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Chemical Composition and Larvicidal Activity of Essential Oil Extracted from *Anemia tomentosa* (SAV.) Sw: Study Conducted *in vitro* and *in silico*

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The essential oil extracted from *Anemia tomentosa* (EOAT) has shown larvicidal potential against *Aedes aegypti*, based on methods *in vitro* and *in silico*. Chromatographic and spectrometric techniques (gas chromatography-mass spectrometry (CG-MS), gas chromatography-flame ionization detector (GC-FID)), as well as mono and bidimensional nuclear magnetic resonance spectroscopy (NMR) were used to assess 10 essential oil components. Chemical composition of essential oil comprised 87.51% sesquiterpenes, with emphasis on presilphiperfolan-1-ol (42.13%) and silphiperfol-5-ene (19.47%). Larvicidal assay conducted *in vitro* with EOAT has evidenced potential cytotoxic activity up to 48 h exposure to it; mortality rate observed for *A. aegypti* larvae exposed to essential oil reached 100%. Study conducted *in silico* with chemical compounds deriving from the herein investigated plant species has evidenced its potential to inhibit acetylcholinesterase in *A. aegypti*. Activity of triquinane sesquiterpenes ranging from −6.8 to −8.2 kcal mol^{-1} stood out in comparison to that of temephos (−7.5 kcal mol^{-1}). Chemical compounds identified in the investigated essential oil presented low human and environmental toxicity, as observed in absorption, distribution, metabolism and excretion, and toxicity (ADMETox) predictions.

Keywords: Anemiaceae, *Aedes aegypti*, molecular docking, acetylcholinesterase inhibitors, sesquiterpens

Introduction

Anemia tomentosa (SAV.) Sw. (Anemiaceae) is a recurrent plant species that has highly aromatic leaves and grows in rocky regions. It is used in folk medicine to improve individuals' digestion, as expectorant and anti-flu drug, as well as to treat bronchitis. It naturally grows in South America, mainly in mountainous regions of Northeastern and Central Brazil, as well as in Bolivia, Paraguay and Argentina.[1] The current study used *A. tomentosa* leaves collected in the Caatinga biome, in an

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area located on a rocky outcrop in Jequié County, Bahia State, Brazil.

Essential oils (EO) are secondary metabolites formed by volatile and semi-volatile compounds. They are produced by more than 17,500 plant species and are stored in different plant organs, such as leaves, fruits, flowers, roots and seeds. EOs are chemically featured by hydrocarbons and terpenes found in them.[2,3] Sesquiterpene compounds of the EOs have fifteen carbon atoms. In addition, they can have double bonds and (or) oxo/oxy functions with broad action spectrum. EO extracted from *A. tomentosa* presented activity against tuberculosis bacillus, among other bacteria, as well as repellent activity against mosquitoes.[4]

Aedes aegypti mosquito is one of the main vectors of pathogens capable of affecting humans. Dengue, Zika and Chikungunya viruses are examples of pathogens transmitted by female individuals belonging to this species.[5] Several studies[6] conducted with essential oils have evidenced their larvicidal activity, which was associated with their composition mostly comprising sesquiterpenes.

Several molecular targets have been explored in studies focused on finding inhibitors to be used to develop new sustainable larvicides associated with natural active compounds.[7] Acetylcholinesterase (AChE) is the main molecular target with larvicidal action, because of interference generated by larvicides that block larval movements through mechanotropic receptors that prevent larvae from feeding. This feature emphasizes its cytotoxic potential and chemical control ability.[8]

There is a limited number of studies in the literature focused on investigating *A. tomentosa* composition and the activity of its essential oil. Studies[9-11] have described compositions rich in mono- and sesquiterpenes, as well as highlighted major compounds, such as pinocarvone, α-bisabolol and presilphiperfolan-8-ol. Presilphiperfolan-8-ol was found in *A. tomentosa* essential oil analyzed in previous studies.[9-11] With respect to its biological activity, the investigated essential oil has shown antimycobacterial potential to be used against *Mycobacterium tuberculosis* and *M. smegmatis* strains, as well as its repellent action against *A. aegypti*.[4,12]

Studies carried out with essential oils have evidenced the ability of volatile compounds to kill *A. aegypti* larvae, which was constantly attributed to high sesquiterpene activity in the composition of EOs.[13,14] Caryophyllene, α-muurolene, as well as trichinan sesquiterpenoids, were identified in these oils' composition. They play a key role in the larvicidal activity of these volatile mixes since they inhibit the activity of the AChE enzyme in these insects' larvae.[15,16]

Thus, given the larvicidal potential of essential oils, mainly due to the action of sesquiterpenes in inhibiting AchE enzyme of the insects, in addition to the lack of studies focused on investigating essential oil extracted from *A. tomentosa* (EOAT), it is necessary assessing a new aspect of the action of EOATs, based on the following steps: essential oil identification, quantification and featuring as a potential larvicidal agent.

The aim of the current study was to perform the chemical and pharmacological assessment of essential oil extracted from species *A. tomentosa* grown in Jequié County, Bahia State, Brazil. Gas chromatography techniques (gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID)), biological assays *in vitro* and studies *in silico* were used to assess both the chemical composition and larvicidal potential of the investigated essential oil against *Aedes aegypti*. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data have confirmed the identity of the major compounds found in EOAT.

Experimental

Plant material collection

A. tomentosa shoots were collected in Poço Dantas region (latitude (W): –13.8575, longitude (S): –40.0836), rural area of Jequié County, Bahia State, Brazil, in February 2021. Two vouchers were deposited in the Herbarium of Southwestern Bahia State University and received registration and botanical identification code of HUESB-8934.

Essential oil extraction and analyses

Fresh *A. tomentosa* leaves (1,300 g) were subjected to hydrodistillation for EOAT (5.1 g) extraction at crude yield close to 0.4%. The extracted essential oil presented yellow color and typical citrus-woody odor. The obtained crude oil was passed through a column filled with anhydrous sodium sulfate to rule out moisture. EOAT (density measured at 0.951 g mL^{-1}) was stored in freezer at approximately –18 °C.

