MARILZA DA SILVA COSTA

ACETOGENIN A TOOL TO CONTROL *Aedes aegypti*: A PERSPECTIVE OF TOXICITY AND GENE REGULATION

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia para a obtenção do título de Doctor Scientiae.

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APROVADA: 12 de agosto de 2016.

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Gustavo Costa Bressan	Luis Carlos Martinez Castrillón
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José Eduardo Serrão
(Orientador)

“Aprendi a transformar o medo em respeito, o respeito em confiança. Descobri como é bom chegar quando se tem paciência para se chegar, onde quer que seja. Aprendi que não é preciso dominar a força, mas a razão”.

Amyr Klink

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Aos meus pais Adelino e Cíçera, pelo carinho, confiança e exemplo de dignidade que levarei para toda a vida. Aos meus irmãos Claudio, Carli, Edinéia, Edileuza e meus sobrinhos pela força e compreensão em todos os momentos desta e de outras caminhadas.

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SUMÁRIO

RESUMO

COSTA, Marilza da Silva, D. Sc., Universidade Federal de Viçosa, agosto de 2016. **Acetogenina como instrumento de controle do Aedes aegypti: uma perspectiva de toxicidade e regulação gênica**. Orientador: José Eduardo Serrão.

Dengue, Chikungunya e Zika são arboviroses transmitidas principalmente pelo mosquito Aedes aegypti. Embora seu controle seja realizado através da eliminação dos criadouros, controle biológico, controle genético e uso de inseticidas, são necessárias formas alternativas de manejo integrado desta praga para diminuir a ocorrência de resistência e que propiciem menores riscos ambientais e toxicológicos. Este trabalho avaliou um metabólito secundário de Annonaceae, com propriedades inseticidas, no controle de larvas de A. aegypti. Foi isolada e caracterizada uma acetogenina das sementes de Annona mucosa e testou-se sua toxicidade contra larvas de A. aegypti (inseto-alvo), seus predadores Culex bigotti e Toxorhynchites theobaldi (insetos não-alvo) e leucócitos humanos. Posteriormente avaliou as alterações na morfologia celular no intestino médio das larvas tratadas com acetogenina e determinou a influência desta molécula na expressão de genes para autofagia (Atg1 e Atg8), V-H^+-ATPase (proteína transportadora), e para aquaporina-4 (Aqp4) (proteína de canal de água). E por fim, obteve informações sobre a influência da acetogenina na papila anal de larvas de A. aegypti, através da descrição das alterações morfológicas e influência desta na expressão de V-H^+-ATPase e Aqp4. A molécula isolada foi a esquamocina, que apresentou alta toxicidade às larvas, porém não apresentou toxicidade aos insetos não alvos e leucócitos humanos, indicando a seletividade desta molécula. No intestino médio, esquamocina teve ação citotóxica com modificações nas células digestivas, aumento na expressão de genes para autofagia (Atg1 e Atg8), diminuição da expressão de V-ATPase e de Aqp4 na CL_{20}, bem como inibição da expressão em CL_{50}, demostrando ter múltiplos modos de ação neste órgão. Nas papilas anais, esquamocina causou alterações morfológicas no epitélio, diminuição dos níveis de transcrição de AaAQP4 e inibição da transcrição de AaV-H^+-ATPase influenciando no processo de osmorregulação deste órgão. Os modos de ação da esquamocina em níveis morfo-fisiológico e molecular são descritos pela primeira vez. Os resultados mostram que esquamocina pode ser um protótipo de produtos naturais com potencial no controle de larvas de A. aegypti contribuindo ao estabelecimento de estratégias alternativas para o controle de insetos vetores com eficiência e baixo impacto ambiental.

ABSTRACT

COSTA, Marilza da Silva, D. Sc., Universidade Federal de Viçosa, August, 2016. **Acetogenin as tool to control Aedes aegypti: a perspective of toxicity and gene regulation**. Adviser: José Eduardo Serrão.

Dengue, Chikungunya and Zika are arboviruses transmitted mainly by the mosquito Aedes aegypti. Although the control of this insect vector has been accomplished by eliminating rearing sites, use of biological control, genetic methods and the use of insecticides, it is important the use alternative control strategies for the integrated management of this pest. Thus it is expected decrease in the emergence of resistant mosquito strains and in the environmental risks and toxicology. This work evaluated a secondary metabolite of Annonaceae plant with insecticidal properties in the control of A. aegypti larvae. In the first step an acetogenin of Annona mucosa seeds was isolated and tested against A. aegypti larvae (target insect), its predators Culex bigotti and Toxorhynchites theobaldi (non-target insects) and on human leukocytes. The second step evaluated the morphology of the midgut cell in larvae exposed to acetogenin and the effect of this molecule in the expression of genes for autophagy (Atg1 and Atg8), V-H+-ATPase (membrane carrier protein) and aquaporin -4 (Aqp4) (water channel protein). The third step tested the effect of acetogenin on anal papilla of A. aegypti larvae, on the morphology expression of V-H^+-ATPase and Aqp4. The isolated molecule was squamocin that showed high toxicity to the A. aegypti larvae, but without toxicity to non-target insects and human leukocytes, indicating the selectivity of this molecule. In the midgut, squamocin showed cytotoxic effects in the digestive cells, increased expression of genes for autophagy (Atg1 and Atg8), decrease of V-ATPase and Aqp4 expression in LC_{20} as well as their inhibition in LC_{50}, showing multiple modes of action in this organ. In anal papillae, squamocin caused morphological damages in the epithelium, decreased transcription levels of AaAQP4 and inhibited the AaV-H + -ATPase transcription, suggesting effect in the osmoregulation process of this organ. The modes of action of squamocin at morpho-physiological and molecular levels are described for the first time. Our results indicated that squamocin can be a prototype of natural products with potential to control A. aegypti larvae, contributing to the establishment of alternative strategies for the control of insect vectors with high efficiency and low environmental impact.

INTRODUÇÃO GERAL

No Brasil, Aedes aegypti (Linnaeus, 1762) (Diptera: Culicidae) é o principal inseto vetor do vírus da Dengue, Chikungunya e Zika (Depoli et al. 2016; Pustiglione 2016) cuja situação é caracterizada principalmente pela infestação generalizada em todas as regiões. O controle deste vetor é feito pela eliminação ou limpeza dos locais de reprodução (Who 2003), por meio de controle biológico (i.e. Bacillus thuringiensis israelenses) (Andrade e Modolo 1991; Vilarinhos e Monnerat 2004), controle genético (i.e. machos inférteis) (Fu et al. 2010) e, principalmente, inseticidas sintéticos que constituem, ainda, a base dos programas de controle (Campos e Andrade 2001). No entanto, o emprego de inseticidas associados às ações educativas e de manejo ambiental não tem suprimido suficientemente as populações do vetor, e por consequência, os índices de infestação e incidência dos vírus supracitados. Tal fato se deve, em muitos casos, a frequente exposição aos produtos químicos, que pode propiciar seleção de resistência aos principais ingredientes ativos usados (i.e. temephos, fenitrothion) (Campos e Andrade 2003; Lima et al. 2003; Macoris et al. 2003; Braga et al. 2004; Silva et al. 2015; Chediak et al. 2016) e até ao B. thuringiensis israelenses (Tabashinik 1994). Além disso, esses inseticidas têm elevado custo e podem gerar riscos ambientais (i.e. desequilíbrio ecológico) e toxicológicos (Tauil 2002).

Opcionalmente a estes métodos de controle vigentes, a indústria de inseticidas busca continuamente fontes naturais de novos fitoquímicos com propriedades inseticidas, visando sua utilização direta ou como matéria prima para preparação semisintética de novos produtos, além de novos modelos para síntese completa de produtos com atividade inseticida potencializada (Balandrin et al. 1993; Isman 2015). Neste ponto de vista, as plantas podem ser uma alternativa como agentes de controle, uma vez que constituem uma fonte rica de substâncias químicas bioativas.

Os inseticidas botânicos já teve uma posição de importância no arsenal de produtos fitofarmacêuticos, no entanto, foram substituídos pelos inseticidas sintéticos na década de 1950 e 1960, principalmente nos países industrializados. O aumento de documentações que relatam negativamente os impactos dos inseticidas sintéticos para o ambiente e para a saúde, juntamente com regulamentação cada vez mais rigorosa, tem renovado o interesse no desenvolvimento e uso de produtos botânicos

para o controle de pragas e vetores como uma estratégia biorracional mais adequada ambientalmente, embora ainda controversos.

Entre as principais famílias botânicas com sucesso como fonte de substâncias inseticidas destaca-se a Annonaceae pela sua diversidade de espécies e valor econômico (frutos, sementes e madeira) (Chatrou et al. 2014). Plantas desta família apresentam uma série de produtos (compostos/substâncias) naturais, com evidência para as acetogeninas registrada inicialmente em 1965 (Jolad et al. 1982), mas somente em 1988, houve o isolamento da asimicina, a primeira acetogenina registrada com propriedades inseticidas (Rupprecht et al. 1986). Desde então, os registros sobre estas moléculas incluem discussões referentes aos métodos de extração, isolamento, purificação, elucidação da estrutura molecular, técnica de determinação via espectrometria, atividade biológica, fontes de origem e registros de novas acetogeninas (Rupprecht et al. 1990; Zeng et al. 1996; Alali et al. 1999; Bermejo et al. 2005; McLaughlin 2008).

Acetogeninas de anonáceas são metabólitos secundários derivados de ácidos graxos de cadeia longa, contendo de 35 a 37 átomos de carbono (Alali et al. 1999). São caracterizadas pela presença de uma cadeia alifática longa com grupos funcionais hidroxila, acetila e carbonila e um anel γ-lactona terminal, metilo substituído α, β-insaturado, e por vezes um arranjo ceto-lactona (Alali et al. 1999). Em consequência da reatividade dos grupos metileno e biossíntese de compostos fenólicos, as acetogeninas possuem estruturas moleculares diretamente dependentes do número de unidades de acetato conectadas e tais características são de extrema importância para a eficácia destas moléculas. A cadeia pode ter um, dois ou três anéis tetrahidrofurânicos (THF) (Yang e Kitahara 2000), com alguns substituintes oxigenados (hidroxila, cetonas, e epóxidos) (Nattrass et al. 2005), e em menor grau, podem possuir anéis tetrahidropirânicos (THP) (Shi et al. 1995). Desta forma, de acordo com a quantidade de anéis tetrahidrofurânicos e de subunidades de γ-lactona, as acetogeninas podem ser classificadas como mono-THF, bis-THF adjacentes, bis-THF não adjacentes, lineares e com anel THP.

A relação entre estrutura da molécula de acetogenina e sua atividade biológica tem sido reportada (Rupprecht et al. 1990; Alali et al. 1999; Crisóstomo et al. 2006; Barrachina et al. 2007; Liu et al. 2012). As acetogeninas bis-THF com anéis adjacentes são as mais potentes biologicamente (He et al. 1997), seguidas em ordem decrescente pela bis-THF não adjacentes, a mono-THF e por fim, por aquelas que

não possuem anéis (Rupprecht et al. 1990; Fang et al. 1993). O grupo hidroxila também tem relação com a potencialização da acetogenina, pois aquelas com três grupos hidroxila, dois vizinhos aos anéis THF e outro em qualquer posição ao longo da cadeia hidrocarbônica, favorecem a polaridade necessária para potencializar a atividade das acetogeninas (Blessing et al. 2012).

Apesar dos estudos sobre a relação entre estrutura e atividade das acetogeninas, seus mecanismos de ação para insetos não estão totalmente elucidados. Em mamíferos as acetogeninas são potentes inibidores do complexo mitocondrial I, por inibir a NADH ubiquinona oxidorredutase, enzima essencial no complexo I (Bermejo et al. 2005; Grandic et al. 2004). Elas inibem também a NADH oxidase ligada a ubiquinona, peculiar às membranas plasmáticas das células cancerígenas (Bermejo et al. 2005). Estas justificativas são as atualmente aceitas para a toxicidade em insetos (Zafra-Polo et al. 1996).

Como as moléculas de acetogeninas são geralmente lipofílicas, há uma hipótese de que podem se associar às membranas lipídicas e adotar uma conformação favorável no seu interior de forma a atingir seus locais alvo por meio da difusão através da membrana (Shimada et al. 1998). Os anéis THF da molécula, juntamente com seus grupos hidroxila, podem agir como uma âncora hidrofílica na membrana lipídica, e a posição de anel de THF ao longo da cadeia da acetogenina determina a profundidade do grupo funcional lactona, que então atua diretamente com o sítio receptor, talvez, mimetizando a ubiquinona (Takada et al. 2000). No entanto, não está estabelecidos se há qualquer ligação entre a inibição do complexo I e a apoptose e/ou citotoxicidade provocada por acetogeninas em células, contudo, sugere-se que acetogeninas podem apresentar, além de ação mitocondrial (complexo I), modos de ação secundários de citotoxicidade tais como, ação citosólica e sobre enzimas associadas ao retículo endoplasmático (Derbré et al. 2008).

