Biomonitoring the *Vitex gardneriana* Shauer (Lamiaceae) Toxic Effects to Shed Light on Bioactive Compounds against a Major Coconut Pest Mite

Anderson Soares de Almeida¹, Caroline Rabelo Coelho², Mirele Santana de Sá³, Edson de Souza Bento⁴, Alberto Wisniewski Junior³, Adenir Vieira Teodoro⁵, Haroudo Sátiro Xavier⁶, Jose Guedes de Sena Filho*

¹Departamento de Farmácia, Universidade Federal de Sergipe, Cidade Universitária Professor José Aloísio de Campos, Avenida Marechal Rondon s/n, Bairro Jardim Rosa Elze, São Cristóvão, Brazil
²Universidade Estadual do Maranhão, Cidade Universitária Paulo VI s/n, Bairro Tirirical, São Luís, Brazil
³Petroleum and Energy from Biomass Research Group (PEB), Department of Chemistry, Federal University of Sergipe, São Cristóvão, Brazil
⁴Instituto de Química e Biotecnologia, Universidade Federal de Alagoas, Cidade Universitária, Campus Aristóteles Calazans Simões, Avenida Lourival Melo Mota, s/n, Bairro Tabuleiro do Martins, Maceió, Brazil
⁵Embrapa Tabuleiros Costeiros, Bairro Jardins, Aracaju, Brazil
⁶Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco, Cidade universitária, Recife, Brazil

Email: anderson123soares@outlook.com, carolinecoelho7@gmail.com, mirelesantana@live.com, edson.iqb@gmail.com, albertowi@ufs.br, adenir.teodoro@embrapa.br, *jose-guedes.sena@embrapa.br


Received: September 15, 2021
Accepted: November 2, 2021
Published: November 5, 2021

Abstract

The coconut mite, *Aceria guerreronis* Keifer (Acari: Eriophyidae), is a major pest of coconut plantations (*Cocos nucifera* L.) worldwide. Here, we conducted a bioguided phytochemical approach using toxicity and repellency bioassays of nonpolar extract and its fractions of *Vitex gardneriana* Shauer (Lamiaceae) leaves to this pest. Nonpolar crude extract was fractionated by column chromatography using solvents with increased polarity and binary mixtures, resulting in five semipurified groups. The biomonitoring bioassay provided active fractions and led to the isolation and characterization of the bioactive compound squalene, a biosynthetic precursor of 20-hydroxyecdysone, which plays an important role in plant defense against arthropods. The LC₅₀ of the crude extract of *V. gardneriana* for *A. guerreronis* was estimated to be 0.185 mg·mL⁻¹ and LC₈₀ = 4.123 mg·mL⁻¹. Also, the extract was highly repellent to this pest for up to 24 h. The fractions of *V. gardneriana*, and also squ-
alene, caused mortality to *A. guerreronis*. The potential of *V. gardneriana* fractions/squalene as biopesticides for controlling *A. guerreronis* in coconut plantations is discussed herein.

**Keywords**

Bioassay, Toxicity, Phytochemistry, Terpene, Biopesticides, Biorational Control

1. Introduction

The Lamiaceae family constitutes taxa composed of about 236 genera and around 7000 plant species widely distributed worldwide [1] [2] [3]. Species of this family are known for their medicinal and insecticidal properties, serving as raw material for the pharmaceutical and agrochemical industry [3] [4] [5].

In this context, the *Vitex* genus consists of about 250 species from trees and shrubs, producing a high content of terpenes and ecdysteroids compounds, the latter being natural hormones that participate in biochemical processes in insects [6] [7] [8]. *Vitex gardneriana* Shauer (Lamiaceae) is restricted to the semiarid caatinga biome of Brazil’s Northeast, found on the riverbanks in Paraíba, Bahia and Pernambuco states and further dispersed in the states of Sergipe and Alagoas [9] [10]. Its main constituents are terpenes, mainly sesquiterpenes, which are promising secondary compounds for the development of biopesticides [5] [10] [11].

