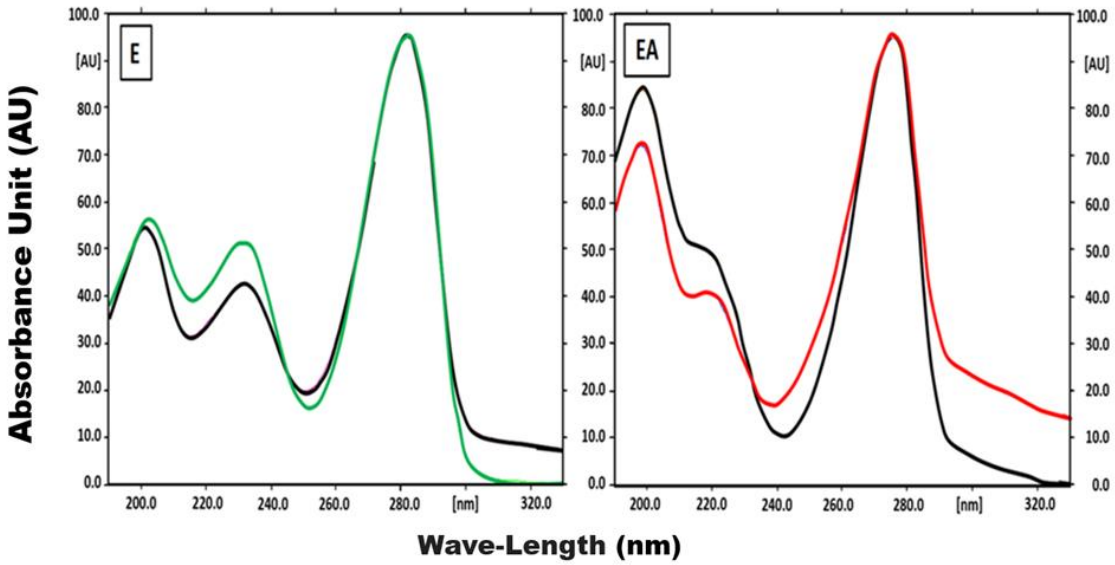

FIGURE 4. Overlaying of the UV spectra of the sample (black lines) and standards of eugenol (green line) and eugenyl acetate (red line).

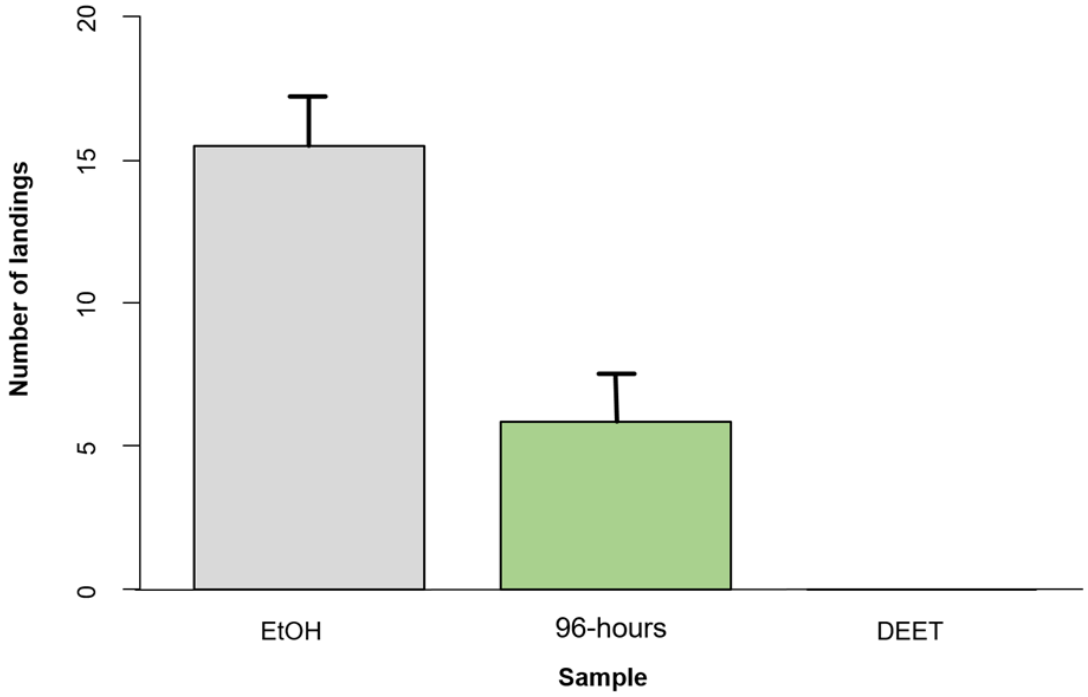

FIGURE 5. Number of landings observed in volunteers exposed to *Aedes aegypti for* 1minute after *treatments*. Values correspond to average ± *standard error (n*=6).

