Journal of Medical Entomology, 57(2), 2020, 463–476
doi: 10.1093/jme/tjz171
Advance Access Publication Date: 31 October 2019
Research

Design of a Repellent Against *Aedes aegypti* (Diptera: Culicidae) Using in silico Simulations With AaegOBP1 Protein

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Subject Editor: Julian Hillyer

Received 24 June 2019; Editorial decision 4 September 2019

Abstract

Skin irritation has been reported to be the main adverse effect of excessive use of *N*,*N*-diethyl-*m*-toluamide (DEET) and ethyl 3-acetyl(butyl)amino (IR3535) commercial repellents. Therefore, there is an interest in alternatives of natural origin such as essential oils (EOs) and major compounds, which have repellent effects but have no contraindications. The main purpose of the present study was to identify the repellent effect of selected terpenes on *Aedes aegypti* Linnaeus, 1762 (Diptera: Culicidae) by in silico analysis based on their affinity with the odorant protein AaegOBP1. The protein-metabolite interactions in 20 terpenes were analyzed using the SwissDock tool. Terpenes presenting the highest affinity compared with commercial repellents were selected to evaluate repellent activity at concentrations 0.1, 10, and 25% against *Ae. aegypti*. Different periods (0–2, 2–15, 15–60 min) were evaluated with DEET as a positive control. The toxicity of terpenes was verified through Osiris and Molinspiration Cheminformatics Software, and cytotoxicity assays in *Vero* and *HepaRG* cells were performed using the MTT method. Two formulations were prepared with polyethylene glycol to evaluate skin long-lasting in vivo assay. The results showed four terpenes: geranyl acetate, nerolidol, α-bisabolol, and nerol, with affinity to AaegOBP1 comparable with DEET and IR3535. Geranyl acetate, nerolidol, and their mixtures showed no cytotoxicity and protection percentages close to 100% during the test at concentrations 10 and 25%. Long-lasting assays with geranyl acetate and nerolidol formulate showed 3 h as maximum protection time with 100% protection percentage. These metabolites and their mixtures are candidates to repellent formulations with times and protection percentages similar to DEET.

Key words: repellent, molecular docking, secondary metabolite, odorant-binding protein, protection time

Aedes aegypti (L.), a mosquito species, is the primary vector of arboviruses such as dengue, chikungunya, and Zika. These diseases affect particularly human populations located in tropical zones, where up to 2.7 million cases are reported annually (Murray et al. 2013, Yactayo et al. 2016, Colón-González et al. 2017). The most usual prevention strategy against these diseases consists in the use of personal protection items such as long-sleeved clothing or clothing impregnated with synthetic substances such as permethrin, or installing mosquito nets. Additionally, the use of topical repellents containing synthetic substances is recommended, e.g., *N*,*N*-diethyl-*m*-toluamide (DEET) or ethyl 3-acetyl(butyl)amino (IR3535) propanoate (Cisak et al. 2012, Orsborne et al. 2016, Achee et al. 2019). However, the effectiveness of these synthetic repellents contrasts with reports on their adverse effects, such as nervous system toxicity and skin irritation in children and older adults (Department of Health Toxicology Unit 2002, Faiman et al. 2010, Tavares et al. 2018). For this reason, the search for alternative protection based on naturally occurring compounds has focused on the use of essential oils (EOs) produced by plants, e.g., by species from the genera *Citronella*, *Citrus*, *Eucalyptus*, *Cymbopogon*, and *Thymus*, whose repellent effect has been noted both by science and cultural tradition (Nerio et al. 2010, Maia and Moore 2011, Hsu et al. 2013, Diaz 2016, Misni et al. 2016, Castillo et al. 2017).

The repellent effect generated by some molecules is due to the insect's capacity to detect a variety of chemical signals via its sensory receptors, located mainly on the antennae and maxillary palps. A specific behavioral response of attraction or repellence is generated depending on the type of chemical signal captured by the insect; this mechanism is reflected by the insect's loss of sensory capacity (Bohbot and Dickens 2010, Liu et al. 2016, Guo and Smith 2017, Jacob 2018). Although the mechanism of action of repellent compounds on insects of medical importance is poorly understood at the molecular level, this mechanism can be expected to be similar to the process by which odorant substances are perceived. Similar processes have been observed in the fruit fly *Drosophila melanogaster* (Xu et al. 2005, Guo and Smith 2017). The behavioral effect begins with the reception of volatile organic compounds (VOCs) produced by plants and animals and captured by trichoid sensilla, specialized chemoreceptors present in the antennae. These VOCs pass through the sensilla to the sensillar lymph (hydrophilic medium at pH close to 7), where they are transported by odorant-binding proteins (OBPs). These OBPs are characterized by a binding pocket where molecules are bound via different types of molecular interactions (Leal 2013, Bohbot and Pitts 2015, Leal and Leal 2015, Northey et al. 2016).

The main OBP in *Ae. aegypti* is known as AaegOBP1, and it is located in chemoreceptor neurons of maxillary antennae and palps (Bohbot and Dickens 2012). This globular protein is composed of 125 amino acid residues of an approximate cavity depth of 2.3 nm, and the odorant-binding site is composed of arginine 23 (Arg23), tyrosine 54 (Tyr54), and isoleucine 125 (Ile125) (Zhou et al. 2008, Leite et al. 2009). These residues interact with odorant molecules via hydrophobic and hydrophilic interactions, facilitating their transport through the sensillar lymph. The protein-ligand complex, formed by the OBP and the odorant molecule, extends to the cellular membrane of olfactory receptor neuron (ORN) dendrites, where odorant receptors (ORs) are found. Interactions between OBPs and ORs take place in a low-pH medium favorable for the conformational change of OBPs and the subsequent release of the odorant molecule in receptors (Bohbot and Pitts 2015). ROs are specialized proteins formed by receptor-coreceptor heterodimers that allow for the passage of ions through the neuron membrane (Benton 2006, Xu et al. 2014), which transforms a chemical signal into an electrical signal (Guo and Smith 2017) (Fig. 1). The response produced by the mosquito will be of attraction or repellence depending on the odorant perceived by the insect (Seenivasagan et al. 2012, Xu et al. 2014).

In silico studies have predicted interactions among different compounds of biological interest and identified substances with a high probability of interacting at the binding sites responsible for specific biological functions. This information has allowed for the design of molecules targeting biological structures that are difficult to extract or purify, such as proteins and enzymes (Alonso et al. 2006, Gopal and Kannabiran 2013, Devillers et al. 2014). The scarce computational studies focused on the search for molecules with a potential repellent effect against *Ae. aegypti* have used knowledge of physicochemical and molecular properties to predict their biological activity (Natarajan et al. 2008, Oliferenko et al. 2013, Andrade-Ochoa et al. 2018); for this reason, the present study sought to evaluate the repellent effect of different secondary metabolites (terpenes) selected based on the analysis of multiple in silico simulations of their physicochemical properties and molecular coupling with the AaegOBP1 protein. In addition, repellency tests against *Ae. aegypti* were carried out in vivo, as well as cytotoxicity tests in vitro, to verify the computational results.

