Research Article

SCI

Received: 16 November 2015 Revised: 22 June 2016 Accepted article published: 1 July 2016 Published online in Wiley Online Library: 4 August 2016

(wileyonlinelibrary.com) DOI 10.1002/ps.4350

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 ($LC_{50} = 0.01$ μg mL^{-1} and $LC_{90} = 0.11$ μg 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.
© 2016 Society of Chemical Industry

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*.

* Correspondence to: JE Serrão, Laboratory of Ultrastructural Cell, Department of General Biology, Federal University of Viçosa, Viçosa 36570–000, Minas Gerais, Brazil. E-mail: jeserrao@ufv.br

a Department of Entomology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

b Institute of Chemistry and Biotechnology, Federal University of Alagoas, Maceió, Alagoas, Brazil

c Department of General Biology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

d Laboratory of Ultrastructural Cell, Department of General Biology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

© 2016 Society of Chemical Industry

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 ($\lambda = 244$ 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 10 μL 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 100 μL 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

© 2016 Society of Chemical Industry

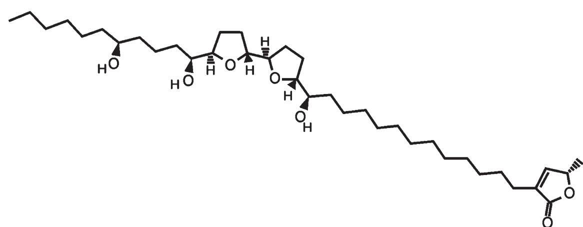

Figure 1. Chemical structure of squamocin isolated from *A. mucosa* seeds.

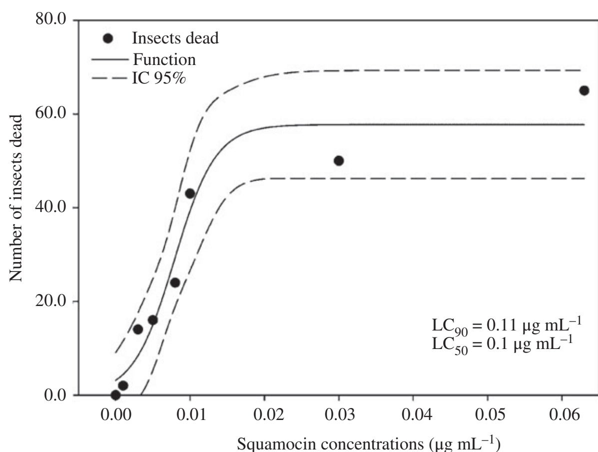

Figure 2. Concentration–response curve of *A. aegypti* larvae treated with squamocin from *A. mucosa* seeds.

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 ^{1}H 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

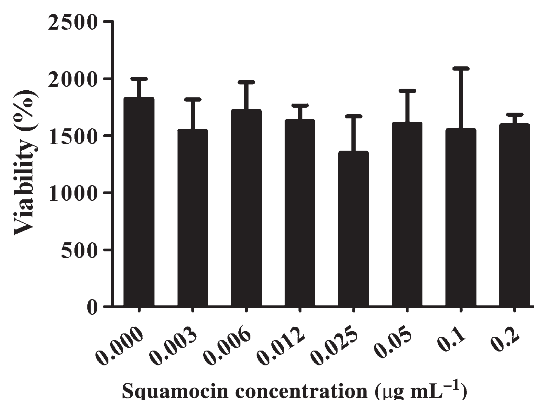

Figure 3. Absorbance spectrum (mean ± SD) for metabolic cell viability of human leukocytes when treated with different concentrations of squamocin.

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.

