Original Article

Larvicidal Activity of Citrus Limonoids against *Aedes albopictus* Larvae

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(Received 16 Nov 2011; accepted 24 Sep 2012)

Abstract

Background: Development of insecticide resistance occurred due to the continuous and misuse of synthetic insecticides therefore, the recent study was conducted to explore eco-friendly plant extracts that have some potential to suppress mosquito larval population.

Methods: WHO recommended mosquito larval bioassay method for insecticide was used while for the analysis of citrus oils for limonin and nomilin content HPLC was used.

Results: Among the two citrus cultivars tested as larvicide against *Aedes albopictus*, valencia late (*Citrus sinensis*) was the best in terms of LC_{50} (297 ppm), % mortality (97%) and LT_{50} (18.49 hours) then freutrall early (*Citrus reticulate*) with LC_{50} (377.4 ppm), % mortality (88%) and LT_{50} (31 hours), While nomilin gave lowest LC_{50} (121.04 ppm) than limonin (382.22 ppm) after 72 hours of exposure. Valencia late also had more limonin and nomilin (377 µg/ml and 21.19 µg/ml) than freutrall early (5.29 µg/ml and 3.89 µg/ml) respectively.

Conclusion: Valencia late showed best results in term of LC_{50}, LT_{50} and percentage mortality against *Aedes albopictus* as it has more amount of nomilin then freutrall early, however further evaluation in the field conditions is required.

Keywords: Limonin, nomilin, citrus, *Aedes* larvae

Introduction

Plants have a variety of small organic molecules called secondary metabolites (Sarker et al. 2005), which are required by them for interaction with the environment (Kutchan and Dixon, 2005). Secondary metabolites from plants are confirmed to have biological activity and that can be helpful in protecting the plants from a pathogen, herbivore or competitor. Secondary metabolites normally produce low concentrations of minor compounds. These metabolites show structural similarities to primary products; these secondary metabolites can be divided into the different chemical groups like alkaloids, terpenoids, phenolic, plant amines, rare amino acids and glycosides (Rohloff 2003). These compounds play an important role as anti-nutritional components of food and animal feed with a number of phenolic compounds. These include cell wall phenolic components, lignification of cells and the presence of polyphenols such as condensed tannins. Plant terpenoids have been studied for their activities against a number of insects (Gutierrez et al. 1997). Defense strategy of plant against insect pest and pathogens depends on the presence of volatile essential oils or monoterpenes (Langenheim 1994). Plant also used these volatile terpenoids in plant-plant interactions and serve as attractants for pollinators (Tholl 2006). Cyanogenic glycosides isoflavonoids and alkaloids, these soluble secondary compounds can also be toxic to animals (Morris and Robbins, 1997).

Dengue Fever (DF) is transmitted by *Aedes aegypti* while, *Ae. albopictus* is considered as maintance vector in Southeast Asia (CDC 2001). The incidence and geographical distribution of DF has increased dramatically over the past 30 years. It is estimated that 2.5 billion of the world's population are at risks and

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that 100 million cases arise annually, including 500,000 cases of DHF (Peters and Geoffrey, 2007). In Pakistan, an outbreak of Dengue Hemorrhagic Fever (DHF) was first reported in Karachi in 1994 (Chan et al. 1995). Another outbreak was seen in 2005 (Ali et al. 2006) while in 2010 there were 11,024 confirmed cases of DF including 40 deaths and now in 2011 again dengue outbreak has occurred in Punjab with 20,500 confirmed cases with 304 deaths are reported (Anonymous 2011).

