Tropical Biomedicine 23(2): 180–185 (2006)

Efficacy of botanical extracts from *Callitris glaucophylla*, against *Aedes aegypti* and *Culex annulirostris* mosquitoes

Essam Abdel-Salam Shaalan[1], Deon Vahid Canyon[2], Bruce Bowden[2,] Mohamed Wagdy Faried Younes[3], Hoda Abdel-Wahab[1] and Abdel-Hamid Mansour[1]
[1]Zoology Department, Aswan Faculty of Science, South Valley University, Aswan, Egypt.
[2]Anton Breinl Centre for Public Health and Tropical Medicine, James Cook University, Townsville, Australia.
[3]Zoology Department, Faculty of Science, Menoufia University, Shebin El-Kom, Egypt.
Corresponding author email: Deon.Canyon@jcu.edu.au
Received 8 August 2006; received in revised form 6 September 2006; accepted 8 September 2006

Abstract. Using standard WHO methodology, this study investigated the susceptibility of 4th instar *Aedes aegypti* (L) and *Culex annulirostris* (Skuse) larvae to three extracts from *Callitris glaucophylla* (J. Thompson & L. Johnson) (1: steam distillation extract, 2: liquefied refrigerant gas extract, and 3: methanol reflux extract), lambda-cyhalothrin (a synthetic pyrethroid insecticide) and fenitrothion (an organophosphorous insecticide). *Cx. annulirostris* was significantly more susceptible than *Ae. aegypti* to all tested chemicals except lambda-cyhalothrin. Responses to the three *C. glaucophylla* extracts were exceptional for a botanical compound: *Cx. annulirostris* (LC_{50} = 0.23, 9.53 and 38.95 mg/L) and *Ae. aegypti* (LC_{50} = 0.69, 5.21 and 306.43 mg/L). Both *Cx. annulirostris* and *Ae. aegypti* larvae were significantly more susceptible to lambda-cyhalothrin (LC_{50} = 0.00013 and 0.00016 mg/L) than fenitrothion (LC_{50} = 0.0009 and 0.004 mg/L). As expected, the pyrethroid and organophosphorous insecticides were far more potent than the crude *C. glaucophylla* extracts. The steam distilled extract was fractionated and the major components guaiol and citronellic acid were identified and tested. Activities for these major components were lower than observed for the distillate. Minor components include lactones such as eldanolide, and future testing of minor components may indicate the active component.

INTRODUCTION

Several considerable insecticide-related problems are prompting biodiscovery researchers to reconsider botanical alternatives. These problems include the lack of novel insecticides, the high cost of synthetic pyrethroids, concern for environmental sustainability, the unacceptability of many organophosphates and organochlorines, and increasing insecticide resistance on a global scale. Bioinsecticide research has reported a wide range of acute and chronic toxic effects from a large number of plant species that are globally distributed (Shaalan *et al*., 2005). The essential oils of many plants have shown larvicidal activity against various mosquito species (Kumar & Dutta, 1987; Mwaiko, 1992; Sharma *et al*., 1994; Soliman & El-Sherif, 1995; Jayaprakasha *et al*., 1997; Shalaby *et al*., 1998; Thomas & Callaghan, 1999; Mansour *et al*., 2000). Insecticidal effects of plant extracts vary not only according to plant species, mosquito species and plant parts (Sukumar *et al*., 1991), but also to extraction methodology. As viable synthetic insecticides are depleted, phytochemicals have a significant role to play as alternatives or companions to the synthetic insecticide arsenal. When considering which botanicals to study, it can be useful to investigate those that exhibit anti-insect qualities in the natural setting. One such candidate is *Callitris glaucophylla* (J. Thompson & L. Johnson), commonly known as White Cypress Pine, which is famous in Australia as a termite resistant timber (Charles 1996; Watanabe *et*

al. 2005). This study thus evaluated the larvicidal activity of three extracted *C. glaucophylla* oils against *Aedes aegypti* (L.) and *Culex annulirostris* (Skuse) mosquitoes and compared them to the efficacy of a synthetic pyrethroid, an organophosphate and an insect growth regulator.

MATERIALS AND METHODS

Susceptible *Ae. aegypti* mosquitoes were obtained from a colony initiated from mosquitoes collected in 2002 from Townsville, Australia. *Cx. annulirostris* mosquitoes were bred from a colony maintained by the Queensland Institute of Medical Research in Brisbane, Australia. The colonies were maintained in environmental conditions of 27 ± 2 C° and 70 % ± 5 RH under 14L - 10D light cycles. *Ae. aegypti* larvae were kept in plastic buckets half filled with tap water and fed on a variety of commercial goldfish flakes. *Cx. annulirostris* larvae were reared in aerated plastic trays half filled with dechlorinated tap water that contained pieces of grass. These larvae were fed a mixture of granulated fish food (80%), liver powder (10%) and yeast powder (10%). Water in rearing containers was refreshed every 2 days. Adult mosquitoes were maintained on a 10 % sugar solution and female adults were given the opportunity to feed on rat blood.