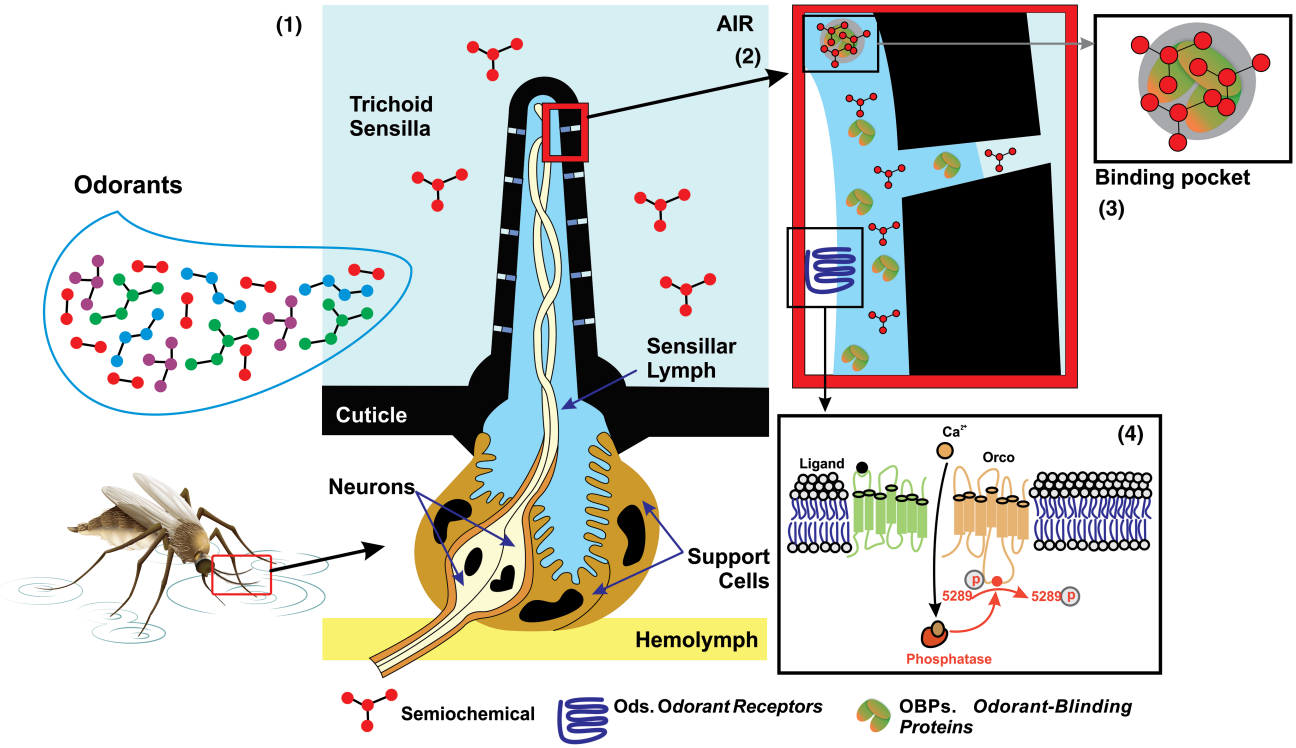

Fig. 1. Mechanism of reception of odorants in insects (Adapted from Guo and Smith 2017 and Leal 2013). Image: (1) Odorant molecules released by animals and plants reach the trichoid sensilla on the maxillary antennae and palps. (2) Odorant molecules reach the sensillar lymph after passing through the sensilla. (3) Odorant molecules bind to OBPs in the sensillar lymph and are transported in a binding pocket toward the ORs. (4)These ROs, located in the cellular membrane of ORNs, transform the chemical signal into an electrical signal, which triggers a behavioral response.

Materials and Methods

Selection and Evaluation of Secondary Metabolites

A literature review was carried out to identify reports on the biological activity of different EOs against mosquitoes of medical importance. The pre-selection criterion for EOs was their repellent activity records; on this basis, we pre-selected EOs whose major compounds were among terpenes and terpenoids (Gillij et al. 2008, Vera et al. 2014, Ríos et al. 2017).

In silico Analysis: Molecular Docking and Toxicity Analysis

Molecular docking was carried out using the SwissDock platform (http://www.swissdock.ch/), which is based on the EADock DSS algorithm and the CHARMM force field (Grosdidier et al. 2011). The structure *Ae. aegypti*'s odorant protein AaegOBP1 was obtained from the Protein Data Bank (PDB), under code 3K1E. The 3K1E. PDB protein consists of two identical A and B chains; for the B chain, the water molecules, Cl^- and Mg^{+2} ions, and heptacosaethylene glycol monomethyl ether were eliminated from the protein. The chemical structures of the 20 pre-selected metabolites and the reference compounds (DEET and IR3535) (Fig. 2) were obtained from the ZINC AC platform, specialized in molecular structures for docking (Grosdidier et al. 2011). UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) software was employed to prepare the protein (elimination of metal ions and irrelevant molecules from the structure obtained from the PDB) and analyze the conformational ensembles of each metabolite in the possible binding sites for the odorant protein AaegOBP1 presenting less Gibbs's free energy; $\Delta G_{binding}$ (kcal/mol). The same procedure was used for DEET and IR3535 commercial repellents.

Metabolites with a lower Gibbs free energy value $\Delta G_{binding}$ (kcal/mol), i.e., higher spontaneity of protein-metabolite coupling, as well as reference compounds, were analyzed for cytotoxic potential using the Osiris (https://www.organic-chemistry.org/prog/peo/) and Molinspiration Cheminformatics (https://www.molinspiration.com/) software packages. Some of Lipinski's parameters were used as quantifiers of biological properties: Logarithm of partition

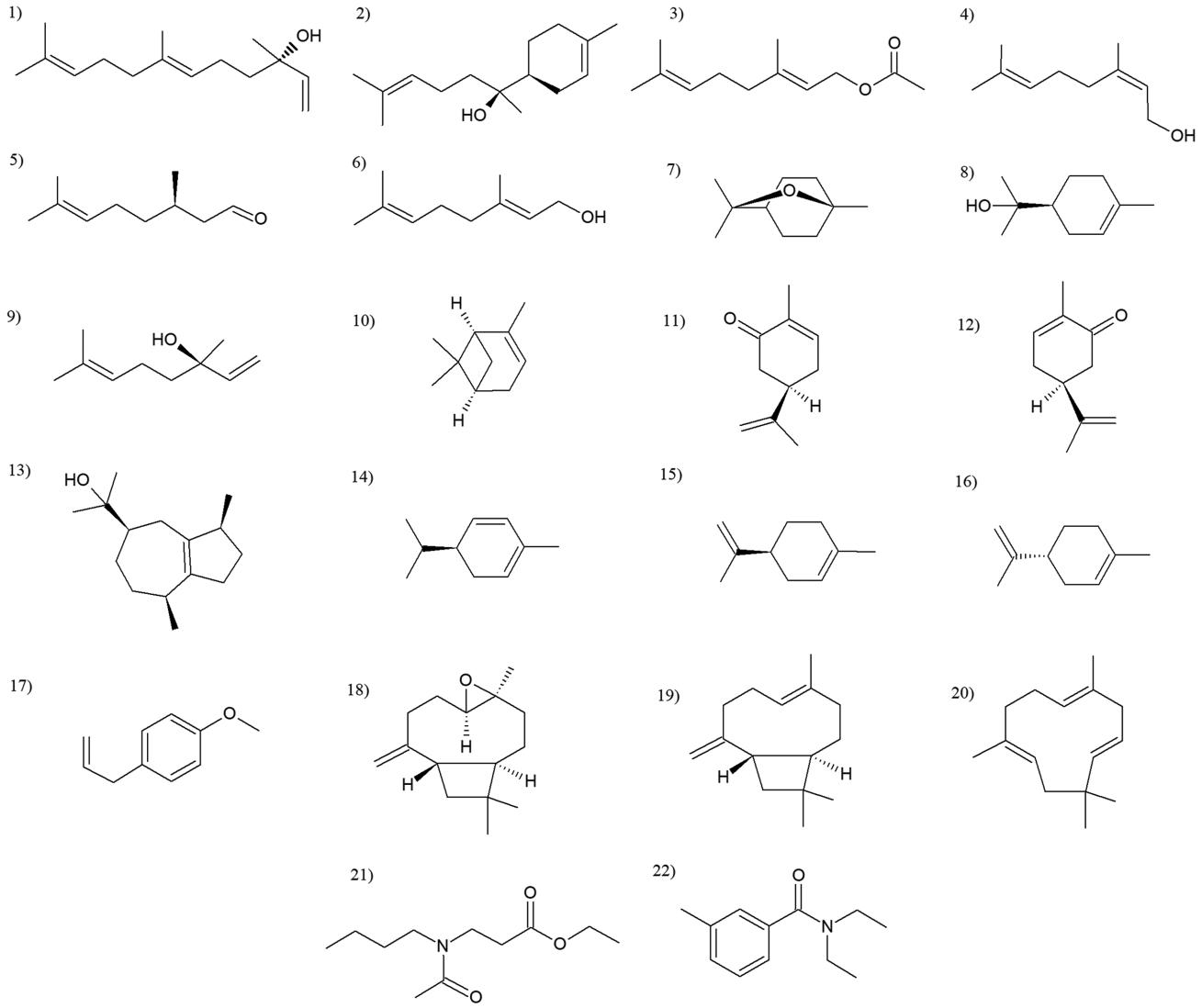

Fig. 2. Chemical structures of the 20 analyzed metabolites and reference compounds (DEET and IR3535). 1) Nerolidol, 2) α-bisabolol, 3) Geranyl acetate, 4) Nerol, 5) Citronellal, 6) Geraniol, 7) Eucalyptol, 8) α-terpineol, 9) Linalool, 10) (S) -(-)-pinene, 11) (-)-(R)-carvone, 12) (+)-(S)-carvone, 13) (-)-guaiol, 14) α-phellandrene, 15) (-)-(S)-limonene, 16) (+)-(R)-limonene, 17) Estragole, 18) (-)-caryophyllene oxide, 19) (-)-trans-caryophyllene, 20) α-humulene, 21) IR3535 (ethyl 3-acetyl(butyl) amino propanoate), and 22) DEET (*N,N*-Diethyl)-*m*-toluamide).

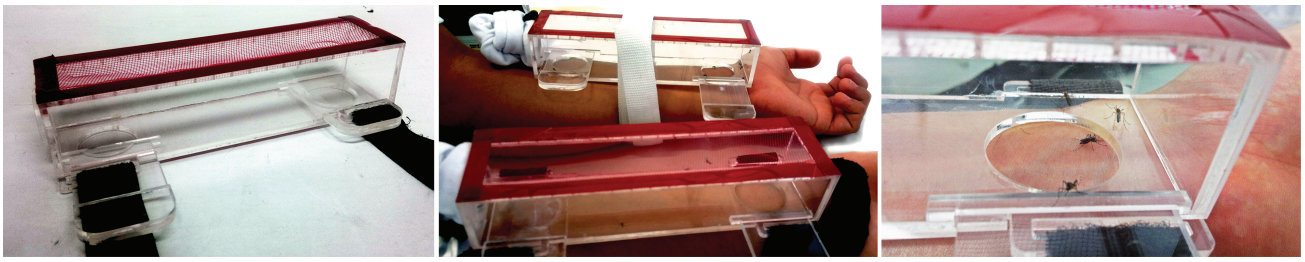

Fig. 3. In vivo repellent activity. Left: Acrylic devices used for tests Center: Location of devices on the forearms of a volunteer. Right: Area of 29 mm in diameter exposed to mosquitoes (Castillo et al. 2017).

coefficient and solubility in lipids and aqueous solutions (LogP); logarithm of solubility in aqueous solutions (LogS), and mutagenic, tumorigenic, and reproductive risk analysis. Metabolites presenting high spontaneous molecular assembly with the odorant protein and had no reported cytotoxic risks in silico were used for in vivo repellency tests.

