Larvicidal activity of leaf extracts and seselin from *Clausena anisata* (Rutaceae) against *Aedes aegypti*

[1*]Mukandiwa L., [1]Eloff, J.N., [2]Naidoo V.

[1]Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, P. Bag X04, Onderstepoort 0110, South Africa

[2]Biomedical Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa

* Corresponding author Email: Lilian.Mukandiwa@up.ac.za , Tel: +27 12 529 8173; Fax: +27 12 529 8321

Highlights

- Hexane leaf extracts of *Clausena anisata* cause *A. eagypti* mosquito larval mortality.
- The larvicidal activity is partially attributed to the pyranocoumarin, seselin.
- The hexane leaf extract retains larvicidal activity after 2 months of storage.

Abstract

The *Aedes aegypti* mosquito is a vector of various diseases in both humans and livestock. Mosquito control focuses on reducing the longevity as well as the population of mosquitoes to lessen their damage on human and animal health. It entails several strategies such as environmental management, insecticide treatments, and molecular entomological approaches. Environmental management centres on elimination of breeding sites, however mosquitoes can breed in sites that cannot be eliminated. Resultantly, focus is turned onto mosquito larvae control. The objective of this study was to evaluate the larvicidal activity of extracts and compounds from *Clausena anisata* against A. *aegypti*. The World Health Organization guidelines for testing of mosquito larvicides were used. The acetone, dichloromethane and hexane crude leaf extracts were evaluated in a preliminary screening for larvicidal activity at

the concentrations of 12.5, 25, 50, 100 and 200 ppm. Batches of 25 third-instar larvae were transferred into cups each containing test solutions and larval mortality was recorded 24 h and 48 h after exposure. Acetone was used as the solvent control while permethrin was used as a positive control. Only the n-hexane extract caused mortality at the tested concentrations, thus it was further tested at 40, 60, 80, 100, and 120 ppm and had LC_{50} values of 68.30 and 59.65 ppm after 24h and 48h respectively. A stored hexane extract, of 2 months, was also evaluated under simulated field conditions to establish stability of extract. It caused about 90% mortality when tested at 100 ppm. The n-hexane extract was subjected to open column chromatography on silica gel to isolate the active compound. The isolated compound was identified as the pyranocoumarin, seselin. Dose dependent mortality was observed in the larvae exposed to seselin. The LC_{50} values at 24 and 48 h were 13.90 and 9.96 ppm respectively. Results obtained from this study indicate a potential of the incorporation of *C. anisata* extracts into the control of mosquito populations.

Keywords: mosquito control; biopesticides; natural products

1 Introduction

The *Aedes aegypti* mosquito(Diptera: Culicidae) has been implicated in the mechanical transmission of various diseases in livestock such as Rift Valley fever (Hoch et al., 1985), lumpy skin (Chihota et al., 2001) and anthrax (Turell and Knudson, 1987). All these diseases are on the OIE 2014 list of notifiable diseases. This list comprises of transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal product (OIE, 2014). Epidemiological evidence indicates the incidence of these diseases is highest during wet periods coinciding with periods of mosquito abundance and wanes with the onset of the dry season (Magori-

Cohen et al., 2012; Caminade et al., 2014). Although protection of livestock from these diseases can be achieved by vaccination, vaccines and veterinary personnel are not always easily accessible in resource poor-communities. As such other preventative methods such as minimising mosquito populations on farms are of paramount importance. *Aedes aegypti* is also a principal vector of viruses that pose a threat to human health such as dengue, chikungunya, and yellow fever viruses (Gubler, 1998). Dengue fever is regarded globally as the most important arthropod-borne viral disease. It is endemic in at least 100 countries in Asia, the Pacific, the Americas, Africa, and the Caribbean, occurring every year during a season when *Aedes* mosquito populations are high. About 50% of the world's population lives in areas where there is a risk of dengue transmission (Murray et al., 2013). Thus the importance of controlling *A. aegypti* mosquito populations cannot be over-emphasized.

Control of mosquito populations involves preventing wastewater that stands for longer than 4 days; keeping weeds down around ponds, in ditches, and in shallow wetlands and irrigating properly so that all surface water is gone within 4 days (Lawler and Lanzaro, 2005). Mosquito larval control becomes important in cases where mosquito breeding sites cannot be eliminated such as the drinking ponds, watering troughs and hoof prints. Mosquito control at larval stage has the advantage of controlling the vector before it can acquire and transmit the disease.