Estas informações sobre estas moléculas abre novos caminhos para a compreensão da citotoxicidade desta classe de metabólito secundário sobre insetos. A elucidação dos mecanismos de toxicidade e dos alvos moleculares de substâncias inseticidas se reveste de grande relevância por fornecer informações básicas necessárias para o aprimoramento e ampliação das estratégias de controle de vetores de doenças, sempre considerando seus efeitos sobre organismos não alvos, inclusive sobre humanos. Assim, este trabalho fornece subsídios para uma nova estratégia de

controle de larvas de A. aegypti com o uso de acetogenina de anonáceas e descreve sua interferência na morfo-fisiologia da larva deste mosquito vetor.

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CHAPTER 1

TOXICITY OF SQUAMOCIN ON Aedes aegypti LARVAE, ITS PREDATORS AND HUMAN CELLS

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Toxicity of squamocin on *Aedes aegypti* larvae, its predators and human cells

Marilza S Costa,[a] Antônio EG Santana,[b] Leandro L Oliveira,[c] José C Zanuncio[a] and José E Serrão[d]*

Abstract

BACKGROUND: The mosquito *Aedes aegypti* transmits a virus that causes diverse human diseases, and control of the vector is an important strategy to avoid disease propagation. Plants in the family Annonaceae are recognised as sources of molecules with uses in the medical and agriculture fields. Molecules of secondary metabolites of Annonaceae plants exhibit insecticidal potential against insect pests and vectors, especially acetogenins, showing high toxicity at low doses, which has encouraged research into producing new insecticide molecules. Herein, we identify an acetogenin from *Annona mucosa* seeds (chemical analysis) and provide the results of toxicity tests against larvae of *A. aegypti* (target insect) and its predators *Culex bigoti* and *Toxorhynchites theobaldi* (non-target insects) and cytotoxicity to human leukocytes.

RESULTS: We identified squamocin ($C_{37}H_{66}O_7$), a fatty acid with a *bis* -tetrahydrofuran ring. In *A. aegypti*, this compound caused behavioural disturbance before larval death and high mortality at low concentrations ($LC_{50} = 0.01\ \mu g\ mL^{-1}$ and $LC_{90} = 0.11\ \mu g\ mL^{-1}$). However, in predators and human leukocytes, squamocin showed no toxicity effect, indicating the selectivity of this molecule for non-target organisms.

CONCLUSION: We identified squamocin from *A. mucosa* seeds, which exhibited lethal action against *A. aegypti* and showed selectivity for non-target insects and low cytotoxicity to human cells.

Supporting information may be found in the online version of this article.

Keywords: acetogenin; selectivity; target insect; non-target insect; *Annona mucosa*

1 INTRODUCTION

Acetogenins are secondary metabolites produced by members of the family Annonaceae, derived from fatty acids, with carbon atoms (35 to 39).[1,2] These compounds are found in *Annona* spp.,[3–5] *Asimina* spp.,[6,7] *Disepalum* spp.,[8,9] *Goniothalamus* spp.,[10,11] *Uvaria* spp.[12,13] and *Xylopia* spp.[14–16]

The acetogenins have insecticide, fungicide, herbicide, acaricide, antitumour, anthelmintic, antibacterial, antiprotozoal and molluscicide activities,[17–20] and at least 44 acetogenins have insecticide activity against larvae of *Aedes aegypti* (Diptera: Culicidae), an important vector of yellow fever, dengue[21,22] and chikengunya fever.[23]

Acetogenins with two tetrahydrofuran rings have high insecticide activity against *A.* aegypti,[24,25] probably owing to their effect as potential inhibitors of mitochondrial respiration, interfering in ATP synthesis.[20] Thus, acetogenins have been studied for the control of *A. aegypti*. The control of this insect is critical in the prevention of diseases given the increasing problems of resistance to the larvicides used (e.g. pyrethroids), requiring the use of new effective and ecologically safe molecules.[26,27] In this context, molecules of plant origin have been considered an an alternative method for the control of insect vectors.[28] Botanical insecticides may have multiple sites of action and may reduce the potential for insect resistance.[29,30] Biopesticides may be used in association with other conventional control practices,[31,32] increasing the number of agents with different modes of action and reducing the selection of insect resistant populations.

In addition to artificial control, populations of *A. aegypti* are affected naturally by the predators *Culex bigoti* Bellardi, 1862 (Diptera: Culicidae)[33] and *Toxorhynchites theobaldi* (Dyar & Knab) (Diptera: Culicidae).[34,35] However, there are scant data on the effect of acetogenins on non-target insects, which may provide important information for the compatibility of alternative and biological control methods[36] contributing to the integrated pest management of *A. aegypti*.

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We tested the toxicity of an acetogenin from *Annona mucosa* (Annonaceae) seeds against *A. aegypti*, non-target predators *C. bigoti* and*T. theobaldi*and human leukocytes, in order to contribute to the future use of this molecule for safe control of insect vectors.

2 EXPERIMENTAL

2.1 Plant material, isolation and identification of acetogenin

Seeds of *A. mucosa* were collected from fruits in natural areas in the municipality of Tangará da Serra (14° 39′ S, 57° 26′ W) in the state of Mato Grosso, Brazil. Voucher specimens of the plants collected were deposited in the herbarium of the Universidade do Estado do Mato Grosso (Tangará da Serra) and were identified by the number 964.

The seeds of *A. mucosa* were dried and ground in a laboratory mill to a fine powder (mesh 2.5 mm). The dry powder (2.0 kg) was extracted with methanol (3.0 L) at room temperature (25–27 °C) for 3 days and filtered. The residue was extracted twice more by a similar procedure. The solvent was removed by distillation under low pressure in a rotatory evaporator. The concentrated extract was further dried in a freeze drier, resulting in 152.50 g (7.62%) of a crude methanol extract. The crude methanol extract (149.0 g) was suspended in methanol and extracted with *n*-hexane (4 ×400 mL). Water was added to the methanol solution to produce a methanol–water (1:1 v/v) mixture. The solution was partitioned at room temperature successively with chloroform (400 mL× 4) and ethyl acetate (400 mL × 4), resulting in the fractions hexane (56.6 g; 37.9%), chloroform (85.4 g; 57.3%), ethyl acetate (3.9 g; 2.6%) and hydromethanol (0.35 g; 0.23%) after removal of the solvents. The chloroform-soluble fraction (80.0 g) was incorporated into silica gel (350.0 g) and extracted successively with hexane (1.0 L) (5.6 g, 7.0%), hexane–chloroform (1:1 v/v, 1.0 L) (25.0 g, 31.25%), chloroform (1.0 L) (31.0 g, 38.75%), ethyl acetate (1.0 L) (12.0 g, 15.0%), ethyl acetate–methanol (9:1 v/v, 1.0 L) (2.5 g, 3.12%) and methanol (1.0 L) (2.1 g, 2.62%) fractions. The hexane–chloroform-soluble fraction (20.0 g) and the chloroform-soluble fraction (25.0 g) were compared by thin-layer chromatography (TLC) and put together, and part of it (10.0 g) was further purified by column chromatography over a C-18 column by eluting with increasingly polar hexane–ethyl acetate solutions (120 mL each fraction) (7:3; 7:4; 7:5; 3:7 v/v) and with ethyl acetate alone on medium-pressure liquid chromatography apparatus (Shimadzu, Columbia, MD). The fractions eluted with hexane–ethyl acetate 7:4 and 7:5 were combined (8.0 g) into major fractions which were purified by column chromatography over Sephadex LH-20 (three repetitions with 2.0 g each) by eluting with 25 methanol fractions (15 mL each fraction) to 2.1 g of the compound from fractions 9 to 15. Analytical TLC was carried out on aluminium sheets precoated with 60 F254 silica gel, layer thickness 0.2 mm (Merck, Darmstadt, Germany), eluted with chloroform and visualised by a UV lamp (λ=244 and 365 nm) and after spraying with 3% ceric sulphate and heating at 100 °C for 3–5 min. The compound was identified on the basis of ^{1}H and ^{13}C NMR spectra (Bruker Avance 400 MHz; Bruker, Coventry, UK), mass spectrometry (Shimadzu), IR (PerkinElmer, São Paulo, Brazil) and UV (Shimadzu) data (see supporting information Table S1).

2.2 Insects

*A. aegypti*larvae from the susceptible PPCampos strain (Campos Goytacazes) were reared at 25 ±2 °C, 54 ± 2% relative humidity and a photophase of 12 h. The predators *C. bigoti* and*T. theobaldi* were collected from Atlantic rainforest in Viçosa (20° 45′ S, 42° 51′ W) in the state of Minas Gerais, Brazil, with oviposition traps placed at ground level. The traps were made from plastic black polystyrene containers containing 100 mL of dechlorinated water and were visited every 15 days to verify the presence of larvae, which were transferred to the laboratory. Insects without amputations or apparent defects were used in bioassays.

2.3 Toxicity tests against *A. aegypti*

For dose–response tests, an isolated compound of *A. mucosa* seeds was presolubilised in 2% Tween-20 and dissolved in water, resulting in a stock solution of 10 μg mL^{-1}, and from this, increasing concentrations were obtained from 0.001, 0.003, 0.005, 0.008, 0.01 and 0.03 μg mL^{-1}. Twenty third-instar larvae were added in 25 mL of each concentration in quadruplicate, following the methodology of the World Health Organisation.[46] Distilled water and 2% Tween-20 were used as a negative control. Mortality was assessed every 3 h of exposure to different concentrations of the compound to define the lethal concentration (LC) and lethal time (LT). In this bioassay, behavioural changes were also observed in the treated larvae at 1 h intervals.

2.4 Toxicity tests against *C. bigoti*and *T. theobaldi*

Previous tests using LC_{90} for *A. aegypti* had no toxic effect against the predators *C. bigoti* and*T. theobaldi* . Thus, to determine the toxicity of the compound against these predators, their larvae were exposed to 50, 100, 200, 500 and 1000 μg mL^{-1} of the compound. Assays for each predatory insect species were done in quadruplicate with 15 third-instar larvae. The experimental design was completely randomised, and larval mortality was evaluated after 24, 48 and 72 h of exposure. The surviving larvae were observed until adult emergence to evaluate possible morphological changes.

2.5 Cytotoxicity in human leukocytes

A suspension of normal human leukocytes was distributed in a 96-well plate with 90 μL per well and incubated at 37 °C together with 10μ L of the *A. mucosa* compound. In this study the concentrations were 0.003, 0.006, 0.012, 0.025, 0.05, 0.1 and 0.2 μg mL^{-1} of the compound. The suspensions of leukocytes were incubated for 48 h in triplicate. After the incubation period, 10 μL of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (5 mg mL^{-1}) was added, and the cells were further incubated for 4 h.[37] Then, the medium was carefully removed, followed by the addition of 100μ L of dimethyl sulphoxide for solubilisation of the formazan crystals. The plates were shaken for 5 min, and the absorbance corresponding to each sample was measured in an ELISA (Enzyme-Linked Immunosorbent Assay) reader at 550 nm.[37] The absorbance obtained from the cells treated with *A. mucosa* compound was compared with the absorbance of control cells not exposed to the compound. To verify a possible inactivation of the *A. mucosa* compound by the serum in the cell culture medium, an acute cytotoxicity assay was carried out at concentrations of 100, 10, 1, 0.1, 0.01 and 0.001 μg mL^{-1} for 6 h in medium without serum.

2.6 Statistical analysis

The dose–response curve was determined for *A. aegypti*, and to estimate the lethal concentrations LC_{50} and LC_{90}, data were submitted to probit analysis.[38] The absorbance data of leukocytes were subjected to tests for normality (Shapiro–Wilk) and

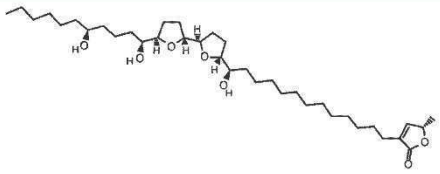

Figure 1. Chemical structure of squamocin isolated from *A. mucosa* seeds.

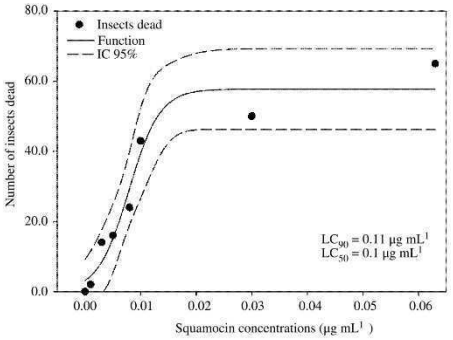

Figure 2. Concentration–response curve of *A. aegypti* larvae treated with squamocin from *A. mucosa* seeds.

homoscedasticity (Bartlett), followed by analysis of variance (one-way ANOVA) and a *post hoc F*-test (P=0.05), using R software v.3.1.1 (R-Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2014).