Biological Material

Bioactivity assays were carried out using an *Ae. aegypti* Rockefeller strain, which was placed in safety cages and stored in an insectarium at a temperature of 25 ± 5°C, relative humidity of 70 ± 5%, and 12:12 (L:D) h photoperiod. Males were continuously fed with 10% (v/v) honey solution (carbohydrates). To promote egg production, females were periodically fed with albino Wistar rat (WI IOPS AF/Han strain) blood, provided by the Industrial University of Santander's bioterium, in compliance with Colombian Congress Act 84 (1989) and Colombian Ministry of Health Resolution 8430 (1993). The hatched larvae were stored in plastic containers and fed 0.5 g Tetramin Tropical Flakes fish feed three times a week.

In vivo Repellent Activity Bioassays

Compounds selected from in silico analysis for biological tests were commercially acquired from Sigma–Aldrich (St. Louis, MI). Repellent activity essays were conducted per the method proposed by Barnard et al. 2006. Men and women between 18 and 35 yr of age volunteered for the study and participated after having signed a consent agreement approved by the Ethics Committee Scientific Research (CEINCI) Act No. 3/2013.

An area of approximately 4 cm^2 was marked on the volunteers' forearms; these areas were impregnated with 50 µl of each metabolite selected using bioinformatics tools at concentrations of 0.1, 10, and 25% (v/v) dissolved in acetone. Once the treatments were applied, a prudent time of 5 min was allowed for the solvent to evaporate and to control for possible allergic reactions to the substance. Participants' right forearms were treated with the terpenes under study, and their left forearms were used as a negative control (50 µl acetone at 99% v/v). The repellent substance DEET was used as a positive control at a concentration of 25% (v/v). Subsequently, 20 *Ae. aegypti* females aged 5–10 d, both nulliparous and parous, were placed in rectangular acrylic containers (18 × 4 × 5 cm) (Castillo et al. 2017). Each container had a 29-mm diameter opening at its lower side intended to expose only a section of the volunteer's forearm to mosquitoes (Fig. 3).

Each trial was carried out in triplicate on different days and with different volunteers. The number of bites during the periods from 0 to 2 min, 2–15 min, and 15–60 min were counted in order to estimate the protection percentage for each interval using a modified version of the equation proposed by Phasomkusolsil and Soonwera (2010):

Table 1. Gibbs free energy values of the coupling, $\Delta G_{binding}$ (kcal/mol), and number of hydrogen bridges available for the most spontaneous conformation (lower Gibbs free energy $\Delta G_{binding}$) of secondary metabolites and repellent compounds DEET and IR3535 containing the odorant protein AaegOBP1

Molecule		$\Delta G_{binding}$ (kcal/mol)	Number of hydrogen bonds
Positive control	IR3535	−8.233	0
	DEET	−7.507	0
Metabolites	Nerolidol	−8.252	1
	α-bisabolol	−8.184	1
	Geranyl acetate	−7.975	0
	Nerol	−7.402	1
	Citronellal	−7.294	0
	Geraniol	−7.269	0
	Eucalyptol	−7.242	0
	α-terpineol	−7.213	0
	Linalool	−7.207	0
	(-)-(*S*)-pinene	−7.202	0
	(-)-guaiol	−7.095	0
	(-)-(*R*)-carvone	−7.081	0
	α-phellandrene	−7.069	0
	(+)-(*S*)-carvone	−7.041	0
	(-)-(*S*)-limonene	−7.040	0
	(+)-(*R*)-limonene	−7.039	0
	Estragole	−6.917	0
	(-)-caryophyllene oxide	−6.920	0
	(-)-*trans*-caryophyllene	−6.707	0
	α-humulene	−6.524	0

$$\% \text{ Protection} = \frac{Nc - Nt}{Nc} * 100\ \%$$

Nc = Number of bites in control arm; Nt = Number of bites in treated arm.

Evaluation of Metabolite Mixtures

Metabolites whose percentages of protection showed values similar to those obtained using commercial repellent DEET were used to prepare four mixtures in a 1:1 ratio by volume using acetone as a solvent to evaluate their repellent activity in vivo under the conditions described above.

Evaluation of Formulations

After the evaluation of metabolites and mixtures, those presenting the highest protection percentages in vivo during the test period (1 h) and lowest in vitro cytotoxicity values were selected to prepare a formulation. Substances were dissolved in a solution composed of 10% or 15% polyethylene glycol (PEG 400), 10% milli-Q water,

and 65–70% ethanol. In vivo tests were carried out to count the number of bites during non-adjacent periods of 15 min per hour until the first bite in the treated forearm was observed; the protection percentage was calculated. The negative control used the amounts of PEG 400, milli-Q water, and ethanol mentioned above.

In vitro Cytotoxicity Tests

Cell lines *Vero* (normal epithelial cells from the kidney of an African green monkey) and *HepaRG* (normal human hepatocyte cells) were cultivated in EMEM (Eagle's Minimum Essential Medium: 7% bovine fetal serum at a pH of 7.2 and 112.5 mg gentamicin) and Williams' medium (10% fetal bovine serum at a pH of 7.2, 1% glutamine, and 112.5 mg/liter gentamicin), respectively. Cells were separated from the culture dishes using a trypsin-EDTA solution and then placed on a 96-well plate (1×10^4 cells per well) and incubated at 37°C in a 5% CO_2 atmosphere for 24 h for total adhesion to the plate. Under optimal metabolic conditions, cells were treated with the best metabolites, their mixtures, and the synthetic repellent compound DEET at a concentration of 200 µM for (maximum reference concentration with a potential cytotoxic effect on normal *Vero* and *HepaRG* cells) for 24 h.

Cell viability was determined by MTT method, as described by Mosmann (1983) and Riss et al. (2013). The supernatant of the 96-well plate was discarded after the treatment, and 200 µl MTT (500 µg/ml) was added to Hank's Balanced Salt Solution (HBSS) for subsequent incubation for 3 h. The MTT solution was removed, and 200 µl of dimethyl sulfoxide (DMSO) was added to solubilize the resulting crystals. Absorbance reading was carried out using a microplate reader (Thermo Scientific) at 570 nm using DMSO as a target. Viable and metabolically active cells reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt, forming DMSO-soluble formazan crystals. The LC_{50} value was calculated by comparing cell viability between treated and untreated cells for each of the evaluated compounds. Replications were carried out on the 96-well plate. Three wells were used for each substance and concentration, 9 wells as control, and 3 wells as target (DMSO).

Statistical Analysis

Data were subjected to Kolmogorov–Smirnov, Shapiro–Wilk, and Lillieford normality tests. When the data presented normal distribution, they were subjected to one-way ANOVA means comparison tests and a Tukey test. Non-parametric tests (Kruskall-Wallis) were applied when the distribution was not normal. Comparisons were considered statistically significant if $P < 0.05$. The software used for these analyses was Statistic V11 (Statsoft).

Results

In silico Analysis: Molecular Docking and Toxicity Analysis

Twenty metabolites derived from plant EOs were pre-selected based on the literature review; all of these EOs have been reported as presenting repellent-like protection percentages greater than 80% (Yang and Ma 2005, Gillij et al. 2008, Vera et al. 2014, Castillo et al. 2017, Ríos et al. 2017). Molecular assembly spontaneity was determined as Gibbs free energy, $\Delta G_{binding}$ (kcal/mol) in the metabolites interacting with the odorant protein AaegOBP1. Results showed

