Acta Tropica 229 (2022) 106367

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Screening organic repellent compounds against *Lutzomyia longipalpis* (Diptera: Psychodidae) present in plant essential oils: Bioassay plus an in silico approach

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ARTICLE INFO

Keywords:
Pharmacophore model
Molecular docking
Biological activity
Terpenoids
Ethnobotanical repellent

ABSTRACT

In the Americas, *Lutzomyia longipalpis* is the most relevant sand fly species for the transmission of visceral leishmaniasis. For its vector control in Brazil, insecticide spraying has not shown persistent reduction in disease prevalence while some sand fly populations are reported resistant to the insecticides used in spraying. The usage of repellents and personal protection behavior can reduce vector borne diseases prevalence. Therefore, the search for new repellent compounds is needed to use together with insecticide spraying, especially from natural sources to overcome the resistance developed by some sand fly populations to the compounds commercially used. In silico strategies have been applied together with repellency bioassays successfully identifying new bioactive compounds from natural sources. Thus, the present study aimed to screen repellent potential of neem (*Azadirachta indica*), citronella (*Cymbopogon winterianus*), bushy matgrass (*Lippia alba*) and 'alecrim do mato' (*Lippia thymoides*) essential oils against L. *longipalpis* and to identify potential repellent compounds by chemical analysis and in silico approach. Plant essential oils were extracted from leaves and repellency bioassays were performed on volunteers using colony reared L. *longipalpis*. Aside from neem oil, all other tested essential oil has shown a reduced number of sand fly bites using higher concentrations. Chemical composition from oils was assessed and its compounds were screened on a pharmacophore model using odorant binding protein 1 (OBP1). All essential oils were majorly composed of either oxygenated monoterpenes, except for the oil extracted from neem which was composed of sesquiterpene hydrocarbons. Molecular docking was performed with the compounds that best superimposed in the OBP1 pharmacophore model, identifying those binding to OBP4, which is associated with insect repellency behavior. Citronellol, Citronellol acetate, Citronellal and Geranyl acetate showed similar interactions with OBP4 binding site as DEET. Thus, it is suggested that these compounds are able to bind to L. *longipalpis* OBP4 generating repellent behavior in sand flies.

1. Introduction

Sand flies are responsible for the transmission of protozoan of the species *Leishmania infantum*, etiologic agent of human and canine visceral leishmaniasis (VL) (McCall et al., 2013). In Brazil the lack of effectiveness in VL control is attributed to late human case diagnosis, dog culling delay after a positive diagnosis and low residual insecticide spray efficacy on the most relevant sand fly species, *Lutzomyia longipalpis*

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https://doi.org/10.1016/j.actatropica.2022.106367
Received 8 November 2021; Received in revised form 9 February 2022; Accepted 10 February 2022
Available online 12 February 2022

(Menon et al., 2016).

Regarding vector control, the use of residual spray has shown to be less effective over time, which may lead to insecticide resistant populations in the urban environment (Sherrard-Smith et al., 2018). Alternatively to this vector control method personal protection behavior (PPB) together with localized insecticide spraying at community level seems to be more efficient than a vector eradication focused approach (Johnston et al., 2020; Omodior et al., 2018). Furthermore, it has been demonstrated that practicing two or more PPBs such as wearing covering clothes and repellent application has reduced the risk of West Nile virus by more than 50% (Loeb et al., 2005).

Currently, repellents commonly used in PPBs have been composed of only one synthetic active compound whereas plant essential oil based repellents encompass a botanical mixture of chemical compounds (Asadollahi et al., 2019). Pyrethroid synthetic products have been broadly distributed in the market and its origin is related to ethnobotanical studies that evaluated the repellent activity of natural pyrethrum extracts from pyrethrum daisy (*Tanacetum cinerariaefolium*) flowers (Matsuo, 2019). This exploratory approach associated with in silico methods, e.g. ligand and structure-based virtual screening, can contribute to identifying chemical compounds that have high affinity with receptors related to insect's repellent behavior (Neto et al., 2020). However, there are few studies that use this kind of approach for the identification of new repellent organic compounds against arthropod pathogen vectors such as mosquitoes (de Brito et al., 2021; Thireou et al., 2018).

In the last decades, studies on the interaction between plants and insects have assayed essential oils containing volatile organic compounds (VOCs) with insecticide and/or repellent activities. In this context, plant essential oil from *Cymbopogon* and *Lippia* genus as well as neem (*Azadirachta indica*) have been extensively assayed exhibiting repellent activity against *Anopheles gambiae, Aedes aegypti, Culex quinquefasciatus* and *Anopheles dirus* mosquitoes (Maia and Moore, 2011). Despite the lack of ethnobotanical studies evaluating the essential oil's repellent activity of the aforementioned plant species on sand flies, neem oil has shown protection against *Phlebotomus orientallis* bites both in laboratory and field environments (Kebede et al., 2010). Additionally, lemon grass (*Cymbopogon citratus*) essential oil has presented repellent activity against another old world sand fly species of the same genus, *P. duboscqi* (Kimutai et al., 2017).