Three *C. glaucophylla* extracts were supplied by the Queensland Forestry Research Institute, Department of Primary Industries, Queensland Government, Australia in 2002, who obtained them from a sawmill that steam-extracted the oil from a mixture of sapwood and hardwood sawdust and stored it in sealed drums. The first extract was obtained by steam distillation from sawdust in water at atmospheric pressure. In this method, the most volatile components of the wood float on the condensate water as an oil. The second extract from sawdust was obtained using liquefied refrigerant gas under pressure. The refrigerant was removed by flashing off by reducing the pressure. This extract contained a mixture of more volatile and less volatile components. The third extract from sawdust was obtained using methanol under reflux followed by solvent removal by distillation and then under vacuum in a rotary evaporator, and freeze drying of the remainder. This extract contained much less of the more volatile components. These extracts were sealed and stored in a refrigerator away from light for up to several months before use.

Technical grade organophosphorous and synthetic pyrethroid insecticides (fenitrothion 96.8 % and lambda-cyhalothrin 90.99 %) were provided by Nufarm Ltd (103-105 Pipe Road, Laverton, 3026 North Victoria, Australia). Technical grade (s)-methoprene, an insect growth regulator, was obtained from Wellmark International (1100 East Woodfield Road, Suite 500 Schaumburg, IL 60173, USA).

Crude extracts (the essential oils) were first screened in a descending series of concentrations (1000, 500, 100, 50 and 5 mg/L) to identify the lowest dose that killed 100% of mosquito larvae. Then standard WHO methodology (WHO, 1981) was used to produce data for lethal concentration calculations. Approximately 25 newly molted 4th instar larvae were released into glass beakers containing 99 ml of de-ionized water. 1 ml of either the extracts or insecticides was added to each beaker while 1 ml of ethanol was added to control beakers. Four replicates for each concentration were conducted. Larvae in both test and control were sourced from different rearing containers to avoid the effect of differently reared batches of larvae (WHO, 1996). Percentage mortality was recorded after 24 h and Abbott's formula (Abbott, 1925) was applied to correct percentage mortality if control mortality was between 5% and 20 %.

$$\text{Corrected mortality} = \frac{\text{\% test mortality} - \text{\% control mortality}}{100 - \text{\% control mortality}} \times 100$$

In the methoprene bioassay, dead larvae, pupae and adults were recorded daily until the death of the last larva or pupa, or emergence of the last mosquito (WHO, 1996). Only methoprene results for *Ae. aegypti* were obtained due to unavoidable high control mortality while testing *Cx.*

annulirostris larvae. The Probit Regression program in SPSS version 12 was used to determine lethal concentration values and corresponding 95 % confidence intervals.

In an attempt to identify the active components of the steam distilled extract, fractionation was first achieved by preparation of a diethyl ether solution that was extracted with 1M NaOH. Acidification of the basic extract with 1M HCl and extraction with diethyl ether afforded (after solvent removal) the major acidic component, which was identified using 1D and 2D NMR techniques. The solvent was removed from the ether solution that had been extracted with base to afford the neutral components from the steam distillate. This fraction was chromatographed on silica gel using a solvent gradient from hexane through diethyl ether to ethyl acetate, to afford fractions that contained various neutral components.

RESULTS

Preliminary screening of *C. glaucophylla* extracts against *Ae. aegypti* and *Cx. annulirostris* larvae showed the lowest concentrations causing 100% mortality were 5 mg/L for the steam distillated extract and 50 mg/L for the liquefied refrigerant gas extract. The lowest effective concentrations of the methanol reflux gas extract that caused 100% larval mortality were 100 against *Cx. annulirostris* and 500 mg/L against *Ae. aegypti*.

According to calculated LC_{50} values for the *Callitris* extracts (Tables 1 and 2), the steam distillated extract was the most toxic (LC_{50} = 0.23 and 0.69 mg/L against *Cx. annulirostris* and *Ae. aegypti* larvae, respectively), followed by the liquefied refrigerant gas extract (LC_{50} = 9.53 and 5.21 mg/L against *Cx. annulirostris* and *Ae. aegypti* larvae, respectively), while the methanol reflux extract was the least effective (LC_{50} = 38.95 and 306.43 mg/L against *Cx. annulirostris* and *Ae. aegypti* larvae, respectively). Slope values of the 3 extracts were significantly different indicating different responses of larvae to these extracts.