3 RESULTS

The compound isolated from the *A. mucosa* seeds was the acetogenin squamocin (Fig. 1). This substance showed a consistent peak (*m/z* 604, 586 and 568) to the formula $C_{37}H_{66}O_7$ (molecular weight 622) with characteristic signals in α,β-unsaturated γ-lactone (1755 cm^{-1}) in the ^1H and ^{13}C NMR spectra. The presence of a *bis*-tetrahydrofuran ring with two hydroxyls (3585 and 3460 cm^{-1}) on adjacent carbons was confirmed by correlation of the hydrogen and carbon signals in the IR spectrum and the UV maximum at 215 nm, with data in accordance with Fujimoto *et al.*[39] (supporting information Table S1).

The toxic effect of squamocin on*A. aegypti* larvae was shown in dose–response tests ($\chi^2 = 71.22$, df = 5, $P < 0.001$), where lethal concentrations (IC 95%) were estimated after 3 h of exposure, with $LC_{50} = 0.01$ µg mL^{-1} (0.01–0.02 µg mL^{-1}) and $LC_{90} = 0.11$ µg mL^{-1} (0.05–0.34 µg mL^{-1}) (Fig. 2). The lethal time (LT_{90}) for 0.03 µg mL^{-1} of squamocin was 43.01 h, and at lower concentration (0.001 µg mL^{-1}) the estimated time was 285.45 h. The LT_{50} was 17.13 h with 0.03 µg mL^{-1} of squamocin and 103.26 h with 0.001 µg mL^{-1} of squamocin (Table 1).

At squamocin concentrations of 0.03 and 0.01 µg mL^{-1}, 1 h after the treatment the larvae showed erratic and violent twitching movements. This behaviour pattern persisted for approximately 2 h, followed by gradual reduction in intensity and resting of the larvae on the surface of the water. After 4 h, the *A. aegypti* larvae showed a high level of lethargy. These behavioural patterns were similar in insects treated with 0.008, 0.005, 0.003 and 0.001 µg mL^{-1} of squamocin, although the time for onset of this behaviour was concentration dependent – 5, 8, 10 and 18 h after exposure respectively.

Table 1. Estimated lethal time (h) for 50% (LT_{50}) and 90% (LT_{90}) and their 95% confidence intervals, in parentheses, for *A. aegypti* larvae treated with different concentrations of squamocin from *A. mucosa* seeds

Squamocin (µg mL^{-1})	LT_{50}	LT_{90}	χ^2	*P*-value
0.03	17.13 (16.01–18.49)	43.01(36.73–53.11)	162.20	<0.05
0.01	21.24 (19.68–23. 27)	49.81(41.50–64.31)	125.75	<0.05
0.008	34. 04 (28.66–43.29)	89.35 (63.45–156.53)	63.27	<0.05
0.005	35.81 (30.23–49.27)	69.17 (50.02–129.76)	36.46	<0.05
0.003	40.24 (32.61–59.77)	92.62 61.73–202.55	37.25	<0.05
0.001	103.26 (46.58–259.27)	285.45 (80.99–309.90)	5.23	<0.05

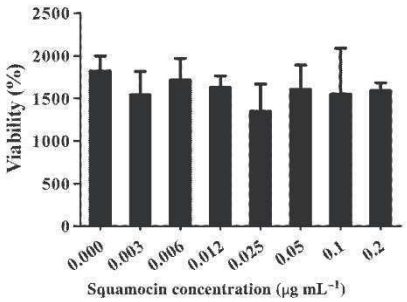

Figure 3. Absorbance spectrum (mean ± SD) for metabolic cell viability of human leukocytes when treated with different concentrations of squamocin.

Squamocin caused no mortality for larvae of the predators *C. bigoti* and*T. theobaldi* until 1000 µg mL^{-1} of squamocin, and nor was any change found in their behaviour patterns. In tests to evaluate the cytotoxicity of squamocin to human leukocytes, there was no reduction in cell viability 48 h after exposure to all tested concentrations of squamocin when compared with the control in medium with serum (Fig. 3) and without serum (Fig. 4).

4 DISCUSSION

The squamocin from *A. mucosa* seeds has a larvicidal effect on *A. aegypti* at low concentrations, which is similar to reports for other acetogenins[1,40,41] The toxic action of squamocin may be due to the tetrahydrofuran (THF) rings, as suggested by Miyoshi *et al.*[42] The squamocin obtained here has two tetrahydrofuran rings, thus it is a*bis* -THF, which has a high inhibitory effect on mitochondrial NADH-ubiquinone oxidoreductase,[43] probably owing to partial competition with the ubiquinone analogue.[42,44,45] In insects, the action of acetogenins occurs in mitochondrial electron transport systems.[46] The changes in the behaviour pattern of *A. aegypti* larvae exposed to squamocin may be due to suppression of ATP. Acetogenins block ATP synthesis, which affects insect metabolism, and in*A. aegypti* larvae exposed to squamocin the low rate of ATP might result in ataxia and progressive paralysis.

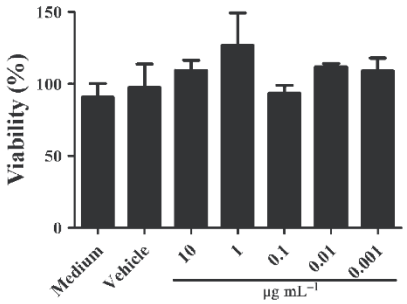

Figure 4. Absorbance spectrum (mean ±SD) for metabolic cell viability of human leukocytes when treated with acute doses of squamocin in medium without serum. Vehicle=Tween-20 (0.02%).

An intriguing finding was that, until a concentration of 1000 μg mL^{-1}, almost 10 000 times higher than the lethal concentration for *A. aegypti*larvae, squamocin had no effect on *C. bigoti* and*T. theobaldi* larvae, as there was no mortality or behavioural changes in these predators. This shows that squamocin from *A. mucosa* is selective, probably owing to the specificity of their target site. This result shows that the use of this molecule is advantageous in acting on the target insect while preserving its natural enemies. In addition, squamocin has no cytotoxicity to human leukocytes, suggesting that it may be non-toxic to mammals. Generally, plant molecules have low toxicity to mammals, and some molecules at the recommended doses are non-toxic to human and other non-target organisms,[47] like the squamocin studied herein.

For squamocin from *A. mucosa* seeds, as well as other phytochemicals, toxicity may be specific for the target species. The selectivity of squamocin for non-target insects may be physiological and is directly related to the high tolerance of these insects in relation to *A. aegypti*. This selectivity may be due to reduced intake of the molecule across the insect integument or its degradation by the detoxification system of *C. bigoti, T. theobaldi* and human leukocytes. Similar results have been reported for*Annona crassiflora* extract, which is selective to the parasitoid *Trissolcus urichi* (Hymenoptera: Platygastridae), a natural enemy of the soybean stink bug *Euschistus heros* (Hemiptera: Pentatomidae) which is affected by this phytochemical.[48]

Our findings show that squamocin may be a prototype derived from natural products with the potential to control *A. aegypti* larvae, as it is toxic to the target insect and selective for non-target insects and humans, contributing to the establishment of alternative strategies for controlling insect vectors with substances of botanical origin that have low environmental impact compared with synthetic insecticides.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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CHAPTER 2

Multiple Modes of Action of the Squamocin in the Midgut Cells of Aedes aegypti Larvae

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RESEARCH ARTICLE

Multiple Modes of Action of the Squamocin in the Midgut Cells of*Aedes aegypti*Larvae

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Abstract

Annonaceous acetogenins are botanical compounds with good potential for use as insecticides. In the vector,*Aedes aegypti*(L.) (Diptera: Culicidae), squamocin (acetogenin) has been reported to be a larvicide and cytotoxic, but the modes of action of this molecule are still poorly understood. This study evaluated the changes in the cell morphology, and in the expression of genes, for autophagy (*Atg1*and*Atg8*), for membrane ion transporter *V-ATPase*, and for water channel aquaporin-4 (*Aqp4*) in the midgut of*A.aegypti*larvae exposed to squamocin from*Annona mucosa*Jacq. (Annonaceae). Squamocin showed cytotoxic action with changes in the midgut epithelium and digestive cells of*A.aegypti*larvae, increase in the expression for autophagy gene*Atg1*and*Atg8*, decrease in the expression of*V-ATPase*, decrease in the expression of*Aqp4*gene in LC_{20} and inhibition of*Apq4* genes in the midgut of this vector in LC_{50}. These multiple modes of action for squamocin are described for the first time in insects, and they are important because different sites of action of squamocin from*A.mucosa*may reduce the possibility of resistance of*A.aegypti* to this molecule.

Introduction

Some natural products from plants has been identified and isolated with insecticidal against various insects. These have been obtained from plants and have been identified and isolated (for review see Okwute) [1]. However, the focus of these studies is to find molecules with potential for the synthesis of new insecticides, which act against several molecular targets in the insects [2].

Among these natural compounds, acetogenins from Annonaceae plants are compounds with good potential for use as insecticides [3]. It is quite likely that there are other botanical compounds whose modes of action of acetogenins against insects are poorly understood. In mammals, acetogenins are potent inhibitors of mitochondrial complex I, inhibiting nicotinamide adenine dinucleotide hydride (NADH) ubiquinone oxidoreductase, an essential enzyme in the complex I [4]. Acetogenins bind on the catalytic site of ubiquinone in the complex I as well as in the microbial glucose dehydrogenase inhibiting NADH oxidase in cancer cells [4].

Because acetogenins affect cellular respiration, especially during the conversion of energy [5], they may be important components in management programs to avoid insect resistance to these molecules. Molecules with specific or multiple sites of action may be a power tool in mitigating insect resistance to insecticides. In addition, unexplored compounds may have different sites of action, may be selective in safety to humans and the environment [6]. In general, secondary plant metabolites play some role of interference in critical components of the cell signaling system, nervous system (e.g., neurotransmitter synthesis, receptors activation and signal transduction), metabolic pathways and the enzymes activities [7].

The search for alternatives to control disease vectors, especially *Aedes aegypti* (Diptera: Culicidae), the main vector for yellow fever, dengue [8], chikengunya fever [9] and zika virus [10] is urgently required. This mosquito is difficult to control because it adapts well to the environment due to its resilience and ability to overcome population disturbance caused by human interventions [11,12]. In addition, *A. aegypti* has developed rapid resistance for conventional insecticides, and to date, its resistance is reported for 35 active ingredients [13]. In Brazil, the main strategies used in the control of *A. aegypti* are based on the chemicals that act as acetylcholinesterase inhibitors, axonic nerve poisons, and insect growth regulators [14,15]. Thus, the use of alternative molecules with multiple sites of action is important to control this resistant species of mosquito.

In *A. aegypti*, different classes of acetogenins have been tested [16 18], and squamocin (acetogenin from *A. squamosa* seed) had cytotoxic and larvicidal activities against this vector [18]. However, as with many other botanical molecules, there are no data about its modes of action in this insect. In this study, we tested a squamocin isolated from *A. mucosa* seeds against the larvae of *A. aegypti*, and describe the changes in the cell morphology, and in the expression of genes involved in cell death processes and in plasma membrane transport in the midgut, thus increasing our understanding of the possible modes of action of this molecule.

Materials and Methods

Acetogenin

The squamocin was obtained from the Research Laboratory of Natural Resources (LPqRN) of the Federal University of Alagoas, Maceió, Alagoas, Brazil. Squamocin (CAS number: 120298-30-8) is a white solid wax obtained from a methanolic extraction of *Annona mucosa* seeds following by a successive partition with chloroform (85.4 g; 57.3%). This compound was pre-solubilized in 1% dimethylsulfoxide and dissolved in distilled water, resulting in a stock solution of 10 μg/mL.

Insects

Third instar larvae of *A. aegypti* previously fed with cat food (Whiskas) were obtained from mass rearing in the insectary of the Laboratory of Molecular Biology of Insects of the Federal University of Viçosa (UFV), Minas Gerais, Brazil. The insect colonies and the bioassays were performed at 25 ± 2°C, with a 12 hours photoperiod.

Preliminary tests were conducted to determine the toxicity of squamocin, and to set its lethal concentration (LC) and lethal time (LT) against third instar larvae of *A. aegypti*, and the bioassays were performed with the sublethal concentrations LC_{20} and LC_{50}, because higher concentrations these larvae have a high level of lethargy.

Midgut cytotoxicity

The *A. aegypti* larvae were exposed to the acetogenin at concentrations of 0.004μL/mL (LC_{20}) and 0.01μL/mL (LC_{50}) for 6 and 12 hours, along with control with untreated larvae at the

same times. For analysis in light microscope, ten larvae per treatment were dissected in 2% paraformaldehyde fixative solution and stored for 12 hours at 4°C. Then, the samples were dehydrated in a graded ethanol series (30 100%) and embedded in historesin (JB4 Polysciences). Slices of 5μm thickness were stained with hematoxyline and eosin and, examined and photographed in light microscope (Olympus BX60).