Regarding new world sand fly species, neem and *Eucalyptus* spp. essential oils have shown larvicide and adulticide effects on L. *longipalpis* (Maciel et al., 2010a; 2010b). However, little is known about their repellent activity against new world sand fly species, especially L. *longipalpis*. In silico strategies have been applied for the identification of new bioactive compounds from natural sources (Rodrigues et al., 2021), demonstrating to be a practical and objective way to explore new repellent compounds.

Thus, the present study aimed to screen vegetal extracts obtained from four plants for their repellent potential against L. *longipalpis* sand flies as well as to identify VOCs as potential bio repellents by means of chemical analysis followed by in silico approach to hypothesize mechanism of action.

2. Materials and methods

2.1. Plant selection and essential oil extraction

The repellency potential of phytochemical oils was evaluated against *L. longipalpis* phlebotomine sand flies. Therefore, four plant species were selected based on previous knowledge about their repellent and insecticide efficacy found in the literature, their kinship with plants known to have repellent and/or insecticide activity as well as phytotherapeutic popular knowledge by the local community. All plant material was collected in the morning from garden trees in Barra's urban area (S 11° 08′ 71′′ / W 43° 14′ 24′′), between January and April of 2019. Leaves were undamaged without apparent fungi and bacteria contamination, after macroscopic inspection. Experts at the herbarium of Federal University of West Bahia (Campus Barra) confirmed the taxonomic identification. The time between leaf collection and oil extraction was less than 12 h.

Oils were extracted from neem, 'alecrim do mato' (*Lippia thymoides*), citronella grass (*Cymbopogon winterianus*) and bushy matgrass (*Lippia alba*) leaves by hydrodistillation using a Clevenger apparatus. On average, extraction took two hours for each essential oil and it was followed by the addition of a small amount of anhydrous sodium sulfate to absorb water remnants. Using micropipette, the oil was collected and then transferred to microtubes and stored at room temperature away from luminous incidence until usage for dilutions.

2.2. Oil characterization

Gas chromatography mass spectrometry (GC–MS) has been used to characterize the essential VOCs using a dilution of 1μL of oil in 1μL of methanol. Then, 1μL of the mixture was injected in the GC–MS system (Shimadzu GCMS- QP2010 Plus high performance single quadrupole, Kyoto, Japan) equipped with a split/splitless injector in the split mode (25.0 ratio) at 250 °C during the chromatographic run. VOCs were separated in a capillary column (Rxi-5 MS 95% dimethyl polysiloxane; 30 $m\times$ 0.25 mm ID $\times$ 0.25 μm, Restek, Bellefonte, USA) using helium (99.999%) as carrier gas at 0.81 mL min^{-1} flow rate. Oven temperature started at 40 °C, then heated to 80 °C (2 °C min^{-1}) holding for 3 min, 100 °C (1.0 °C min^{-1}) also holding for 3 min, 200 °C (5 °C min^{-1}) and ending at 300 °C (10 °C min^{-1}), totalling 79 min. Mass detector conditions were: transfer line temperature at 250 °C, ion source temperature at 250 °C and ionization mode with electron impact at 70 eV.

VOCs identification was achieved by means of comparing the GC retention times and mass spectra with those of pure standard compounds, when available and all mass spectra were also compared with the data system library (NIST 147 Database). Kovats retention índex (KI) values were determined using a homologous series of n-alkanes C_8–C_{24} which were compared with values reported in the literature for similar chromatographic columns. The percentage of individual peaks was achieved by peak area normalization measured without correction factors.

2.3. Repellent assay

Before the experiments, volunteers have been enlightened on the assay procedures and signed an informed consent. The study has been approved by the human research ethics committee from Universidade Federal do Oeste da Bahia, UFOB (CAAE n° 16,451,719.5.0000.8060). Each evaluated repellent extract has been tested on three different volunteers totaling 12 people recruited for the study. Volunteers were included if they were between 18 and 50 years old and presented healthy physical conditions. If they had a history of allergic reaction to insect bites, ingested alcoholic beverages or spicy food or used perfumes 12 h before the experiment, they would be excluded from the repellent assays. They were exposed to colony reared L. *longipalpis* unfed female sand flies from a colony held at Laboratório de Interação Parasito-Hospedeiro e Epidemiologia (LAIPHE), Instituto Gonçalo Moniz (FIOCRUZ-Bahia). L. *longipalpis* strain comes from Camaçari, Bahia/Brazil, an endemic area for visceral leishmaniasis and it had been three years since colony establishment using golden sirius hamster (*Mesocricetus auratus*) as blood source when experiments were performed. Sand flies were transported to Laboratório Multidisciplinar, UFOB, where all experiments were conducted. The exposure was performed inside 30 $\times$ 30 $\times$ 30 cm mesh cages containing 50, 3–7 day old, unfed females which were previously sugar starved for 24 h. Before each experiment, sand flies were transferred to the cages and maintained for at least 2 h at the testing site for acclimatation.