Relative potency was calculated as LC_{50} of synthetic insecticide / LC_{50} of plant extract. Both mosquito colonies were extremely susceptible to the synthetic insecticides with relative potency values ranging from 0.004 to 0.00016. *Cx. annulirostris* larvae were more susceptible to the synthetic insecticides (LC_{50} = 0.00013 & 0.0009 mg/L for lambda-cyhalothrin and fenitrothion, respectively) than *Ae. aegypti* larvae (LC_{50} = 0.00016 & 0.004 mg/L for lambda-cyhalothrin and fenitrothion, respectively). Methoprene was even more toxic to this colony of *Ae. aegypti* (LC_{50} = 0.003 ppb). Furthermore, lambda-cyhalothrin was more toxic to both mosquito species than fenitrothion. Slope values for regression

Table 1. Dose responses of *Callitris glaucophylla* extracts and synthetic insecticides tested against newly molted 4th instar *Aedes aegypti* larvae using a Probit analysis

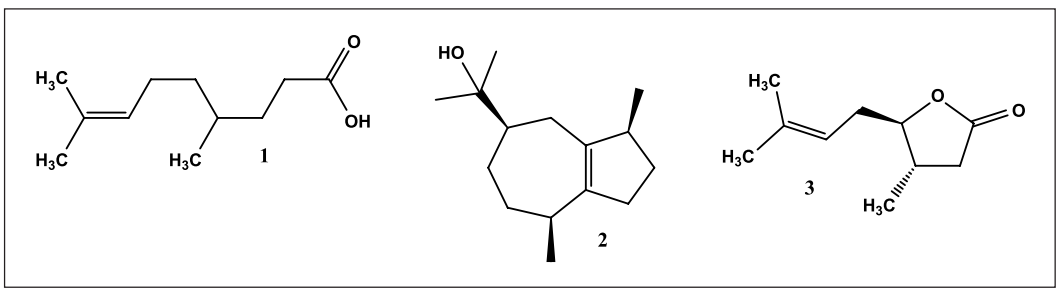
Callitris glaucophylla extracts and insecticides	Slope ± SE	LC_{50} mg/L	95 % FL	LC_{90} mg/L	95 % FL
Steam distillation extract	1.74 ± 0.16	0.69	0.55 – 0.85	1.43	1.19 – 1.87
Liquefied refrigerant gas extract	0.07 ± 0.007	5.21	2.131 – 7.54	23.31	20.75 – 26.82
Methanol reflux extract	0.008 ± 0.0006	306.43	282.97 – 333.55	457.24	417.27 – 516.58
Lambda-cyhalothrin	7533 ± 594.28	0.00016	0.00013 – 0.0002	0.00033	0.00027 – 0.00044
Fenitrothion	361.30 ± 20.14	0.0044	0.0036 – 0.0052	0.0079	0.0069 – 0.0094
Methoprene	263.36 ± 20.83	0.003*	0.0023 – 0.0041	0.008*	0.0065 – 0.0103

* ppb.

Table 2. Dose responses of *Callitris glaucophylla* extracts and synthetic insecticides tested against newly molted 4th instar *Culex annulirostris* larvae using a Probit analysis

Callitris glaucophylla extracts and insecticides	Slope ± SE	LC_{50} mg/L	95 % FL	LC_{90} mg/L	95 % FL
Steam distillation extract	4.73 ± 0.40	0.23	0.18 – 0.29	0.5	0.42 – 0.63
Liquefied refrigerant gas extract	0.22 ± 0.015	9.53	7.77 – 11.13	15.22	13.33 – 18.44
Methanol reflux extract	0.06 ± 0.004	38.95	35.48 – 42.57	57.72	52.71 – 65.28
Lambda-cyhalothrin	10021.46 ± 778.29	0.00013	0.00011 – 0.00015	0.0002	0.00023 – 0.00029
Fenitrothion	1051.02 ± 92.62	0.00093	0.00033 – 0.00135	0.002	0.00167 – 0.00322

Figure 1. Compounds from *Callitris glaucophylla*.



lines of both insecticides against both mosquito species were significantly different and reflected the huge difference in larval dose-response to tested concentrations of both insecticides.