Mosquito larvae can be controlled by substances added directly to the water. These substances may be organisms that consume them such as larvivorous fish (Chandra et al., 2013), biological compounds that poison them or cause fatal infections that are specific to them (*Bacillus thuringiensis israelensis*) (Ramírez-Lepe and Ramírez-Suero, 2012), chemicals that disrupt their development or physiology such as the insect growth regulator,

methoprene (Lawler and Lanzaro, 2005; Karunamoorthi, 2011), or oils and films that suffocate them (Bukhari et al., 2011). Biological compounds tend to be more expensive than chemical controls but they affect fewer non-target organisms (Gillette, 1988). Chemical controls are typically very effective against mosquitoes but they are toxic to non-target organisms and inaccessible due to high cost and limited availability in nearby markets, particularly in resource-poor communities. In addition, in cases where the chemicals are continuously used mosquitoes develop resistance. Resistance to temephos, the commonly used larvicide, has been widely reported (Grisales et al., 2013). Oils and films also suffocate non-target aquatic life and cause bird feathers to mat, and matted feathers cannot keep young birds warm and dry. Thus, against the facts presented, there is a need for a continual search of larvicides that can be used on farms.

Many plant extracts have been tested against various species of mosquitoes, focusing on larvicidal action (Shaalan et al., 2005). The use of plant extracts against noxious insects has the advantage that the closely related compounds within these complex mixtures often act synergistically (Isman, 1997). The exposure of a target organism to a group of phytochemicals, rather than to a single active principle, lowers the probability for that organism to develop resistance or behavioural desensitization. *Clausena anisata* is one of the ethnomedicinal plants that have been reportedly used traditionally to repel or kill mosquitoes (Okunade and Olaifa 1987; Mavundza et al., 2011). It has also been reported to have insecticidal and repellent activities against various insects in ethnoveterinary medicine (Chavunduka, 1976) and *in-vitro* laboratory studies (Boeke et al., 2004; Ndomo et al., 2008). It contains compounds that interfere with larval feeding and the neuroendocrine control mechanisms in the blowfly (Mukandiwa et al., 2012, 2013). We therefore investigated the

biological activity of *C. anisata* extracts and the isolated compound against *Aedes eagypti* mosquito larvae.

2 Materials and methods

2.1 Collection of Plants

The leaves of *Clausena anisata* (Wild) Hook. f. ex. Benth were collected in autumn from the Pretoria National Botanical Garden, South Africa and dried at room temperature in a well-ventilated room. The plant species was identified by tree name tags and were authenticated by the Guide at the National Botanical Garden. The voucher specimen of the plant species, numbered PMDN317, is kept at the Medicinal Plant Collection Herbarium of the Department of Paraclinical Sciences, University of Pretoria, South Africa. Collection, drying and storage guidelines of the plant material followed were as outlined by McGaw and Eloff (2010).

2.2 Extraction

Dried leaf material was ground to fine powder (c. 1 mm diameter) using an IKA-WERKE M20 mill (GMBH & Co., Germany). Three different extractants were used, namely: acetone, dichloromethane and hexane (all technical grade, Merck). To prepare the extracts, 25 g of the leaf material were shaken vigorously for 1 hour in 250 ml of the respective extractants on an orbital shaker (Labotec®, model 20.2, South Africa). The extracts were allowed to settle, centrifuged at 2000 g for 10 min and the supernatant filtered through Whatman No. 1 filter paper into pre-weighed glass vials. The extraction process was repeated 3 times for three samples of the plant material. The extracts were dried in a stream of cold air at room temperature and the mass extracted with each solvent was determined. The dried extracts were reconstituted in acetone for use in the bioassays.

2.3 Test Organism

Aedes aegypti mosquito eggs were obtained from the Pesticide Trial Section of the South African Bureau of Standards (SABS). The eggs were placed in distilled water to hatch. The emerging larvae were reared and tested at 28 ± 2 ◦C temperature, ≥45± 10% relative humidity, and a 12:12 (light:dark) photoperiod and were fed tropical fish flakes

2.4 Larvicidal bioassay

2.4.1 Laboratory evaluation

The larvicidal activity of the plant extracts was evaluated according to the World Health Organization guidelines for laboratory and field testing of mosquito larvicides (WHO, 2005). The acetone, dichloromethane and hexane crude extracts were evaluated in a preliminary screening at the concentrations of 12.5, 25, 50, 100, and 200 ppm. Batches of 25 third-instar larvae were transferred to a small disposable test cups, each containing 100 ml of distilled water. Then one ml aliquots of the plant extracts at the concentrations ranging from 1.25 to 20 mg/ml were added, producing final concentrations ranging from 12.5 to 200 ppm. The acetone and dichloromethane extracts had no larvicidal activity at all the concentrations tested. The larvae exposed to these extracts underwent the subsequent developmental stages successfully. Therefore, only the activity of the hexane extract was further evaluated at 40, 60, 80, 100, and 120 ppm to enable the determination of the LC_{50} value. Four replicates were set up for each concentration and the tests were repeated 3 times on different days. Permethrin (0.1ppm; technical grade dissolved in acetone) was used as a positive control. It was selected as a positive control because it is based on plant compounds, the pyrethrins from the chrysanthemum flower, and it has been reported to be among the pyrethroids with best activity against mosquito larvae (Mulla and Schaefer, 1980). Acetone was used as the solvent control.