For ultrastructural analyses of the midgut in *A.aegypti* larvae treated with acetogenin at LC_{20} and LC_{50} for 6 and 12 hours, the organs were transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 for 2 hours. The samples were post-fixed in 1% osmium tetroxide for 2 hours at room temperature in the dark, dehydrated in a graded of ethanol series and embedded in LR White resin [19]. Ultrathin sections were stained with 2% aqueous uranyl acetate and 1% lead citrate and examined in a Zeiss EM 109 or LIBRA 120 transmission electron microscope.

RNA preparation

The midgut from 10 larvae exposed to each squamocin concentration of 0.004μL/mL (LC_{20}) and 0.01μL/mL (LC_{50}) for 0.5, 1, 2, 3 and 4 hours for *Atg1* and *Aqp4* and 5, 10, 15 and 17 hours for *Atg8* and *V-ATPase*, and the control, were dissected and transferred to RNAlater (Sigma-Aldrich). Then the samples were transferred to 500μL of Tri-reagent (Sigma), homogenized and centrifuged at 12,000 ×*g* for 10 minutes at 4°C. To the supernatant was added 100μ L of chloroform, following incubation for 10 minutes and centrifugation at 12,000 ×*g* for 15 minutes at 4°C. The aqueous phase was transferred to 250μL of 2-propanol, incubated for 10 minutes in ice and centrifuged at 12,000 ×*g* for 10 minutes. The pellet was washed twice with 500μL of 75% ethanol and centrifuged at 12,000 ×*g* for 5 minutes. The pellet was then air dried and resuspended in 20μL of ultrapure water. The amount of RNA was determined with a NanoDrop Lite Spectrophotometer (Thermo Scientific), and its sample integrity was verified by agarose gel electrophoresis in Tris/Borate/EDTA buffer.

Synthesis of cDNA

The RNA (500 ng) obtained from the midgut of *A.aegypti* larvae treated with squamocin at each concentration and time and control was transferred to 1μL of 2.5 mM dNTP mix (dATP, dGTP, dCTP and dTTP), 1μL 100μM primers oligo (dT) and ultrapure water to 10μL final volume. After mild vortexing, the samples were incubated for 3 minutes at 70°C and cooled in ice. Next, were added 4μL of buffer (500 mM Tris-HCl pH 8.3, 500 mM KCl, 30 mM $MgCl_2$, 50 mM DTT), 1μL of M-MuLV reverse transcriptase enzyme (Invitrogen) and ultrapure water to 20μL final volume. These samples were incubated for 1 hour at 37°C, following enzyme inactivation at 72°C for 15 minutes. The cDNA obtained was quantified in a spectrophotometer NanoDrop Lite.

Real Time qPCR (RT-qPCR)

The genes tested were *Atg1* and *Atg8* for autophagy, *Aqp4* for aquaporin, *V-ATPase* type for membrane ion transport, and the *rp7* Sribosomal protein as reference from their primers (Table 1) [20].

For the determination of gene expression, cDNA samples from the midgut of *A.aegypti* larvae treated at different concentrations and times of acetogenin and control were submitted to RT-qPCR (Eco Real-Time PCR System- Illumina) in quadruplicate using the quantitation fluorescence kit GoTaq®Master Mix (Promega). The final primer concentration was 0.1μM.

The relative expression of the genes was obtained using the Cycle Threshold method. The Ct values were subjected to $2^{-\Delta\Delta Ct}$ to determine the gene expression [21].

Table 1. Primers used in the bioassay of gene expression by real-time quantitative PCR.

Gene	Forward primer	Reverse primer
Atg1	5 CCTGACTGGTAAGGCACCAT 3	5 GTTGTTGCTGCTGGAGTTGA3
Atg8	5 GGAAGAACACCCATTCGAGA3	5 AGCCGATGTGGTGGAAT3
V-ATPase	5 GTTGTTCTGGCTCTGCTGTTA3	5 GAGTGTTCTCGATAAGCCATAATC 3
Aqp4	5 ATGCCACTGCTTGTCCCTAC 3	5 TTTCCGAAATGACCTTGGAG 3
rp7S	5 TCAGTGTACAAGAAGCTGACCGGA 3	5 TTCCGCGCGCGCTCACTTATTAGATT 3

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Statistical analysis

The gene expression data for *Atg1*, *Atg8*, *Aqp4* and *V-ATPase* were subjected to one-way analysis of variance, considering as factors the lethal concentration, and the exposition time followed by a post-hoc Tukey HSD test at 5% significance level.

Results

Squamocin at sublethal concentrations LC_{20} and LC_{50} showed cytotoxic effect in the midgut cells of *A. aegypti* larvae exposed for 6 and 12 hours, although these changes were not dose dependent.

In the control insects, the midgut digestive cells were columnar, with homogenous cytoplasm, median spherical nucleus with nucleoli (Fig 1A), and with a well-developed apical brush border (Fig 1B).

All larvae exposed to squamocin, independently of doses and exposure times, showed damaged digestive cells (Fig 2A), with presence of vacuoles in the apical and basal cytoplasm (Fig 2B and 2C), and disorganized brush border (Fig 2B and 2D).

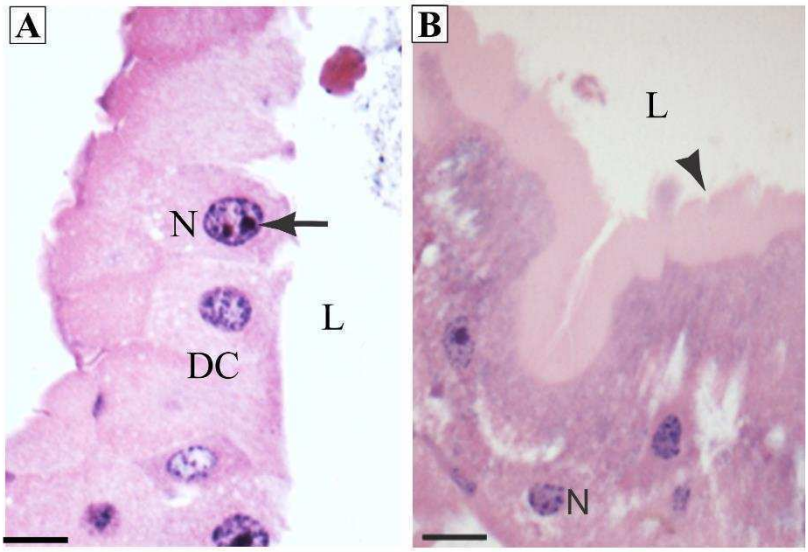

Fig 1. Photomicrographs of the midgut of *Aedes aegypti* third instar larvae (control). (A) Epithelium with a single layer of columnar digestive cells (DC), with spherical nucleus (N) containing nucleolus (arrow). (B) Epithelium showing well-developed apical brush border (arrowhead). L = lumen. Bar = 5μm.

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Ultrastructural analyses of the midgut cells in the *A. aegypti* larvae showed that control ones had digestive cells with well-developed mitochondria and microvilli (Fig 3A), whereas those

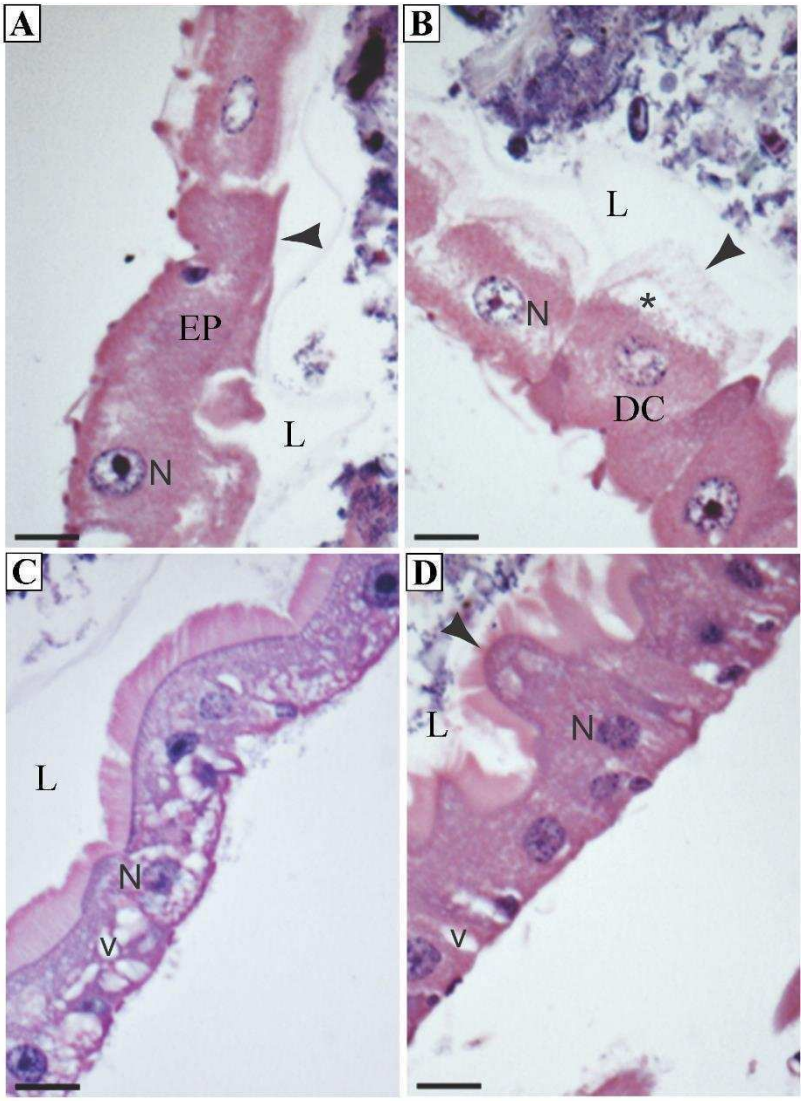

Fig 2. Photomicrographs of the midgut in *Aedes aegypti* third instar larvae exposed to sublethal doses LC_{20} and LC_{50} of squamocin. (A) Disorganized midgut epithelium (EP) (LC_{20}). (B) Digestive cells (DC) with vacuoles (*) in the apical cytoplasm and disorganized brush border (arrowhead) (LC_{50}). (C) Digestive cells with vacuoles (v) in the basal cytoplasm (LC_{20}). (D) Digestive cells with damaged striated border (arrowhead) and vacuoles (v) (LC_{20}). N = nucleus, L = lumen. Bars = 5μm.

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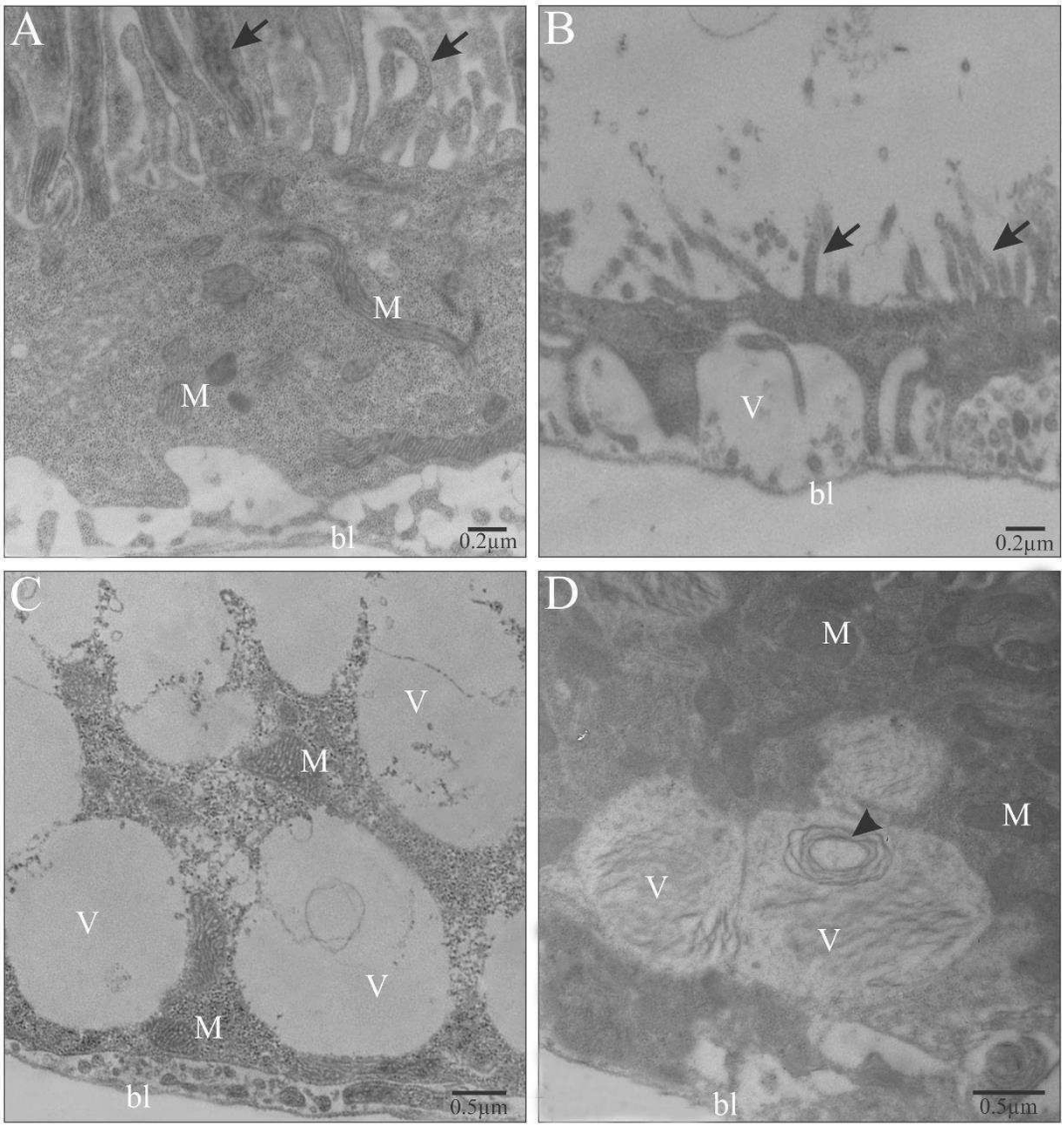