In an attempt to identify the active components of the steam distilled extract, fractionation of the steam distillate was first achieved by separation into acidic and neutral components. The acidic fraction was identified as citronellic acid, approximately 30% of the steam distillate (Fig 1-1), using 1D and 2D NMR techniques. The neutral fraction, after chromatography on silica gel afforded guaiol, which comprised approximately 40% of the original steam distillate (Fig 1-2), and a number of minor components that included the lactone, eldanolide (Fig 1-3).

Neither citronellic acid nor guaiol, which are documented components of Callitris steam volatile extracts (Yazaki, 1983), exhibited activity comparable to that observed for the original steam distillate.

DISCUSSION

This study confirmed the effects of mosquito species and extraction method on extract efficacy as reviewed by Shaalan *et al.* (2005). As expected, *Cx. annulirostris* was more susceptible to all tested substances than *Ae. aegypti*. Several investigations have mentioned that larvae of different mosquito species display different levels of susceptibility to the same extract and *Culex* larvae are generally more susceptible than *Aedes* larvae (Amonkar & Reeves, 1970; Sukumar *et al.*, 1991; Pizzaro *et al.*, 1999; Rey *et al.*; 1999, 2001; Park *et al.*, 2002). Extracting methods that produced more volatile compounds (Steam distillation extract) produced material with superior activity compared with the other extracts against both mosquito species. Similarly, Chahad & Boof (1994) found that both methanol and acetone extracts of *Piper nigrum* (black pepper) that were obtained by Soxhlet extraction produced more

toxicity than was exhibited by extraction using a maceration method against *Cx. quinquefasciatus* larvae.

In a 2005 review of mosquitocidal botanicals by Shaalan *et al.*, the lowest LC_{50}s that could be found for fractionated botanical extracts and their phytochemicals were 0.001 mg/L recorded for a hexane fraction of *Curcuma longa* extract against *Ae. aegypti* larvae (Roth *et al.*, 1998), 0.0026 mg/L recorded for dichloromethane fraction of *Abuta grandifolia* fruit extract against *Ae. aegypti* larvae (Ciccia *et al.*, 2000) and 0.004 mg/L recorded for pipercide, a constituent of *Piper nigrum* fruits, against *Cx. pipiens pallens* larvae (Park *et al.*, 2002). Most raw or crude botanical extracts fail to cause 100% mortality at less than 10 mg/L and it is very rare to find one that is effective at less than 1 mg/L. In its raw form, a petroleum ether extract of *Piper nigrum* only had an LC_{50} of 2.6 mg/L (Moawed, 1998). Compared with these results, the performance of the steam distillated extract of *C. glaucophylla* against *Ae. aegypti* and *Cx. annulirostris* larvae looks highly promising despite it being a crude extract. Fractionation could lead to the discovery of a more active compound comparable to findings of other researchers (Ciccia *et al.*, 2000; Park *et al.*, 2002). The liquefied refrigerant gas extract was less effective but still performed well compared to most botanicals.

Although the botanical extracts were far less active than the synthetic insecticides in this study, the steam distilled extract still outperformed several other economically viable synthetic insecticides, such as temephos, malathion, propoxur and fenthion (Canyon & Hii, 1999). Slope values for each of the test substances were quite different, which suggested the presence of different compounds and/or sites of activity rather than differences in compound concentration and dose-response (Tables 1 and 2).

Further fractionation and compound isolation, particularly of the minor components of the steam distillate, will hopefully reveal a potent phytochemical or synergistic mixture that will be comparable to or even better than synthetic insecticides.

Acknowledgements. We are grateful to Dr Michael Kennedy at the Queensland Forestry Research Institute, Department of Primary Industries, Queensland Government, Australia, for providing us with extracts of *C. glaucophylla*.

REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18:** 256–257.

Amonkar, S.V. & Reeves, E.L. (1970). Mosquito control with active principle of garlic, *Allium sativum*. *Journal of Economic Entomology* **63:** 1172-1175.

Canyon, D.V. & Hii, J.L.K. (1999). Insecticide susceptibility status of *Aedes aegypti* (Diptera: Culicidae) from Townsville. *Australian Journal of Entomology* **38:** 40-43.

Chahad, S. & Boof, M.I.C. (1994). Effect of black-pepper extracts on the larvae of *Culex* (Culex) *quinquefasciatus* Say (Diptera: Culicidae). *Anais da Sociedade Entomologica do Brasil* **23:** 13- 18.

Charles, P.B. (1996). *Aspects of management of subterranean termites in Queensland*, Ph.D. dissertation, University of Queensland, Brisbane, Australia.

Ciccia, G., Coussio, J. & Mongelli, E. (2000). Insecticidal activity against *Aedes aegypti* larvae of some medicinal South American plants. *Journal of Ethnopharmacology* **72:** 185-189.