Repellent activity assays were performed as recommended by WHO's

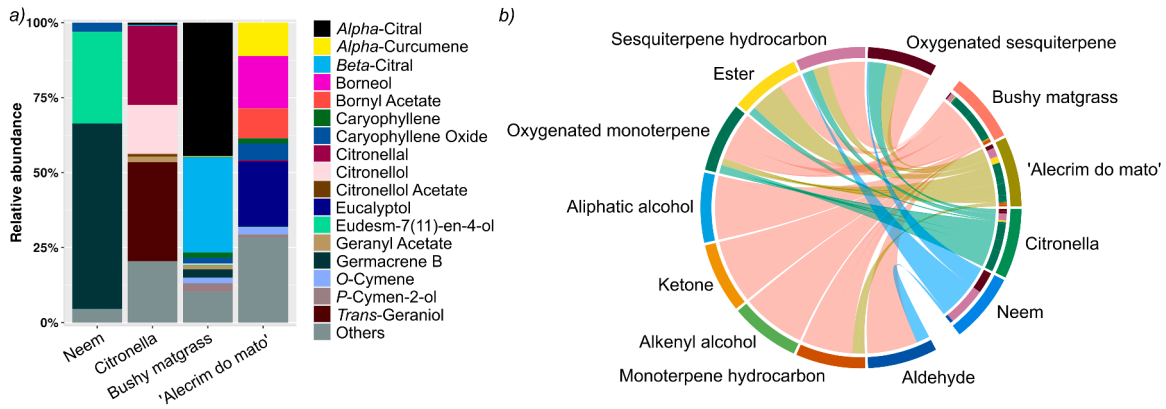


Fig. 1. Chemical composition of neem, citronella, bushy matgrass and 'alecrim do mato' essential oils extracted from leaves. (a) Stacked bar plot showing volatile organic compounds (VOCs) relative abundances for each tested essential oil. "Others" represents the sum of 37 compounds with overall abundance lower than 15%. (b) Circular plot showing proportions of chemical groups present in each plant essential oil.

Table 1
Mean protection rates (%) against sand fly bites, from all replicates, obtained for neem, citronella, bushy matgrass and 'alecrim do mato' essential oils in cumulative tested doses, being: 1 - 1.25 mg mL^{-1}; 2 - 3.75 mg mL^{-1}; 3 - 8.75 mg mL^{-1}; 4 - 18.75 mg mL^{-1}; 5 - 38.75 mg mL^{-1} and effective doses 50, 90 and 99.9 for each essential oil.

Dose	Mean protection rates (%) using different essential oils			
	Neem	Citronella	Bushy matgrass	Alecrim do mato
1	−79.2[a]	−44.8	37.3	61.9
2	20.8	62.1	62.7	64.3
3	20.8	93.1	49.1	66.7
4	−4.2	93.1	94.9	90.5
5	41.7	93.1	94.9	90.5
ED50 (mg mL^{-1})	0.9	0.7	9.2	9.4×10^{-3}
ED90 (mg mL^{-1})	1.4	2.7	9.8×10^{7}	3.0
ED99.9 (mg mL^{-1})	3.5	46.2	5.8×10^{16}	7.3×10^{5}

[a] Negative protection means that there were more bites than the control arm.

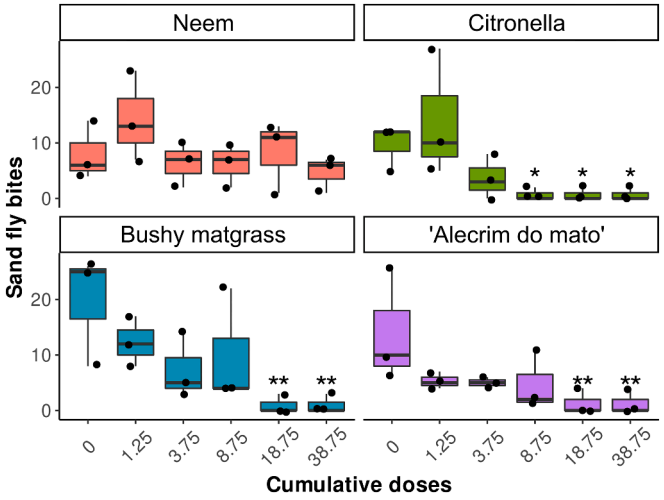
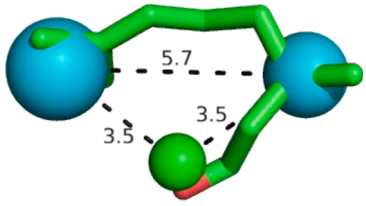
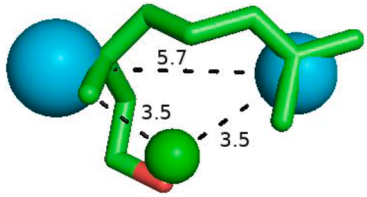
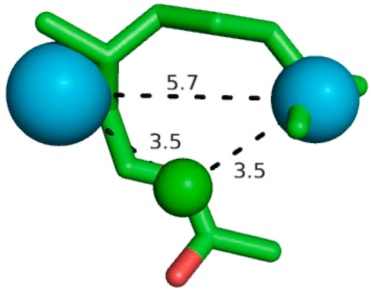
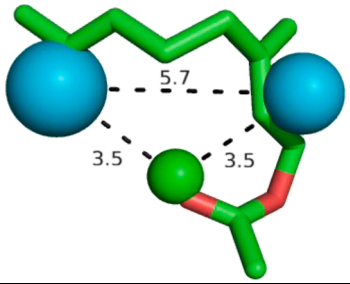


Fig. 2. Number of L. *longipalpis* bites distribution for each tested treatment using essential oils in cumulative doses, obtained for neem, citronella, bushy matgrass and 'alecrim do mato' leaves. Herein are represented the results of three replicates per dose and per plant species. Negative controls are shown as "0" in the x axis. * shows statistically significant differences in comparison with controls by Kruskal-Wallis test followed by Dunn's post hoc test, being: $p < 0{,}05$ *; $p < 0{,}01$ **. In boxplot are represented: min, 1st quartile, median, 3rd quartile and max values.