Fig 3. Transmission electron micrographs of the digestive cells in the midgut of *Aedes aegypti* third instar larvae. (A) Midgut cell with long microvilli (arrows) associated with mitochondria (M) in the control larvae. (B) Midgut cell showing disorganized microvilli (arrows) in larvae exposed to LC_{50} acetogenin. (C) Middle-basal cell region showing mitochondria (M) and large vacuoles (V) in the larvae exposed to LC_{20} acetogenin. (D) Middle-basal cell region showing presence of large vacuoles (V) with lamellar content (arrowhead) in larvae exposed to LC_{20} acetogenin. bl basal lamina.

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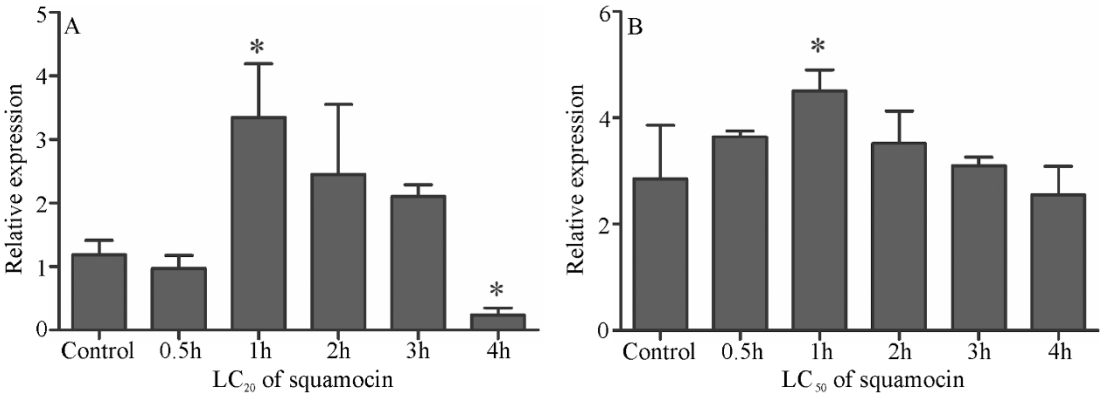

Fig 4. Relative mRNA levels of *Atg1* in the midgut of *Aedes aegypti* third instar larvae exposed to sublethal doses of squamocin and at different times. (A) Lethal concentration of 20% of population (LC_{20}). (B) Lethal concentration of 50% of population (LC_{50}). The *y-axis* indicate the relative gene expression, corresponding to the *Atg1* mRNA levels relative to ribosomal protein rp7S (reference) gene mRNA level (mean±se). *$p<0.05$.

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exposed to LC_{20} and LC_{50}, independently of exposure times, showed damages in the cell surface with microvilli loses (Fig 3B) and cytoplasm containing many large vacuoles with lamellar content (Fig 3C and 3D).

To verify whether morphological changes in the digestive cells of *A. aegypti* larvae exposed to sublethal concentrations of squamocin results from the action of this molecule in cell death processes and/or membrane transporters, some gene expressions were evaluated.

The *Atg1* expression in the midgut of *A. aegypti* larvae in LC_{20} was higher after 1 hour of exposure to squamocin when compared to the other times and the control ($F_{5,12} = 11.16$; $p = 0.0004$) (Fig 4A) in the same manner as in LC_{50} ($F_{5,12} = 4.571$; $p = 0.0145$) (Fig 4B).

The expression of *Atg8* in the midgut of *A. aegypti* larvae in LC_{20} was similar to control in all exposure times ($F_{4,15} = 0.7548$, $p = 0.5704$) (Fig 5A). In the LC_{50}, after 15 hours of exposure to squamocin there was higher *Atg8* expression in comparison with other periods and control ($F_{4,15} = 3.900$; $p = 0.0230$) (Fig 5B).

The expression of the gene for *V-ATPase* in the midgut of *A. aegypti* larvae in the LC_{20} was lower than in control at all periods tested ($F_{4,15} = 7.581$, $p = 0.0015$) (Fig 6A). Larvae treated with LC_{50} for 17 hours had lower expression than other animals ($F_{4,15} = 3.736$, $p = 0.0266$) (Fig 6B).

The expression of aquaporin gene *Aqp4* decreased when larvae were treated with LC_{20} squamocin in all periods in comparison with control ($F_{5,12} = 6.204$; $p = 0.0046$) (Fig 7). However, when subjected to LC_{50} expression was inhibited in the midgut of *A. aegypti* third instar larvae

Discussion

Our results show that squamocin from *A. mucosa* seed affect cell morphology and physiology of *A. aegypti* third instar larvae in sublethal doses, and these effects may negatively affect the fitness of this vector. In the midgut digestive cells, squamocin causes loss of microvilli and intense cytoplasm vacuolization with lamellar bodies. Similar to that found for squamocin, other chemical compounds have been reported to have toxic effects against larvae of *A. aegypti* and other mosquitoes, with damages in the gut cells, mitochondrial cristae, brush border

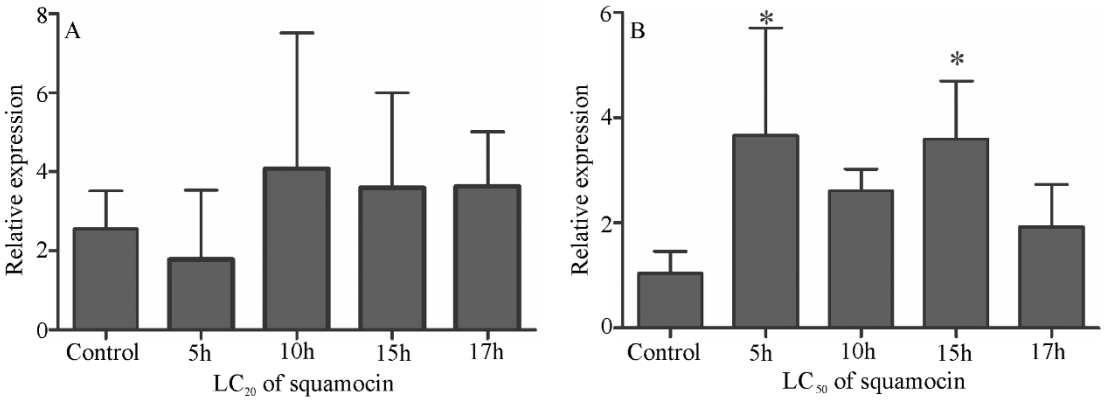

Fig 5. Relative mRNA levels of *Atg8* in the midgut of *Aedes aegypti* third instar larvae exposed to sublethal doses (LC_{20} and LC_{50}) of squamocin and control at different times. (A) Lethal concentration of 20% of population (LC_{20}). (B) Lethal concentration of 50% of population (LC_{50}). The y axis indicates the relative gene expression corresponding to the *Atg8* mRNA levels relative to ribosomal protein rp7S (reference) gene mRNA level (mean±se).* $p<0.05$.

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alterations, increase in autophagic vacuoles, membrane cell rupture, cell detachment from basal membrane, and presence of large cytoplasm vacuoles [22 27].

The presence of many vacuoles with lamellar content in the digestive cells of *A. aegypti* larvae treated with acetogenin, suggests a possible occurrence of cell death [25,28]. Although the presence of vacuoles with lamellar content occurs in the midgut cells of some insects [26,27], the intense cytoplasm vacuolization has been characterized as cell death process by autophagy [29 31]. Cell death by autophagy in the digestive cells of *A. egypti* larvae after exposure to squamocin is supported by the increase in the *Atg1* and *Atg8* expression in these insects. There was a higher expression of *Atg1* after 1 hour of exposure to squamocin in both LC_{20} as the LC_{50}, suggesting that after this short period, cells undergo autophagy. *Atg1* is a gene regulatory of autophagy induction and its is sufficient to induce hight leves of autophagy [32]. The putative complex ATG1 (serine/threonine kinase) is a protein involved in the autophagy induction [28], and its activation promotes the recruitment of other ATG proteins forming preautophagosomes [33]. The protein, ATG8, is an ubiquitin involved in the expansion of autophagosome vesicle used as a molecular marker for the autophagy [29], and the increase of *Atg8* mRNA level in the midgut of *A. aegypti* larvae exposed to LC_{50} squamocin indicates the occurrence of autophagy in these cells.

Neither cytotoxic effect nor changes in the *Atg8* expression are dose dependent in the midgut of *A. aegypti* larvae. The main function of ATG8 in the autophagy is to bind regulatory and/or autophagy-regulated proteins and guide them to their site of action in the autophagosome membrane[33,34]. This process may occur in *A. aegypti* larvae in the first 5 hours of squamocin exposure, or, in this period, may occur in the formation of larger autophagosome, because the amount of ATG8 determines the size of an autophagosome vacuole [35]. ATG8 is the unique structural protein known to remain in autophagosome membrane after its formation [29,35], which may explain the expression of this gene in the digestive cells of *A. aegypti* larvae even 17 hours after exposure to squamocin. Anyway, the presence of ATG8 occurs only after ATG1 synthesis[34 38]. Kamada *et al*. [36] supports that in the midgut digestive cells of *A. aegypti* larvae, squamocin promotes an increase in autophagy rate.

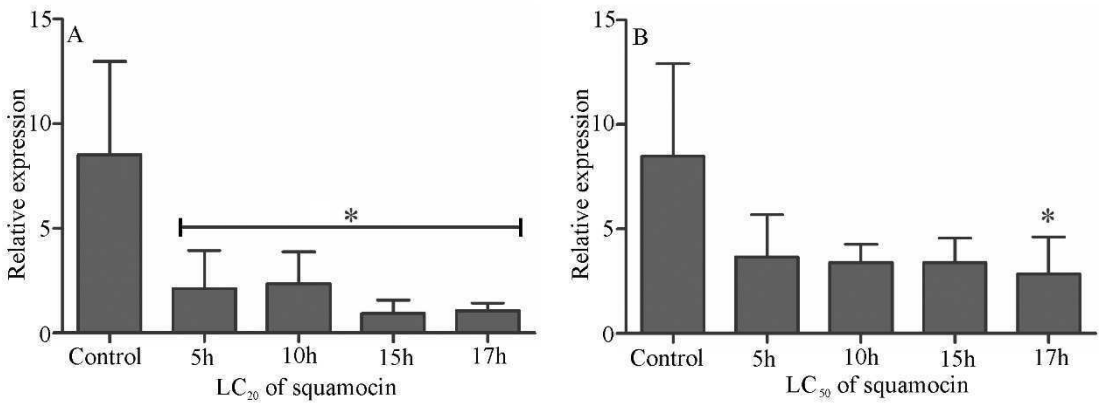

Fig 6. Relative mRNA levels of *V-ATPase* in the midgut of *Aedes aegypti* third instar larvae exposed to sublethal doses (LC_{20} and LC_{50}) of squamocin and control at different times. (A) Lethal concentration of 20% of population (LC_{20}). (B) Lethal concentration of 50% of population (LC_{50}). The *y* axis indicates the relative gene expression, corresponding to the *V-ATPase* mRNA levels relative to ribosomal protein rp7S (reference) gene mRNA level (mean±se). *$p<0.05$.