The coconut mite *Aceria guerreronis* Keifer (Acari: Eriophyidae) is the most expressive pest to affect coconut plantations in tropical America, Asia, and Africa [12] [13] [14] [15]. *A. guerreronis* populations are found in restricted spaces under fruit bracts [16] [17]. This microhabitat makes it difficult for predatory mites to enter, making *A. guerreronis* less vulnerable to attack by predators [18]. The bracts also act as physical barriers against acaricide spraying [17] [19]. The attack of this pest leads to premature fruit drop, reduced fruit size, weight and water volume. Aesthetic skin necrosis also reduces the value of fruits intended for the fresh market [12] [19].

The control of *A. guerreronis* relies heavily on preventive spraying with acaricides, which potentially leads to several problems such as environmental and food contamination, in addition to resistance and death of natural enemies [20] [21] [22]. From this perspective, botanical insecticides may play an important role in controlling *A. guerreronis* without most of the harmful effects of chemical pesticides [23]. Based on this, we conducted a bioguided phytochemical study using toxicity and repellency as parameters to shed light on the bioactivity of crude extract/fractions and isolated compound from the leaves of *V. gardneriana* against the coconut mite *A. guerreronis*. 
2. Materials and Methods

2.1. Living Material

_V. gardneriana_ leaves were collected in the municipality of Patos, Paraíba, Brazil. (07°01’28”S; 37°16’48”W). Voucher specimens were deposited in the herbarium of the Federal University of Southwest Bahia under number 8126. This species and _A. guerreronis_ were also registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the codes A7E13BA and A5DD7EA, respectively. _A. guerreronis_ adults were collected from fruits of a green dwarf coconut plantation located in the city of Aracaju (10°57’S; 37°03’W), Sergipe, Brazil.

2.2. Chemical Study

2.2.1. Extraction and Isolation of Chemical Constituents

The dried and crushed leaves (1400 g) were extracted four times through maceration with hexane for 48 h and the extracts were filtered with filter paper and evaporated to dryness at 40℃ under reduced pressure. Column chromatography was performed on silica gel (70 - 230 and 230 - 400 mesh, Merck). The hexane extract (1.0 g) was fractionated on a column of silica gel (100 g, 70 - 230 mesh) with pure solvents and binary mixtures of cyclohexane, hexane and ethyl acetate (AcOEt) in increasing polarity. The chromatographic analyses were made by Thin Layer Chromatography (TLC) on Si gel (MERCK-Germany) developed by different solvent systems and spots were visualized using a sulfuric acid-vanillin (2%) spray reagent and then grouped into five groups: cyclohexane 100 (Fr1), cyclohexane-hexane 50:50 (Fr2), hexane 100 (Fr3), hexane-AcOEt 99.8:0.2 (Fr4) and hexane-AcOEt 99.5:0.5 (Fr5). The Fr2 group was concentrated and rechromatographed as before and eluted with cyclohexane-hexane (50:50) to generate the subfraction (6 - 14) as a colorless oil (13.0 mg).

2.2.2. Instruments

Nuclear magnetic resonance (NMR) and 13C and 1H spectra were obtained using a spectrometer (Bruker ASCEND 600). Chemical shifts were expressed in ppm (d) and coupling constants in Hz, using a residual solvent peak or tetramethylsilane as a reference for the 1H NMR spectra.

Chemical characterization of the isolated compound was analyzed by gas chromatography/mass spectrometry (GC/MS) using a TRACE 131 gas chromatograph and TSQ-9000 mass spectrometer with a TriPlus RSH autosampler (Thermo Scientific, Bremen, Germany). The separations were carried out using a 60 m × 0.25 mm × 0.25 μm 5 MS column (5% diphenyl- and 95% dimethylpolysiloxane) with oven programming (50℃, 3 min; 5℃ min⁻¹ up to 150℃ and 15℃ min⁻¹ up to 280℃, 15 min) and carrier gas and helium flow at 1.0 mL min⁻¹. A volume of 0.5 μL was injected with a split ratio of 1:20. The injector and interface temperature was 280℃ and the ion source temperature was 300℃, with an electronic energy of 70 eV and an analysis time of 46.7 min. The com-
pound was identified by comparing the spectra obtained with those in the equipment database and by the retention index [24].