Chromatographic analyses aimed at identifying the main EOAT constituents were carried out in Shimadzu gas chromatograph, model QP2010-SE (Shimadzu company, Kyoto, Japan), coupled to mass spectrometry detector (GC-MS) equipped with a simple quadrupole ion filter detector, which operates by electron impact ionization (EI, 70 eV.). The equipment was set at scan speed of 1,000 amu s^{-1} and *m/z* fragment detection ranging from 45 to 800 Da. Chromatographic separation applied to EOAT components used an Agilent SLB-5ms poly-(5% diphenyl/95% dimethyl siloxane) phase capillary

column (length: 30 m × internal diameter: 0.32 mm × film thickness: 0.32 μm). Helium provided by White Martins (Salvador, Brazil) was used as carrier gas at linear velocity of 48.4 cm s^{-1} and at pressure of 22.2 kPa. Samples were dissolved (1%) in dichloromethane and injected (1 μL) in triplicate, in split mode (1:40). Both the injector and the detector were adjusted to operate at 220 and 240 °C, respectively. The oven was set at heating rate of 3 °C min^{-1}, temperature increased from 40 to 280 °C, and this temperature was kept for additional 4 min. The ion source and the interface were kept at 240 and 280 °C, respectively. Essential oil components were identified based on comparison to their retention indices (RI), which were calculated in a series of *n*-alkanes (C_8-C_{36}) provided by Sigma-Aldrich (São Paulo, Brazil), as well as by comparing their mass spectra to the NIST-14 library (match > 90%).

EOAT constituents were quantified in Shimadzu gas chromatograph, model GC-2010 Plus (Shimadzu, Kyoto, Japan), equipped with flame ionization detector (GC-FID). Column type, oven and inlet temperature adjustment parameters were the same as the ones adopted in GC-MS. The flow rates of gases used as carriers (column, split vent, purge and H_2), FID flame (H_2) and make-up (N_2) by White Martins (Salvador, Brazil) were 14.0, 40 and 30 mL min^{-1}, respectively. Synthetic air flow of 400 mL min^{-1} was used to feed the FID flame. Sample split ratio was 1:10. Operating parameters were established after checking the best resolution. Injections were performed in triplicate, at volume of 1.0 μL.

Phytochemical study applied to methanolic extract

In total, 1 kg of *A. tomentosa* leaves was subjected to EO extraction through maceration with methanol (Synth, Salvador, Brazil) in three repetitions (48 h period, each). The aliquot of 39.8 g of *Anemia tomentosa* methanolic extract (ATME) was obtained after solvent evaporation.

In total, 30.0 g of ATME were subjected to preliminary fractionation in filtering column (silica gel 60 70-230 Mesh) (Synth, Salvador, Brazil), from which 4 different fractions (F1 to F4) were obtained based on polarity: hexane (Synth, Salvador, Brazil) (F1, 1.7 g), chloroform (Synth, Salvador, Brazil) (F2, 0.3 g), ethyl acetate (Synth, Salvador, Brazil) (F3, 2.1 mg) and methanol (F4, 20.7 mg), consecutively. The chromatographic profiles fractions were obtained through thin-layer chromatography (TLC) (Synth, Salvador, Brazil).

Given its best chromatographic profile and highest yield, F3 was subjected to column fractionation by liquid chromatography in a column (35 mm diameter) packed with silica gel 60 (70-230 Mesh). It was done based on using the polarity gradient with hexane:ethyl acetate solvents, at the 10:0, 9.5:0.5, 9:1, 8:2 and 7:3 ratios. In total, 95 fractions (10 mL, each) were collected. Fractions 37, 38 and 39 presented a yellow substance with oily appearance (mass = 4.6 mg), which was soluble in chloroform and dichloromethane, and presented retention factor (Rf) close to 0.5 in the TLC analysis conducted with eluent mix of hexane:ethyl acetate solvents (9:1). Later on, the aforementioned substance was identified as presilphiperfolan-1-ol (**8**).

Spectrometric and optical analyses

Infrared and ^{1}H and ^{13}C magnetic resonance spectrometric analyses were used to improve and resolve ambiguities in the process to identify chemical constituents of EOs through GC-MS. Fourier transform infrared (FTIR) spectra (4,000 to 450 cm^{-1}) were obtained through PerkinElmer attenuated total reflectance (ATR)-FTIR spectrometer, model Spectrum Two (PerkinElmer, Brazil). One and two-dimensional ^{1}H and ^{13}C NMR spectra were recorded in Varian Inova 500 NMR spectrometer (Varian, USA), (500 MHz); tetramethylsilane (TMS) was used as internal standard, whereas chloroform-*d* was applied as solvent. Optical rotations were measured at 25 °C in Anton Paar MCP 300 polarimeter (Anton Paar, USA)

Larvicidal assay *in vitro*

A. aegypti 3rd and 4th instar larvae were obtained from Rockefeller-strain eggs, which were kindly provided by the Toxicology Research Laboratory of the Antibiotics Department of Federal University of Pernambuco (UFPE). They were placed in glass container filled with 30 mL of 0.1% EOAT and solubilized in deionized water and Tween 80 (0.5%) to carry out the tests. Each test used 30 larvae and comprised five repetitions (150 larvae, in total). Control solutions comprised (*i*) 0.5% Tween 80, and (*ii*) distilled and deionized H_2O. Larval death (nine dead larvae, in total) was assessed 1-12 h (2 h intervals), as well as 24 and 48 h after the beginning of the experiment. Experiments were carried out under laboratory conditions and followed a completely randomized design (CRD).

Homology model prediction

AChE1 enzyme deriving from *A. aegypti* was subjected to homology modeling based on using Swiss-Modeller PDB and AlphaFold. Protein sequence was retrieved from the *A. aegypti* GenBank (ID: ABN09910.1). The best scoring model was assessed in Swiss-Modeller based on the Global QMEAN closest to 1.0 and on identity close to 100%.[17] PDB 6ARY was the model identified and used for

homology modeling purposes. The modeled structure was subjected to energy minimization process based on using Gromacs 2018 package[18] with GROMOS96 force field.[19,20] Hydrogen atoms were added to finalized target-protein model and used for molecular docking analysis with MGL Tools.[21] All images and the electrostatic potential of the enzyme surface were visualized in PyMOL[22] and Discovery Studio 2021 software.[17,23]

Dataset, virtual screening, molecular docking and ADMETox tools

All molecules were checked and designed in Marvin Sketch software.[24] Molecular structures were downloaded in SMILES format and converted into 3D *sdf* format in Open babel software for docking calculation purposes.[25] The acethycolinesterase (AChE) crystallographic structure (6ARY) of *Anopheles gambiae* was obtained from the Protein Data Bank (PDB). All docking simulations were performed in AutoDock Vina software.[26] Docking was performed between the proposed ligands (10 compounds deriving from EOAT, acetylcholine (PubChem CID 187), temephos (PubChem CID 5392) and the receptor), which were prepared and converted into pdbqt format in Autodock tools software. Docking results and the assessment of each receptor-ligand complex, such as affinity energy (kcal mol^{-1}) and ligand positioning inside the acethycolinesterase active site, were analyzed in PyMOL 2.1 software. Nine ligand poses were generated for each complex and returned their respective affinity energies. Subsequently, all best selected ligand-protein poses were graphically plotted in PyMOL 2.1 software. Their respective 2D interaction maps were plotted in Discovery Studio 4.5 software. ADMETox properties were assessed in Data Warrior,™ [27] PkCSM™ [28] and ToxCast™ [29] software.