Table 2
Superposition of essential oil compounds in *Aaeg*OBP1 pharmacophore model.

Compound	Superposition	QFIT
21 (Citronellal)		26.8
29 (Citronellol)		11.3
37 (Citronellol acetate)		52.2
39 (Geranyl acetate)		10.3

Carbons are green, and oxygen is red. Spheres: Green spheres represent the H-bond acceptor group, and cyan spheres are hydrophobic groups. The size of spheres varies according to the tolerance radius calculated using GALAHAD™. All distances are measured in Angstrom.
Superposition pictures and table adapted from Brito et al., 2021.

Table 3
Essential oil's compound affinity to OBP4 binding site of *L. longipalpis* proved from the DOCK 6.7 program.

Compound	Energy (kcal mol^{-1})	Compound	Energy (kcal mol^{-1})
***Beta*-Farnesene**	−35.1	**Limonene**	−23.6
***Alpha*-Curcumene**	−33.9	**Eucalyptol**	−23.6
Citronellol acetate	**−34.8**	***Gamma*-Terpinene**	−24.2
Geranyl acetate	**−32.4**	**1-octen-3-ol**	−24.6
***Alpha*-Selinene**	−30.4	**Germacrene B**	−31.1
***Beta*-Cadinene**	−31.5	**3-octanol**	−25.5
***Beta*-Elemol**	−34.8	**Carvone**	−25.2
***Delta*-Cadinene**	−30.8	**Carveol**	−25.0
***Delta*-Guaiene**	−31.9	***Alpha*-Thujene**	−23.9
Caryophyllene	−29.9	**Isopulegol**	−25.3
***Trans*-Geraniol**	−28.5	***Gamma*-Eudesmol**	−32.6
***Cis*-Geraniol**	−28.5	**3-carene**	−23.1
Linalool	−28.0	**O-cymene**	−21.8
Citronellol	**−28.9**	**6-methyl-5-hepten-2-one**	−24.4
***Alpha*-Cadinol**	−33.3	**Caryophyllene oxide**	−30.6
Copaene	−31.3	***L*-Pinocarveol**	−23.9
***P*-Cymen-8-ol**	−24.9	**Bornyl acetate**	−30.7
***Delta*-Cadinol**	−33.3	**Camphor**	−23.1
Germacrene D	−31.6	**Eudesm-7(11)-em-4-ol**	−32.4
***Beta*-Citral**	−27.6	***Alpha*-Pinene**	−21.5
***Beta*-Myrcene**	−26.5	***Beta*-Pinene**	−21.8
4-terpineol	−26.2	**Pinocarvone**	−22.7
***Alpha*-Terpineol**	−25.6	**Verbenone**	−23.5
***Alpha*-Citral**	−27.6	**Camphene**	−22.1
Citronellal	**−28.5**	**2-Hexenal**	−19.0
***Beta*-Ocimene**	−24.6	**Borneol**	−23.7
Sabinene	−22.8		

guideline for efficacy testing of mosquito repellents for human skin with adaptations (Kimutai et al., 2017; Nieves et al., 2010; WHO, 2009). These assays aimed to screen the essential oils with potential repellent activity. Therefore, no positive controls were used, as the oils with statistically significant differences in number of bites in comparison with the negative controls were further evaluated by in silico approaches. An arm-in-cage approach was used and before each test volunteers had cleaned their arms with neutral soap and put one arm inside the cage wearing a bovine rectal palpation glove which had a squared hole, 10 × 5 cm, whereby the skin was exposed to sand fly bites with or without essential oils. Essential oils at 1.25, 2.5, 5, 10 and 20 mg mL^{-1} were diluted in ethanol and applied on the above-mentioned skin area and let dry for one minute before insertion in the cage. At first the arm was exposed only with ethanol for five minutes to certify that sand flies were eager to feed. If less than 10 bites were counted, a new cage would be prepared for the experiment, as aforementioned. Then, all concentrations were tested starting with the lowest, 1.25 mg mL^{-1}, to the highest, 20 mg mL^{-1} exposing the skin area for five minutes for each concentration, applying the next concentration right after exposure time, only waiting one minute to let the diluted essential oil dry. At last, the other arm was also exposed with ethanol for five minutes as negative control and to certify that sand flies were still eager to feed. If the same criteria as in the experiment's beginning have not been met, the replicate would be invalidated.

2.4. Statistical analysis

Data were analyzed using probit regression to estimate effective doses (ED) corresponding to 50% (ED_{50}), 90% (ED_{90}) and 99.9% ($ED_{99.9}$). Protection was expressed by a proportion (P%) of the number of sand fly bites on the treated arm (T) in relation to the number of bites on the control arm (C) of the same individual: $P\% = \frac{(C-T)}{C} \times 100$. Median number of sand fly bites for each essential oil cumulative dose were compared with control using Kruskal-Wallis test and Dunn test as a post hoc test to evaluate differences between treatments. For such estimations and comparisons, the packages DRC v3.0–1 and stats v4.0.2 in R environment version 4.0.2 were used.