2.3. Toxicity to A. guerreronis

The crude hexane extract, the five groups of fractions obtained from the chromatographic fractionation and the isolated compound from the most active fraction were evaluated for their toxicity to A. guerreronis. Ten discs were made with pieces of meristematic tissue of young coconut fruits, placed in a petri dish (10 cm deep) and covered with a mixture of 5% agar, 0.3% methylparaben (Nipagim, as a fungicide) and distilled water. After solidification of the medium, ten discs of 1 cm in diameter were cut with a mold [25].

Concentration–mortality bioassays were performed to estimate the lethal concentration (LC₅₀) of the crude extract. In short, six concentrations of the extract (0.025, 0.5, 1.0, 1.5, 2.0 and 2.5 mg·mL⁻¹) were prepared and the preliminary toxicity of the fractions was at the standard concentration of 1.0 mg·mL⁻¹, with two replicates. The samples were dissolved in acetone and sprayed on Petri dishes; spraying was performed at a pressure of 34 kPa (0.34 bar) and a spray aliquot of 9.3 mL using a Potter tower device (Burkard, UK) [26].

After spraying, the discs were left to dry before 20 adults of A. guerreronis were transferred onto each disc. Ten repetitions (discs) were used for each concentration, totaling 200 mites per plate. The plates were kept in a biochemical oxygen demand (BOD) chamber at 27˚C ± 2˚C and relative humidity of 70% ± 10%. The mortality of A. guerreronis was assessed after 24 h of exposure and the mites were considered dead when unable to move on touching gently with a brush [25].

2.4. Repellency to A. guerreronis

The LC₅₀ of the hexane extracts determined in the toxicity bioassays was used in the repellency bioassay. The procedures followed the description above but only half of each disc was sprayed (half was covered with adhesive tape). The discs were left to dry for 1 h before one adult of A. guerreronis was placed on the center of each disc and its position recorded after 1 and 24 h. Each treatment comprised three replicates and each replicate had 20 mites (60 mites per treatment) [26].

2.5. Statistical Analysis

The mortality data were subjected to Probit analysis to estimate lethal concentrations capable of killing 50% (LC₅₀) of target organisms using the PROC PROBIT procedure in SAS (Version 5.2.38), with p > 0.05 (SAS Institute, 2013). To assess the toxicity of the fractions obtained from fractionation, analysis of variance (ANOVA) and the Tukey test (p < 0.05) were performed to compare the groups with each other, and the toxicity of squalene was assessed by descriptive statistics, expressed as mean, standard error and standard deviation, using the
BioEstat 5.0 software. The repellency of hexane extracts to *A. guerreronis* was determined by frequency analysis according to the chi-square test using Procfreq from the SAS software [27].

3. Results

3.1. Phytochemical Investigation

Phytochemical approach of *V. gardneriana* hexanic extract was able to identify, isolated and characterized squalene as the bioactive compounds (from the most active fraction-Fr2). Squalene, was a colorless oil (13 mg). MS: molecular ion peak at m/z 410.

3.2. Toxicity to *A. guerreronis*

The toxicity assay (Table 1) shows the estimated concentrations of the crude hexanic extract (EBH) of *V. gardneriana* that killed 50% (LC50 = 0.185 mg/mL) of *A. guerreronis* (*p* = 0.0863) and that killed 80% (LC80 = 4123 mg/mL), (*p* = 0.0829).

The five groups of fractions (Fr1, Fr2, Fr3, Fr4 and Fr5) also were toxic to the mite in two replicates: *p* < 0.0001 and *F* = 83.48.06 and *p* < 0.0001 and *F* = 10.94.71 (Table 2). The Fr2 group was the most toxic as it presented the highest mortality of mites. The Fr2 group also isolated squalene, which killed up to 44% of *A. guerreronis* at a concentration of 0.50 mg/mL (Table 3).