Results and Discussion

Chemical composition analysis of EOAT

The herein extracted EOAT (0.4%) presented yellowish color, citrus-woody odor and density (0.951 g mL^{-1}) typical of essential oil rich in terpenoids. In addition, its physicochemical data and organoleptic features were similar to those observed in previous studies.[4,11] GC-MS and GC-FID analyses (Table 1) enabled identifying and quantifying the ten main chemical constituents of the essential oil extracted from fresh *A. tomentosa* leaves, which recorded prevalence of 87.5% sesquiterpenes.

The main EOAT constituent is a triquinane sesquiterpene called presilphiperfolan-1-ol (**8**, t_R at 33.671 min, 42.13%). The ^{13}C NMR spectra analysis has evidenced the most intense ^{13}C (C–OH) signal at 84.90 ppm, it referred to compound **8**.[4,30] Compound **8** was isolated from *A. tomentosa* leaves (ethyl acetate extract) through liquid chromatography on silica gel column eluted with hexane:ethyl acetate (9:1). The analysis applied to spectrometric data (optical activity $[\alpha]_D^{25}$, FTIR, ^1H and ^{13}C NMR) on compound **8** has confirmed it as presilphiperfolan-1-ol[31] (see Table 2).

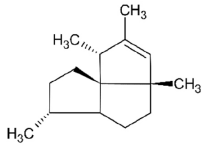
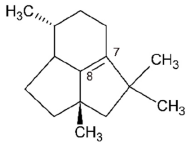
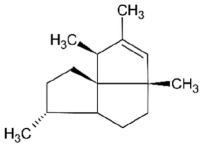
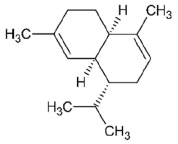
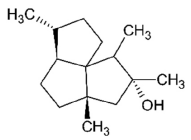
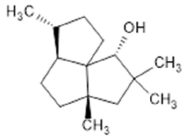
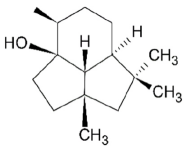
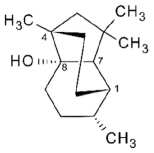
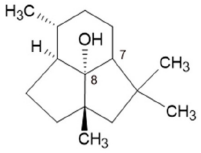
Complementary analysis applied to the ^1H and ^{13}C NMR spectra of EOAT identified signals typical of

Table 1. Chemical constituents of EOAT identified through GC-MS, ^1H and ^{13}C NMR and FTIR

t_R / min	Chemical constituent	Class	Area / %	RI_{calc}	RI_{lit}
5.924	hexanal (**1**)	AL	1.47 ± 0.66	802	801
25.475	7-*epi*-silphiperfol-5-ene (**2**)	SH	0.56 ± 0.02	1329	1328
25.849	presilphiperfol-7-ene (**3**)	SH	5.72 ± 0.12	1333	1336
26.291	silfiperfol-5-ene (**4**)	SH	19.47 ± 0.26	1350	1348
30.288	α-muurolene (**5**)	SH	4.02 ± 0.13	1496	1500
33.229	silphiperfolan-6-α-ol (**6**)	OS	3.52 ± 0.16	1509	1507
33.423	cameroonan-7-α-ol (**7**)	OS	4.15 ± 0.01	1512	1511
33.671	presilphiperfolan-1-ol (**8**)	OS	42.13 ± 0.23	1515	1518[4]
35.925	prenopsan-8-ol (**9**)	OS	4.69 ± 0.06	1570	1577
36.365	presilphiperfolan-8-ol (**10**)	OS	3.25 ± 0.03	1586	1586
		Total	88.98 ± 0.19		
Aldehydes (AL)			1.47 ± 0.66		
Sesquiterpene hydrocarbons (SH)			29.77 ± 0.13		
Oxygenated sesquiterpenes (OS)			57.74 ± 0.10		

RI_{calc}: calculated chromatographic retention index with series of alkanes (C8-C26); RI_{lit}: reported chromatographic retention index (NIST14 mass spectral database and literature).

Table 2. Structures of EOAT-constituent sesquiterpenes elucidated by GC-MS, ^{1}H and ^{13}C NMR (500 MHz, chloroform-*d*), FTIR, and comparison to spectral data available in the literature

		
(**2**) MS: M^+ *m/z* 204 (35%), 189 (33%), 175 (64%), 161 (24%), 133 (54%), 122 (100%); NMR δ_H 4.94 (H-5, s-br), δ_C 133.11 (C-5), 142.43 (C-6)	(**3**) MS: M^+ *m/z* 204 (20%), 189 (38%), 161 (53%), 147 (51%) 119 (100%), 105 (58%); NMR δ_C 131.55 (C-8), 161.07 (C-7)	(**4**) MS: M^+ *m/z* 204 (21%), 189 (38%), 161 (53%), 147 (51%), 133 (35%), 119 (100%); NMR δ_H 4.90 (H-5, s-br), δ_C 133.46 (C-5), 140,80 (C-6)
		
(**5**) MS: M^+ *m/z* 204 (13%), 189 (21%), 161 (40%), 148 (74%), 133 (63%), 91 (100%); NMR: δ_H 0.71 and 0.79 (H-12 and H-13, d, *J* 7 Hz), δ_C 120.63 and 134.18 (C-5 and C-4), δ_C 124.38 and 135.93 (C-9 and C-10)	(**6**) MS: M^+ *m/z* 222 (12%), 207 (23%), 175 (5%), 151 (55%), 135 (%), 107 (28%); NMR δ_H 2.18 (H-1, dd, 8.0, 5.5 Hz); δ_C 9.49 (7-Me), δ_C 78.48 (C-6)	(**7**) MS: M^+ *m/z* 204 (9%), 166 (19%), 135 (100%), 124 (36%), 109 (32%), 96 (45%); NMR δ_H 3.58 (H-7, t, *J* 6.5 Hz), δ_C 89.69 (C-7)
		