2.5. Pharmacophore model

Pharmacophore-based virtual screening was performed according to previous studies (Neto et al., 2020). A dataset with 54 compounds of essential oils with repellent potential screened in the repellency assays was employed for virtual screening. Marvin® Sketch 16.9.5 software (ChemAxon, 2015) was used to select the most reliable tautomers in L. *longipalpis* lymph (pH = 7.5). Next, the CONCORD module implemented on SYBYL® -X 2.0 package (Tripos Associates, 2012) was employed to convert the 2D structures to 3D format. An energy-minimized protocol using Conjugate Gradient (CG), a convergence criterion of 0.001 kcal mol^{-1} and Tripos force field (dielectric constant = 80.0; maximum number of iterations = 50.000) (Clark et al., 1989) was used in all structures. Partial atomic charges were calculated using GasteigerHuckel method (Halgren, 1996), as available on SYBYL X 2.0.

A previously validated OBP1 pharmacophore model (Neto et al., 2020) was employed in a flexible alignment with a dataset of essential oils compounds, as available in GALAHAD™ module, to prioritize the best superimposed compounds in OBP1 pharmacophore model and, thus, compounds with requirements to repellency activity. Compounds were ranked according to QFIT values and those with positive values were selected for molecular docking.

2.6. Molecular docking

Docking-based virtual screening was performed according to previous studies (Santana et al., 2018). The conformational search and scoring evaluation were performed by the DOCK 6.7 package using the default parameters (Allen et al., 2015). The compounds prioritized on pharmacophore-based virtual screening were employed on molecular docking.

3. Results and discussion

3.1. Chemical composition

All essential oils demonstrated to be different from one another as seen in Fig. 1a. An overview of all compound composition data can be seen on supplementary Table 1. The majority of the compounds present in citronella, bushy matgrass and 'alecrim do mato' essential oils were oxygenated monoterpenes while sesquiterpene hydrocarbons and oxygenated sesquiterpenes represented most of the compounds in neem oil (Fig. 1b).

Limonoids such as Salanin, Nimbin and Azadirachtin are the main compounds found in neem seed essential oils (Benelli et al., 2017). However, in our study, the main compounds found in neem leaves were Germacrene B (61.8%) and Eudesm-7(11)-em-4-ol (30.5%). Both compounds are sesquiterpenes which is a group of terpenes that have been shown to have repellent activity against mosquitos as well as monoterpenes (Alvarez Costa et al., 2017).

Citral represented about 76.4% (*alpha*-Citral with 44.5% and *beta*-Citral with 31.9%) of all compounds detected in bushy matgrass essential oil. Citral is a monoterpene known to have antifungal, antimicrobial, insecticidal activities as well as repellent activity against mosquitos and aphids (Dancewicz et al., 2020; Muema et al., 2017).

Regarding citronella oil's chemical composition, we have found *trans*-Geraniol (33%), Citronellal (26.3%) and Citronellol (16.3%) as the main compounds corroborating what was found by other studies on its chemical composition and effective repellent activity against mosquitos (Deletre et al., 2015; Eden et al., 2020). All three compounds are monoterpenes which are commonly found in plant essential oils and