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Table 1. Lethal concentrations (LC50) and (LC80) of the crude extract of leaves of *V. gardneriana* to *A. guerreronis*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC50 (95% CI) (mg/mL)</th>
<th>X²</th>
<th>Df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>EH</td>
<td>0.18539 (0.068 - 0.387)</td>
<td>2.0372</td>
<td>4</td>
<td>0.0863</td>
</tr>
<tr>
<td>EH</td>
<td>LC80 (95% CI) (mg/ML)</td>
<td>X²</td>
<td>Df</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.123</td>
<td>2.0620</td>
<td>4</td>
</tr>
</tbody>
</table>

Average values obtained (*n* = 7 discs with 20 adult mites each). CI = 95% confidence interval; X² = chi-square test (*p* > 0.05).

Table 2. Percentage toxicity of apolar fractions from *V. gardneriana* to *A. guerreronis*.

<table>
<thead>
<tr>
<th>Time 24 (h)</th>
<th>Dose (1.0 mg/mL)</th>
<th>Fr (1)</th>
<th>Fr (2)</th>
<th>Fr (3)</th>
<th>Fr (4)</th>
<th>Fr (5)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicates 1</td>
<td></td>
<td>52.20</td>
<td>64.40</td>
<td>56.10</td>
<td>7.20</td>
<td>30.00</td>
<td>0.0001</td>
</tr>
<tr>
<td>Replicates 2</td>
<td></td>
<td>51.80</td>
<td>64.50</td>
<td>56.00</td>
<td>7.00</td>
<td>29.00</td>
<td>0.0001</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>52.00</td>
<td>64.45</td>
<td>56.05</td>
<td>7.10</td>
<td>29.50</td>
<td></td>
</tr>
</tbody>
</table>

(*n* = 10 discs with 20 adult mites each).
Table 3. Squalene toxicity to *A. guerreronis*.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Dose mg/mL</th>
<th>Average percentage mortality %</th>
<th>Standard error</th>
<th>Detuor error</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.15</td>
<td>15.6000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>29.3500</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>44.3500</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Percentage average values obtained by descriptive statistics (n = 8 discs with twenty adult mites each).

3.3. Repellency to *A. guerreronis*

The LC50 of the hexanic extract of leaves of *V. gardneriana* repelled adults of *A. guerreronis* after 1 and 24 h of exposure (Figure 1).

4. Discussion

Previous studies have reported the isolation and identification of *V. gardneriana* chemical compounds and their biological activity. For example, the following have been obtained: ursolic and oleanolic acids; the wood acids 2α,3α,19α-trihydroxyursan-12-en-oic, 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate, 4-hydroxy-3-methoxybenzoic acid and 4-hydroxybenzoic acid; from the leaves, the flavonoid 5-hydroxy-3,7,4-trimethoxyflavone; from the roots, 3,4-dihydroxybenzoic acid; and from the stem bark, the ecysteroid 20-hydroxyecdysone and the glycosylated iridoid aucubine [10] [28]. There are reports of the chemical study of essential oils in the leaves by GC/MS, with a predominance of the sesquiterpenes cis-calamenene, 6,9-guaiadiene, karyophyline oxide and α-cadinol [5] [11].

Chromatographic treatment of the most active fraction, Fr2, in the bioassays provided colorless oil. The 1H NMR proton spectrum (600 MHz, CDCl₃) showed the presence of five signals: δH 1.28 singlet attributed to methylenic groups; δH 1.63 singlet associated with water present in the deuterated chloroform solvent; δH 1.70 singlet attributed to methyl hydrogens linked to olefinic carbons; δH 1.98 - 2.07 multiplet associated with methylene hydrogens linked to olefinic carbons; and δH 5.10 - 5.20 multiplet associated with the presence of olefinic hydrogens. Analysis of the 13C NMR spectrum (150 MHz, CDCl₃) showed the presence of 15 signals: three δC signals attributed to olefinic non-hydrogenated carbons (131.2, 134.8 and 135.1: C); three δC signals attributed to methylenic carbons next to quaternary carbons (126.4, 126.3 and 134.4: CH₂); six δC signals attributed to methylene groups (26.7, 26.8, 28.3, 29.7, 39.7 and 40.0: CH₃); and four δC signals attributed to methyl carbons (16.1, 16.2, 17.6 and 25.1: CH₃). These observations, associated with the presence in the mass spectrum of a molecular ion peak at m/z 410, and comparison of the data described in the literature [29] [30], allowed us to propose the structure of squalene, a polyunsaturated triterpene with six isoprene units, described here in *V. gardneriana* for the first time (Figure 2). Squalene has been reported to be a biochemical precursor of...
Figure 1. Expanded spectrum m/z 317-415.