The twelve fractions of the crude hexane extract (section 2.4) and the isolated compound (section 2.5) were also evaluated for larvicidal activity in the same manner as described above. The fractions were tested at 50 ppm. Only one concentration was used as this step was only meant to guide further fractionation of the extract. The isolated compound was tested at concentrations of 10, 20, 30, 40 and 50 ppm. Larval mortality was recorded 24 h and 48 h after exposure. Moribund larvae were counted and added to dead larvae for calculating percentage mortality. Dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region whilst moribund larvae are those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed (WHO, 2005).

After data collection at 48 hours food was added into the test containers and every other day thereafter for 7 days. During this period observations were made on the development of the larvae to pupae stage.

2.4.2 Small-scale simulated field trial

According to WHO guidelines larvicides that show promise in laboratory studies (Phase I) should be subjected to Phase II. In Phase II, field trials of formulated products are performed on a small scale against target mosquitoes, preferably in representative natural breeding sites or, where such trials are not feasible, under simulated field conditions. For this phase of the study, 2L beakers, filled to half capacity with water were placed on 2 different sites on the grounds of the University of Pretoria. We decided not to use water from the field, because there could be too many variables between different water bodies that would hinder repeatability of the results. At this stage we needed to develop a proof of concept, i.e. that the extract is effective against mosquito larvae. The water was allowed to age for 24 hours, after

which, batches of 100 laboratory-reared third instar larvae of *A. aegypti* were released into each container with larval food. After 2–3 h of larval acclimation, 1 ml of the hexane *C. anisata* extract dissolved in acetone at 100 mg/ml was added into one of the beakers per site to a final concentration of 100 ppm. The selection of the test concentration was guided by the results from the laboratory assay; the 100ppm concentration caused larval mortality of over 90%. The containers were covered with nylon mesh to prevent other mosquitoes or other insects from laying eggs and to protect the water from falling debris. Two replicates of the treatment and two controls were used in each test. The containers were examined after 48 h and live larvae were counted to score post-treatment larval mortality. The test was repeated four times. The extract used in this study was intentionally 2 months old, to get an indication of the stability of the extract. The extract was stored in closed glass jar in a cupboard at room temperature with an average minimum of 15°C and maximum of 32°C and humidity of ≥ 25%. The isolated compound was also evaluated under simulated field conditions in the same manner described above. It was evaluated at 25 ppm, which was selected as a concentration most likely to cause 90% mortality based on the laboratory assay.

2.5 Fractionation of the crude n-hexane extract

Three (3) grams of the crude *n*-hexane extract was fractionated by column chromatography using silica gel (Kieselgel 60, 70–230 mesh, 0.063–0.200mm, Merck), with a gradient solvent of n-hexane: ethyl acetate 100:0, 98:2, 95:5, 90:10, 85:15, 80:20 to 70:30 (hexane: ethyl acetate). The resulting fractions were combined based on thin layer chromatography (TLC) analysis to give a total of 12 fractions.

2.6 Isolation of an active component from the n-hexane fraction

From the bioactivity assays of the different fractions mentioned above, only 2 fractions, 1/11 and 12/13, caused larval mortality with Fraction 12/13 being the most active. It was therefore subjected to repeated column chromatography. Open column chromatography was undertaken on silica gel (Kieselgel 60, 70–230 mesh, 0.063–0.200mm, Merck) using n-hexane: ethyl acetate at 100:0, 98:2, 96:4, 94:6, 98:2, and 90:10 (hexane: ethyl acetate) and led to the isolation of Compound **A**.

2.6.1 Structural analysis of the isolated active compound

Spectroscopic techniques, ^{1}HNMR, ^{13}C NMR and 2DNMR(HMBC, HSQC, COSY, DEPT), were used for the elucidation of the structure of isolated active compound using a Bruker ARX-400 nuclear magnetic resonance (NMR) spectrometer (in deuterated chloroform ($CDCl_3$)).Chemical shifts were reported with reference to the respective residual solvents or deuterated solvent peaks. The structure of the isolated compound was confirmed by comparison of its NMR data with that in literature. ESI-MS were obtained on Waters Synapt HDMS spectrometer.

2.7 Data analysis

Data from the larvicidal assays for all replicates was pooled together for analysis. The data was analysed using the pharmacology software Kinetica version 5 (Thermo Scientific). The concentration required to kill 50 % of the larvae (LC_{50}) was determined using the Sigmoid Emax model (Hill).