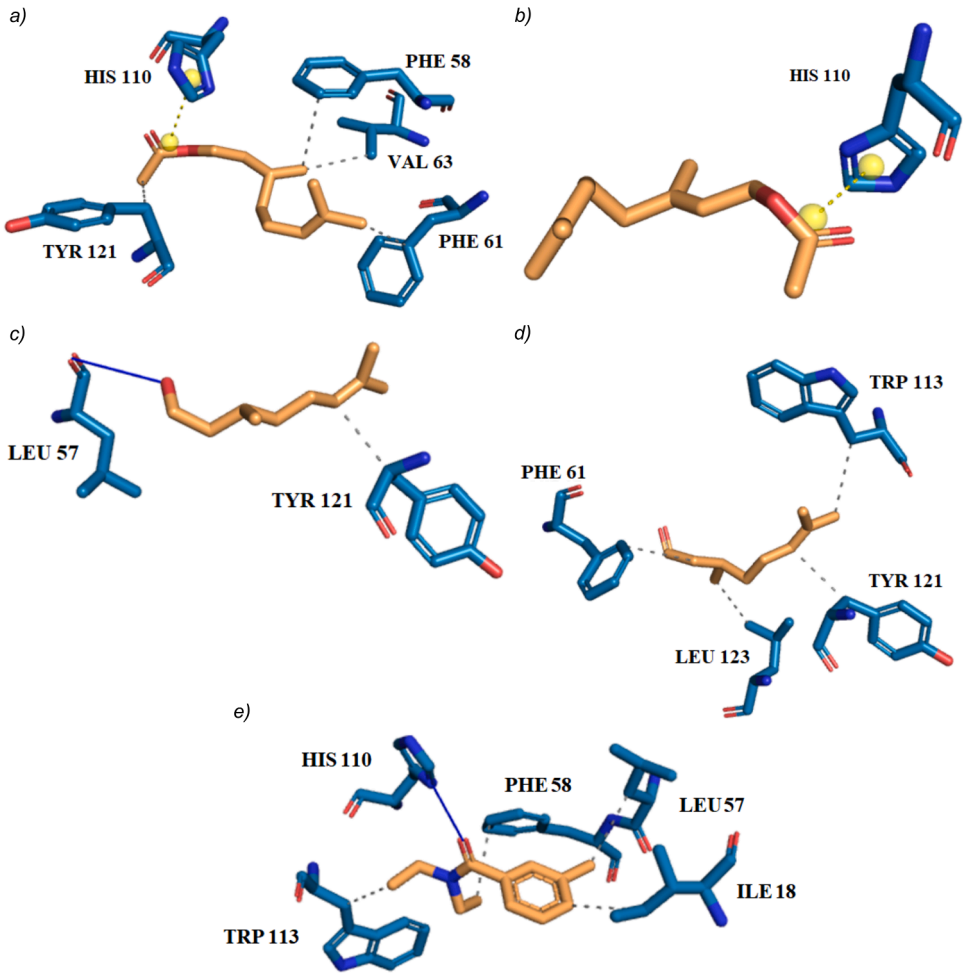


Fig. 3. Interaction maps of Citronellol acetate (a), Geranyl acetate (b), Citronellol (c), Citronellal (d) and DEET (e) in *Lutzomyia longipalpis*' OBP4 binding site generated by PLIP online server. Dashed gray lines represent hydrophobic interactions. Blue lines represent hydrogen bonds. Dashed yellow lines represent saline bridges.

have been broadly studied as a repellent and insecticide against insects and ticks with positive results (Lima et al., 2019; Saad et al., 2019).

'Alecrim do mato' essential oil's chemical composition seems to be highly diverse, containing Eucalyptol (21.5%), Borneol (17.4%), *alpha*-Curcumene (11.2%) and Bornyl acetate (10%) as its main prevalent compounds. Aside from the sesquiterpene *alpha*-Curcumene, all other above-mentioned compounds are monoterpenes. Eucalyptol is one of the most prevalent compounds found in essential oils extracted from *Eucalyptus* plants, which has demonstrated adulticide and larvicide activity against L. *longipalpis* (Maciel et al., 2010b). Besides Eucalyptol, none of 'alecrim do mato' compounds have been isolated tested on sand flies. However, our findings together with the activity against other arthropods found in the literature (Ali et al., 2015; Lee et al., 2009; Liu et al., 2012) highlight their potential to act as repellent or insecticide on L. *longipalpis*.

3.2. Repellency bioassays

Besides neem, all essential oils demonstrated protection rates above 90% especially when cumulative concentration was higher than 18.75 mg mL^{-1} (table 1). Citronella oil presented mean protection rates above 90% for concentrations higher than 8.75 mg mL^{-1} while 'alecrim do mato' and bushy matgrass reached such protection rates with cumulative concentration of 18.75 mg mL^{-1} and higher. Aside from neem essential oil, any of the tested oils reached protection rates of 100% for at least one replicate. Neem essential oil fluctuated its protection rate reaching the highest, 41.67%, with cumulative dose of 38.75 mg mL^{-1}. Using the probit model the effective dose 50 was lower than 1 mg mL^{-1} in all cases except for bushy matgrass which was 9.16 mg mL^{-1}. The effective dose 99.9 varied from 3.54 mg mL^{-1} for neem to 5.83 $\times$ 10^{16} mg mL^{-1} for bushy matgrass.

We have observed a variable mean protection rate of neem's leaves essential oil against L. *longipalpis* sand flies, even though it had the lowest $ED_{99.9}$ when compared with other tested plant oils (table 1). In the literature, neem essential oil from leaves has not yet been tested against L. *longipalpis*. However, its seed oil has demonstrated adulticide and larvicide effects against such sand fly species (Maciel et al., 2010a) as well as repellent activity against sand fly species of the genus *Phlebotomus* (Kebede et al., 2010). Such finding together with the common usage of plant leaves to extract natural repellents, has encouraged us to test neem leaves instead of seeds as in other studies. The repellent activity of leaves and seed extracts have been evaluated and compared in a study that observed a lower larvicidal effect from leaves extract against *Anopheles stephensi* mosquitoes (Nathan et al., 2006). Therefore, our findings reinforce the importance of choosing the plant component used to obtain essential oils. Insecticide or repellent activity of a plant may vary due to composition and concentration of main essential oil compounds which change according to oil origin and plant component used (Burt, 2004). Thus, collection procedures, season of collection as well as plant components and solvent used for oil extraction should be considered.

Regarding bushy matgrass bioassay results of the present study, a

95% protection rate has been observed on 18.75 mg mL^{-1} cumulative dose and higher. Due to its predominance, citral might be associated with such a protective result. To our knowledge, it has been the first time that bushy matgrass essential oil was tested for its repellent activity against sand flies. Thus, our results are promising against L. *longipalpis* sand flies, although such protection has not been seen when bushy matgrass essential oil was tested for its effect to avoid *A. aegypti* bites (Castillo et al., 2017), even with previously larvicidal activity observed in another study (Vera et al., 2014).