Rational control of mosquitoes lies in personal protection and community education as the most economical method in eradicating breeding sites and application of eco-friendly larvicides for the control of mosquito larvae (Certin et al. 2004). Synthetic insecticides are no doubt having quick actions but due to their adverse effects to the environment received wide public concern (St leger et al. 1996), like insecticide resistance (Severini et al. 1993), environmental pollution, toxic hazards to human and other non-target organisms (Forget 1989). To mitigate these problems, a major emphasis has recently been explored which includes the use of natural plant based products as larvicides which can provide an alternate to synthetic chemical insecticides (Junwei et al. 2006). Many plants contain chemicals which are helpful for the control of insects and are useful for field applications in mosquito control programmes (Kalyanasundaram and Das, 1985, Isman 1999) such as *Solanum villosum* berry (Nandita et al. 2008) and fenugreek (Halawa 2001) have shown better insecticidal effects. Citrus fruits posses a wide variety of bioactive compounds with health promoting disease preventing properties that have been shown to be effective against Cancer. Moreover limonoids from Rutaceae particularly citrus (Klocke and Kubo, 1982) have attracted greater concern due to their growth regulating activity (Champage et al. 1992) besides having anti-carcinogenic effects (Sohail et al. 2005). They have some potential as insecticide (Akram et al. 2010).

In view of the recently increased interest in developing plant origin insecticides as an alternative to chemical insecticide, this study was undertaken to assess the larvicidal potential of the citrus seed crude extracts as well as limonin and nomilin against the dengue vector *Aedes albopictus* Skuse.

Materials and Methods

Collection of Citrus Cultivars

Feutrall early (*Citrus reticulate*) and Valencia late (*Citrus sinensis*) were collected from Sargodha (32°5′1″N 72°40′16″E).

Extraction of oil

The seeds of citrus cultivars were washed with tap water to remove the pulp and then dried in the oven for 48 hours at 60^{o} C and later were grounded in an electric grinder (Anex, Germany). The grounded material was put in thimble and kept in extraction tube of Soxhelt apparatus with extractor ID 38 mm, extractor volume 85 ml and flask volume 250 ml (Vogel 1978) for the extraction of oil by steam distillation method using Diethyl-ether as solvent (250 ml/20 g sample). The cycle time for one sample was 4–5 hours. Oil with Solvent was kept at room temperature for the evaporation of solvent, leaving oil which was then collected.

HPLC analysis

HPLC apparatus comprised of a Shimadzu (Japan) pump (LC-10AT) with a Shimadzu ultraviolet (UV) detector (SDD-10AV) and Shimadzu degasser unit (DGU-12A). Limonoids were quantified on C18 reverse phase Shim Pack CLC-ODS column, 5 µm particle size (15 cm× 4.6 mm) and detected at 210 nm.

Analytical or HPLC grade solvents were used. Methanol and acetonitrile were obtained from Merck (Germany) and standards (nomilin and limonin) from MP Biochemicals (France). Mobile phase consisted of methanol, acetonitrile and water with the following ratio

10:41:49. 5 mg of limonin and nomilin in 100 ml of mobile phase were used for the preparation of stock solution UV absorption at 210 nm with 1 ml/min flow rate was used to monitor the elution. 0.45-µm Millipore filter was used for the filtration of both standards and samples and the compounds were quantified by chromatography station software CSW 32, version 1. 4.11.89. The linearity of method was estimated by analyzing standards. Elution was carried out isocratically and peak responses with their respective retention time were obtained by injecting twenty micro liters of each standard in HPLC. Filtered samples (20µl) were injected in C18 reverse phase column, eluted isocratically with acetonitrile, methanol, and water (10:41: 49). The limonoid aglycones were identified on the basis of retention times (Ohta et al. 1993).

Colection and Rearing of mosquitoes

Aedes albopictus larvae and pupae were collected round the year from all artificial containers and natural habitats from Faisalabad (31°21′52″N 72°59′40″E) and brought to the mosquito rearing room in Biosystematics lab of department of Agri. Entomology, University of Agriculture Faisalabad, reared in steel trays of 3 inch depth and fed on TetraMin® (fish food) Akram et al. (2010). Adult population was maintained in transparent plastic cages (70× 35× 35 cm), with cotton wicks soaked in 10% sucrose solution while females were also fed with blood of white rats every alternate day for egg development (Shaalan et al. 2006). A glass beaker with strips of moistened filter paper was provided for gravid females to lay their eggs. The population was maintained at lab conditions of 30±2 °C, 75±5% RH and L14: D10 photoperiod.