3 Results

3.1 Larvicidal activity of crude hexane extract of Clausena anisata

3.1.1 Larvicidal activity under controlled conditions

Larval mortality was observed for only the larvae exposed to the hexane extract of *C.anisata* in the preliminary screening of the extracts. Therefore further evaluation of the hexane extract was conducted at concentrations selected based on the observations from the preliminary screening. Mortality increased with the increase in concentration of the extract from 40 ppm to 120 ppm with the LC_{50} values after 24 h and 48 h being 68.30(60.293- 73.736) and 59.67 (53.148- 67.092) ppm respectively (figures in parenthesis are the lower confidence limit (LCL) and the upper confidence limit (UCL)). Mortality was also time dependent, being higher at 48 hours compared to at 24 hours (Figure 1). Larvae exposed to the hexane extract at 40 and 60 ppm that did not die completed the rest of the developmental stages. However, those exposed to 80 ppm and above, all eventually (>48 h) died at larval stage.

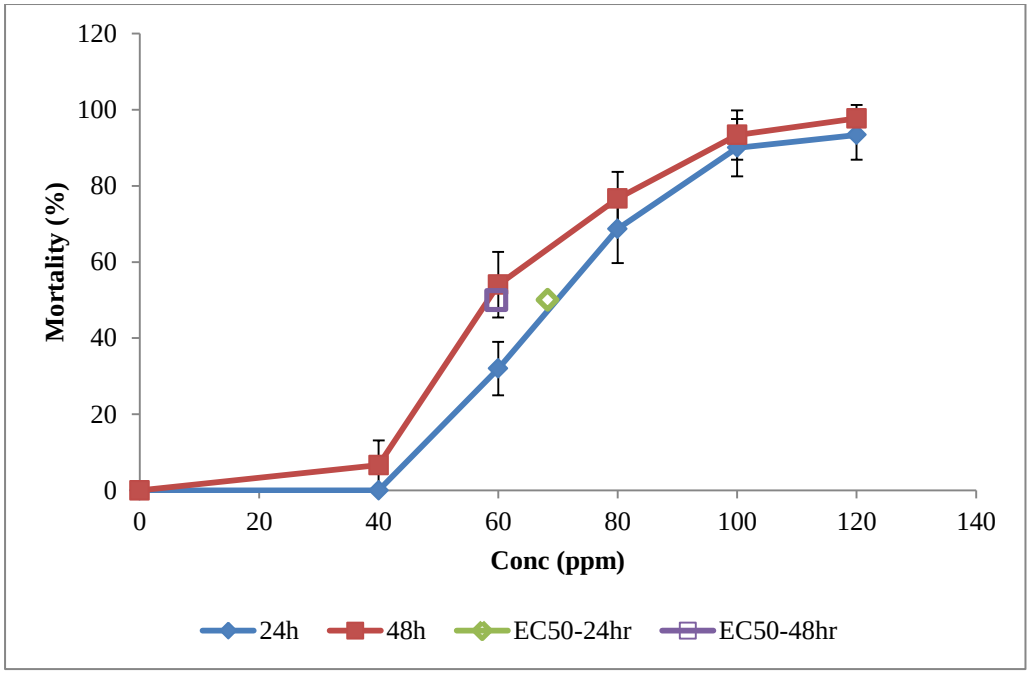

Figure 1: Larvicidal activity of the different concentrations of hexane extract of *Clausena anisata* against *Aedes eagypti larvae*

3.1.2 Larvicidal activity under small-scale simulated field conditions

The hexane extract was still active after 2 months of storage resulting in 89 % ± 8.61 mortality of exposed larvae when tested at 100 ppm. The larvae that did not die also failed to develop to the next life stage.

3.2 Isolation and identification of larvicidal compounds

The total amount of SFr. 12/13 extracted from 3.0g of the crude hexane extract was 820 mg thus the fraction yield was 27.3%. The structure of the isolated active compound was elucidated by NMR and MS as the pyranocoumarin, seselin, chemically called 2', 2'-dimethylpyranocoumarin. A detailed description of this compound is available elsewhere (Mukandiwa et al., 2013). The total amount isolated from 3g of crude hexane extract was 473 mg thus an isolation efficiency of 15.77%. Based on the starting plant material from which the crude hexane extract was obtained (25g), the compound percentage yield was 1.89%.

3.3 Mosquito larvicidal activity of Seselin

Dose dependent mortality was observed in the larvae exposed to seselin (Figure 2). The LC_{50} values at 24 and 48 h were 13.90 and 9.96 ppm respectively. Larvae exposed to less than 10ppm did not die and successfully underwent the rest of the developmental stages. Exposure to seselin at 25 ppm resulted in 100% mortality when evaluated under simulated field conditions similar to what was observed under laboratory conditions at the same concentration.