 © 2016 Society of Chemical Industry

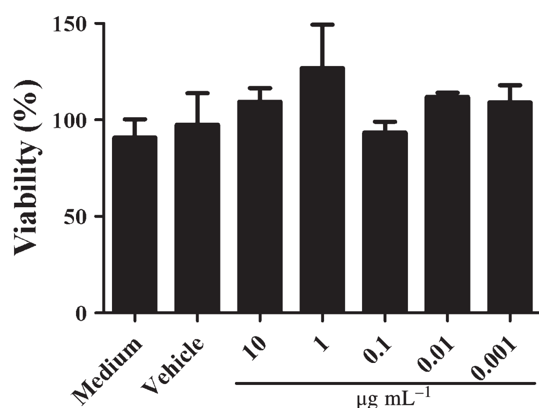

Figure 4. Absorbance spectrum (mean ± SD) for metabolic cell viability of human leukocytes when treated with acute doses of squamocin in medium without serum. Vehicle = Tween-20 (0.02%).

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.

ACKNOWLEDGEMENTS

This research was supported by Brazilian research agencies CNPq, CAPES and FAPEMIG. The authors thank Dr Mateus Ramos de Andrade for assistance in the field collections.

SUPPORTING INFORMATION

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

REFERENCES

1 Alali FQ, Liu XX and McLaughlin JL, Annonaceous acetogenins: recent progress. *J Nat Prod* **62**:504 (1999).
2 Gleye C, Laurens A, Laprévote O, Serani L and Hocquemiller R, Isolation and structure elucidation of sabadelin, an acetogenin from roots of *Annona muricata*. *Phytochemistry* **52**:1403–1408 (1999).
3 Chang FR, Chen JL, Lin CY, Chiu HF, Wu MJ and Wu YC, Bioactive acetogenins from the seeds of *Annona atemoya*. *Phytochemistry* **51**:883–889 (1999).
4 Chen Y, Chen JW, Wang Y, Xu SS and Li X, Six cytotoxic annonaceous acetogenins from *Annona squamosa* seeds. *Food Chem* **135**:960–966 (2012).
5 Myint SH, Cortes D, Laurens A, Hocquemiller R, Leboeuf M, Cavé A *et al.*, Solamin, a cytotoxic mono-tetrahydrofuranic γ-lactone acetogenin from *Annona muricata* seeds. *Phytochemistry* **30**:3335–3338 (1991).
6 Zhao GX, Gu ZM, Zeng L, Chao JF, Koslowski JF, Wood KV *et al.*, The absolute configuration of trilobacin and trilobin, a novel highly potent acetogenin from the stem bark of *Asimina triloba* (Annonaceae). *Tetrahedron* **51**:7149–7160 (1995).
7 Woo MH, Kim DH and McLaughlin JL, Asitrilobins A and B: cytotoxic mono-THF annonaceous acetogenins from the seeds of *Asimina triloba*. *Phytochemistry* **50**:1033–1040 (1999).
8 Ee GCL, Chuah CH, Sha CK and Goh SH, Disepalin, a new acetogenin from *Disepalum anomalum* (Annonaceae). *Nat Prod Lett* **9**:141–151 (1996).
9 Éparvier V, Nguyen VH, Thoison O, Martin MT, Sévenet T and Guéritte F, Cytotoxic monotetrahydrofuran acetogenins from *Disepalum plagioneurum*. *J Nat Prod* **69**:1289–1294 (2006).
10 Gu ZM, Fang XP, Zeng L and McLaughlin JL, Goniocin from *Goniothalamus giganteus*: the first tri-THF annonaceous acetogenin. *Tetrahedr Lett* **35**:5367–5368 (1994).
11 Lekphrom R, Kanokmedhakul S and Kanokmedhakul K. Bioactive styryllactones and alkaloid from flowers of *Goniothalamus laoticus*. *J Ethnopharmacol* **125**:47–50 (2009).
12 Chen Y, Chen RY and Quan Y, Six acetogenins from *Uvaria tonkinesis*. *Phytochemistry* **43**:793–801 (1996).
13 Hisham A, Pieters LAC, Claeys M, Esmans E, Dommisse R and Vlietinck AJ, Squamocin-28-one and panalicin, two acetogenins from *Uvaria narum*. *Phytochemistry* **30**:545–548 (1991).
14 Colman-Saizarbitoria T, Gu Z-M, Zhao G-X, Zeng L, Kozlowski JF and McLaughlin JL, Venezenin: a new bioactive annonaceous acetogenin from the bark of *Xylopia aromatica*. *J Nat Prod* **58**:532–539 (1995).
15 Colman-Saizarbitoria T, Montilla L, Rodriguez M, Castillo A and Hasegawa M, Xymarginatin: a new acetogenin inhibitor of mitochondrial electron transport from *Xylopia emarginata* Mart., Annonaceae. *Rev Bras Farmacogn* **19**:871–875 (2009).
16 Moreira IC, Roque NF, Vilegas W, Zalewski CA, Lago JHG and Funasaki M, Genus *Xylopia* (Annonaceae): chemical and biological aspects. *Chem Biodivers* **10**:1921–1943 (2013).
17 Santos AF and Sant'Ana AEG, Molluscicidal properties of some species of *Annona*. *Phytomedicine* **8**:115–120 (2001).
18 Vairappan CS and TanKai L, C-15 halogenated acetogenin with antibacterial activity against food pathogens. *Malaysian J Sci* **28**:263–268 (2009).
19 Zafra-Polo MC, González MC, Estornell E, Sahpaz S and Cortes D, Acetogenins from Annonaceae, inhibitors of mitochondrial complex I. *Phytochemistry* **42**:253–271 (1996).
20 Zeng L, Ye Q, Oberlies NH, Shi G, Gu ZM, He K *et al.*, Recent advances in annonaceous acetogenins. *Nat Prod Rep* **13**:275–306 (1996).
21 Barrett ADT and Higgs S, Yellow fever: a disease that has yet to be conquered. *Annu Rev Entomol* **52**:209–229 (2007).
22 Halstead SB, Dengue virus–mosquito interactions. *Annu Rev Entomol* **53**:273–291 (2008).
23 Ligon BL, Reemergence of an unusual disease: the chikungunya epidemic. *Semin Pediatr Infect Dis* **17**:99–104 (2006).
24 Costa MS, Cossolin JFS, Pereira MJB, Sant'Ana AEG, Lima MD, Zanuncio JC *et al.*, Larvicidal and cytotoxic potential of squamocin on the midgut of *Aedes aegypti* (Diptera: Culicidae). *Toxins* **6**:1169–1176 (2014).
25 He K, Zeng L, Ye Q, Shi G, Oberlies NH, Zhao GX *et al.*, Comparative SAR evaluations of annonaceous acetogenins for pesticidal activity. *Pest Manag Sci* **49**:372–378 (1997).
26 Rahuman AA, Gopalakrishnan G, Venkatesan P and Geetha K. Larvicidal activity of some Euphorbiaceae plant extracts against *Aedes*