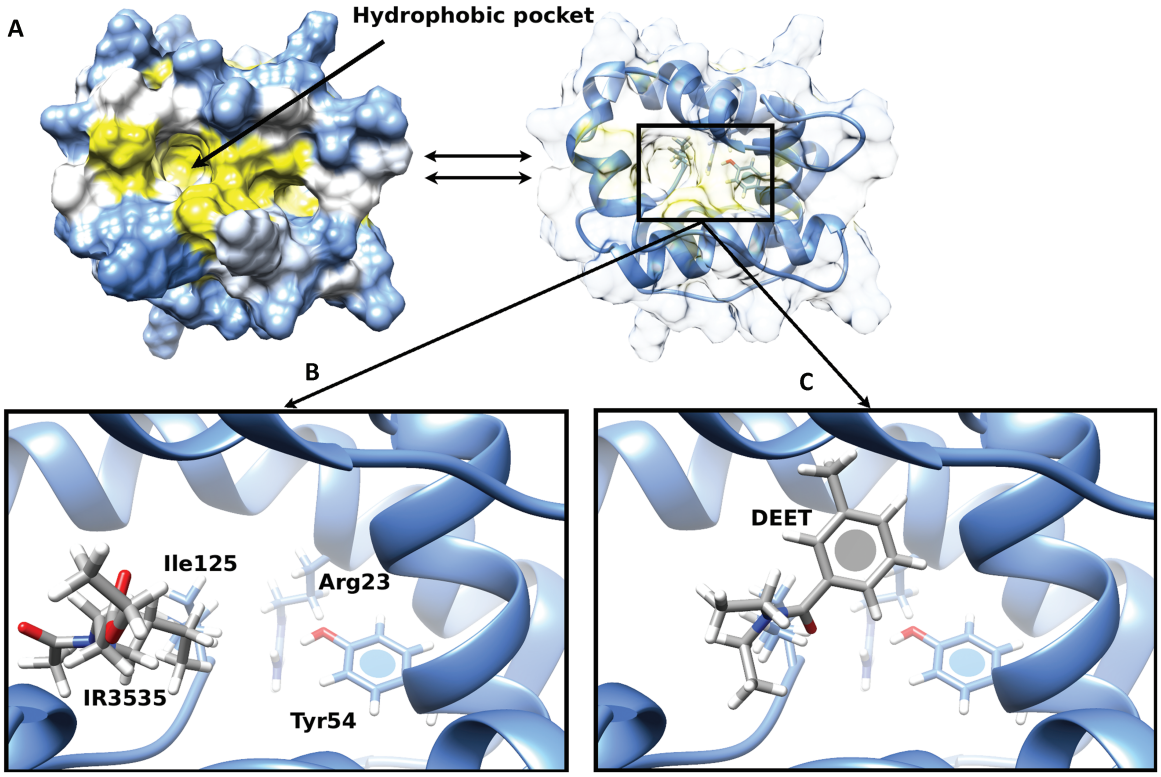

Fig. 4. Results of molecular docking: odorant protein - reference compounds. (A) Representation of AaegOBP1's hydrophobic surface and ribbons (surface at 40% transparency). Surface colors correspond to the Kyte-Doolittle scale (Yellow: hydrophobic zone; Blue: hydrophilic zone) (Kyte and Doolittle 1982). (B) IR3535 in hydrophobic cavity of protein at distances of 2.8 Å, 7.5 Å, and 9.6 Å from residues Ile125, Tyr54, and Arg23, respectively. (C) DEET in hydrophobic cavity of protein at distances of 2.6 Å, 8.1 Å, and 10.5 Å from residues Ile125, Tyr54, and Arg23, respectively. Images generated by UCSF Chimera (Pettersen et al. 2004).

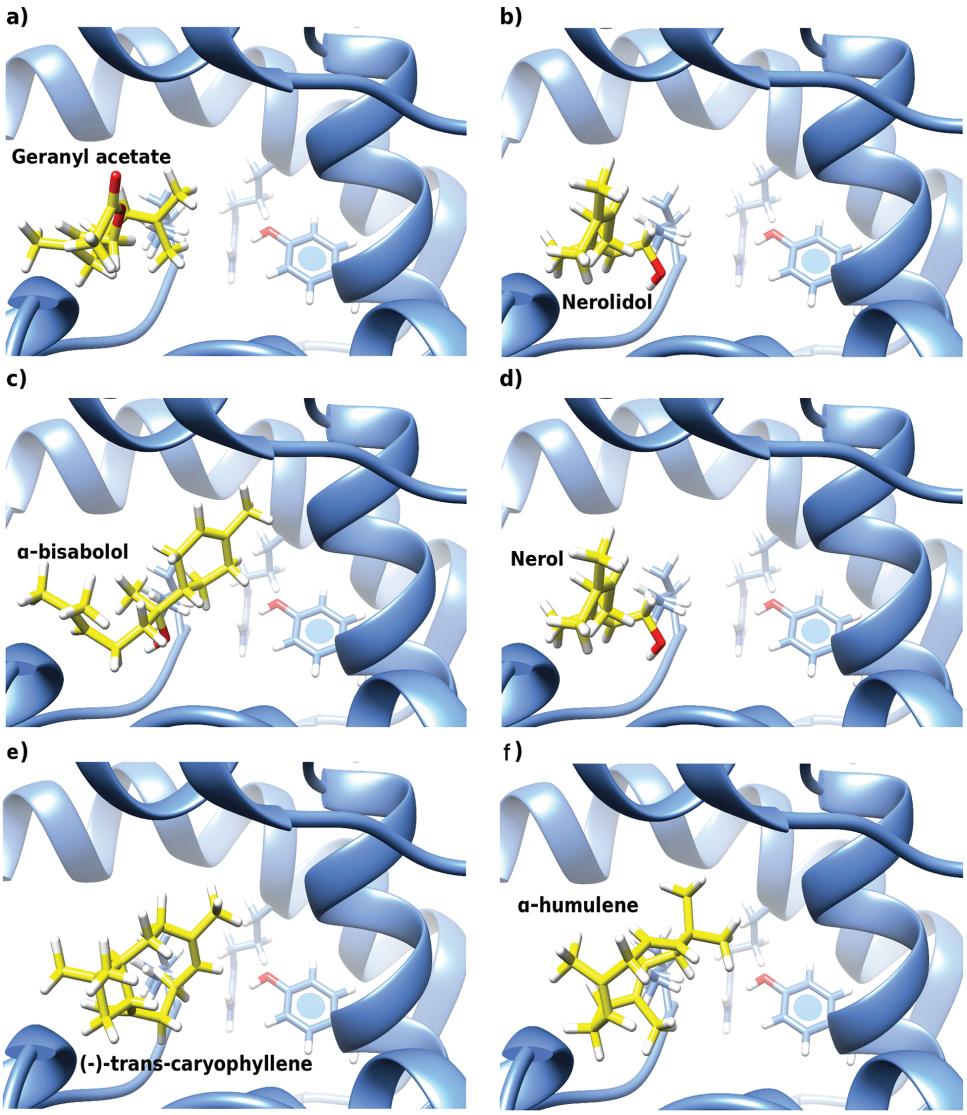

Fig. 5. Results of molecular docking: odorant protein - plant metabolites. (a) Geranyl acetate 2.7 Å from Ile125; (b) Nerolidol 4.2 Å from Ile125, and 6.9 Å from Tyr54; (c) α-bisabolol 2.0 Å from Ile125 and 7.3 Å from Tyr54; (d) Nerol 3.9 Å from Ile 125. Images generated by UCSF Chimera (Pettersen et al. 2004).

that the binding energies of four of the metabolites (geranyl acetate, nerolidol, α-bisabolol, and nerol) are similar to those produced by the binding of the reference compounds with the protein (Table 1). In addition, the protein-metabolite conformations with the lowest value of Gibbs' free energy corresponded to the location of the reference compounds in the odorant protein cavity formed by the residues Arg23, Tyr54, and Ile125 (Figs. 4 and 5), which have a compound transport function (Leite et al. 2009). On the other hand, the observed conformations showed that nerolidol, α-bisabolol, and nerol can present intermolecular interactions such as hydrogen bonds and London dispersion with the protein binding site, whereas DEET, IR3535, and geranyl acetate can display intermolecular dipole–dipole interactions and London dispersion with the odorant protein cavity.

Additionally, Lipinski's parameters determined for the selected metabolites (geranyl acetate, nerolidol, α-bisabolol, and nerol) showed that the octanol/water partition coefficient (LogP) values were higher than the values of the reference compounds (≥2.521), and the logarithm of solubility in aqueous solutions (LogS) values for geranyl acetate, nerolidol, and α-bisabolol were lower than those obtained for reference compounds DEET (−2,203) and IR3535 (−1,402). In addition, no mutagenic, tumorigenic, or reproductive risks were detected by metabolite toxicity predictions (Table 2).

In vivo Repellent Activity Bioassays

Percentages of protection and durability against *Ae. aegypti* female mosquito bites were determined by in vivo repellency tests, to verify the results obtained by the bioinformatics tools. In all cases, the metabolites geranyl acetate, nerolidol, α-bisabolol, and nerol showed protection percentages higher than 70% at concentrations of 10% and 25% (v/v) for each of the protection periods (0–2, 2–15, and 15–60 min). The highest percentages of protection were obtained in the 0–2 min interval (100%). Similar protection percentages (≥90%) were obtained with the two concentrations (10 and 25%) in the 60-min interval (Table 3).

The number of bites observed when evaluating the terpene-based treatments (geranyl acetate, nerolidol, α-bisabolol, and nerol) at different concentrations during the different exposure periods (0–2, 2–15, 15–60 min) was compared. The number of bites was observed to be higher during the first interval (0–2 min) when the

Table 2. Values of Lipinski's parameters for secondary metabolites geranyl acetate, nerolidol, α-bisabolol, nerol, and the repellent substances DEET and IR3535

Molecule	Lipinski's parameters		Molecular weight (g/mol)	Risk profiles:		
	LogP	LogS		1	2	3
IR3535	1.655	−1.402	222.86	NO	NO	NO
DEET	2.521	−2.203	191.27	YES	YES	YES
Geranyl acetate	3.970	−2.299	196.29	NO	NO	NO
Nerolidol	5.403	−3.125	222.37	NO	NO	NO
α-bisabolol	4.471	−3.162	222.37	NO	NO	NO
Nerol	3.485	−1.889	154.25	NO	NO	NO

Risk profiles: 1. Mutagenic 2. Tumorigenic 3. Reproductive.