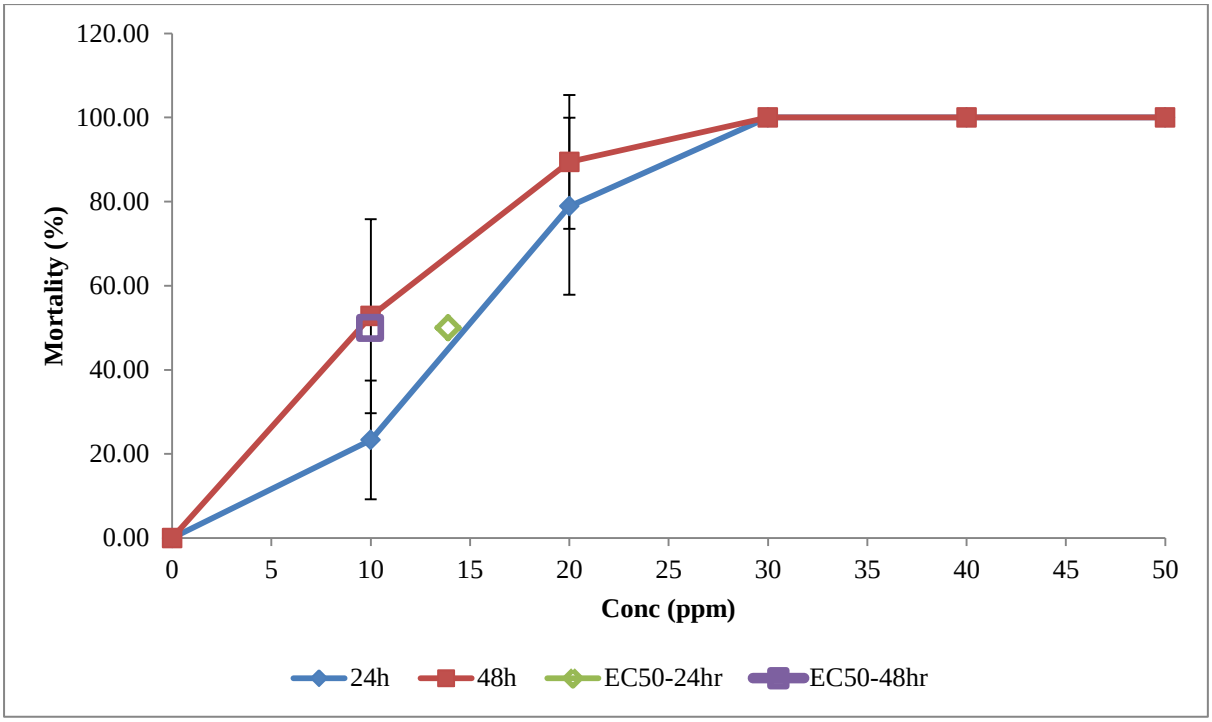

Figure 2: Larvicidal activity of different concentrations (ppm) of seselin against *Aedes eagypti* mosquito larvae

4 Discussion

The aim of this study was to establish the larvicidal activity of *Clausena anisata* against the *Aedes eagypti* mosquito and identify the compound(s) responsible for the observed activity. This work adds to the current efforts worldwide to discover new mosquito control agents. Plant bioactive chemicals are generally considered as nontoxic, easily available at affordable prices, biodegradable and show broad-spectrum target-specific activities against different species of vector mosquitoes (Ghosh et al., 2012).

The hexane extract of *Clausena anisata* had promising LC_{50} values of 68.30 and 59.65 ppm after 24 h and 48 h respectively. In a previous research by Mavundza et al. (2013), the ethanolic extract of *C. anisata* had an LC_{50} value of 112.7 ppm against *Anopheles arabiensis* mosquitoes. Our findings and those of previous researchers indicate that C. *anisata* certainly

contains mosquito larvicidal compounds. The observed difference in activity can be attributed to the different mosquito species and extract types of the C. *anisata* leaves used in the 2 studies. The fact that mosquito larvae exposed to 80% and higher concentrations of the crude hexane extract that did not die failed to complete the subsequent life stages suggests that the extract may also act as an insect growth regulator.

In this study, we went further than just screening the plant for larvicidal activity to isolate and identify the larvicidal compound in *Clausena anistata*. In addition to the isolated seselin the researchers hypothesize that there may be more active compounds in the extract. Several studies have focused on the larvicidal activities of various plant species in preliminary screenings (Hardin and Jackson, 2009; Ghosh et al., 2012) but only a few have gone further to determine the active principles. As a result few botanicals have moved from laboratory to field use. The observed activity of seselin against *A. eagypti* is comparable to what other researchers have reported for other isolated plant compounds against the same species which have LC_{50} values that range from 0.25 to 14.7 ppm (Ghosh et al., 2012). The identified compounds include lapachol, (E)-6-hydroxy-4, 6-dimethyl-3-heptene-2-one, α-terpinene, *N*-methyl-6β-(decal', 3', 5'-trienyl)-3-β-methoxy-2-β-methylpiperidine, Methyl-*p*-hydroxybenzoate, β-sitosterol, and Pipernonaline. This clearly supports the notion that bioactive constituents of plants have potential to be employed as larvicides useful in controlling mosquito vectors. Although inferior to methoprene and temephos which have LC_{50} values ranging between (0.00278 to 0.0195ppm) (Braga et al., 2005); Silva and Mendes, 2007) and (0.006 to 0.038ppm) (Loke et al., 2010; Lek-Uthai et al., 2011) respectively, these compounds could still be important as alternatives considering that resistance to both compounds has been reported already (Grisales et al., 2013).