Bioassay

The extracted oils were used in six different concentrations (300,400,500,600,700,800 ppm). There were three replicates for each treatment, each replicate containing 200ml of the oil solution placed in 250ml glass beakers (WHO 2005). A batch of 30 early third instar larvae of the *Aedes albopictus* were exposed in each beaker containing oil solution (Mohtar et al. 1999). Larvae in control were only treated with acetone. The experiment was conducted using CRD (Complete Randomized Design) under lab conditions at 30±2 °C and 75±5% relative humidity. The percentage mortality was calculated from the average of three replicates using the following formula (Sumroiphon et al. 2006).

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae} \times 100}{\text{Number of larvae tested}}$$

Data analysis

Abbot's formula (Abbot 1925) was used for corrected mortality and the data so obtained was analyzed by probit analysis (Finney 1989) by using the software Minitab-15 for dose and time mortality regression lines.

$$\text{Corrected mortality\%} = \frac{\text{Observed mortality\%} - \text{Control mortality\%} \times 100}{100 - \text{Control mortality\%}}$$

Results

Citrus fruits contain certain compounds (limonoids) that are extremely bitter which are mainly accumulated in seeds. Peaks were recorded with standards at retention time of 8.15 min (limonin) and 12.66 min (nomilin) as shown in Fig 1. Non-volatile collection through HPLC analysis was done and the responsive peaks at specific retention time were recorded. Limonin and nomilin contents in freutrall early were 5.29 and 3.89 µg/ml respectively, while Valencia late had 377 and 21.19 µg/ml respectively as indicated in Table 1.