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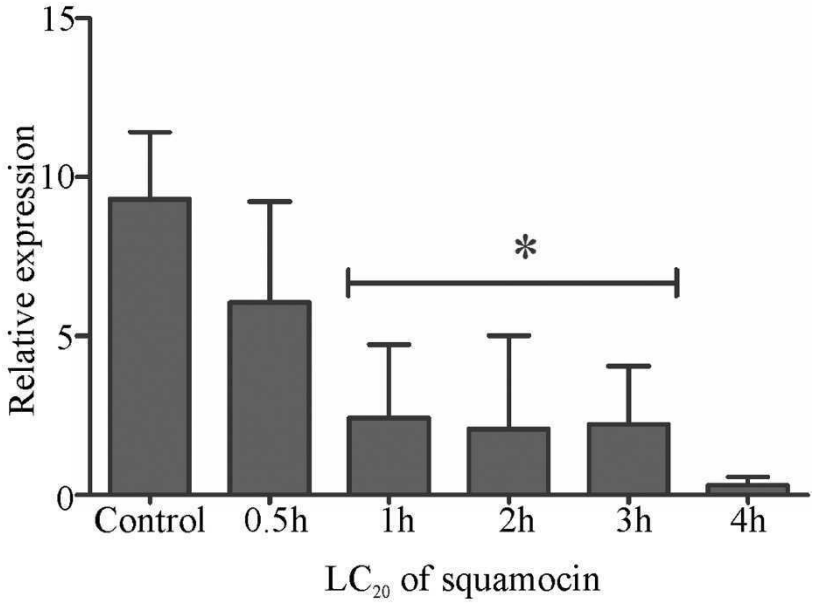

Fig 7. Relative mRNA levels of *Aqp4* in the midgut of *Aedes aegypti* third instar larvae exposed to sublethal dose (LC_{20}) of squamocin and control at different times. The *y-axis* indicates the relative gene expression corresponding to the *Aqp4* mRNA levels relative to ribosomal protein rp7S (reference) gene mRNA level (mean±se). *$p<0.05$.

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Changes in the brush border, and the vacuolization of apical cytoplasm of digestive cells in *A.aegypti* larvae exposed to sublethal doses of squamocin, may be due to changes in the water balance, as suggested for some neurotoxic insecticides in nontarget organs such as the gut [26,39 41]. The squamocin decreases the expression of *V-ATPase* gene in the midgut of *A. aegypti* larvae compared with the control. ATPases are cell membrane enzymes, especially in the gut epithelium, which mediate absorption and transport of metabolites and nutrients, as well as ions and nonelectrolytes [42]. In *A.aegypti* larvae, the V-ATPase is located in the basolateral membrane of the anterior midgut and in the apical cell membrane of the posterior midgut region [43,44]. Thus, the decrease in the expression of *V-ATPase* may be also associated with autophagy in the midgut of larvae studied here. The relationship between autophagy and *V-ATPase* inhibition observed in *A.aegypti* larvae exposed to squamocin has been found in cancer cells exposed to the V-ATPase inhibitor archazolid [45].

Another effect of squamocin in the midgut in *A.aegypti* larvae is in the expression of aquaporin gene *Aqp4*. When treated with a low squamocin concentration (LC_{20}) of the *Aqp4* expression decreased in the first hour of treatment, whereas after high concentration (LC_{50}) the gene is inhibited. AQP4 is highly produced in the midgut of *A.aegypti*, and it is an aquaglyceroporin of the aquaporin superfamily [46] playing an important role in water transport and in the absorption of polyols, urea and trehalose, which shows that AQP4 is a multifunctional membrane transporter [47]. Together, decrease and inhibition of *Aqp4* and decrease of *V-ATPase* expression in the midgut of *A.aegypti* larvae exposed to squamocin, suggest that this molecule may affect water and ion transport, which are important for the maintenance of cell osmotic balance, resulting in digestive cell injuries. The block of substances transported across the midgut plasma membrane may activate autophagy in these cells, which, despite its essential role for the maintenance of cell homeostasis, when activated in excess, may destroy cellular proteins and organelles, resulting in cell collapse [48]. Our findings show that squamocin increases the cytoplasm vacuolization in the digestive cells probably due to the action of *Atg8* that is increased and triggered by inhibition of *V-ATPase* and *Aqp4* genes responsible for coding two important membrane carriers.

Our study shows that squamocin in sublethal doses causes severe reactions in the midgut digestive cells of *A.aegypti* larvae, linked with dowregulation of *V-ATPase* and *Aqp4* genes for osmoregulatory proteins resulting in cytological abnormalities and cell death by autophagy. Thus, the squamocin from *A.mucosa* herein studied in sublethal doses affects physiological processes essential to the insects development. Generally, sublethal residues of insecticides cause effects on insect development, because for its survival it is essential that many physiological events are accurately coordinated [49].

This is the first report that an acetogenin induces autophagy, decreases *V-ATPase*, and inhibits *Aqp4* genes expression in insects. Overall, this finding is important because squamocin has multiple modes of action in the midgut of an insect larva, which may reduce the possibility of resistance of *A.aegypti* larvae exposed to this molecule.

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Conceptualization: MSC JES.

Formal analysis: MSC AEGS JES.

Funding acquisition: JES.

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CHAPTER 3

Modes of action of squamocin in the anal papillae of Aedes aegypti larvae

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Abstract

Squamocin is an annonaceous plant acetogenin with potential for use in the control of the dengue vector Aedes aegypti, although the target organs of this molecule are still poorly understood. This study evaluated the action of a squamocin from Annona mucosa (Annonaceae) seeds on anal papillae, organs that play roles in the osmo and ion-regulation of A. aegypti larvae. The morphological and the primary transcript levels of the genes for aquaporin AQP4 (AaAQP4) and the membrane transporter V-type-H^+-ATPase (AaV-H^+-ATPase) in the anal papillae of A. aegypti larvae exposed to squamocin were evaluated. Squamocin caused great vacuolization in the anal papillae wall, basal lamina disruption with loss of canalicular spaces, and disorganization of the cuticular layers. There was also decrease of AaAQP4 transcript levels and inhibition of AaV-H^+-ATPase in the anal papillae of larvae exposed to squamocin in comparison with control ones. Morphological together with decrease in the AaAQP4 and AaV-H+-ATPase primary transcript levels suggest that squamocin may affect osmoregulation and ion-regulation of this insect followed by death. This study shows that the anal papilla of A. aegypti is a target organ for squamocin, contributing to future studies of the mechanism of action of this promising insecticide molecule.

Key-words: Acetogenin, aquaporin, V-type-H^+-ATPase, membrane transport, yellow fever.

Key message

- There is an urgent need to develop alternative methods of chemical control of Aedes aegypti, an insect vector of many diseases.
- Squamocin affects the morphology and primary transcript levels of genes for proteins that mediate the water and ions flow in anal papillae of A. aegypti larvae affecting the homeostasis in these insects
- Squamocin is a bioactive molecule that could be used to develop sustainable control for this insect vector.

Introduction

Squamocin is an annonaceous acetogenin found in plants of Annona squamosa L. (Khalequzzaman and Sultana 2006; Costa et al. 2014), Annona cherimolia Mill. (Álvarez-Colom et al. 2006; Álvarez-Colom et al. 2008; Tolosa et al. 2014), Annona muricata L. (Guadaño et al. 2000) and Annona cornifolia A. St.-H (Lima et al. 2010). Acetogenins have potential as natural organic pesticides (McLaughlin et al. 1988) and in insects the action of these molecules occurs in the mitochondrial electron transport systems (Zafra-Polo et al. 1998). Some acetogenins such as bullatacin and trilobacin are more powerful than rotenone, a classic inhibitor of mitochondrial complex I (He et al. 1997).

Insecticide action of squamocin has been reported against Spodoptera frugiperda (J.E. Smith) (Lepidoptera) (Álvarez-Colom et al. 2006; Tolosa et al. 2014), Myzus persicae (Sulzer) (Hemiptera), Leptinotarsa decemlineata (Say) (Hemiptera) (Guadaño et al. 2000) and Tribolium castaneum (H.) (Coleoptera) (Khalequzzaman and Sultana 2006). In the vector mosquito Aedes aegypti L. (Diptera: Culicidae) this molecule showed larvicide action (Costa et al. 2014, 2016) with possible effect on midgut cell disorders causing the insect death (Costa et al. 2014). However, there are no data on the effects of squamocin in other organs of insects.

Among the target organs for squamocin, anal papillae of A. aegypti are important, because changes in this organ affect the osmotic regulation and ion concentration in the haemolymph that can cause larval death (Insunet al. 1999; Kumar et al. 2010).

Mosquito larvae have four anal papillae extending from the terminal abdominal segment (Edwards and Harrison 1983; Becker et al. 2010). The anal papillae wall has an epithelial syncytium of cells and the lumen is continuous with the hemocoel (Wigglesworth 1933a, b; Donini et al. 2007).

Anal papillae on mosquito larvae together with hindgut and Malpighian tubules play important roles in osmoregulation and ion-regulation controlling the levels of ions and water in the body fluids (Bradley, 1987, Becker et al. 2010). Anal papillae are associated with the absorption of environmental ions, being the primary site of Na^+, Cl^- and K^+ intake (Wigglesworth 1938; Treherne 1954; Stobbart 1960; Del Duca et al. 2011; Chasiotis et al. 2016) and H^+ and $NH4^+$ excretion mediate by membrane V-type-H^+-ATPase transporter (Donini and O'Donnell 2005). Associated with ion transport, in the anal papillae occurs intensive water movement by the presence of aquaporin, especially aquaporin-4 (AQP4) (Marusalin et al. 2012).

The ability to successfully osmoregulate is an important physiological mechanism for survival of mosquito larvae and disrupting the osmoregulatory capacity may serve as a potential strategy for population control, since this organ may be a target for insecticides. This study verified the hypothesis that squamocin affects the morphology and the primary transcript levels of genes for proteins that mediate the water and ions flow in the anal papillae of A. aegypti larvae affecting the homeostasis in these insects. This study evaluated the effects of a squamocin isolate from Annona mucosa (Annonaceae) seeds in the morphology and primary transcript levels of the genes for aquaporin-4 (AaAQP4) and the membrane transporter V-type-H^+-ATPase (AaV-H^+-ATPase) in the anal papillae of A. aegypti larvae, contributing to the comprehension of the modes of action of squamocin on this vector.

Materials and Methods

Acetogenin

The squamocin (CAS number: 120298-30-8) is a white solid wax that was obtained from methanolic extraction of Annona mucosa seeds followed by successive partition with chloroform and identified by NMR in Research Laboratory of Natural Resources (LPqRN) of the Federal University of Alagoas, Maceió, Alagoas, Brazil (Costa et al. 2016). This compound was pre-solubilized in 1% dimethylsulfoxide and dissolved in distilled water, resulting in a 10 µg/mL stock solution.

Experimental animals

Third instar larvae of A. aegypti insecticide susceptible stream were obtained from mass rearing in the insectary of the Laboratory of Molecular Biology of Insects of the Federal University of Viçosa (UFV), Minas Gerais, Brazil. The insect colonies and the bioassays were performed at 25 ± 2 °C, with 12:12h (light:dark) photoperiod. The bioassays were performed with the sublethal concentrations LC_{20} and LC_{50} of squamocin (Costa et al. 2016).

Cytotoxicity

The A. aegypti larvae were exposed to the acetogenin at concentrations of 0.004 µL/mL (LC_{20}) and 0.01 µL/mL (LC_{50}) for 5, 10, 15 and 17 h, and untreated larvae as control at the same

periods. For analysis in light microscope, 10 larvae survivors after each treatment (LC_{20} or LC_{50}) were dissected in saline solution for insects (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4) pH 7.3, and the anal papillae transferred to 2% paraformaldehyde fixative solution for 12 h. Then, the samples were dehydrated in a graded ethanol series (30-99%) and embedded in historesin JB4 (Leica Biosystems Nussloch, Heidelberger, Germany). Slices of 3 µm thickness were stained with hematoxyline and eosin and examined under a light microscope (OlympusBX60, Tokyo, Japan).

For ultrastructural analyses of the anal papillae of A. aegypti larvae, 10 treated with squamocin at LC_{20} and LC_{50} for 6 and 12 h and controls, were dissected and transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 4 h. The samples were post-fixed in 1% osmium tetroxide for 2 hours at room temperature in the dark, dehydrated in a graded of ethanol series and embedded in LR White resin. Ultrathin sections were stained with 2% aqueous uranyl acetate and 1% lead citrate and examined in a Zeiss EM 109 or LIBRA 120 transmission electron microscope.