(**8**) MS: M^+ *m/z* 222.05 (< 1%), 207 (20%), (%), 189 (3%), 165 (57%), 123 (100%); NMR δ_H 1.64 (H-8, d, *J* 8.5 Hz), δ_C 84.90 (C-1); FTIR δ / cm^{-1} 3450 (OH), 2950 (CH), 1450 (CH_3), 1370 (CH_2); $[\alpha]_D^{25}$ = −52,0 ($CHCl_3$, 2.0)	(**9**) MS: M^+ *m/z* 222 (15%), 207 (27%), 137 (100%), 111 (78%), 95 (45%), 83 (63%); NMR: δ_H 2.02 (H-2_{ax}, ddd, 15.0, 12.0, 8.0 Hz); δ_C 82.11 (C-8)	(**10**) MS: M^+ *m/z* 222 (< 1%), 207 (%), 189 (%), 161 (%), 149 (%), 119 (100%); NMR δ_H 2,74 (H-1, dd, *J* 9.5, 3.0 Hz), δ_C 96.93 (C-8)

chemical sesquiterpenes' shifts with alcohol hydroxyls and double bond (C=C),[32] which enabled featuring the structure of its nine sesquiterpene constituents. Shift values observed at δ_H 1.64 (d, *J* 8.5, H-8) and δc 84.90 (C-1) were assigned to presilphiperfolan-1-ol (**8**), which was the most abundant EOAT constituent.[31] Proton-shift values observed at δ_H 4.94 (H-5, s-br), 4.90 (H-5, s-br) and 5.43 (H-5 and H-9, m) were assigned to four olefinic protons in sesquiterpenes (**2**), (**4**) and (**5**), respectively.[33,34] With respect to the ^{13}C NMR spectrum, four other signals of oxygenated sesquiterpenes were also observed at δ_C 78.48 (C-6), 89.69 (C-7), 82.11 (C-8), and 96.93 (C-8). They were assigned to oxygenated sesquiterpenes, such as silphiperfolan-6-α-ol (**6**), cameroonan-7α-ol (**7**), prenopsan-8-ol (**9**) and presilphiperfolan-8α-ol (**10**), respectively.[35] Five pairs of ^{13}C olefinic carbon NMR signals observed at δc 133.11 and 142.40 (C_5=C_6), δc 141.55 and 161.07 (C_8=C_7), δc 133.46 and 140.80 (C_5=C_6), δc 120.63 and 134.18 (C_9=C_{10}), δc 124.38 and 135.93 (C_5=C_4), were assigned to EOAT compounds **2**, **3**, **4** and **5**, respectively. Compound **5** presented two double bonds[30,33,34] (Table 2).

Assessing the larvicidal activity of EOAT *in vitro*

Results in the current study have evidenced broad larvicidal activity of EOAT (Figure 1). In total, 71.31% *A. aegypti* larvae were dead after 24 h experiment, whereas all larvae (100%) were dead after 48 h.

Chemical diversity of terpenes leads to variation in their polarity degree. Thus, they can increase the absorption of both lipophilic and hydrophilic substances by the membranes, and it contributes to the synergistic action resulting from the combination among oil components. Thus, lipophilicity gradient variation resulting from the mix of neutral and functionalized sesquiterpenes can explain the larvicidal activity observed for this class of compounds.[13]

Sesquiterpenes found in essential oils can show activity in larvae, at different target sites. Oviposition inhibition can be achieved through interaction with odorant-binding protein 1 (OBP1), which is one of the most important players in the olfactory system.[36] Activities observed for these EO constituents were reported in other receptors and proteins, such as γ-aminobutyric acid (GABA) receptor. Among

them, one finds death by nervous system overactivation; blockade of the protein accounting for cholesterol transport in insects (AeSCP2), which is found at high concentrations in larvae; and acetylcholinesterase (AChE) inhibition, which leads to a typical neurotoxic effect produced by organophosphorus and carbamate insecticides.[37]

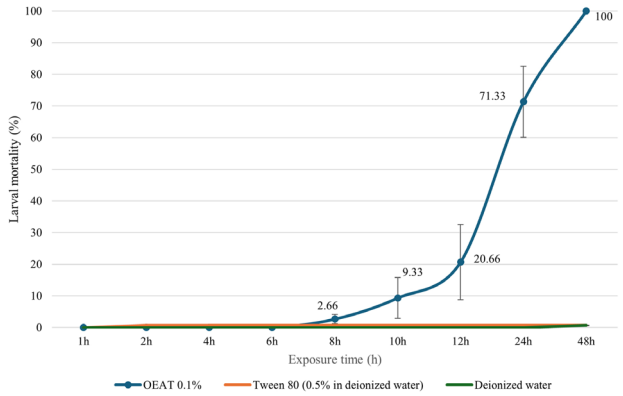


Figure 1. Cumulative mortality rate (%) observed for *Aedes aegypti* larvae, based on time exposed to EOAT treatment at 0.1%.

Triquinane sesquiterpenes, such as (**3**), (**7**) and (**10**), were described in the literature[38] as having larvicidal activity. Pavela *et al.*[39] reported these larvicidal action of the compounds against species *Culex quinquefasciatus* Say, which is the filariasis transmission agent. Silphiperfolanes, presilphiperfolanes and cameroonane are sesquiterpene groups deriving from caryophyllene.[40] The investigated EOAT was rich in compounds presenting caryophyllene skeleton. This finding corroborates the high larvicidal activity presented by this natural product.[6,38]

Molecular docking of major *A. tomentosa* (SAV.) Sw essential oil compounds against acetylcholinesterase (AChE) from *Aedes aegypti*

The best scoring model used in the current study was assessed in Swiss-Modeller, based on Global QMEAN of 0.92 (the closer to this value, the higher the accuracy of the model) and identity of 92.58%. Results have evidenced that the modeled structure maintained high homology degree to AChE deriving from *A. aegypti*. Molecular docking results have indicated that all sesquiterpenes described for the investigated EOAT interacted with amino acids in the active site of AChE deriving from *A. aegypti*. In addition, they showed affinity energy ranging from –4.0 to –8.2 kcal mol^{-1}, and it featured their potential to inhibit the enzyme at energies higher than that of larvicide temephos (–7.5 kcal mol^{-1}), except for (**1**) (–4.3 kcal mol^{-1}), which showed similar energy to that of endogenous ligand acetylcholine (–4.0 kcal mol^{-1}). The active site of this enzyme has ion channel shape. Moreover, it comprises the 10 most representative amino acid residues; among them, sesquiterpene compounds bind to the active site of the AChE enzyme, which presents a quite characteristic behavior at the entrance of the chanel (Figure 2).