Seselin has been previously identified as the antifeedant compound in *Clausena anista* deterring blowfly larval feeding (Mukandiwa et al., 2013). It has been isolated from plants particularly those belonging to the Rutaceae family (Keating and O'Kennedy, 1997; Borges et al., 2005). It has several activities including vasodilatory (Lima et al., 2006); antitumor and anti-HIV(Huang et al., 1994); antifungal (Bandara et al., 1991; Cardenas-Ortega et al., 2007); ovicidal against *Tetranynchus urticae* (red spider mite) (Tanaka et al., 1985); weak to moderate cytotoxicity (Gunatilaka et al., 1994); peripheral anti-inflammatory and antinociceptive (Lima et al., 2006); inhibits phytohemagglutinin-stimulated cell proliferation in human blood mononuclear cells (Tsai et al., 2008); inhibitory activity in both indole acetic acid oxidase and peroxidase enzyme systems (Goren and Tomer, 1971) and autotoxicity in citrus trees (Singh et al., 1999). However this is the first report on seselin having mosquito larvicidal activity.

The crude hexane extract of *C. anisata* at 100 ppm resulted in mortalities of 90.00 % ± 7.53 and 93.33 % ± 6.46 after 24 and 48 h respectively thus only 0.1mg of the extract per ml of water was required to cause over 90% mortality. Accordingly, 1g of the crude hexane extract (which requires 31.25g of plant material) will kill mosquito larvae in 10 litres of water. Thus it may be feasible to use the crude hexane *C. anisata* extract as a larvicide on farms as part of an integrated pest management system. The 2 month-old extract was able to give similar results in the simulated field study as a fresh extract in the laboratory study. This is noteworthy in light of the fact that one of the major drawbacks to the use of plant extracts is that they are unstable under environmental conditions and quickly lose their activity. A more detailed stability study will be conducted in further studies in which differently aged extracts will be tested. Safety evaluation of *C. anisata* in animal drinking water will also be a focus of our next study.

5 Conclusion

Results obtained from this study have initiated on-going investigations into the incorporation of *C. anisata* into the control of mosquito populations, with a view to developing an environmentally acceptable product of value in integrated vector control. The use of a plant extract that reduce mosquito populations at the larval stage can provide many associated benefits to vector control.

6 Acknowledgements

The University of Pretoria and the National Research Foundation provided the financial support for this research. The curator of the Pretoria National Botanical Garden gave us permission to collect the plant material. The authors are grateful to Mr Vincent Nel and Mr Heinz Loots (SABS) for supplying us with the mosquito eggs and giving technical advice on rearing the mosquitoes. L. Mukandiwa gratefully acknowledges the financial support from German Academic Exchange Service, DAAD, during the period of this study.

7 References

Bandara, B.M.R., Gunatilaka, A.A.L., Wijeratne, E.M.K., MacLeod, J.K., 1991. Acridone alkaloids and coumarins from *Pleiospermium alatum*. Phytochemistry 29, 297 – 301.

Boeke, S.J., Baumgart, I.R., van Loon, J.J.A., van Huis, A., Dicke, l. M., Kossou, D.K., 2004. Toxicity and repellence of African plants traditionally used for the protection of stored cowpea against *Callosobruchus maculatus*. Journal of Stored Products Research 40, 423-438.

Borges, F., Roleira, F., Milhazes, N., Santana, L., Uriarte, E., 2005. Simple coumarins and analogues in medicinal chemistry: Occurrence, synthesis and biological activity. Current Medicinal Chemistry 12, 887-916.

Braga, I.A., Mello, C.B., Montella, J.B.P.L., Martins Júnior, A.J., Medeiros, P.F.V., Valle, D., 2005. Effectiveness of Methoprene, an insect growth regulator, against Temephos-resistant *Aedes aegypti*

*p*opulations from different Brazilian localities, under laboratory conditions. Journal of Medical Entomology 42, 830-837.

Bukhari, T., Takken, W., Githeko, A.K., Koenraadt, C.J.M., 2011. Efficacy of Aquatain, a monomolecular film, for the control of malaria vectors in rice paddies. PLoS ONE 6, doi:10.1371/journal.pone.0021713

Caminade, C., Ndione, J.A., Diallo, M., MacLeod, D.A., Faye, O., Ba, Y., Dia, I., Morse, A.P., 2014. Rift valley fever outbreaks in Mauritania and related environmental conditions. International Journal of Environmental Research and Public Health 11, 903–918.