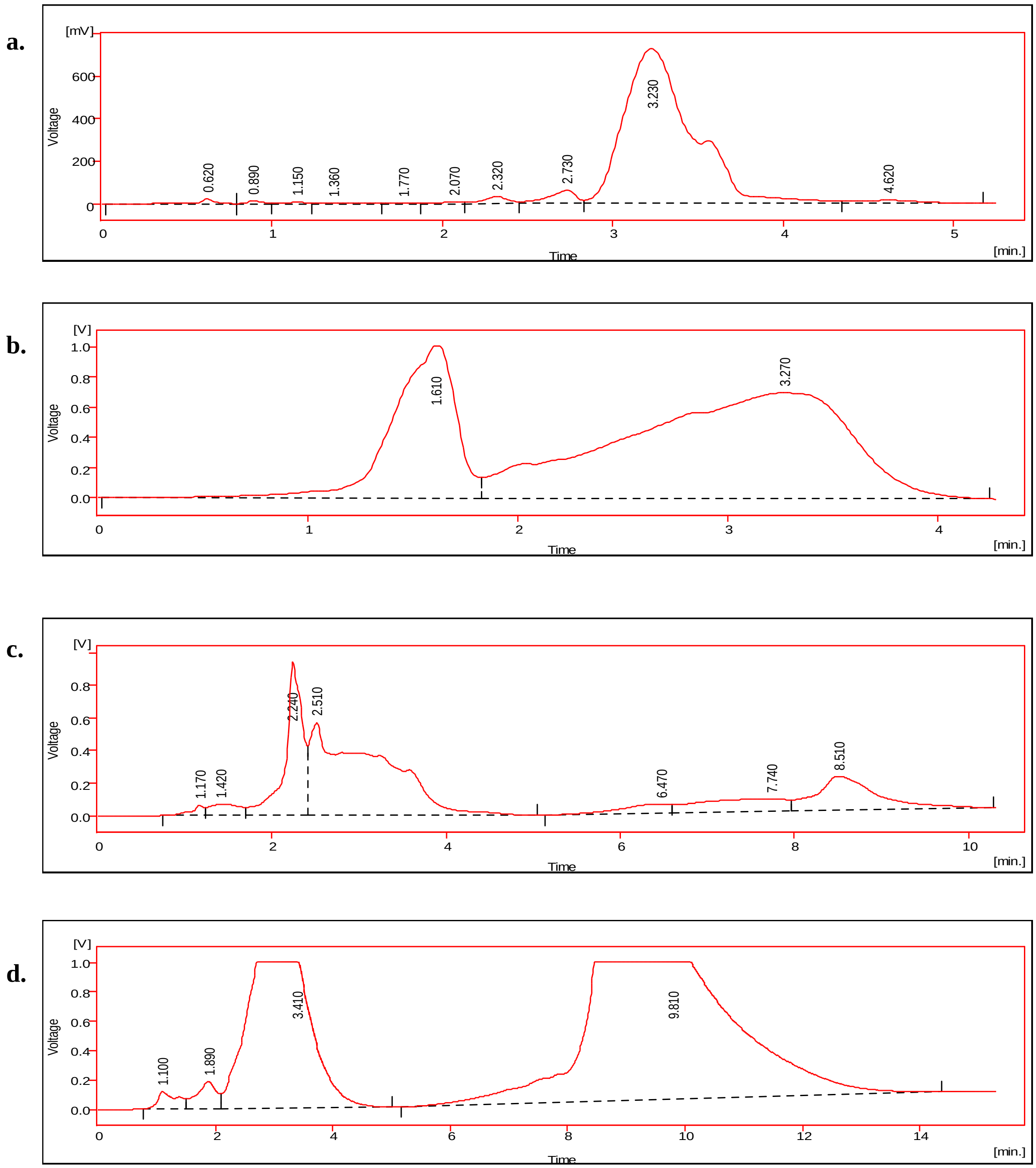

Fig. 1. HPLC analysis of a) Limonin, b) Nomilin, c) Freutrall early d) Valencia late

In terms of LC_{50} Valencia late had the lowest value (368 ppm) after 24 hours when compared with freutrall early, it gave 564.7 ppm value. After 48 hours Valencia late gave 333.3 ppm and freutrall early gave 465.6 ppm while after 72 hours Valencia late had 297 ppm and freutrall early had 377.4 ppm values, which clearly indicated that Valencia late had good results. Furthermore lethality of limonin and nomilin was also evaluated; results showed that nomilin is best as shown in Table 1.

Freutrall early and Valencia late both exhibited strong larvicidal activity against *Aedes albopictus*, with respect to percent mortality, Valencia late caused highest mortality (81%) after 24 hours of exposure then freutrall early (68%), while after 48 and 72 hours again Valencia late exhibited highest mortality (88 and 97%) then freutrall early (74 and 88%) as shown in Fig. 2.

Lethal time for mortality of 50% tested population was recorded at different concentration, when both citrus cultivars were compared Valencia late had the lowest time (18.49 hours) to kill the 50% tested population, while freutrall early took more than a day as is clear from Table 2.

Table 1. Lethal concentration recorded at different concentration on two citrus cultivars, Limonin and Nomilin against larvae of *Aedes albopictus*

Citrus Cultivars	Limonin (µg/ml)	Nomilin (µg/ml)	Time	LC_{50} (ppm)	Slope±SE	χ^2	*P*
Freutrall early	5.29	3.89	24	564.7	1.17±0.17	0.44	0.97
Citrus reticulate			48	465.6	1.06±0.16	0.60	0.96
			72	377.4	1.31±0.17	2.57	0.63
Valencia Late	377	21.19	24	368	1.09±0.17	0.17	0.99
Citrus sinensis			48	333.3	1.11±0.17	2.40	0.66
			72	297	1.32±0.18	8.78	0.06
Limonin			24	836.70	1.22±0.15	0.60	0.99
			48	548.01	1.18±0.17	0.41	0.83
			72	382.22	1.27±0.17	1.21	0.04
Nomilin			24	289.12	1.30±0.18	5.01	0.91
			48	163.28	1.33±0.17	3.22	0.52
			72	121.04	1.29±0.17	8.01	0.21

Table 2. Lethal time of citrus cultivars against larvae of *Aedes albopictus*

Citrus Cultivars	LT_{50} (hours)	Slope±SE	P	χ^2
Feutrall early ***Citrus reticulate***	31.07	0.41± 0.06	2.1(	0.14
Valencia Late ***Citrus sinensis***	18.49	0.72± 0.07	7.9:	0.005