AChE found in arthropods has important physiological functions, such as maintaining neuronal transmission and locomotion mechanisms accounting for favoring environmental exploration and feeding processes. In addition, this active site of the enzyme is evolutionarily conserved in mosquito species belonging to genera *Aedes* and *Anopheles*.[41] AChE is a major target for insecticides. Thus, this disruption of the receptor leads to ACh accumulation, which, in its turn, triggers neurotransmission hyperstimulation and eventual blockage in insects (Figure S1, Supplementary Information (SI) section).[7]

Overall, EOs are almost fully composed of sesquiterpenes, which have similar chemical structures that, in their turn, present small variations. This feature justifies their close affinity energies. Most sesquiterpenes, (**4**) (–8.2 kcal mol^{-1}) and (**8**) (–7.6 kcal mol^{-1}), have shown better affinity than the temephos larvicide (–7.2 kcal mol^{-1}). Results of assay conducted *in silico* corresponded to data

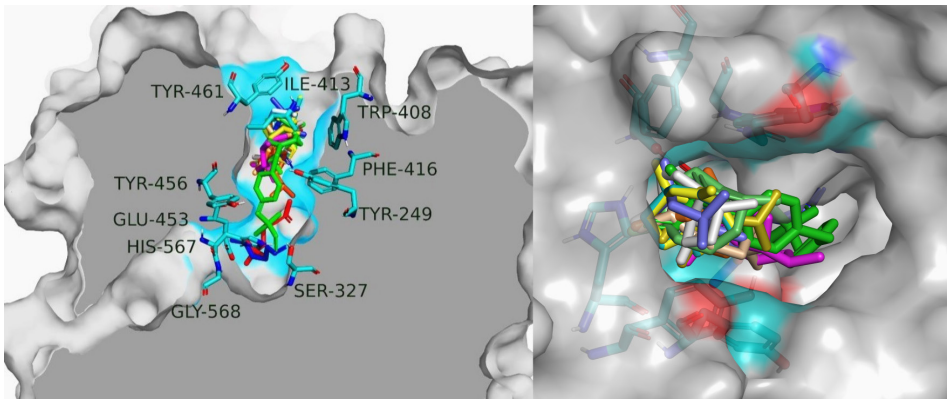


Figure 2. In gray: the active "gorge"-shaped site of the AChE deriving from *Aedes aegypti* stands out; red: acetylcholine; green: temephos; blue: hexanal; yellow: silphiperfol-5-ene; magenta: presilphiperfol-7-ene, cyan: α-muurolene, orange: silphiperfolan-6α-ol; and beige: cameroonan-7α-ol.

deriving from assay conducted *in vivo*, according to which, larvicidal activity recorded 100% larval death within 48 h. Computational and biological data integration enabled inferring that biological activity may be associated with the individual potential of isolated compounds (most of them), as well as with the synergistic action observed among them. Terpenoid compounds found in EOs have great neurotoxic potential. They can cause paralysis followed by death in insects, and this factor features them as bioinsecticides. Table 3 shows the affinity energies of all ligands.

Family Anemiaceae has been investigated as acknowledged source of terpenoid compounds, mainly of triquinane sesquiterpenes, besides being considered a standard taxonomic chemomarker for genus *Anemia*. These compounds present the larvicidal and anticholinesterase potential described in other species that are rich in terpenoid compounds, such as the ones belonging to genera *Lantana*, *Pomacea*, *Gyraulus*, *Aframomum*, *Dichrostachys* and *Echinops*.[39] Understanding the best molecular mechanism of interaction with these residues enables building a library of analogs, as drug design strategy applied to compounds with bioinsecticidal activity against *A. aegypti*.

Notably, studies focused on investigating dengue vector insecticide-resistance mechanisms are of paramount importance, since this disease is a public health issue in several countries.[43] The large-scale production and the frequent use of insecticides have caused their accumulation in ecosystems, and it led to environmental contamination, as well as to toxicity in several species, including humans. Many vector control strategies are used, in an integrated manner, to fight *A. aegypti*. Among them, one finds the use of biopesticides, such as microorganisms, viruses, biological toxins and natural products. Combining different approaches can help maximizing vector control effectiveness and avoiding the outspread of mosquito-borne diseases.[44]

Anticholinesterase insecticides currently used for biological vector control purposes are inhibitors capable of forming a covalent bond with a conserved serine in the active site of AChE. This active site involves a serine residue: Ser203, in humans and Ser397, in *A. aegypti*. It is located at the basis of a deep and narrow gorge, where it forms part of the catalytic triad along with His447 and Glu334 residues, among other aromatic residues that form the basis of the structural gorge in this active site of the enzyme (Figure 2).[41]

All compounds found in the investigated EOAT interacted at the entrance of the AChE "gorge" and showed potential to develop new natural inhibitors as alternative to increasing resistance of the insect to organophosphate larvicides. The following factors may be associated with the high potential larvicidal activity observed for EOAT: the relative concentration of each constituent in the mix found in EO,[45] spatial size of the molecules[46] and the presence of fewer rings in its structure associated with higher larvicidal activity.[47] Therefore, the inhibitory activity observed in AChE is associated with the synergistic potential observed among its compounds and with its selective and irreversible ability to bind to the active site of the target enzyme.

Table 3. Intermolecular interactions of sesquiterpenes found in *Anemia tomentosa* (SAV.) essential oil in the *Aedes aegypti* acetylcholinesterase site

Compound	EF / (kcal mol^{-1})	His567	Tyr460	Tyr456	Phe416	Trp408	Gly247	Gly246	Gly245
Lig1	–8.2	–	PiA	PiA	PiA	PiA	–	–	–
Lig2	–8.2	–	PiA	PiA	PiA	PiA	–	–	–
Lig3	–7.9	–	PiA	PiA	Vw	PiA	–	–	–
Lig4	–7.9	–	PSg	PiA	Vw	PiA	–	–	–
Lig5	–7.7	–	PSg	PiA	VW	PiA	–	–	–
Lig6	–7.6	–	PiA	PiA	PiA	PiA	–	–	–
Lig7	–7.6	–	PiA	PiA	PiA	PSg	–	–	–
Lig8	–7.6	–	PiA	PiA	Vw	PSg	–	–	–
Lig9	–7.5	–	TS	Vw	PSu	PSu	–	Vw	Pid
lig10	–6.8	–	PiA	PiA	PiA	Vw	Vw	–	–
Lig11	–4.3	–	–	PSg	–	–	–	–	–
Lig12	–4	–	–	Vw	Vw	–	HB	Vw	Vw
Lig13	–6.4	Vw	–	TS	PiA	–	CH	Vw	Vw

EF: energy affinity; His: histidine; Tyr: tyrosine; Phr: phenylalanine; Trp: tryptophan; Gly: glycine; Vw: van der Waals interaction; TS: Pi-Pi T-shaped interaction; HB: hydrogen bound; A: alkyl interaction; PiA: Pi-alkyl interaction; PSg: Pi-sigma bond; CH: carbon-hydrogen bond. PSu: Pi-sulfur; PiD:Pi-donor hydrogen bond; Lig1: 7-*epi*-silphiperfol-5-ene; Lig2: silphiperfol-5-ene Lig3: cameroonan-7α-ol; Lig4: silphiperfolan-6α-ol; Lig5: presilphiperfol-7-ene; Lig6: presilphiperfolan-1-ol; Lig7: prenopsan-8-ol; Lig8: presilphiperfolan-8-ol ; Lig9: temephos; Lig10: α-muurolene; Lig11: hexanal; Lig12: acetylcholine; Lig13: 2-methyl-1-[1-(pentan-3-yl)-1*H*-pyrazol-4-yl]propan-1-one.[42]