Cardenas-Ortega, N.C., Perez-Gonzalez, C., Zavala-Sanchez, M.A., Hernandez-Ramirez, A.B., Perez-Gutierrez, S., 2007. Antifungal activity of seselin in protecting stored maize from *Aspergillus flavus*. Asian Journal of Plant Sciences 6, 712-714.

Chandra, G., Ghosh, A., Bhattacharjee, I., Ghosh, S.K., 2013. Use of larvivorous fish in biological and environmental control of disease vectors. In: Cameron MM, Lorenz LM (eds) Biological and Environmental Control of Disease Vectors. CABI, UK, pp 25-41.

Chavunduka, D.M., 1976. Plants regarded by Africans as being of medicinal value to animals. Rhodesian Veterinary Journal **7,** 6–12.

Chihota, C.M., Rennie, L.F., Kitching, R.P., Mellor, P.S., 2001. Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae). Epidemiology and Infection 126, 317-321.

Ghosh, A., Chowdhury, N., Chandra, G., 2012. Plant extracts as potential mosquito larvicides. Indian Journal of Medical Research 135, 581-598.

Gillette, B., 1988. Controlling mosquitoes biologically. BioScience 38, 80-83.

Goren, R., Tomer, E., 1971. Effects of seselin and coumarin on growth, indole acetic acid oxidase, and peroxidase, with special reference to cucumber (Cucumissativa L.) radicles. Plant Physiology 47, 312-316.

Grisales, N., Poupardin, R., Gomez, S., Fonseca-Gonzalez, I., Ranson, H., Lenhart, A., 2013. Temephos resistance in *Aedes aegypti* in Colombia compromises dengue vector control. PLoS Neglected Tropical Diseases 7, doi:10.1371/journal.pntd.0002438.

Gubler, D.J., 1998. Resurgent vector-borne diseases as a global health problem Emerging Infectious Diseases 4, 442-450.

Gunatilaka, A.A., Kingston, D.G., Wijeratne, E.M., Bandara, B.M., Hofmann, G.A., Johnson, R.K., 1994. Biological activity of some coumarins from Sri Lankan Rutaceae. Journal of Natural Products 57, 518 – 520.

Hardin, J.A., Jackson, F.L.C., 2009. Applications of natural products in the control of mosquito-transmitted diseases. African Journal of Biotechnology 8, 7373-7378.

Hoch, A.L., Gargan, T.P., Bailey, C.L., 1985. Mechanical transmission of Rift Valley fever virus by hematophagous diptera. The American Journal of Tropical Medicine and Hygiene 34, 188-193.

Huang, L., Kashiwada, Y., Cosentino, L.M., Fan, S., Lee, K.H., 1994. 3′, 4′-Di-*o*-(−)-camphanoyl-(+)-*cis*khellactone and related compounds: A new class of potent anti-HIV agents. Bioorganic and Medicinal Chemistry Letters 4, 593–598.

Isman, M.B., 1997. Neem and other Botanical insecticides: Barriers to commercialization. Phytoparasitica 25, 339-344.

Karunamoorthi, K., 2011. Vector control: a cornerstone in the malaria elimination campaign. Clinical Microbiology and Infection 17, 1608–1616.

Keating, G.J., O'Kennedy, R., 1997. The chemistry and occurrence of coumarins. In: O'Kennedy, R., Thornes, R.D. (Eds), Coumarins: Biology, Applications and Mode of Action. Wiley, New York, pp. 23-66.

Lawler, S.P., Lanzaro, G.C., 2005. Managing mosquitoes on the farm. ANR publication 8158. http://anrcatalog.ucdavis.edu/pdf/8158.pdf . Accessed 3 October 2014

Lek-Uthai, U., Rattanapreechachai, P., Chowanadisai, L., 2011. Bioassay and effective concentration of temephos against *Aedes aegypti* larvae and the adverse effect upon indigenous predators: *Toxorhynchites splendens* and *Micronecta sp*. Asia Journal of Public Health 2, 67-77.

Lima, V., Silva, C.B., Mafezoli, J., Bezerra, M.M., Moraes, M.O., Mourao, G.S., Silva, J.N., Oliveira, M.C., 2006. Antinociceptive activity of the pyranocoumarin seselin in mice. Fitoterapia 77, 574 – 578.

Loke, S.R., Andy-Tan, W.A., Benjamin, S., Lee, H.L., Sofian-Azirun, M., 2010. Susceptibility of field-collected *Aedes aegypti* (L.) (Diptera: Culicidae) to *Bacillus thuringiensis israelensis* and temephos. Tropical Biomedicine 27, 493–503.

Magori-Cohen, R., Louzoun, Y., Herziger, Y., Oron, E., Arazi, A., Tuppurainen, E., Shpigel, N.Y., Klement, E., 2012. Mathematical modelling and evaluation of the different routes of transmission of lumpy skin disease virus**.** Veterinary Research 43, doi:10.1186/1297-9716-43-1.