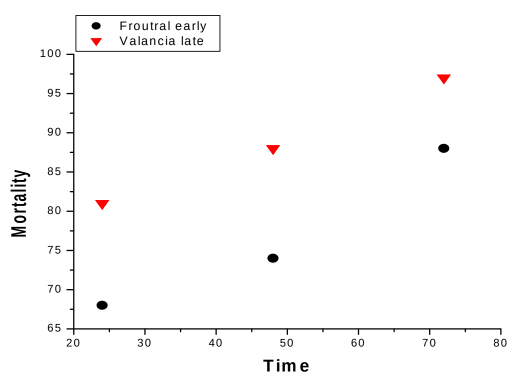

Fig. 2. Percent mortality of Freutrall early and Valencia late against *Aedes albopictus* larvae after 24, 48 and 72 hours

Discussion

Plant products (Rutaceae) combat problems associated with public health especially mosquitoes that provide valuable replacement of dangerous chemicals (Silva et al. 2003), therefore the present study was planned and ether extract of two citrus cultivars (freutrall early and Valencia late) have been selected against *Ae. albopictus* larvae instead of synthetic insecticides as *Ae. albopictus* is reported resistant (Khan et al. 2011). Plants are well known to contain a complex of chemicals with bioactive potential (Farnsworth and Bingel, 1977) like deterrents or attractants (Fisher 1991). Secondary metabolites like limonoids contain larvicidal and anti-feedant activity, therefore, the citrus oils that contain limonoids possess more potential than citrus varieties without limonoid contents (Roy and Saraf, 2006) as they arrest the metabolic activities of the larvae (Senthilkumar et al. 2009). Our results clearly indicate that nomilin is better of the two tested limonoids (Limonin and Nomilin) (Table 1). Ruberto et al. (2002) reported limonin as the best of all extracted limonoids against *Spodopter* spp. Our results on the larvicidal effect is comparable with the study of Akram et al. (2010), Din et al. (2011) that citrus cultivars have potential to kill the larvae of *Ae. albopictus*. The findings of our results are also in collaboration with the findings of Sumroiphon et al. (2006) who have reported that the effect of water extract of citrus seed extract showed LC_{50} values of 135, 319.40 and 127, 411.88 ppm against the larvae of *Culex quinquefasciatus* and *Ae. aegypti*. Similarly Bagavan et al. (2008) have reported that peel chloroform extract of *Citrus sinensis*, leaf ethyl acetate extracts of *O. sanctum* and *O. canum*, and leaf chloroform extract of *Rhinacanthus nasutus* against the larvae of *Anopheles subpictus* (LC_{50}= 58.25, 88.15, 21. 67 and 40.46 ppm, LC_{90}= 298.31, 528.70, 98. 34 and 267.20 ppm) and peel methanol extract of *Citrus sinensis*, ethyl acetate extracts of *O. sanctum*, leaf methanol extract of *O. canum* and *R. nasutus* against the larvae of *Cu. tritaeniorhynchus* (LC_{50}= 38.15, 72.40, 109.12, and 39.32 ppm, 90= 184.67, 268.93, 646.62, and 176.39 ppm), respectively and the extracts from peel of different citrus varieties had good larvicidal effect with lemon peel oil as the best (Mwaiko et al. 1994). However further studies are required to confirm its action.

Acknowledgements

The funds provided by Higher Education Commission (HEC) Pakistan to carry out this project are highly acknowledged. The authors declare that there is no conflict of interest.

References

Abbot SW (1925) A method of competing the effectiveness of an insecticide. J Econ Entomol. 18: 265–267.

Akram W, Hafiz AAK, Faisal H, Hazrat B, Yeon KK, Jong-jin L (2010) Potential of citrus seed extracts against dengue Fever mosquito, A*edes albopictus* (skuse) (Diptera: Culicidae). Pak J Bot. 42(4): 3343–3348.

Ali N, Nadeem A, Anwar M, Tariq WU, Chotani RA (2006) Dengue fever in malaria endemic areas. J College Phy Surg Pak. 16: 340–342.