Protection rates above 90% with cumulative dose of 8.75 mg mL^{-1} and higher has been shown by citronella's essential oil against L. *longipalpis* bites. Such a high protective effect has been demonstrated by the oil of another plant of the same genus, lemon grass, against old world sand fly species *P. duboscqi (Kimutai et al., 2017)*. Indoor protection using Citronella oil based and Geraniol candles have been demonstrated to avoid both mosquito and sand fly bites being geraniol candles five times as effective as citronella candles (Müller et al., 2008). These findings highlight the potential usage of citronella's essential oil as repellent against L. *longipalpis* bites which could be integrated into PPBs practiced in VL endemic areas.

Similar to citronella essential oil, 'alecrim do mato' oil has shown a protection rate above 90% against L. *longipalpis* bites, however with higher cumulative dose, i.e. 18.75 mg mL^{-1}. This plant species is endemic in Brazil's northeast and recently several studies have demonstrated pharmacological properties (Silva et al., 2016; S.G. 2019).

When the number of sand fly bites on each cumulative dose was compared with the bites in the control arm, there were statistically significant differences for the highest cumulative doses, 18.75 mg mL^{-1} and 38.75 mg mL^{-1}, for citronella, bushy matgrass and 'alecrim do mato' as shown in Fig. 2. All comparisons between the control and neem's essential oil concentrations showed no statistically significant difference both in the Kruskall-Wallis and Dunn's tests. As the aim of the study was to screen the essential oils with potential to reduce the number of bites, i.e. statistically significant differences in comparison with the negative control, no positive controls were applied. The chemical composition of the oils that achieved such criteria were further evaluated by in silico approaches.

3.3. Pharmacophore model

Among all 54 essential oil compounds of the subset, four compounds have superposed on the pharmacophore model herein employed with partial stereo-electronic requirements ($10.28 < QFIT > 52.22$) (Table 2). According to superposition results, essential metabolites from 'alecrim do mato', bushy matgrass and citronella have partial stereo-electronic requirements of known repellents that modulate the mosquito behavior by *Aaeg*OBP1 (*A. aegypti* odorant-binding protein 1). Based on global and local sequential identity (58.97/100%) of *Aedes aegypti* OBP1 and L. *longipalpis* OBP2, the *Aaeg*OBP1 pharmacophore model was employed (Neto et al., 2020). Thus, we believe that due to repellency behavior associated with *Aaeg*OBP1, a similar response would be observed in L. *longipalpis*.

This strategy has been applied in our group together with structure-based drug design approaches such as molecular docking with bioactive compounds from natural sources (de Brito et al., 2021), in order to find new promising insect behavior modulators. Thus, all molecules were evaluated by molecular docking.

Despite an increase in studies on vector-borne disease control over the last decades, the mechanism of repellency is not yet fully elucidated, as is observed in traditional strategies of vector control (e.g. larvicide and adulticide mechanisms). A theory that explains the repellency mechanism is the interference in insect chemosensory system that governs the behavioral patterns of repellency (e.g. fleeing from chemical irritants) and attraction (e.g. host-seeking and oviposition) (Oliferenko et al., 2013). Studies have shown that several insects share the same molecular filter in odor selection of the olfactory system (Neto et al., 2020; Santana et al., 2018; Tsitsanou et al., 2012).

Computational studies of specific targets in the olfactory system of mosquito OBP1 and repellency activity of known repellents have been attempted far less frequently than for drug discovery and, thus, the improvement of these studies can be useful to elucidate the repellency activity of essential oils. This way, the employment of strategies that search the stereo-electronic requirements of repellents can be useful to show which compounds in an essential oil have the same requirements and, probably, the same repellency mechanism of known molecules.

Pharmacophore-based approaches can help in prioritization of compounds with the same stereo-electronic features of known active compounds and, thus, with the same mechanism (Mascarenhas et al., 2020; Ramos et al., 2020; Santos et al., 2020). In this manner, the flexible search of stereo-electronic features by pharmacophore model is directly related with potent compounds, which can be useful to elucidate the compounds related with repellency activity in essential oils.

The compounds found in citronella essential oil that superposed *Aaeg*OBP1 were Citronellal (QFIT = 26.77), Citronellol (QFIT = 11.29), Citronellol acetate (QFIT = 52.22) and Geranyl acetate (QFIT = 10.28). The repellency activity is related with the synergic contributions of majoritarian compounds Citronellal (22.98%) and Citronellol (14.24%) with Citronellol acetate (0.79%) and Geranyl acetate (1.61%) compounds.

The *Aaeg*OBP1 superposed compounds found in bushy matgrass essential oil were Citronellol (0.27%) and Geranyl acetate (1.01%) and, thus, a synergic contribution between the compounds was observed. While the only one compound found in 'alecrim do mato' essential oil with superposition on *Aaeg*OBP1 was Citronellal (0.32%). This way, the repellency activity of citronella, bushy matgrass and 'alecrim do mato' essential oils should be highly related to the presence of Citronellal, Citronellol, Citronellol acetate and Geranyl acetate compounds with a synergic or individual activity. These four compounds that superimposed in the pharmacophore model have stereo-electronic requirements to binding in *Aaeg*OBP1 and thus might be modulating the sand fly behavior (Neto et al., 2020).