Tissue collection for RT-PCR

The anal papillae from 10 larvae (survivors) exposed to each squamocin concentration of 0.004 µL/mL (LC_{20}) and 0.01 µL/mL (LC_{50}) for 5, 10, 15 and 17 h, and the control, were dissected and transferred to RNA-Later. Then the samples were transferred to 500 µL of Tri-reagent (Sigma), homogenized and centrifuged at 12.000 × g for 10 min at 4 °C. To the supernatant was added 100 µL of chloroform, following incubation for 10 minutes and centrifugation at 12.000 × g for 15 min at 4 °C. The aqueous phase was transferred to 250 µL of 2-propanol, incubated for 10 minutes in ice and centrifuged at 12,000 × g for 10 min. The pellet was washed twice with 500 µL of 75% ethanol and centrifuged at 12,000 × g for 5 min. The pellet was then air dried and resuspended in 20 µL of ultrapure water. The amount of RNA was determined with a NanoDrop™ 2000 (Thermo Scientific, Wilmington, Delaware USA), and its sample integrity was verified by agarose gel electrophoresis in Tris/Borate/EDTA buffer.

Synthesis of cDNA for RT-PCR

The RNA (500 ng) obtained from the anal papillae of A. aegypti larvae treated with squamocin at each concentration and time and control was transferred to 1 µL of 2.5 mM dNTP mix (dATP, dGTP, dCTP and dTTP), 1 µL 100 µM primers oligo (dT) and ultrapure water to 10 µL final

volume. After mild vortexing, the samples were incubated for 3 min at 70 °C and cooled in ice. Next, were added 4 µL of buffer (500 mM Tris-HCl pH 8.3, 500 mM KCl, 30 mM $MgCl_2$, 50 mM DTT), 1 µL of M-MuLV reverse transcriptase enzyme (Promega, Madison-USA) and ultrapure water to 20 µL final volume. These samples were incubated for 1 h at 37 °C, following enzyme inactivation at 72 °C for 15 min. The cDNA obtained was quantified in a NanoDrop™ 2000 (Thermo Scientific, Wilmington, Delaware USA).

RT-qPCR

The genes tested were AaAQP-4 for aquaporin (XM_001647996), AaV-H^+-ATPase for membrane ion transport (AF092934), and Aarps7 for rp7S ribosomal protein as reference from their primers (Table 1) (Bryant and Raikhel 2011). The cDNA samples from the anal papillae of A. aegypti larvae treated at different concentrations (LC_{20} and LC_{50}) and times (5, 10, 15 and 17 h) of acetogenin and control (without squamocin) was added forward and reverse primers to a final concentration of 1.0 mM and then were submitted to Real-Time PCR System (Illumina Eco™Uniscience). Each cDNA sample was analyzed 20 µL reactions assembled in quadruplicate using GoTaq qPCR Master Mix (Promega, USA). The PCR reaction involved initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 2 min. The relative expression of the genes was obtained using the Cycle Threshold method. The Ct values were subjected to $2^{-\Delta\Delta Ct}$ to determine the transcript levels of gene (Ginzinger 2002).

Table 1. Primers used in the bioassay of gene expression by real-time quantitative PCR

Gene	Forward primer	Reverse primer
AaV-ATPase	5'GTTGTTCTGGCTCTGCTGTTA3'	5'GAGTGTTCTCGATAAGCCATAATC 3'
AaAqp4	5'ATGCCACTGCTTGTCCCTAC 3'	5'TTTCCGAAATGACCTTGGAG 3'
Aarp7S	5'TCAGTGTACAAGAAGCTGACCGGA 3'	5'TTCCGCGCGCGCTCACTTATTAGATT 3'

Statistics

The transcript levels of gene data for AaAQP-4 and AaV-H^+-ATPase were submitted to one-way analysis of variance, considering as factors the lethal concentration, and the exposition time

followed by a post-hoc Tukey HSD at 5% significance level, using the R software version. 3.1.1 (R Development Core Team 2014).

Results

The anal papillae of third instar larvae of A. aegypti had a thin cuticle covering a thick syncytial epidermis in both control and squamocin treated larvae (Fig 1).

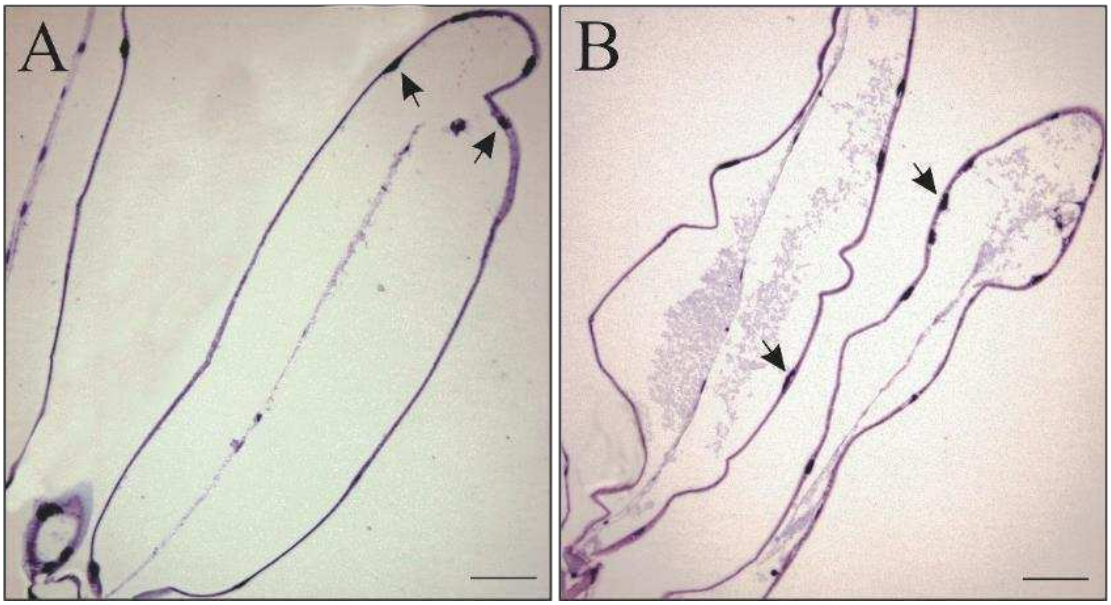

Fig. 1 Photomicrograph of the anal papillae of third instar of Aedes aegypti larvae, with a single layer of epithelial cells into characteristic syncytia (arrows). A: control group; B: larvae treated with squamocin.

At ultrastructural level the anal papillae syncytium showed the basal plasma membrane with infoldings forming large extracellular canals (canalicular spaces) reaching to the median region of the wall (Fig 2a, 2b). The apical region had some short plasma membrane infoldings, but with narrow intercellular space (Fig 2b). The syncytium was rich in well-developed mitochondria and free ribosomes (Fig 2b, 2c). The syncytium was seated on a thin basal lamina and covered by a multi-layered cuticle with a thin epicuticle (Fig 2b).

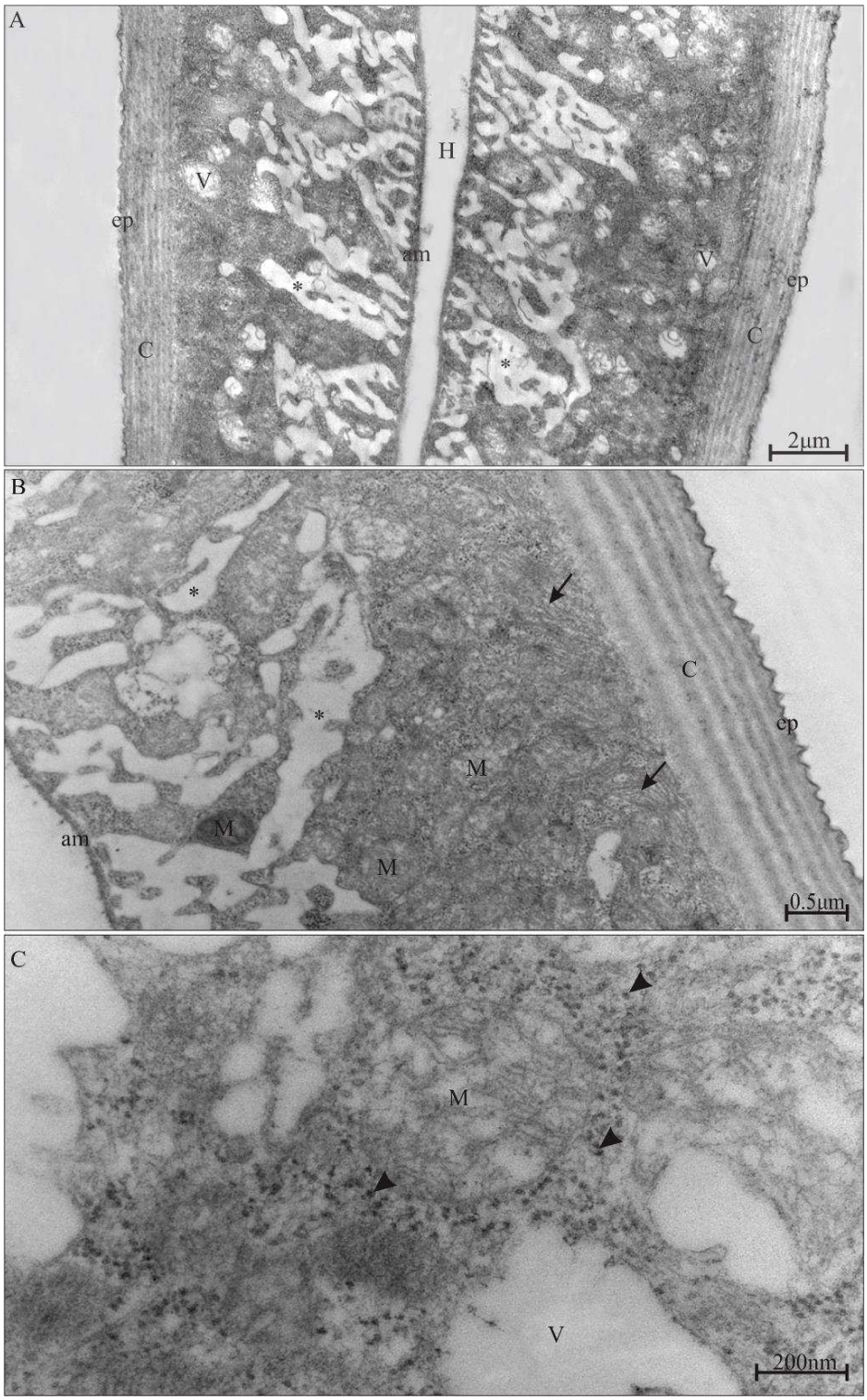

Fig. 2 Photomicrographs of the anal papillae Aedes aegypti third instar larvae control. A: Anal papilla with cuticular layer (C) well-defined, visible canalicular spaces (*) and preserved apical membrane (am). B. Apical region of anal papillae, to note short narrow intercellular space (arrows). C. Syncytium rich in well-developed mitochondria (M) and free ribosomes (arrowhead). H= hemolymph, C=cuticule, ep=epicuticle, V=vacuoles.

In the larvae treated with squamocin, in both concentrations and times, the anal papillae showed a strong disorganization of the syncytium, characterized by many large vacuoles, loss of mitochondria and free ribosomes, disappearance of the basal lamina and canalicular space and disorganization of the cuticle layers (Fig 3a, 3b).