Anonymous (2011) Disease early warning system and response in Pakistan. Weekly E-pidemiol Bulletin Special Suppl. 2(3): 1.

Bagavan A, Kamaraj C, Abdul RA, Elango G, Abduz-Zahir A, Pandiyan G (2008) Evaluation of larvicidal and nymphicidal potential of plant extracts against *Anopheles subpictus* Grassi, *Culex tritaeniorhynchus* Giles and *Aphis gossypii* Glover. Parasitol Res. 104(5): 1109–1117.

CDC (Centers for Disease Control) (2001) Information on *Aedes albopictus*. Arboviral Encephalitides. Available at: http://www.cdc.gov/ncidod/dvbid/arbor/albopic_new.html. (15 March, 2009).

Certin H, Erler F, Vanikoglu A (2004) Larvicidal activity of botanical natural products, AkseBio2, against *Culex pipens*. Fitoterapia. 75: 724–728.

Champage DE, Koul O, Isman MB, Scudder GGE, Towers GHN (1992) Biological activity of limonoids from rutales. Phytochemist. 31: 377–394.

Chan YC, Salahuddin NI, Khan J, Tan HC, Seah CL, Li J (1995) Dengue haemorragic fever outbreak in Karachi Pakistan 1994. Tran Roy Soc Trop Med Hyg. 89: 619–620.

Din S, Waseem A, Hafiz AAK, Aftab H, Faisal H (2011) Citrus Waste-Derived Essential Oils: Alternative Larvicides for Dengue Fever Mosquito, *Aedes albopictus* (Skuse) (Culicidae: Diptera). Pak J Zool. 43(2): 367–372.

Farnsworth NR, Bingel AS (1977) Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutic Activity. Springer Press, Berlin.

Finney DJ (1989) Probit analysis, 3rd ed. Cambridge Great Britain University Press, UK.

Fisher PR (1991) The role of gaseous metabolites in phototaxis by *Dictyostelium discoideum* slugs. FEMS Microbiol Letter. 77: 117–120.

Forget O (1989) Pesticides, necessary but dangerous poisons. The IDRC Reports. 18: 7–13.

Gutierrez C, Fereres A, Reina M, Cabrera R, Gonzalez-Coloma A (1997) Behavioral and sublethal effects of structurally related lower terpenes on *Myzus persicae*. J Chem Ecol. 23: 1641–1650.

Halawa SM (2001) Studies on the use of some plant extracts as factors in pest management. [PhD thesis]. Faculty of Agriculture, Moshtohor Zagazig University, Benha Branch.

Isman MB (1999) Pesticides based on plant essential oils. Pesti Outlook. 2: 68–72.

Junwei Z, Xiaopeng Z, Yanma Z, Ting L, Kuen Q, Yuhua H, Suqin X, Tucker B, Schultz G, Coats J, Rowley W, Aijun Z (2006) Adult repellency and larvicidal activity of five plant essential oils against mosquitoes. J Am Mosq Control Assoc. 3: 515–522.

Kalyanasundaram M, Das PK (1985) Larvicidal and Synergistic activity of plant extracts for mosquito control. The Indian J Med Res. 82: 19–23.

Khan HAA, Waseem A, Khurram S, Shaalan EA (2011) First report of field evolved resistance to agrochemicals in dengue mosquito, *Aedes albopictus* (Diptera: Culicidae), from Pakistan. Parasit and Vect. 4: 146.

Klocke JA, Kubo I (1982) Citrus Limonoids by product as insect control agent. Entomol Experiment et Applicata. 32: 299–301.

Kutchan T, Dixon RA (2005) Secondary metabolism: nature, chemical reservoir under deconvolution. Current Opinion in Plant Biol. 8: 227–229.

Langenheim JH (1994) Higher plant terpenoids: a phytocentric overview of their ecological roles. J Chem Ecol. 20: 1223–1280.

Mohtar M, Yarmo M, Kardri A (1999) The effects of *Warium indicum* leaf extract on *Aedes aegypti* larvae. J Trop Forest and Prod. 5: 87–92.