Figure 2. Repellency of the hexanic extract (LC50) from the leaves of V. gardneriana to A. guerreronis.

Figure 3. Preference of A. guerreronis (%)

cholesterol and other steroids. Interestingly, cholesterol is the biosynthetic precursor of ecdysone and 20-hydroxyecdysone (Figure 3), previously reported in V. gardneriana [10] [31] [32].

The results of the present study showed that the hexanic extract and fractions obtained from V. gardneriana were able to kill A. guerreronis adults. These findings suggest that extracts and fractions may have potential in the management of A. guerreronis. Similar findings have been reported in other studies showing the toxic effects of extracts from other plants of the Vitex genus against
Figure 3. Chemical structure of squalene.

mites and insects [33]. For example, different extracts of the leaves of Vitex negundo L. (Lamiaceae) were active against several arthropods: the ethanolic extracts were toxic to the tick Rhipicephalus (Boophilus) microplus (Acarina: Ixodidae), the hexane extract showed larvicidal action against Culex tritaeniorhynchus Giles (Culicidae) and Anopheles subpictus Grassi (Culicidae) larvae, and petroleum ether extract also had larvicidal action against C. tritaeniorhynchus Giles [33] [34].

Repellency bioassays indicated that the hexanic extract of V. gardneriana was highly repellent to A. guerreronis after 1 and 24 h of exposure. The ability to repel A. guerreronis suggests the irritability of these substances when the mites are in direct contact with them [35] [36]. In a parallel study, the essential oils of V. gardneriana, Vitex capitata Vahl and Vitex megapotamica (Spreng.) also repelled A. guerreronis [5].

Regarding the triterpene squalene isolated from the most active fraction, its toxicity towards A. guerreronis could be expected due to its long-chain aliphatic structure and/or the presence of alternating double bonds, as shown by other studies in which aliphatic compounds are active against cockroaches; however, the mechanism of action is uncertain [37] [38].

Interestingly, the literature reports the bioactivity of vegetable oils rich in fatty acids against A. guerreronis, such as crude oils from palm [25], babassu, coconut and degummed soybean, all rich in aliphatic fatty acids and showing structural similarities with squalene, which partially explains the activity against this pest mite [26]. Some enzymes are described as targets for the control of mites, such as acetylcholinesterase (AChE). Several terpenes inhibit this enzyme in the cholinergic and neuromuscular synapses of arthropods, increasing the cholinergic pathway and leading to muscle paralysis, with greater action for nonpolar lipophilic compounds that cross the lipid membranes of cells [39] [40] [41] [42].

5. Conclusion

The hexane crude extract from leaves of V. gardneriana and its bioactive fractions showed high toxicity to A. guerreronis. The bioguided study provided active fractions and led to the isolation of squalene, which proved to be bioactive against A. guerreronis possibly because of its aliphatic chain. Squalene is a biosynthetic precursor of 20-hydroxyecdysone, which plays an important role in plant defense against arthropods. Furthermore, the extracts/fractions of V. gardneriana, as well as squalene, proved to be candidates for new biopesticide formulations based on their bioactivity against A. guerreronis, a major pest of coconut plantations worldwide.
Acknowledgements

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 405485/2016-6) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. To CLQM (Center of Multi-users Chemistry Laboratories) from Federal University of Sergipe for the facilities.

Ethical Approval

All applicable international, national, and institutional guidelines for the care and use of animals were considered in the present investigation.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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