Structural differences in amino acid position may be strategies leading to selectivity of the compounds to the active site of *A. aegypti* AChE over the same enzyme in humans. Divergences in the "gorge" entry between mosquitoes (free cysteine) and humans (Phe295) may be a potential target for selective covalent inhibitors.[48] Catalytic serine residue has evidenced the likelyhood of finding high selective inhibition rates for mosquito enzymes over vertebrate enzymes.[49] Although the potential of covalent inhibitors has been investigated in several studies, inhibitors with different action modes, such as non-covalent inhibitors, are poorly explored in the literature.[48,49] Furthermore, one of the barriers hindering the development of new insecticides lies on overcoming increasing resistance of the insects to AChE inhibitors due to mutations in the active site. Despite these mutations, it is possible targeting resistant mutant enzymes with non-covalent inhibitors, such as those obtained from EO.[37]

Triquinane sesquiterpenes presented similar chemical structure since they were obtained through the same chemosynthetic route. They showed similar affinity energy features and interaction with the enzyme site.[50] Thus, all molecules have formed a complex with the active site of AChE, as shown in Figure 3. The 3 ligands showing the best energies: (**2**) (–8.2 kcal mol^{-1}), (**4**) (–8.2 kcal mol^{-1}) and (**6**) (–7.9 kcal mol^{-1}), recorded the best intermolecular interactions, with emphasis on van der Waals, π-alkyl and polar (π-alkyl) interactions with amino acid residues found in the catalytic site of the enzyme, such as Cys414, Glu415, Phe416 and Phe457 (Figure 3). Temephos interacted with 19 aa residues, acetylcholine interacted with 13 aa residues, whereas natural compounds recorded from 9 to 11 interactions. They were the ones showing trichinane structures. All compounds, except for (**1**), interacted with at least 8 amino acids similar to temephos, mainly with those involved in the catalytic site of the enzyme (Tyr460, Tyr456, Tyr249, Tyr461, Ile413, Gly412, Glu415, Phe416, Phe457 and Trp408). Figure S1 shows all ligand-receptor complexes (SI section).

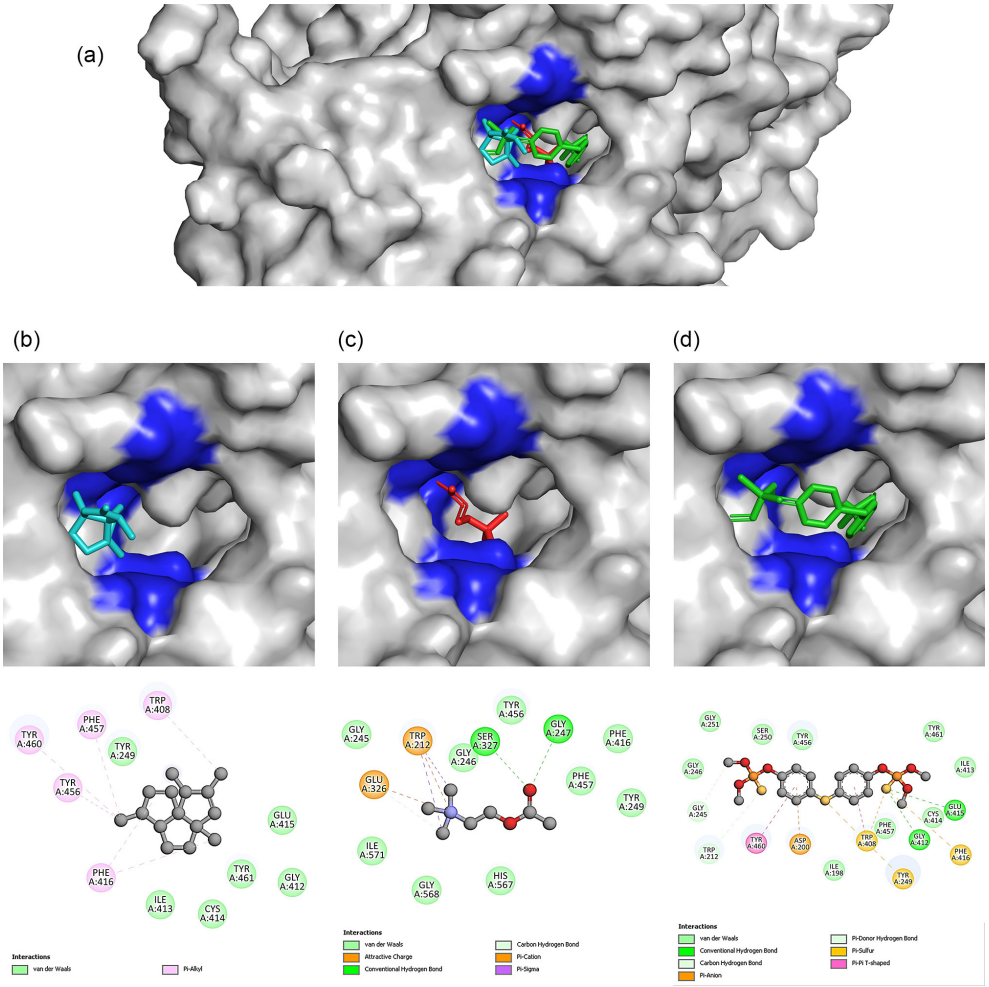


Figure 3. 2D map depicting *Aedes aegypti* AChE channel binding pocket-amino acid interactions. (a) AChE complex with the main ligands; (b) complex receptor-7-*epi*-silphiperfol-5-ene AChE enzyme; (c) complex receptor- acetylcholine AChE enzyme; (d) complex receptor-temephos AChE enzyme.

Predicting ADMETox properties *in silico*

Predictions *in silico* carried out for physicochemical, pharmacokinetic and toxicological parameters of sesquiterpenes identified in EOAT have shown that these compounds presented good oral absorption and bioavailability, according to Lipinski's rule of 5 (Ro5) (Table 4). Sesquiterpenes presented small polar surface area (159.6-181.9), high intestinal absorption rate (94.1-98.6%) and the best liposolubility profile (cLogP), in comparison to temephos (88.8%). Because they have similar chemical structures, they presented distribution volumes (VDs = 0.78) and high binding to plasma proteins as similar features. These molecules presented high distribution-to-tissues ability, as shown by the low fraction of unbound drug in blood plasma (Fu = 0.18) in comparison to temephos (Fu = 0.0), which, in its turn, can induce higher toxicity at higher concentrations in tissues as opposed to blood. Thus, triquinane sesquiterpenes presented the best distribution profile in human tissues. Sesquiterpenes did not show hepatotoxicity. They presented good renal excretion profile in comparison to Temephos. This feature may help their metabolization and excretion in case they come in contact with the human body during their use as larvicide (Table 5).[51]

The herein analyzed triquinane sesquiterpenes did not interfere with the activity of enzyme CYP3A4 (it is the main enzyme in the hepatic microsomal complex accounting for metabolizing most orally administered chemicals) and it indicated easy biotransformation, as well as favored detoxification and CYP3A4 excretion from the body.[44] In addition to low risk of liver damage, these compounds presented higher renal clearance rate (0.43-0.99 log mg kg^{-1} day^{-1}) than that observed for temephos (0.09 log mg kg^{-1} day^{-1}); moreover, they may be more easily excreted. These compounds' lipophilic features (Table 5) enable them to easily attach to, and penetrate, the larval structure. Consequently, it increases their inhibition power and has less impact on humans in comparison to temephos, which, in its turn, can be more easily absorbed through inhalation, through the skin or through ingestion.[52,53] These phamacokinetic properties are shown in Table 5.