Table 3. Protection percentage (% ±SD) of metabolites geranyl acetate, nerolidol, α-bisabolol, and nerol in concentrations of 0.1, 10, and 25% (v/v) during test intervals 0–2, 2–15, 15–60 min

Protection percentage (% ± SD)				
Concentration (% v/v)	Molecule	Test intervals (min)		
		0–2 min	2–15 min	15–60 min
25%	Geranyl acetate	100 ± 0.0	96 ± 0.4	97 ± 0.3
	Nerolidol	100 ± 0.0	100 ± 0.0	100 ± 0.0
	α-bisabolol	100 ± 0.0	90 ± 0.4	91 ± 0.4
	Nerol	100 ± 0.0	100 ± 0.0	72 ± 1.4
10%	Geranyl acetate	100 ± 0.0	100 ± 0.0	100 ± 0.0
	Nerolidol	100 ± 0.0	100 ± 0.0	100 ± 0.0
	α-bisabolol	100 ± 0.0	100 ± 0.0	100 ± 0.0
	Nerol	100 ± 0.0	100 ± 0.0	90 ± 0.7
0.1%	Geranyl acetate	0.0 ± 2.1	0.0 ± 1.6	0.0 ± 1.7
	Nerolidol	0.0 ± 1.7	0.0 ± 1.9	0.0 ± 1.6
	α-bisabolol	56 ± 0.6	12 ± 2.4	3 ± 2.0
	Nerol	57 ± 1.7	58 ± 1.8	28 ± 2.6
Positive control (25%)	DEET	100 ± 0.0	100 ± 0.0	100 ± 0.0

Positive control is 25% DEET (v/v).

concentration was 0.1% (v/v). During the 15- to 60-min interval, the highest number of bites was associated with nerol in the three concentrations evaluated. By contrast, the treatment with nerolidol resulted in lower numbers of bites at concentrations of 10 and 25% in all the intervals (Fig. 6). Statistically significant differences were found between the control (acetone) and the four metabolites in all evaluated intervals: geranyl acetate KW H (Geranyl acetate KW H (3, n = 33) = 26.36142, P < 0.05); (Nerolidol KW H (3, n = 33) = 13.13514, P < 0.05); (α-bisabolol KW H (3, n = 33) = 27.76607, P < 0.05); (Nerol KW H (3, n = 33) = 14.33929, P < 0.05). No statistically significant differences were found between any of the four metabolites and DEET in any of the evaluated intervals (Geranyl acetate KW H (3, n = 33) = 26.36142, P > 0.05); (Nerolidol KW H (3, n = 33) = 13.13514, P > 0.05); (α-bisabolol KW H (3, n = 33) = 27.76607, P > 0.05); (Nerol KW H (3, n = 33) = 14.33929, P > 0.05) (Fig. 6).

The effectiveness of each terpene as a repellent was evaluated by comparing the number of bites registered during the 15- to 60-min interval with each of the evaluated concentrations. When using concentrations of 10 and 25% (v/v), the number of bites observed in treatments with geranyl acetate, nerolidol, and α-bisabolol was lower than the number of bites observed with the control (acetone) with statistically significant differences [Geranyl acetate KW H (3, n = 15) = 12.15228, P = 0.0069]; [Nerolidol KW H (3, n = 15) = 13.74545, P = 0.0033]; [α-bisabolol KW H (3, n = 15) = 12.46737, P = 0.0059], but no statistically significant differences were found in comparison with DEET [Geranyl acetate KW H (3, n = 15) = 12.15228, P > 0.05]; [Nerolidol KW H (3, n = 15) = 13.74545, P > 0.05]; [α-bisabolol KW H (3, n = 15) = 12.46737, P > 0.05]. It is worth mentioning that nerol was the only metabolite with which bites were observed at concentrations of 10 and 25%, and there were no significant statistical differences compared to the control (acetone) [Nerol KW H (3, n = 15) = 9.097403, P > 0.05] (Fig. 7).

Evaluation of Metabolite Mixture

Four mixtures were prepared using a 1:1 ratio (Table 4) with the metabolites that were showing the best protection percentages in vivo (geranyl acetate, nerolidol, and α-bisabolol).

When evaluating the protection percentages of the four proposed mixtures, a protection percentage of 100% was observed with mixtures one and two at concentrations of 5 and 10% in all evaluated time intervals (Table 5). In the cases of mixtures 3 and 4, the protection percentages were over 81% at concentrations of 5 and 10%, and protection effectiveness decreased as exposure time increased. Mixture 4 was not evaluated at the 5% concentration due to the loss of effectiveness observed at 10% (v/v) during the test period (1 h).

The number of bites per interval was registered for each of the concentrations (Fig. 7); fewer bites were observed at concentrations of 5%, even during the interval corresponding to 60-min exposure.

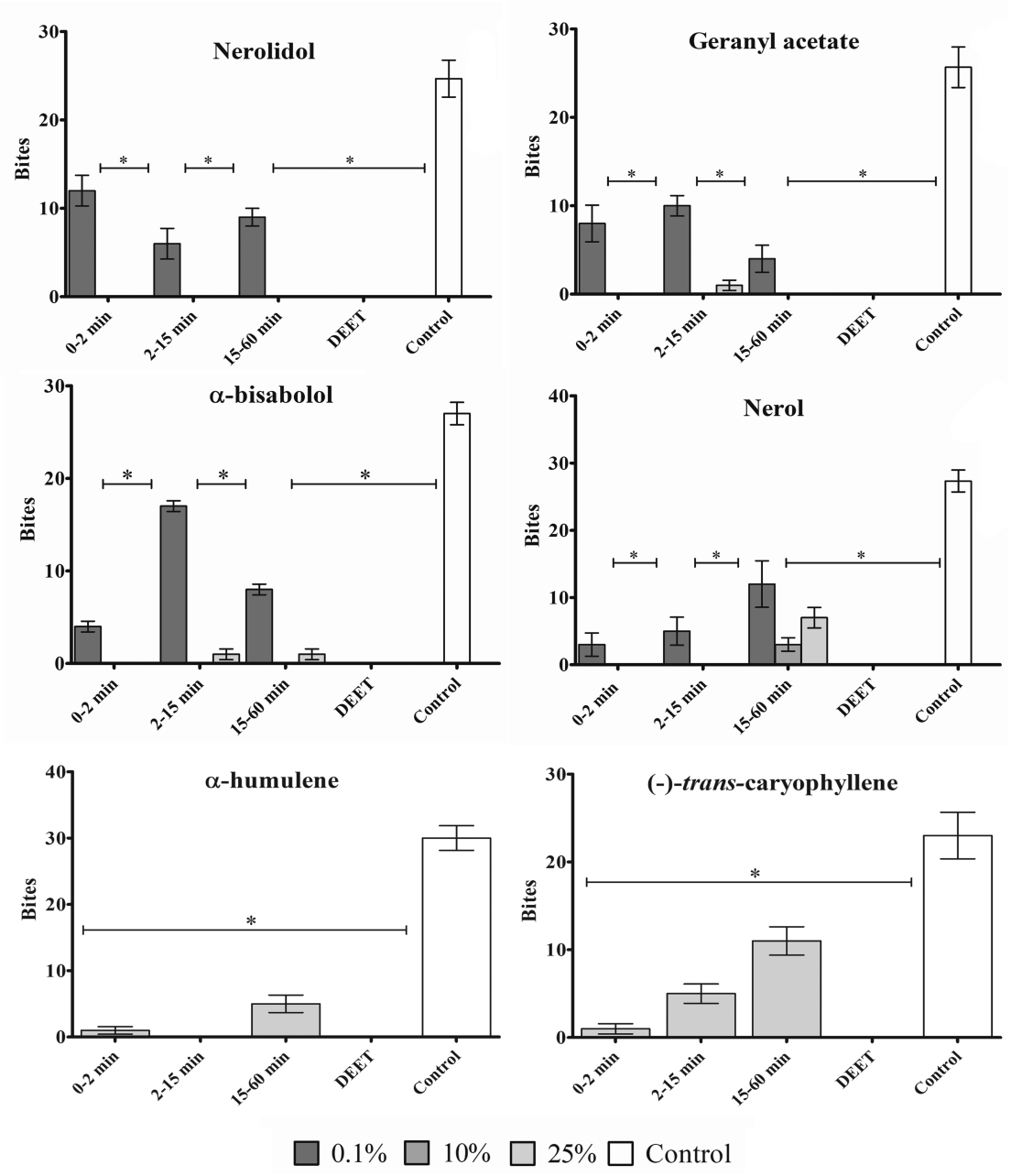

Fig. 6. Comparison of number of bites for each of the evaluated terpenes (geranyl acetate, nerolidol, α-bisabolol, and nerol) at different exposure times (0–2, 2–15, and 15–60 min). *Significant statistical differences between control (acetone) and bites observed during each interval (Geranyl acetate KW H (3, $n = 33$)=26.36142, $P < 0.05$); (Nerolidol KW H (3, $n = 33$) = 13.13514, $P < 0.05$); (α-bisabolol KW H (3, $n = 33$) = 27.76607, $P < 0.05$); (Nerol KW H (3, $n = 33$) = 14.33929, $P < 0.05$).