 © 2016 Society of Chemical Industry

aegypti and *Culex quinquefasciatus* (Diptera: Culicidae). *Parasitol Res* **102**:867–873 (2008).

27 Tauil PL, Aspectos críticos do controle do dengue no Brasil. *Cadernos Saúde Públ* **18**:867–871 (2002).

28 Perumalsamy H, Kim JR, Oh SM, Jung JW, Ahn YJ and Kwon HW, Novel histopathological and molecular effects of natural compound pellitorine on larval midgut epithelium and anal gills of *Aedes aegypti*. *PLoS ONE* **8**:e80226 (2013).

29 Kostyukovsky M, Rafaeli A, Gileadi C, Demchenko N and Shaaya E, Activation of octopaminergic receptors by essential oil constituents isolated from aromatic plants: possible mode of action against insect pests. *Pest Manag Sci* **58**:1101–1106 (2002).

30 Wang Z, Kim JR, Wang MH, Shu S and Ahn YJ, Larvicidal activity of *Cnidium monnieri* fruit coumarins and structurally related compounds against insecticide-susceptible and insecticide-resistant *Culex pipiens pallens* and *Aedes aegypti*. *Pest Manag Science* **68**:1041–1047 (2012).

31 Costa ELN, Silva RFP and Fiuza LM, Efeitos, aplicações e limitações de extratos de plantas inseticidas. *Acta Biol Leopoldensia* **26**:173–185 (2004).