According to the toxicity profile, oral rat acute toxicity (LD_{50}) and maximum tolerated toxicity values in humans (MRTD) observed for sesquiterpenes were within the safe range for human intake. Chronic oral toxicity values observed in rats (LOAEL) were higher than those observed for temephos larvicide. This finding has evidenced that natural compounds have better safety range for chronic dose use (Table 6).

Because they are authentic chemical structures, drug likeness (DL) and drug score (DS) values recorded for sesquiterpenes differed from those observed for temephos. This factor has evidenced their potential to be used to develop new natural and semisynthetic compounds deriving from their structures.[53] None of the natural compounds presented toxicological risks, except for (**1**), which showed potential mutagenic, irritative and aggressive activity in the reproductive system, although its aggressive potential can be assessed in cell cultures (*in vitro*) and in models *in vivo*.[52]

Table 4. Physical-chemical parameters of sesquiterpenes (*A. tomentosa*) and temephos

Molecule	EF / (kcal mol^{-1})	MW / (g mol^{-1})	cLogP	HBA	HBD	RO5	RB	TPSA / Å
Lig1	-8.2	204.35	39.82	0	0	–	5	160.11
Lig2	–8.2	204.35	39.82	0	0	–	15	160.11
Lig3	–7.9	222.37	32.96	1	1	–	14	165.92
Lig4	–7.9	204.35	41.53	0	0	–	15	159.58
Lig5	–7.7	204.35	41.53	0	0	–	18	159.58
Lig6	–7.6	222.37	3.26	1	1	–	0	165.92
Lig7	–7.6	224.39	41.00	1	1	–	0	181.94
Lig8	–7.6	222.37	3.26	1	1	–	0	165.92
Lig9	–7.5	204.35	4.25	0	0	–	0	176.24
Lig10	–6.8	100.16	16.56	1	0	–	0	100.34
Lig11	–4.3	146.21	–31.09	3	0	–	0	123.69
Lig12	–4	466.47	56.17	6	0	–	0	337.01

EF: affinity energy; MW: molecular weight; cLogP: liposolubility; HBA: hydrogen bond acceptors; HBD: hydrogen bond donors; RO5: the Lipinski rule of 05; RB: rotable bonds; TPSA: topological polar surface area; Lig1: 7-*epi*-silphiperfol-5-ene; Lig2: silphiperfol-5-eno; Lig3: cameroonan-7α-ol; Lig4: silphiperfolan-6α-ol; Lig5: presilphiperfol-7-ene; Lig6: presilphiperfolan-1-ol; Lig7: Prenopsan-8-ol; Lig8: presilphiperfolan-8-ol; Lig9: temephos; Lig10: α-muurolene; Lig11: hexanal; Lig12: acetylcholine.

Table 5. Pharmacokinetic properties of sesquiterpenes (*A. tomentosa*) and temephos *in silico*

Molecule	Abs / %	VDss / (L kg^{-1})	Fu	1A2	2C19	2C9	2D6	3A4	Hept.	TC / (mg kg^{-1} day^{-1})
Lig1	96.68	0.75	0.11	yes	no	no	no	no	no	0.99
Lig2	96.68	0.75	0.11	yes	no	no	no	no	no	0.99
Lig3	92.83	0.75	0.21	yes	no	no	no	no	no	0.79
Lig4	98.66	0.78	0.01	yes	no	no	no	no	no	0.97
Lig5	95.43	0.78	0.18	no	no	no	no	no	no	0.82
Lig6	94.14	0.54	0.32	no	yes	yes	no	no	no	0.76
Lig7	93.27	0.4	0.37	no	no	no	no	no	no	0.81
Lig8	95.18	0.57	0.35	yes	yes	yes	no	no	no	0.78
Lig9	88.80	–0.21	0	no	yes	yes	no	no	no	–0.09
Lig10	96.59	0.67	0.22	no	no	no	no	no	no	1.18
Lig11	95.79	0.05	0.58	no	no	no	no	no	no	0.43
Lig12	100	0.14	0.81	no	no	no	no	no	no	0.84

Abs: absorption rate; VDss: static distribution volume; Fu: fraction unbound; 1A2 = CYP1A2; 2C19 = CYP2C19; 2C9 = CYP2C9; 2D6 = CYP2D6; 3A4 = CYP3A4: if molecule is a CYP inhibitor; Hept.: hepatotoxicity; TC: renal clearance; Lig1: 7-*epi*-silphiperfol-5-ene; Lig2: silphiperfol-5-ene; Lig3: cameroonan-7α-ol; Lig4: silphiperfolan-6α-ol; Lig5: presilphiperfol-7-ene; Lig6: presilphiperfolan-1-ol; Lig7: prenopsan-8-ol; Lig8: presilphiperfolan-8-ol; Lig9: temephos; Lig10: α-muurolene; Lig11: hexanal; Lig12: acetylcholine.