However, penalty adjustments were observed in compounds of essential oils dataset on *Aaeg*OBP1 pharmacophore model with flexible alignment, which results in few compounds with low QFIT values. Thus, this approach is not useful to evaluate spatial requirements of molecules at binding sites. Thus, molecular docking was applied to understand the binding mode and affinity of the essential oils dataset in *L. longipalpis* OBP4 binding site.

3.4. Molecular docking

Molecular docking can predict affinity parameters of ligands and help in the prioritization of virtual hits. Among 54 compounds of the essential oil subset, just one of them was not able to perform key interactions at *L. longipalpis* OBP4 binding site (Table 3).

Molecular docking can be employed to predict the binding affinity and adjustment of compounds in L. *longipalpis* OBP4 binding site and, thus, help to understand the binding mode of each compound present in the essential oils with repellency activity. According to molecular docking of hit compounds on pharmacophore-based analysis, Citronellol acetate (energy = −34.77 kcal mol^{-1}) was the best ranked compound, which is related to citronella essential oil. Geranyl acetate (energy = −32.45 kcal mol^{-1}) was the second-best ranked compound, which is related to citronella and bushy matgrass essential oils. Citronellal (energy = −28.52 kcal mol^{-1}) was the worst compound ranked according to docking study and is related to 'alecrim do mato' essential oil. Additionally, molecular docking circumvented the limitation of compounds that did not match on the pharmacophore model and, thus, it showed the affinity of 53 compounds L. *longipalpis* OBP4 binding site.

Besides, affinity energies can depict the order of prioritization. However, this metric alone does not highlight binding mode. In order to visualize the binding mode of hit compounds on both computational

approaches in L. *longipalpis* OBP4 binding site, 2D complexes were generated to discriminate interaction profile (Fig. 3).

According to interaction maps of best ranked compounds in docking, Citronellol acetate (energy = −34.77 kcal mol^{-1}) performs a saline bridge with His110 and hydrophobic interactions with the Tyr121, Phe58, Val63 and Phe61 residues (Fig. 3a), and Geranyl acetate (energy = −32.45 kcal mol^{-1}) performs only a saline bridge with His110 residue (Fig. 3b).

Citronellol compounds (energy = −28.91 kcal mol^{-1}) made a hydrogen bond with Leu57 (acceptor) and hydrophobic interactions with Tyr121 (Fig. 3c). In addition, Citronellal compound (energy = −28.52 kcal mol^{-1}) performs only hydrophobic interactions with Phe61, Leu123, Tyr121 and Trp113 residues (Fig. 3d). Compared with the crystallographic ligand (commercial repellent), DEET performs a hydrogen bond with His110 (donor) and hydrophobic interactions with Ile18, Leu57, Phe58 and Trp113.

The interaction map analysis of compound docking poses shows that the presence of aromatic groups and polar chemical groups with hydrogen bonding acceptor/donor features is an important stereo-electronic requirement in compounds with repellent activity and L. *longipalpis* OBP4 binding site affinity, mainly by the presence of interactions with His110, Leu57, Phe58 and Trp113. In addition, the presence of acetate groups is observed as an important chemical in affinity of Citronellol and Geranyl compounds according to pharmacophore superimposition and docking results.

4. Conclusions

Thus, according to repellent bioassay results and the computational approaches employed, it is suggested that repellent compounds present in citronella, bushy matgrass and 'alecrim do mato', i.e. Citronellol, Citronellol acetate, Citronellal and Geranyl acetate, prioritized in pharmacophore and docking-based approaches, are able to bind to L. *longipalpis* OBP4. Therefore, they generate repellent behavior in sand flies observed herein. Additionally, the interaction with L. *longipalpis* OBP4 binding site is a probable mechanism of repellent compounds found in citronella, bushy matgrass and 'alecrim do mato' essential oils. In addition, the bad performance of repellency assays with neem leaf essential oil corroborate our in silico results.

Supplementary material

Table S1: Complete chemical composition of essential oils and their chemical group composition from neem (*Azadirachta indica*), Citronella (*Cymbopogon winterianus*), Bushy matgrass (*Lippia alba*) and 'Alecrim do mato' (*Lippia thymoides*) leaves analyzed by GC–MS.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. **Claudia I. Brodskyn** is a senior investigator of CNPq and **Tiago F. Mota** received a PhD scholarship from Gonçalo Moniz Institution / FIOCRUZ-BA.

CRediT authorship contribution statement

Tiago F. Mota: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Caliene M. de A. Silva:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft. **Maurício dos S. Conceição:** Methodology, Validation, Investigation, Data curation. **Deborah B.M. Fraga:** Methodology, Resources, Writing – review & editing. **Claudia I. Brodskyn:** Methodology, Resources, Writing – review & editing. **Moysés F. de A. Neto:** Methodology, Validation, Investigation. **Isis B. Santana:** Methodology, Validation, Investigation. **Paulo R.R. Mesquita:** Resources, Formal analysis, Writing – review & editing. **Franco H.A. Leite:** Resources, Formal analysis, Writing – review & editing. **Jairo T. Magalhães-Júnior:** Resources, Writing – original draft, Supervision, Project administration.

Declarations of interest

The authors have no conflicts of interest to declare.

Acknowledgements

The authors are grateful to the sand fly colony staff for providing support and specimens for the bioassays.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106367.

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