32 Isman MB and Grieneisen ML, Botanical insecticide research: many publications, limited useful data. *Trends Plant Sci* **19**:140–145 (2014).

33 Harbach RE, The Culicidae (Diptera): a review of taxonomy, classification and phylogeny. *Zootaxa* **1668**:591–638 (2007).

34 Albeny DS, Martins GF, Andrade MR, Krüger RF and Vilela EF, *Aedes aegypti* survival in the presence of *Toxorhynchites violaceus* (Diptera: Culicidae) fourth instar larvae. *Zoologia (Curitiba)* **28**:538–540 (2011).

35 Pascini TV, Albeny DS, Ramalho-Ortigão M, Vilela EF, Serrão JE and Martins GF, Changes in the fat body during the post-embryonic development of the predator *Toxorhynchites theobaldi* (Dyar & Knab) (Diptera: Culicidae). *Neotrop Entomol* **40**:456–461 (2011).

36 Desneux N, Decourtye A and Delpuech JM, The sublethal effects of pesticides on beneficial arthropods. *Annu Rev Entomol* **52**:81–106 (2007).

37 Shi JF, Wu P, Jiang ZH and Wei XY, Synthesis and tumor cell growth inhibitory activity of biotinylated annonaceus acetogenins. *Eur J Med Chem* **71**:219–228 (2014).

38 Finney DJ, Probit analysis: a statistical treatment of the sigmoid response curve. *J R Statist Soc* **110**:263–266 (1947).

39 Fujimoto Y, Eguchi T, Kakinuma K, Ikekawa N, Sahai M and Gupta YK, Squamocin, a new cytotoxic *bis*-tetrahydrofuran containing acetogenin from *Annona squamosa*. *Chem Pharmaceut Bull* **36**:4802–4806 (1988).

40 Alali FQ, Kaakeh W, Bennett GW and McLaughlin JL, Annonaceous acetogenins as natural pesticides: potent toxicity against insecticide-susceptible and -resistant German cockroaches (Dictyoptera: Blattellidae). *J Econ Entomol* **91**:641–649 (1998).

41 Ratnayake S, Rupprecht KJ, Potter WM and McLaughlin JL. Evaluation of various parts of the paw paw tree, *Asimina triloba* (Annonaceae), as commercial sources of the pesticidal annonaceous acetogenins. *J Econ Entomol* **85**:2353–2356 (1992).

42 Miyoshi H, Ohshima M, Shimada H, Akagi T, Iwamura H and McLaughlin JL, Essential structural factors of annonaceous acetogenins as potent inhibitors of mitochondrial complex I. *Biochim Biophys Acta Bioenerget* **1365**:443–452 (1998).

43 Tormo JR, González MC, Cortes D and Estornell E, Kinetic characterization of mitochondrial complex I inhibitors using annonaceous acetogenins. *Arch Biochem Biophys* **369**:119–126 (1999).

44 Barrachina I, Royo I, Baldoni HA, Chahboune N, Suvire F, DePedro N *et al.*, New antitumoral acetogenin 'Guanacone type' derivatives: isolation and bioactivity. Molecular dynamics simulation of diacetyl-guanacone. *Bioorg Med Chem* **15**:4369–4381 (2007).

45 Oberlies NH, Croy VL, Harrison ML and McLaughlin JL. The annonaceous acetogenin bullatacin is cytotoxic against multidrug-resistant human mammary adenocarcinoma cells. *Cancer Lett* **115**:73–79 (1997).

46 Zafra-Polo MC, Figadère B, Gallardo T, Tormo JR and Cortes D, Natural acetogenins from annonaceae, synthesis and mechanisms of action. *Phytochemistry* **48**:1087–1117 (1998).

47 Mordue AJ and Nisbet AJ, Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. *Anais Soc Entomol Brasil* **29**:615–632 (2000).

48 Turchen LM, Golin V, Butnariu AR and Pereira MJB, Selectivity of *Annona* (Annonaceae) extract on egg parasitoid *Trissolcus urichi* (Hymenoptera: Platygastridae). *Rev Colomb Entomol* **40**:176–180 (2014).

© 2016 Society of Chemical Industry