Table 6. Drug likeness, drug score and toxicity values recorded for sesquiterpenes (*A. tomentosa*) and temephos

Molecule	DL	DS	Mut	Tum	Rep	Irrit	LD_{50} / (mol kg^{-1})	LOAEL / (mg kg^{-1} bw day^{-1})	MRTD / (mg kg^{-1} day^{-1})
Lig1	0.4877	0.4877	none	none	none	none	1.639	1.428	0.07
Lig2	0.4877	0.4877	none	none	none	none	1.639	1.428	0.07
Lig3	–2.336	0.4476	none	none	none	none	1.681	1.219	–0.22
Lig4	–3.490	0.3885	none	none	none	none	1.695	1.438	0.145
Lig5	–2.922	0.3966	none	none	none	none	1.602	1.370	0.075
Lig6	–1.067	0.5206	none	none	none	none	1.771	1.473	0.236
Lig7	–2.963	0.3975	none	none	none	none	1.998	1.609	0.583
Lig8	–1.747	0.4761	none	none	none	none	1.783	1.213	0.363
Lig9	–3.942	0.0281	low	high	high	high	3.279	0.554	0.687
Lig10	–3.433	0.3884754	none	none	none	none	1.543	1.503	0.37
Lig11	–14.29	0.1037099	high	none	high	high	1.762	1.922	0.833
Lig12	–1.438	0.590093	none	none	none	none	2.313	1.922	0.345

DL: drug likeness; DS: drug score; Mut: mutagenicity; Tum: tumorigenicity; Rep: effects on reproductive system; Irrit: irritating effects; LD_{50}: oral rat acute toxicity; LOAEL: oral rat chronic toxicity; MRTD: maximum recommended tolerated dose. Lig1: 7-*epi*-silphiperfol-5-ene; Lig2: silphiperfol-5-ene; Lig3: cameroonan-7α-ol; Lig4: silphiperfolan6-α-ol; Lig5: presilphiperfol-7-ene; Lig6: 9-β-presilphiperfolan-1α-ol; Lig7: prenopsan-8-ol; Lig8: presilphiperfolan-8-ol; Lig9: temephos; Lig10: α-muurolene; Lig11: hexanal; Lig12: acetylcholine.

Reducing the number of mosquito vectors is a consolidated strategy used to control disease transmission. The four main insecticide classes mostly used for mosquito vector control purposes comprise chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids.[41] Environmental toxicity parameters' prediction carried out in the US Environmental Protection Agency (EPA) software[29] has shown that sesquiterpene triquinane compounds may have lower environmental impact than that observed for temephos (organophosphate), as shown in Table 7.

Finding selectivity for these compounds in mosquitoes is a challenge in the process to develop new bioinsecticides, since AChE of vertebrates has similarities to that of mosquito vectors (Yellow Fever and *Aedes*). However, their structural differences, mainly the ones associated with amino acid changes at the entry site, such as free cysteine residues at the entry site of the mosquito active receptor channel, which corresponds to Phe295 in humans, may be a potential target for selective covalent inhibitors, as well as target for the development of new biopesticides that are more selective and less aggressive to humans.[47] Although the potential of covalent inhibitors has been investigated in several studies, inhibitors showing different action modes, such as non-covalent inhibitors, remain poorly explored.[49]

Table 7. Environmental toxicity parameters observed for sesquiterpenes obtained from *A. tomentosa* essential oil, as well as for temephos

Molecule	Lkw	BD	PT	BE	Vw	HLL	TR	TB	TSa	Tair
Lig1	–4.13	0.05	none	none	high	high	2.003	0.695	–0.84	–0.84
Lig2	–0.92	0.22	high	none	low	none	1.849	1.967	0.18	0.18
Lig3	3.04	0.90	none	none	none	none	2.384	1.006	0.465	0.465
Lig4	–25.56	0.21	none	none	none	none	1.951	3.075	–0.627	–0.627
Lig5	–25.56	0.24	none	none	none	none	1.885	2.994	–0.497	–0.497
Lig6	–28.97	0.05	high	high	none	high	1.887	3.066	–0.445	–0.445
Lig7	–25.22	0.18	none	none	none	none	1.933	3.291	–0.452	–0.452
Lig8	–25.22	0.05	high	high	none	high	1.888	3.138	–0.392	–0.392
Lig9	–25.22	0.23	none	none	none	none	1.869	3.062	–0.348	–0.348
Lig10	–25.22	0.09	none	high	none	high	1.855	2.988	–0.293	–0.293
Lig11	–25.22	0.09	none	high	none	high	1.855	2.988	–0.293	–0.293
Lig12	–25.22	0.09	none	high	none	high	1.855	2.988	–0.293	–0.293
Lig13	–25.22	0.09	none	high	none	high	1.855	2.988	–0.293	–0.293

Environmental parameters described by the US International Protection Agency (EPA).[29] Lkw: log Kow; BD: fast biodegradability (environmental biodegradability); PT: persistence time (persistence time in the environment); BE: bioaccumulation estimation (estimation of bioaccumulation in the environment); Vw: volatilization from water (ability to volatilize in water); HLL: half-time lake model (half-life in lake); TR: total removal (ability to remove the substance from the environment); TB: total biodegradation (biodegradation time in the environment); TSa: total sludge adsorption (sludge absorption time); Tair: total to air (time particles' accumulation in the air); Lig1: 7-*epi*-silphiperfol-5-ene; Lig2: silphiperfol-5-ene; Lig3: cameroonan-7α-ol; Lig4: silphiperfolan-6α-ol; Lig5: presilphiperfol-7-ene; Lig6: presilphiperfolan-1-ol; Lig7: prenopsan-8-ol; Lig8: presilphiperfolan-8-ol ; Lig9: temephos; Lig10: α-muurolene; Lig11: hexanal; Lig12: acetylcholine; and Lig13: 2-methyl-1-[1-(pentan-3-yl)-1*H*-pyrazol-4-yl]propan-1-one.[42]

Conclusions

The current study assessed the chemical composition of EOAT, as well as its potential to act as larvicide in tests conducted both *in vitro* and *in silico*. Based on GC-MS, GC-FID, and mono and bidimensional NMR analyses, sesquiterpene triquienanes, such as presylphiperfolan-1-ol, silphiperfol-5-ene and presylphiperfol-7-ene, were the major components of the investigated EO. Analyses performed with 3rd and 4th instar larvae of *A. aegypti in vitro* have evidenced 100% larval mortality 48 h after the beginning of the test. Assessments conducted *in silico* have shown high binding energies between essential oil components and the active site of the AchE enzyme. They were even higher than that observed for organophosphate larvicide temephos. Results in the current study have indicated that the investigated EOAT and its isolated constituents can be explored as potential environmentally and human safe larvicides. Further studies should be conducted with the investigated EOAT, and with its isolated constituents, to assess their effects on non-target organisms, as well as to define their effective concentrations for larvicidal activity. These results are important to help produce new safe, sustainable and biodegradable larvicides.

Supplementary Information

Supplementary information about ligand-receptor complexes of triquinane sesquiterpenes investigated in the current research is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Vilisaimon S. de Jesus was responsible for conceptualization, validation, formal analysis, investigation, writing original draft, visualization; Juliana L. Leite for validation, formal analysis, investigation, writing original draft; Jailan S. Sousa for validation, formal analysis, investigation, data curation, writing original draft; Fabricio S. Barbosa for validation, formal analysis, investigation, data curation, writing original draft; Djalma M. de Oliveira for conceptualization, methodology, validation, formal analysis, investigation, resources, writing review and editing, visualization, funding acquisition; Rubens S. Barreto for validation, formal analysis, investigation, writing review and editing; Wagner R. A. Soares for conceptualization, methodology, validation, formal analysis,

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