Table 4. Composition proposed for mixtures based on geranyl acetate, nerolidol, and α-bisabolol

Mixture	Composition
Mixture 1	Nerolidol, geranyl acetate, and α-bisabolol
Mixture 2	Geranyl acetate and nerolidol
Mixture 3	Nerolidol and α-bisabolol
Mixture 4	Geranyl acetate and α-bisabolol

Statistically significant differences were found between the mixtures and the control (acetone) when comparing the intervals [Mixture 1 KW H (3, $n = 24$) = 22.87895, $P < 0.05$; Mixture 2 KW H (3, $n = 24$) = 22.87895, $P < 0.05$; mixture 3 KW H (3, $n = 24$) = 16.11389, $P = 0.0011$; mixture 4 KW H (3, $n = 15$) = 9,853165, $P = 0.0073$] (Fig. 8).

The number of bites recorded during the 15- to 60-min interval when using each of the evaluated concentrations was compared in order to evaluate the repellent effectiveness of these mixtures (Fig. 8). No bites were recorded for mixtures one, two, three, and four at the 5% concentration. Mixture four was the only one in which biting was observed at a concentration of 10%. Mixtures one, two, and three, at concentrations of 5 and 10% (v/v) showed no statistically significant differences in comparison with DEET [Mixture 1 KW H (3, $n = 12$) = 10.73494, $P < 0.05$; Mixture 2 KW H (3, $n = 12$) = 10.73494, $P < 0.05$; Mixture 3 KW H

Table 5. Protection percentage (% ±SD) of mixtures based on metabolites geranyl acetate, nerolidol, α-bisabolol, and nerol at concentrations of 5 and 10% (v/v) during test intervals 0–2, 2–15, 15–60 min

Protection percentage (% ±SD)				
Concentration (% v/v)	Mixture	Test intervals (min)		
		0–2 min	2–15 min	15–60 min
10% each	Mixture 1	100 ± 0.0	100 ± 0.0	100 ± 0.0
	Mixture 2	100 ± 0.0	100 ± 0.0	100 ± 0.0
	Mixture 3	100 ± 0.0	100 ± 0.0	100 ± 0.0
	Mixture 4	100 ± 0.0	90 ± 0.8	81 ± 1.4
5% each	Mixture 1	100 ± 0.0	100 ± 0.0	100 ± 0.0
	Mixture 2	100 ± 0.0	100 ± 0.0	100 ± 0.0
	Mixture 3	94 ± 0.4	90 ± 0.5	86 ± 0.6
Positive control (25%)	DEET	100 ± 0.0	100 ± 0.0	100 ± 0.0

Mixture one: Nerolidol, geranyl acetate, and α-bisabolol; mixture two Geranyl acetate and nerolidol; mixture three Nerolidol and α-bisabolol and mix four: Geranyl acetate and α-bisabolol. DEET positive control (25% v/v). Mixture 4 was not evaluated at a 5% concentration due to the loss of effectiveness observed at a 10% (v/v) concentration during the test period (1 h).

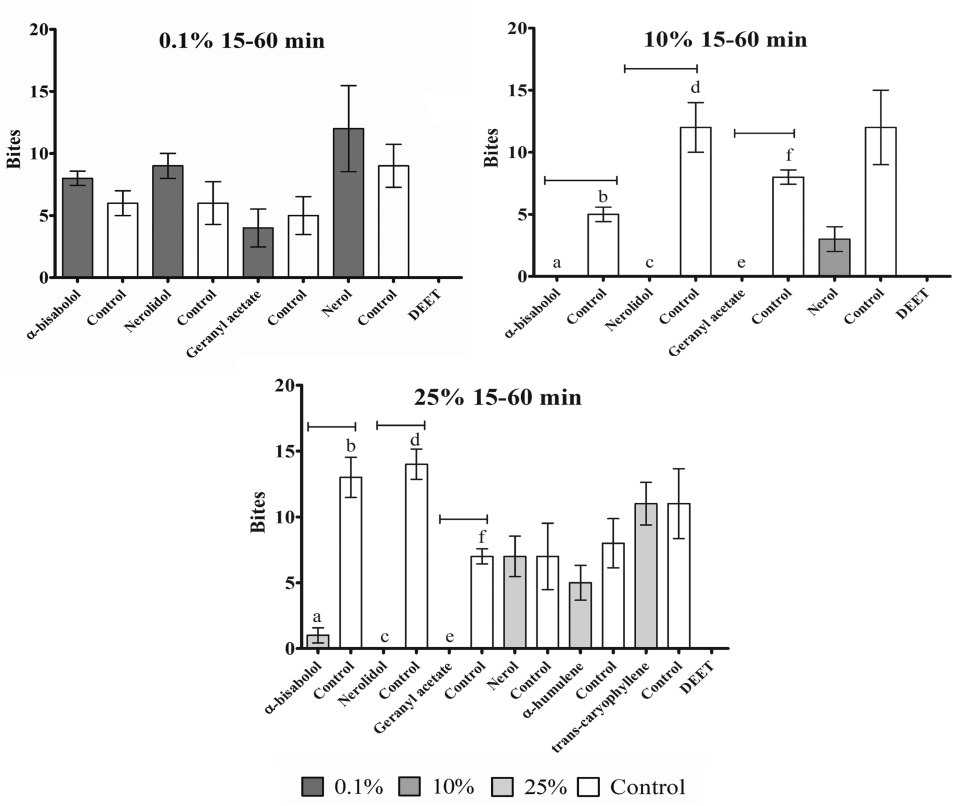

Fig. 7. Number of bites registered in the 15- to 60-min interval at concentrations of 0.1, 10, and 25% (v/v) for each metabolite (geranyl acetate, nerolidol, α-bisabolol, and nerol). (a, b), (c, d), (e, f) Statistically significant differences between the number of bites for each treatment and its control (acetone); (Geranyl acetate KW H (3, $n = 15$) = 12.15228, $P = 0.0069$); (Nerolidol KW H (3, $n = 15$) = 13.74545, $P = 0.0033$); (α-bisabolol KW H (3, $n = 15$) = 12.46737, $P = 0.0059$).

(3, n = 12) = 10.73494, $P < 0.05$], but statistically significant differences were observed with respect to the negative control (acetone) [mixture 1 KW H (3, n = 12) = 10.73494, P = 0.0132; mixture 2 KW H (3, n = 12) = 10.73494, P = 0.0132; Mixture 3 KW H (3, n = 12) = 10.73494, P = 0.0132]. Mixture four presented statistically significant differences with DEET [mixture 4 KW H (2, n = 9) = 6.720000, P = 0.0347], but not with the control (acetone) [mixture 4 KW H (2, n = 9) = 6.720000, $P < 0.05$] (Fig. 8).

Cytotoxicity Tests

In vitro cell viability tests were carried to confirm the toxicity levels of terpenes geranyl acetate, nerolidol, α-bisabolol, their mixtures, and the repellent synthetic substance DEET. The lowest lethal concentration 50 (LC_{50}) was associated with α-bisabolol (138 µM on Vero Cells and 190 µM on HepaRG cells); this terpene was the most cytotoxic against both cell lines. The LC_{50} of geranyl acetate, nerolidol, mixture 2, and DEET were higher than 200 µM on both cell lines (Table 6).