Jayaprakasha, G.K., Singh, R.P., Pereira, J. & Sakariah, K.K. (1997). Limonoids from *Citrus reticulata* and their moult inhibiting activity in mosquito *Culex quinquefasciatus* larvae. *Photochemistry* **44:** 843-846.

Kumar, A & Dutta, G.P. (1987). Indigenous plant oils as larvicidal agent against *Anopheles stephensi* mosquitoes. *Current Science* **56:** 959-960.

Mansour, S.A., Messeha, S.S. & EL-Gengaihi, S.E. (2000). Botanical biocides. 4. Mosquitocidal activity of certain *Thymus capitatus* constituents. *Journal of Natural Toxins* **9:** 49-62.

Mwaiko, G.L. (1992). Citrus peel oil extracts as mosquito larvae insecticides. *East African Medical Journal* **69:** 223-226.

Moawed, H.A.M. (1998). *Joint action of some plant extracts against the mosquito larvae of* Culex pipiens *and their physiological impact*. M.S. thesis, Faculty of Science-Dmietta, Mansoura University, Egypt.

Park, I., Lee, S., Shin, S., Park, J. & Ahn, Y. (2002). Larvicidal activity of isobutylamides identified in *Piper nigrum* fruits against three mosquito species. *Journal of Agriculture, Food and Chemistry* **50:** 1866-1870.

Pizarro, A.P., Oliveira, F.A.M., Parente, J.P., Melo, M.T., dos Santos, C.E. & Lima, P.R. (1999). Utilization of the waste of sisal industry in the control of mosquito larvae. *Reviews of the Society Brazil Medical Tropical* **32:** 23-29.

Rey, D., Cuany, A., Pautou, M. & Meyran, J. (1999). Differential sensitivity of mosquito taxa to vegetable tannins. *Journal of Chemical Ecology* **25:** 537-48.

Rey, D., David, J.P., Besnard, G., Jullien, J.L., Lagneau, C. & Meyran, J.C. (2001). Comparative sensitivity of larval mosquito to vegetable polyphenols versus conventional insecticides. *Entomologia Experimentalis et Applicata* **98:** 361-367.

Roth, G.N., Chandra, A. & Nair, M.G. (1998). Novel bioactivities of *Curcuma longa* constituents. *Journal of Natural Products* **61:** 542-545.

Shaalan, E., Canyon, D.V., Younes, M., Abdel-Wahab, H. & Mansour, A. (2005). A review of botanical phytochemicals with mosquitocidal potential. *Environment International* **31:** 1149-1166.

Shalaby, A.A., Allam, K.A., Mostafa, A.A. & Fahmy, S.M. (1998). Insecticidal properties of citrus oils against *Culex pipiens* and *Musca domestica*. *Journal of the Egyptian Society of Parasitology* **28:** 595-606.

Sharma, R.N., Deshpande, S.G., Tungikar, V.B. & Joseph, M. (1994). Toxicity of natural essential oils to mosquitoes, *Aedes aegypti* and *Culex fatigans*. *Geobios* **21:** 162-165.

Soliman, B.A. & El-Sherif, L.S. (1995). Larvicidal effect of some plant oils on mosquito *Culex pipiens* L. (Diptera: Culicidae). *Journal of the Egyptian German Society of Zoology* **16:** 161-169.

Sukumar, K., Perich, M.J. & Boobar, L.R. (1991). Botanical derivatives in mosquito control: A review. *Journal of the American Mosquito Control Association* **7:** 210-237.

Thomas, C.J. & Callaghan, A. (1999). The use of garlic (*Allium sativa*) and lemon peel (*Citrus limon*) extracts as *Culex pipiens* larvicides: Persistence and interaction with an organophosphate resistance mechanism. *Chemosphere* **39:** 2489-2496.

Watanabe, Y., Mitsunaga, T. & Yoshimura, T. (2005). Investigating antitermitic compounds from Australian white cypress heartwood (*Callitris glaucophylla* Thompson et Johnson) against *Coptotermes formosanus* Shiraki. *Journal of Essential Oil Research* **17:** 346-350.

WHO. (1981). *Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides*. WHO/VBC/81.807. World Health Organization, Geneva.

WHO. (1996). *Report of the WHO informal consultation on the evaluation and testing of insecticides*. CTD/WHOPES/IC/96. World Health Organization, Geneva.

Yazaki, Y. (1983). Volatility of extractive components in white cypress pine (*Callitris columellaris* F. Muell.). *Holzforschung* **37:** 231-235.