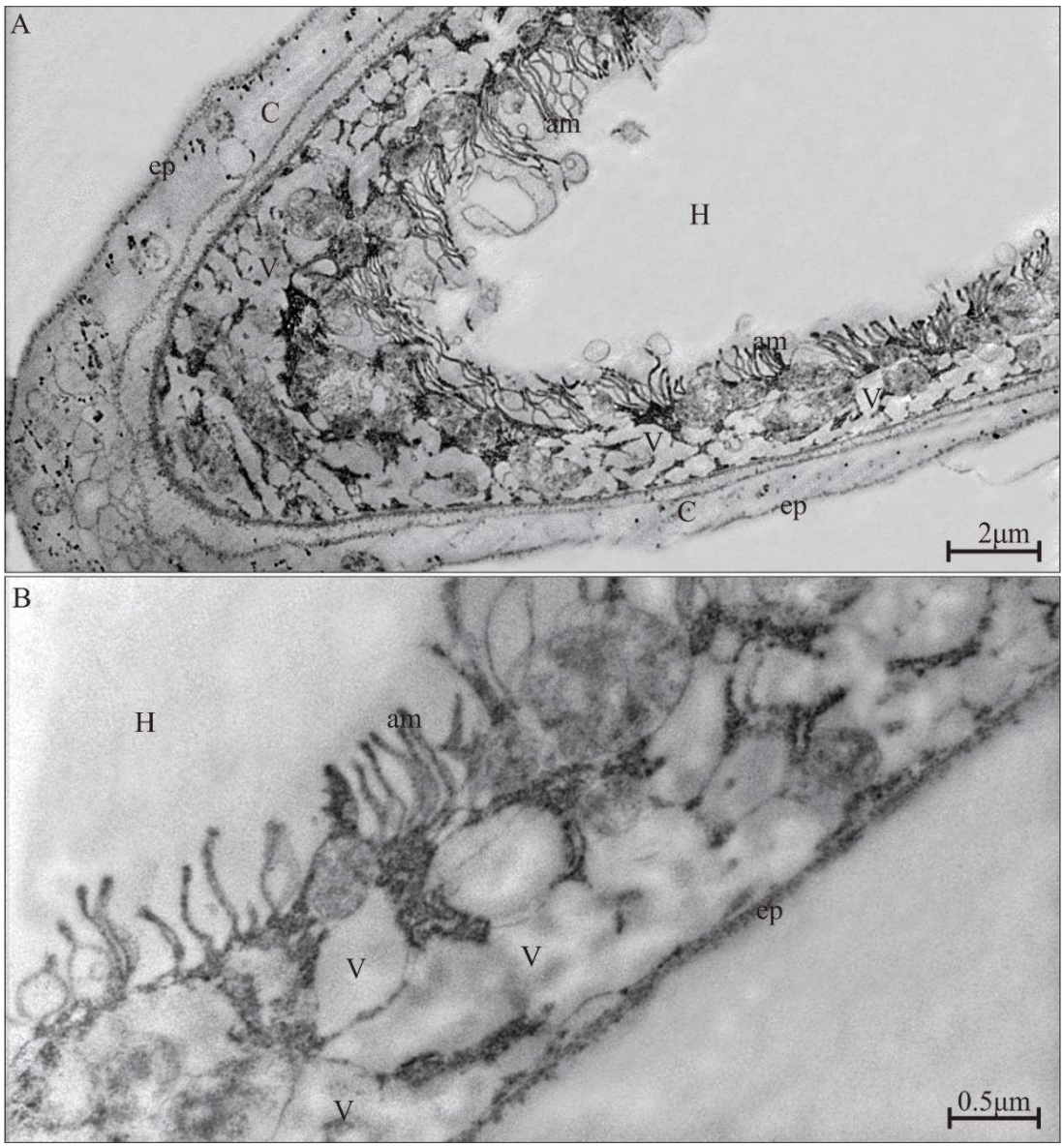

Fig. 3 Photomicrographs of the anal papillae Aedes aegypti third instar larvae exposed to sublethal doses LC_{20} and LC_{50} of squamocin A: Anal papilla treated with squamocin (LC_{50}) with an increase in vacuolization (V) and disruption of the apical membrane (am). B. Anal papilla treated with squamocin (LC_{50}) with in detail to the absence of cuticular layer. H= hemolymph, C=cuticule, ep=epicuticle.

To verify whether when exposed to sub-lethal concentrations of squamocin the osmoregulation of anal papillae was affected, the primary transcript levels for aquaporin-4 and membrane transporter V-type-H^+-ATPase were evaluated. The level of primary transcript for aquaporin in the anal papillae of larvae exposed to squamocin LC_{20} was similar to control (F= 2.166; df=4,19; p=0.1227) (Fig 4a). However at LC_{50} there was decrease in the primary transcript level for aquaporin in all periods evaluated when compared with control (F = 12.4; df =4,19; p <0.0001) (Fig 4b). The primary transcript level for AaV-H^+-ATPase was completely inhibited in the anal papillae of A. Aegypti larvae exposed to both squamocin concentrations with expression only in the anal papillae of control insects (F= 74.88; df = 1,44; p <0.001).

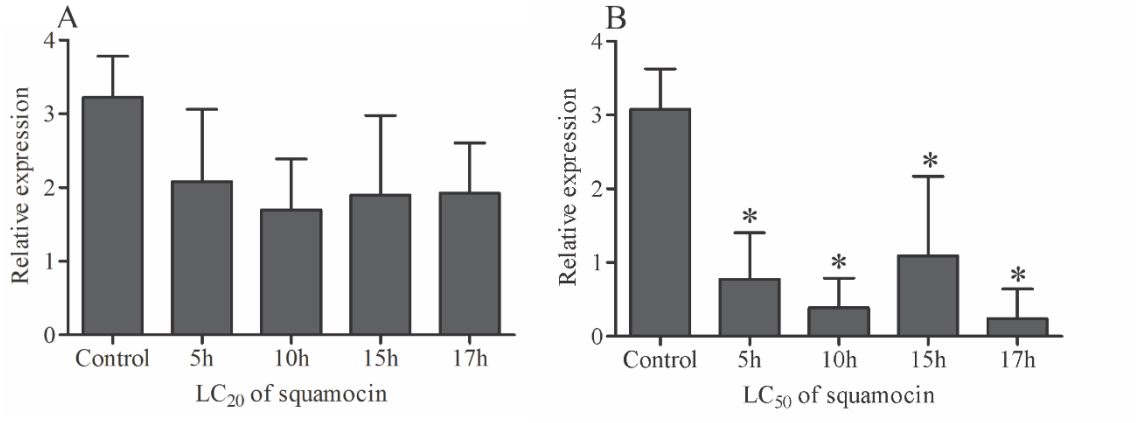

Fig. 4 Relative mRNA levels of AaAQP4 in the anal papillae of A. aegypti third instar larvae exposed to sublethal doses (LC_{20} and LC_{50}) of squamocin and at different times. The y axis indicate the relative gene expression, corresponding to the AaAQP4 mRNA levels relative to ribosomal protein Aarp7S (reference) gene mRNA level (mean ± SE). *p< 0.001.

Discussion

Squamocin cause structural damages in the anal papilla of A. aegypti larvae and decrease the primary transcript level of homeostasis associated genes AaAQP-4 and AaV-H^+-ATPase. The anal papillae of A. aegypti larvae regulates the electrolytes balance necessary for the vital functions (Becker et al. 2010) and changes in this organ, here reported, may be responsible by larval mortality.

Although at light microscopy the anal papillae is similar in treated and control A. aegypti larvae, at ultrastructural level, larvae exposed to squamocin show strong damages in the anal papillae wall, such as increased vacuolization, disorganization of basal lamina and the basal plasma membrane infoldings, loss of mitochondria and disorganization of cuticle layers. The cellular vacuolization

caused by squamocin has been reported in the midgut cells of A. aegypti as an evidence of cell death (Costa et al. 2014), which may be occurring in the anal papillae syncytium. Changes in the cuticle caused by squamocin in this study has been reported for other plant compounds such as Argemone mexicana extract (Warikoo and Kumar 2013), pepper-extracts (Chaithong et al. 2006; Kumar et al. 2010; Warikoo and Kumar 2013) and pellitorine (Perumalsamy et al. 2013). The cuticle changes probably causes dysfunction in the anal papillae of A. aegypti larvae as reported for pepper-extracts in the anal papillae of this insect (Chaithong et al. 2006).

In addition to morphological changes, squamocin decrease the levels of primary transcripts for aquaporin-4 (AaAQP-4) in the anal papillae of A. aegypti larvae exposed to LC_{50} squamocin, which may results in decrease of water and other compounds absorption, since this gene encode an aquaglyceroporin protein in A. aegypti (Finn et al. 2015), which play roles in transport of water, glycerol and trehalose in the gut and Malpighian tubules of adult females of this vector (Drake et al. 2015). Inhibition in the transcription of AaAQP-4 in anal papillae has been reported in A. aegypti larvae exposed to isobutylamide alkaloid pellitorine from Asarum heterotropoides (Aristolochiaceae) (Perumalsamy et al. 2013). Aquaporin-4 is highly expressed in the anal papillae of A. aegypti larvae (Marusalin et al. 2012) and how water transport in these organs is bidirectional and transcellular (Wigglesworth 1933b), this protein mediates the water transport across the anal papillae epithelium (Marusalin et al. 2012), although other compounds such as glycerol may also be transported (Drake et al. 2015). The habits and environmental features of the insects determine their water necessities and type of osmotic stress (Noble-Nesbiti 1990; Song and Brown 1998). Aedes aegypti larvae deal with water excess and achieve osmotic equilibrium by a controlled combination of water and salts and any disturb in this combination cause water gain and loss of vital salts for the insect (Wigglesworth 1933a, b; Noble-Nesbiti 1990; Clark and Bradley 1997). Furthermore, the decline in transcription of AaAQP-4 may disturb the co-transport of Na^+, K^+ and Cl^- which is triggered by an electrochemical gradient established by V-type-H^+-ATPase in the apical plasma membrane of the anal papillae (Patrick et al. 2006), an active membrane transporter, allowing the passive water follow by aquaporins (Gomes et al. 2009).

The transcription of AaV-H^+-ATPase in the anal papillae of A. aegypti larvae is inhibited by squamocin at LC_{20} and LC_{50}, which may contribute to the dysfunction of co-transport system in the anal papillae, resulting in osmotic imbalance. This imbalance is dangerous to the larvae survival since

the ATPase enzyme provides the energy necessary for active transport of ions involved in maintaining the ionic balance and osmoregulation, critical for the homeostasis (Clark and Matsumura 1982).

Squamocin is able to reduce aquaporin and inhibit membrane ion transporter genes in the anal papillae of A. aegypti larvae, which may affect insect osmoregulation that is dependent of ions and water transport with V-type-H^+-ATPase the main driving force for transport of ions in the anal papillae plasma membrane (Patrick et al. 2006).

This is the first report of squamocin effect on anal papillae of mosquito. The cytotoxicity of acetogenins has been reported to inhibit the mitochondrial respiration via complex I NADH: ubiquinone oxidorredutase, blocking mitochondrial oxidative phosphorylation resulting in cell apoptosis (McLaughlin 2008; Chen et al. 2011). However, in A. aegypti larvae, squamocin seems have other targets in the midgut digestive cells causing cell death by autophagy through of several routes as increase in the expression of autophagic genes Atg1 and Atg8, decrease in the expression of V-ATPase and aquaporin-4 genes (Costa et al. 2016). Thus we suggest that changes caused by the squamocin in the anal papillae of A. aegypti larvae, may be others multiple modes of action of acetogenins that limit the survival of this insect vector.

The anal papillae have an apparent trade-off for mosquito larvae, because the apparent disadvantage is due to the high water permeability to the body (Wigglesworth 1933), but the ions influx is essential for the insect survival (Marusalin et al. 2012). Thus, decrease in the aquaporin (AaAQP4) and membrane transporter (AaV-H^+-ATPase) transcription together with ultrastructural damages caused by squamocin in the anal papillae of A. aegypti larvae, shows that this organ is a promising target for this molecule. Thus this study presents new insights for future studies about the modes of action mechanism of this potential insecticide molecule.

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Statement of human rights

For this type of study formal consent is not required.

Statement on the welfare of animals

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

The authors declare that they have no conflict of interest.

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CONSIDERAÇÕES FINAIS

Com o uso de técnicas de cromatografia, espectrometria no ultravioleta, espectrometria no infravermelho e espectrometria de ressonância magnética nuclear de hidrogênio e carbono é possível determinar a estrutura química da esquamocina, presentes nas sementes de A. mucosa. Esta acetogenina ($C_{37}H_{66}O_7$) é classificada como bis-THF adjacente, que de acordo com a relação estrutura e atividade são as acetogeninas mais potentes citotoxicamente.

Esquamocina é altamente tóxica para larvas de A. aegypti em baixas concentrações tornando viável o seu estudo como protótipo de inseticida, uma vez que, não causa mortalidade de seus predadores C. bigoti e T. theobaldi (insetos não-alvos) e tampouco citotoxicidade aos leucócitos humanos. Todavia, sobre no intestino médio das larvas, age independentemente da concentração e tempo de exposição, provocando alterações ultraestruturais tais como desorganização celular, mudança estrutural dos microvilos e vacuolização citoplasmática com presença de conteúdo lamelar, característico de morte por autofagia.

A morte por autofagia provocada é pela esquamocina comprovada, também, pelo aumento nos níveis de transcrição de mRNA para os genes autofágicos Atg1 (indução) após 1 hora de exposição e de Atg8 (expansão autofagossomo) após 5 horas, redução nos níveis de transcrição para o gene V-ATPase que mediam a absorção e o transporte de metabolitos e nutrientes através da membrana, redução nos níveis de transcritos para a aquaporina (Aqp4) em baixa concentração (CL_{20}) e total inibição da expressão na maior concentrações (CL_{50}) influenciando diretamente no transporte de água e íons importantes para a manutenção do equilíbrio osmótico da célula resultando em injuria celular.

A ação de esquamocina pode ser vista também nas papilas anais, órgão que desempenham um papel importante na osmo e ionorregulação das larvas. Ultraestruturalmente, neste órgão, esquamocina provoca intensa vacuolização, supressão dos espaços canaliculares, ruptura da membrana apical e remoção da camada cuticular. A redução dos níveis de transcrição de mRNA para o gene AaAQP-4 ocorre apenas na CL_{50} independentemente do tempo e houve a total inibição do gene AaV-H^+-ATPase em ambas as concentrações.

Em resumo, esquamocina é um larvicida alvo específico com múltiplos modos de ação, e baseado nos promissores resultados observados, é viável sua inserção nos programas de controle de vetores.