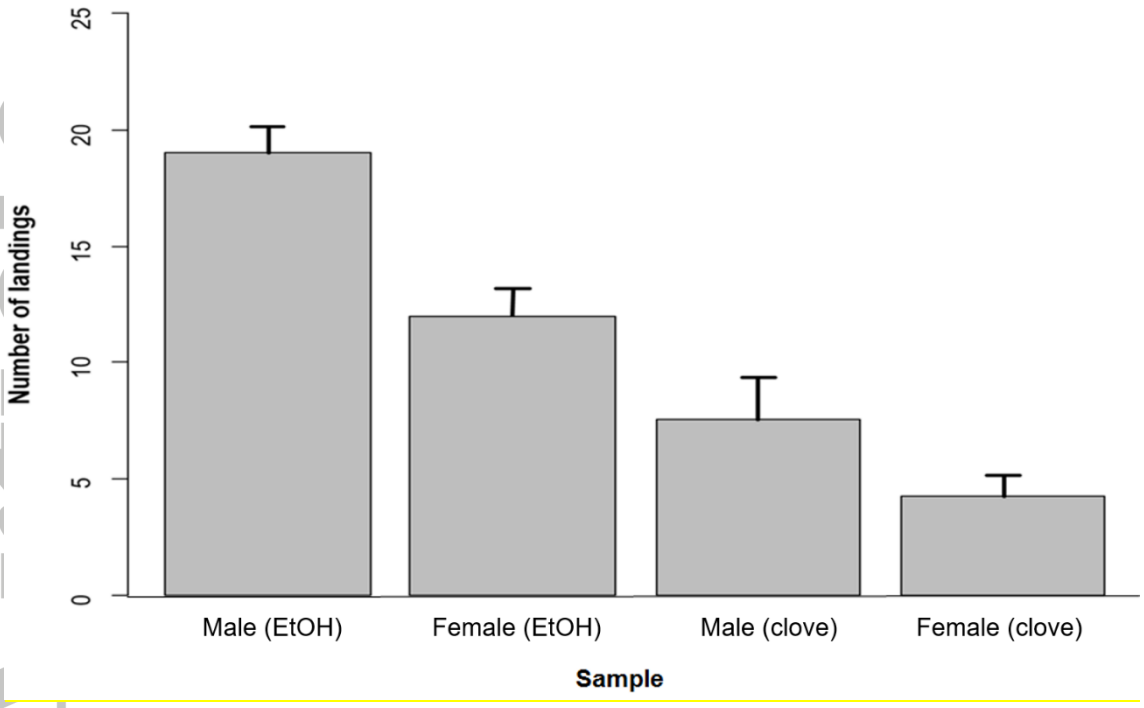

FIGURE 6. Number of landings according to the gender of each volunteer. Values correspond to the average ± standard error (n=6).

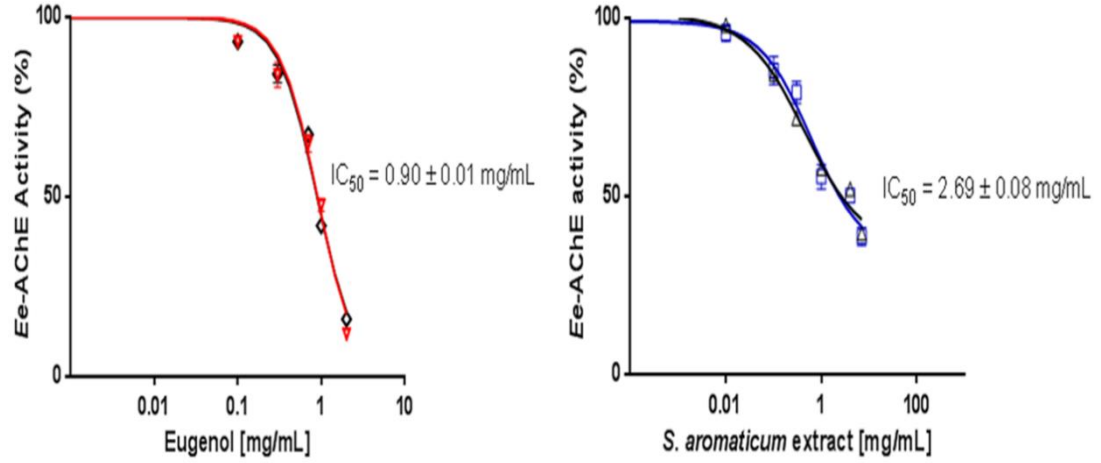

Figure 7. Inhibition of *Ee*AChE by eugenol and the ethanolic extract of *S. aromaticum*. The values are the average ± standard deviation of two independent assays, each one performed in triplicate.