Mavundza, E.J., Maharaj, R., Chukwujekwu, J.C., Finnie, J.F., van Staden, J., 2013. Larvicidal activity against *Anopheles arabiensis* of 10 South African plants that are traditionally used as mosquito repellents. South African Journal of Botany 88, 86-89.

Mavundza, E.J., Maharaj, R., Finnie, J.F., van Staden, J., 2011. An ethnobotanical survey of mosquito repellent plants in uMkhanyakude district, KwaZulu-Natal Province, South Africa. Journal of Ethnopharmacology 137, 1516–1520.

McGaw, L.J., Eloff, J.N., 2010. Methods for evaluating efficacy of ethnoveterinary medicinal plants. In: Katerere DR, Luseba D (eds.) Ethnoveterinary Botanical Medicine: Herbal medicines for Animal Health. CRC Press, London, pp 1-24

Mukandiwa, L., Eloff, J.N., Naidoo, V., 2012. Screening of plants used traditionally to treat myiasis for effect on the survival and development of *Lucilia Cuprina* and *Chrysomya Marginalis* (Diptera: Calliphoridae). Veterinary Parasitology 190, 566-572.

Mukandiwa, L., Ahmed, A., Eloff, J.N., Naidoo, V., 2013. Isolation of seselin from *Clausena anisata* leaves and its effects on the growth and development of blowfly larvae. Journal of Ethnopharmacology 150, 886-891.

Mulla, M.S., Schaefer, C.H., 1980. Developing better larvicides. California Agriculture http://ucanr.edu/repository/a/?get=62729

Murray, N.E.A., Quam, M.B., Wilder-Smith, A. 2013. Epidemiology of dengue: past, present and future prospects. Clinical Epidemiology 5, 299-309.

Ndomo, A.F., Ngamo, L.T., Tapondjou, L.A., Tchouanguep, F.M., Hance, T., 2008. Insecticidal effects of the powdery formulation based on clay and essential oil from the leaves of *Clausena anisata* (Willd.) J. D. Hook ex. Benth. (Rutaceae) against *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae). Journal of Pesticide Science 81, 227–234.

OIE, 2014. OIE-Listed diseases, infections and infestations in force in 2014. http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2014/ Accessed 28 September 2014

Okunade A.L., I. Olaifa, J.I., 1987. Estragole: An Acute Toxic Principle from the Volatile Oil of the Leaves of *Clausena anisata*. Journal of Natural Products 50, 990-991.

Ramírez-Lepe, M., Ramírez-Suero, M., 2012. Biological control of mosquito larvae by Bacillus thuringiensis subsp. Israelensis. In: Perveen F (ed.) Insecticides - Pest Engineering.

http://www.intechopen.com/books/insecticides-pest-engineering/biological-control-of-mosquito-larvae-by-bacillus-thuringiensis-subsp-israelensis . Accessed 4 August 2014.

Shaalan, E.A.S, Canyon, D., Younes, M.W.F., Abdel-Wahab, H., Mansour, A.H., 2005. A review of botanical phytochemicals with mosquitocidal potential. Environment International 3, 1149-1166.

Silva, J.J., Mendes, J., 2007. Susceptibility of *Aedes aegypti* (L) to the insect growth regulators diflubenzuron and methoprene in Uberlândia, State of Minas Gerais. Revista da Sociedade Brasileira de Medicina Tropical 40, 612-616.

Singh, H.P., Batish, D.R., Kohli, R.K., 1999. Autotoxicity: Concept, organisms, and ecological significance. Critical Reviews in Plant Sciences 18, 757-772.

Tanaka, H., Ahn, J.W., Katayama, M., Wada, K., Marumo, S., Osaka, Y., 1985. Isolation of two ovicidal substances against two-spotted spider mite, *Tetranychus urticae* Koch, from *Skimmia repens* Nakai. Agricultural and Biological Chemistry 48, 2189-2190.

Tsai, W.J., Chen, Y.C., Wu, M.H., Lin, L.C., Chuang, K.A., Chang, S.C., Kuo, Y.C., 2008. Seselin from *Plumbago zeylanica* inhibits phytohemagglutin (PHA)-stimulated cell proliferation in human peripheral blood mononuclear cells. Journal of Ethnopharmacology119, 67-73.

Turell, M.J., Knudson, G.B., 1987. Mechanical Transmission of *Bacillus anthracis* by Stable Flies (*Stomoxys calcitrans*) and Mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*). Infection and Immunity 55, 1859-1861.

World Health Organization (WHO), 2005. Guidelines for laboratory and field testing of mosquito larvicides. WHO/CDS/WHOPES/GCDPP/2005.13. http://whqlibdoc.who.int/hq/2005/WHO_CDS_WHOPES_GCDPP_2005.13.pdf?ua=1 Accessed 1 September 2013