Morris P, Robbins M (1997) Manipulating the chemical composition of plants. Iger Innovate. 12–15.

Mwaiko GL, Savaeli ZX (1994) Lemon peel oil extract as mosquito larvicide. East African Med J. 71: 797–799.

Nandita C, Ghosh A, Chandra G (2008) Mosquito larvicidal activities of *Solanum villosum* berry extract against the dengue vector *Stegomyia aegypti*. BMC Comp and Alter Med. 8: 10–15.

Ohta H, Fong CH, Berhow M, Hasegawa S (1993) Thin-layer and high-performance liquid chromatographic analyses of Limonoids and limonoid glucosides in Citrus seeds. J Chromatography A. 639(2): 295–302.

Peters W, Geoffrey P (2007) Atlas of Tropical Medicine and Parasitology. 6th Edition. MOSBAY Elsevier Limited. pp. 1.

Rohloff J (2003) Cultivation of herbs and medicinal plants in Norway-essential oil production and quality control. [PhD Dissertation]. The Plant Biocentre, Department of Biology, Faculty of Natural Sciences and Technology, Norwegian University of Science and Technology, NTNU.77., 57.

Roy A, Saraf S (2006) Limonoids: overview of significant bioactive triterpenes distributed in plants kingdom. Bio and Pharma Bulletin. 29(2): 191–201.

Ruberto G, Renda A, Tringali C, Napoli EM, Simmonds MJ (2002) Citrus limonoids and their semisynthetic derivatives as antifeedant agents against *Spodoptera frugiperda* Larvae. A structure–activity relationship study. J Agri and Food Chemist. 50(23): 6766–6774.

Sarker SD, Latif Z, Gray AI (2005) Natural product isolation. In: Sarker SD, Latifi Z and Gray I (Eds): Natural product isolation. Humana Methods in biotechnology, 2nd edition. 20 Press, Totowa, New Jersey, pp. 1–25.

Senthilkumar N, Varma P Gurusubramanian G (2009) Larvicidal and adulticidal activities of some medicinal plants against the Malarial Vector, *Anopheles stephensi* (Liston). Parasitol Res. 104: 237–244.

Severini C, Rom R, Marinucci M, Rajmond M (1993) Mechanisms of insecticide resistance in field populations of *Culex pipiens* from Italy. J Am Mosq Control Assoc. 9: 164–168.

Shaalan EAS, Canyon DV, Bowden B, Younes MWF, Wahab HA, Mansoin AH (2006) Efficacy of botanical extracts from *Callitris glaucophylla* against *Aedes aegypti* and *Culex annulirostris* mosquitoes. Trop Biomed. 2: 180–185.

Silva I, Zanon G, Silva HHG (2003) Larvicidal activity of *Copaifera reticulata* Ducke Oil-Resin against *Culex quinquefasciatus* say (Diptera: Culicidae). Neotrop Entomol. 32: 729–732.

Sohail E, Ahmed E, Kiku M, and Chae WL (2005) Limonoids are as cancer chemo preventive agents. J Sci Food Agri. 3: 339–345.

St. leger RJ, Joshi L, Bidochka MJ, Roberts DW (1996) Constriction of an improved mycoinsecticide over expressing a toxic protease. Proceed. Nat Academy Sci. USA. 93: 6349–6354.

Sumroiphon S, Yuwaree C, Arunlertaree C, Komalamisra N, Rongsriyam Y (2006) Bioactivity of citrus seed for mosquito-borne diseases larval control. Southeast Asian J Trop Med Publ Health. 37(3): 123–127.

Tholl D (2006) Terpene syntheses and the regulation, diversity and biological roles of terpene metabolism. Current Opinion Plant Biol. 9: 297–304.

Vogel AI (1978) Text Book of Practical Organic Chemistry. The English Language Book Society and Longman, London.

World Health Organization (2005) Guidelines for laboratory and field testing of mosquito larvicides. Communicable Disease Control, Prevention and Eradication, WHO Pesticide Evaluation Scheme, Geneva, Switzerland (WHO/CDS/WHOPES/GCDPP/2005.13).