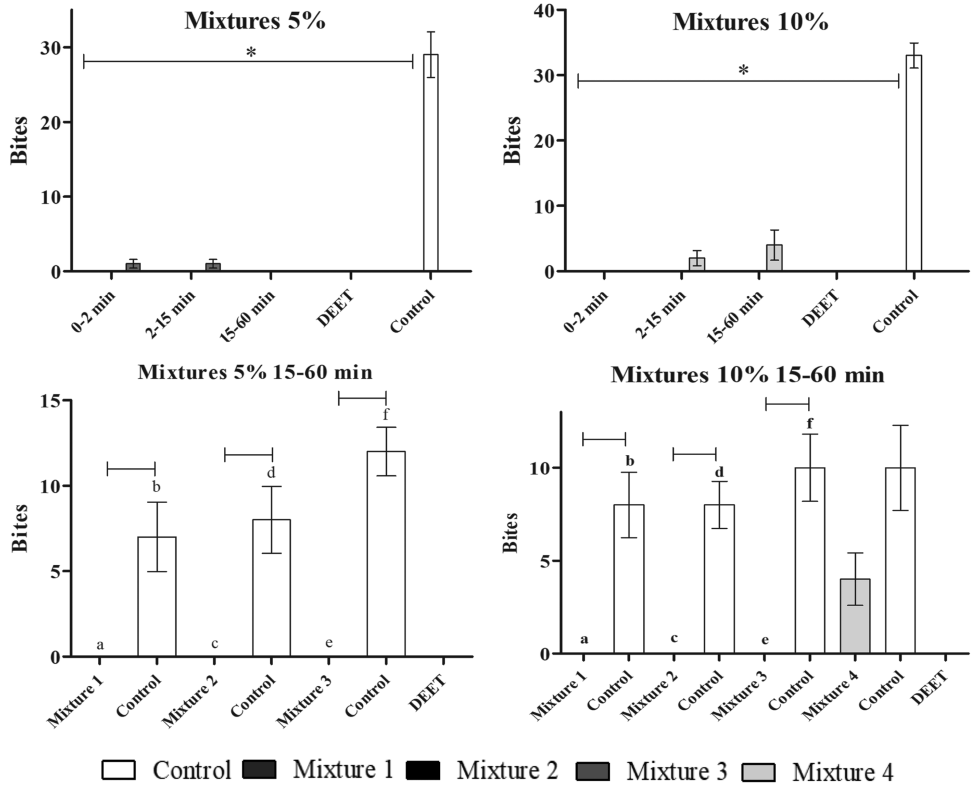

Fig. 8. Mixtures at concentrations of 5 and 10% (v/v). (a and b) Registered bites with mixtures during intervals 0–2, 2–15, and 15–60 min. *Statistically significant differences between treatment and control (acetone) (Mixture 1 KW H (3, $n = 24$) = 22.87895, $P < 0.05$; Mixture 2 KW H (3, $n = 24$) = 22.87895, $P < 0.05$; mixture 3 KW H (3, $n = 24$) = 16.11389, $P = 0.0011$; mixture 4 KW H (3, $n = 15$) = 9.853165, $P = 0.0073$). (c and d) Registered bites for mixtures in the time interval 15–60 min. (a, b), (c, d), (e, f) Statistically significant differences were found between the number of bites registered for each treatment and their control (acetone) (Mixture 1 KW H (3, $n = 12$) = 10.73494, $P = 0.0132$; Mixture 2 KW H (3, $n = 12$) = 10.73494, $P = 0.0132$, Mix 3 KW H (3, $n = 12$) = 10.73494, $P = 0.0132$). Mixture 4 was not evaluated at the 5% (v/v) concentration due to the loss of effectiveness observed at a concentration of 10% (v/v) during the test period (1 h).

Evaluation of Formulations

Two formulations were prepared using the mixture presenting the highest percentage of repellency in vivo and the lowest toxicity in vitro. Formulation 1: 5% (v/v) geranyl acetate, 5% (v/v) nerolidol, 10% PEG 400, 10% milli-Q water, and 70% ethanol. Formulation 2: 5% Geranyl acetate, 5% nerolidol, 15% PEG 400, and 65% ethanol. A protection percentage of 100% was obtained with both formulations for a maximum exposure period of 2 h for formulation 1 and 3 h for formulation 2 (Table 7). No statistical differences were found between the protection percentages of the formulations and DEET (positive control during intervals of 100% protection percentage (Formulations KW H (2, $n = 15$) = 8.990826, $P < 0.05$), but differences were observed with respect to the negative control (PEG 400, milli-Q water, and ethanol) over a 2-h period for formulation 1 (Formulation 1 KW H (2, $n = 15$) = 8.990826, $P = 0.0112$) and over a 3-h period for formulation 2 (Formulation 2 KW H (2, $n = 15$) = 8.990826, $P = 0.0112$).

Discussion

Selection of Secondary Metabolites

Most scientific papers reporting on the bioactivity of EO against *Ae. aegypti* focus on insecticide and repellent products with protection percentages higher than 80%. EOs extracted from plants of the families Lamiaceae (mint-smelling substances), Poaceae (aromatic grasses), and Pinaceae (pines and cedars) have been identified as having insecticide and repellent activity (Amer and Mehlhorn 2006, Maia and Moore 2011, Castillo et al. 2017). However, the possible synergistic interaction among the EO components and its associated mechanism of action remain unexplored (Gleiser et al. 2011, Leal 2013, Bezerra-Silva et al. 2016).

A common characteristic of EOs in the literature is that their major compounds are classified as terpenes. This group of metabolites is characterized by the predominance of carbon and hydrogen in their composition, represented by isoprene units (C_5H_8), membrane lipid precursor molecules such as cholesterol (Ludwiczuk et al. 2016). They can be classified according to the number of isoprene units in the molecule, e.g., monoterpenes (two units), sesquiterpenes (three units), diterpenes (four units), among others. Additionally, each of these subgroups can present functional groups such as alcohols, ketones, ethers, or simply carbon chains (Pandit et al. 2006, Santos et al. 2010). In the present study, terpene compounds used in in silico analyses and in vivo tests were among those classified as monoterpenes and cyclic sesquiterpenes, with conjugated double bonds and ketone, ether, ester, and alcohol groups.

In silico Analyses Molecular Docking and Toxicity Analysis

Molecular docking (with the odorant protein AaegOBP1) results for the 20 pre-selected metabolites showed spontaneous attachment in the cavity whose function is to capture and transport VOCs through the sensillar lymph (Figs. 4 and 5) (Gopal and Kannabiran 2013, Northey et al. 2016), as well as negative values of Gibbs free energy $\Delta G_{binding}$ (Table 1). Additionally, our results

Table 6. Calculation of cytotoxic lethal concentrations 50 (CL_{50}) of metabolites geranyl acetate, nerolidol, α-bisabolol, and their mixtures on cell lines *Vero* and *HepaRG*

Terpene	CL_{50} (μM) *Vero*	CL_{50} (μM) *HepaRG*
Geranyl acetate	>200	>200
Nerolidol	>200	>200
α-bisabolol	138	190
Geranyl acetate + Nerolidol (mixture 2)	>200	235
DEET	>200	>200

Table 7. Protection percentages of formulations 1 and 2, positive control (DEET), and negative control (PEG 400, milli-Q water, ethanol)

Formulation/ Control	Protection percentage (% ±SD)	Maximum protection period (h)
Formulation 1	100 ± 0.0	2
Formulation 2	100 ± 0.0	3
DEET	100 ± 0.0	8
Negative control	0.0 ± 1.6	0

suggest that the spontaneity of the molecular coupling of metabolites in the protein is similar to that of the reference compounds DEET and IR3535 (Table 1). These results suggest that preselected metabolites have a high probability of being transported through the sensillar lymph as a result of their interaction with *Ae. aegypti's* odorant protein AaegOBP1, which plays the role of a vehicle in the sensillar lymph of maxillary palpebral antennas and palps (Leal 2013).

On the other hand, molecular coupling conformations show that the reference compounds (IR3535 and DEET) can present intermolecular dipole–dipole interactions with residues Ile125 and Phe123 near the binding site of protein AaegOBP1 (data not shown). The stabilization of the lowest-energy conformation at the binding site was demonstrated in the cases of nerolidol, α-bisabolol, and nerol; in the vicinity of residues Arg23, Tyr54, and Ile125 (Leite et al. 2009), it may be due to hydrogen bonds-type interactions with the amide functional group of the Phe123 residue and van der Waals interactions with the *sec*-butyl side chain of the Ile125 residue, while geranyl acetate can present intermolecular dipole–dipole interactions between its ester group and the amide functional group of residue Phe123, as well as Van der Waals interactions between its aliphatic chain and the sec-butyl side chain of the Ile125 residue. This evidence confirms that the evaluated terpenes present a potentially effective interaction with the protein with possible biological repellent activity (Oliferenko et al. 2013).

In general, the results of the present study showed that metabolites with polar groups (e.g., hydroxyl and ester) produced less energy when binding with the OBP, an effect that has been reported in numerous studies focused on these systems (Gopal and Kannabiran 2013, Devillers et al. 2014) and is due to the stabilization promoted by the functional hydroxyl group of residue Tyr54 located in the odorant protein cavity. On the contrary, cyclic metabolites with extensive aliphatic chains, i.e., hydrophobic metabolites, presented higher values of free energy $\Delta G_{binding}$.

When analyzing the chemical structures of repellent substances DEET and IR3535 and the metabolites used for in silico analyses and in vivo tests, these substances were found to have hydrophobic properties (Table 2), which make them difficult to be solubilized using hydrophilic media such as the sensillar lymph. This behavior suggests that effective transport through the olfactory system of the mosquito is limited (Pelosi et al. 2014). Nevertheless, the surface of protein AaegOBP1 presents hydrophilic potential energy due to the presence of polar and charged residues in its periphery (Figs. 4 and 5) (Leite et al. 2009), which allows for effective solvation of these hydrophobic compounds and sensillar lymph motility. In addition, the protein possesses a hydrophobic cavity that allows for the attachment of aliphatic odorant compounds inside it, which facilitates their transport in the sensillar lymph toward ORs (Leal and Leal 2015, Yin et al. 2015).

The four metabolites presenting the lowest values of free energy (geranyl acetate, nerolidol, α-bisabolol, and nerol) are classified as terpenes, i.e., they are structurally derived from membrane lipids with cyclic or acyclic hydrocarbon chains, and they can present dipole–dipole and hydrogen bonds-type intermolecular interactions in the binding pocket; this favors protein AaegOBP1-metabolite interaction, and consequently, a more effective transport of these molecules through the sensillar lymph (Bohbot and Pitts 2015, Northey et al. 2016). In addition, the electron cloud of some terpenes with conjugated double bonds or aromatic rings, such as carvacrol and estragole, can establish quadrupolar-π interactions with the side chains of the binding site in the AaegOBP1 protein, whose residues contain heteroatoms such as oxygen and nitrogen (Leal 2013, Bohbot and Pitts 2015).

Higher octanol/water partition coefficients (LogP), as well as lower solubility values in aqueous solutions (LogS), were obtained when comparing the LogP and LogS properties of the metabolites with DEET and IR3535 (Table 2); these results indicate an affinity for lipophilic substances similar to the affinity of synthetic substances DEET and IR3535. The selected metabolites are lipophilic terpenes derived from lipid membrane precursor molecules; therefore, they could have a greater affinity for fatty tissues, which would explain the persistence of these compounds on human skin (Pandit et al. 2006, Silverman and Holladay 2014, Ludwiczuk et al. 2016, Andrade-Ochoa et al. 2018); These values also confirm that the metabolites present hydrophobicity and low solubility in aqueous media, such as the sensillar lymph. On the other hand, it should be taken into account that the reference repellent molecules (DEET and IR3535) have a greater number of heteroatoms such as nitrogen and oxygen in their structure, which facilitates their passage through membranes due to mechanisms facilitating passive transport and diffusion (Wang and Gu 2007, Zhang et al. 2009).

No mutagenic, tumorigenic, or reproductive risks have been reported in connection with the potential cytotoxicity of these four terpenes. Although this type of effect has not been fully demonstrated in commercial repellents, and its potential long-term adverse effects are unknown, commercial products warn of its use in pregnant women and children under 2 yr of age. In this regard, DEET (the most widespread commercial repellent) presented high levels of risk associated with all the parameters evaluated in silico, which has also been reported in previous studies (Cockcroft et al. 1998, Thavara et al. 2001, Department of Health Toxicology Unit 2002, Faiman et al. 2010).

Repellent Activity Bioassays

EOs are composed of numerous metabolites that can give rise to a synergistic effect (increased effectiveness) or an antagonistic effect (decreased effectiveness) when evaluated against *Ae. aegypti*. Studies on EOs containing different proportions of the metabolites evaluated in the present study report decreased effectiveness over

time (Gillij et al. 2008, Gleiser et al. 2011). For instance, Castillo et al. (2017) report low repellent activity for *Swinglea glutinosa* (19% nerolidol), *Cymbopogon citratus* (1% geranyl acetate), and *Cymbopogon flexosus* (10% geranyl acetate). However, evaluating the repellent effect of individual and mixed terpenes resulted in increased protection times against mosquito bites, as shown by our results (Figs. 6–8).

Other authors have reported on the relationship between structure and biological activity in terpenes, establishing that properties such as vapor pressure and molecular weight affect their persistence on the skin over time, which in turn could affect the olfactory capacity of the mosquito (Paluch et al. 2010, Oliferenko et al. 2013). Protective values observed with the 0.1% (v/v) concentration during the different test intervals could be related to the minimum amount of metabolite necessary to inhibit the mosquito's olfactory system: effectiveness is lost in the first minutes (Yin et al. 2015, Northey et al. 2016). This could also explain the loss of effectiveness of nerol at concentrations of 10 and 25% (v/v) during the last test interval (15–60 min). Monoterpenes have higher vapor pressure and lower molecular weight than sesquiterpenes; therefore, they can be less effective as repellents because they volatilize easily over time (Gillij et al. 2008, Paluch et al. 2010). This mechanism was confirmed with the sesquiterpene nerolidol, which presented 100% protection during the test period (1 h) at concentrations of 10 and 25% (v/v), whereas the monoterpene nerol lost effectiveness as a repellent and rapidly evaporated in the last interval (15–60 min) (Table 5).

The presence of oxygen atoms in hydroxyl and ester functional groups in metabolites such as geranyl acetate and nerolidol can play an important role in biological activity due to their intermolecular interactions, e.g., hydrogen bonds, which decrease volatilization (Wang et al. 2008) and increase durability. This was especially evident at concentrations of 10 and 25% (v/v) both for individual metabolites and their mixtures. Different studies evaluating the repellent effect of EOs against mosquito genera such as *Aedes*, *Anopheles*, and *Culex* (Amer and Mehlhorn 2006, Michaelakis et al. 2014, Costa et al. 2017) state that compounds lacking oxygenated functional groups prevent interactions such as hydrogen bonds between molecules. This mechanism results in rapid volatilization of EO components; therefore, the combination of different types of terpenes could result in decreased repellent action, as was observed in mixtures three and four at concentrations of 5 and 10% (v/v), respectively (Table 5).

In vitro Cytotoxicity Tests

MTT cell viability assays revealed the low cytotoxicity of metabolites geranyl acetate, nerolidol, and its mixture against the *Vero* and *HepaRG* cell lines. These experiments allowed for the identification of metabolites lacking toxic effects to be used in formulations. Besides its lack of toxicity, the mixture of geranyl acetate and nerolidol presented a maximum protection period of 3 h, which makes this mixture a potential alternative to DEET, IR3535, or Picaridin, whose protection periods range between three and 8 h in commercial repellent formulations. On the other hand, metabolites geranyl acetate, nerolidol, and α-bisabolol, used in the food and cosmetics industry, have shown no adverse effects in toxicological and dermatological studies on animals and humans (Kamatou and Viljoen 2010, Belsito et al. 2012), as we could confirm in the present study.

Research using the MTT cell viability method and focused on the toxicity of α-bisabolol and some EOs containing this sesquiterpene showed inhibitory concentration values 50 (IC_{50}) lower than 42 μM in human epithelial cells (Van Zyl et al. 2006). Even though these data corroborate that EOs containing α-bisabolol as a major compound are innocuous, it should be taken into account that the results of in vitro cytotoxicity assays depend on the cell viability method and cell line used (Kamatou and Viljoen 2010). Although no cytotoxic effects of DEET were observed on the evaluated cell lines, the literature reports on its tumorigenic effects (Hilairet et al.), ecotoxicity (Guo et al.), and irritating reactions on human skin (Department of Health Toxicology Unit 2002, Swale et al. 2014, Diaz 2016). Lipinski's parameters indicated low toxicity for geranyl acetate, nerolidol and, α-bisabolol, which was corroborated by the LC_{50} obtained in vitro with *Vero* and *HepaRG* cell lines. Therefore, these metabolites are potential candidates to be employed in repellent formulations. Additionally, studies on EOs and their major components have confirmed their low cytotoxicity in animals, normal cells, fibroblasts, *Vero* cells, and hepatocytes, even when their structural chemical properties facilitate permeability across cell membranes (Santos et al. 2010, Costa et al. 2017), which indicates that the topical or medical use of these substances is safe, both individually or in mixtures.

Evaluation of Formulations

Vehicle molecules such as polyethylene glycol 400 (PEG 400) have potential applications in the pharmaceutical and food industries for commercial product formulations. These vehicles decrease diffusion through the skin and allow for the controlled release of volatile molecules by active hydrophilic vehicle-molecule interactions (Nogueira Barradas et al. 2016). Different studies have reported longer protection periods and decreased permeability using vehicles that prevent the rapid volatilization of repellent molecules (Solomon et al. 2012, Balaji et al. 2017). Therefore, considering the results obtained in the present study, it is possible to obtain an effect similar to that of DEET using a lower concentration of active molecules. For example, formulations prepared with molecules derived from EOs and PEG 400 increased the protection period up to 3 h (Table 6), lower concentrations and toxicity levels than synthetic repellent substances DEET, IR3535, and Picaridin.

Conclusions

An in silico analysis was carried out to select metabolites with an affinity for *Ae. aegypti* odorant protein AaegOBP1 (PDB code 3K1E). From these, in vivo repellency tests were subsequently carried out on the metabolites with the highest odorant protein affinity: geranyl acetate, nerolidol, α-bisabolol, and nerol.

Multiple molecular docking simulations were performed with the purpose of predicting and describing the possible passive transport of secondary metabolites from plants as part of the odorant mechanism of the repellent activity against *Ae. aegypti*. Results showed that geranyl acetate, nerolidol, α-bisabolol, nerol, and the reference compounds (DEET and IR3535) interacted spontaneously (measured free energies between −8,252 and −7,402 kcal/mol) with the hydrophobic cavity of OBP AaegOBP1. These data show how these compounds can be easily transported by the sensillar lymph in the odorant process of *Ae. aegypti* at the molecular level.

On the other hand, Lipinski's parameters (LogP, LogS, and risk profiles) showed that the four studied metabolites have a higher affinity for lipids or fatty tissues, which could explain the durability of these terpenes on the skin. Additionally, mixtures of these terpenes showed low toxicity against normal cells with LC_{50} higher than 138 μM using *Vero* and *HepaRG* cell lines. The protection percentages of metabolites geranyl acetate, nerolidol, α-bisabolol, and their mixtures were comparable with those of DEET.

For its part, the metabolite mixture composed of 5% (v/v) geranyl acetate and 5% (v/v) nerolidol in 15% PEG 400 formulation achieved a protection percentage similar to that of DEET (for 3 h), without its toxic effects. Cytotoxicity tests on normal *Vero* and *HepaRG* cells showed that the metabolites with the best repellent effects (geranyl acetate, nerolidol, and α-bisabolol) have low toxicity, which was corroborated by the lack of reports on harmful effects associated with these substances in the literature. The protection periods of formulations with geranyl acetate and nerolidol showed values comparable to those of DEET when using lower concentrations for up to 3 h.

Acknowledgments

This study was carried out thanks to financing provided by the Patrimonio autónomo Fondo Nacional de Financiamiento para la Ciencia, la Tecnología y la Innovación Francisco José de Caldas (COLCIENCIAS), Project number: 110271250633; Contract FP 44842-406-2016. We wish to thank the volunteers for their participation in the in vivo repellency experiments and María Fernanda Vidal for her support designing the images. Molecular graphics and analyses created using UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from National Institutes of Health P41-GM103311.

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