ISSN: 2141 – 3290
www.wojast.com

EVALUATION OF MINIMUM LETHAL CONCENTRATIONS (MLCs) OF PHYTOCHEMICAL LARVICIDE EXTRACTS FROM PEELS, PULP AND SEEDS OF FIVE SPECIES OF *CITRUS* FRUITS.

EFFIOM, O. E.[1] AND AVOAJA, D. A[2]
[1]*Department of Biological Sciences,*
Veritas University, Abuja, Federal Capital Territory.
[2]*Department of Biological Sciences,*
Michael Okpara University, Umudike, Abia State.

ABSTRACT: This collaborative study to evaluate the minimum lethal concentrations (MLCs) of phytochemical larvicide extracts from peels, pulp and seeds of *Citrus sinensis, Citrus limonum, Citrus aurantifolia, Citrus reticulata and Citrus vitis*, was undertaken in support of the on-going global search for phytochemical alternatives to synthetic chemical insecticides which have lost their effectiveness in malaria vector control. Peels, pulp and seeds were obtained from these fruits, processed and air-dried in the laboratory for 21 days avoiding contamination. The materials were ground into powder form, and then subjected to phytochemical extraction using Soxchlet extraction method with Diethyl ether as solvent at temperature range of 60^0C-80^0 Cfor 6 hours. The extracts were left overnight for the remaining ether in them to evaporate. The active compounds were qualitatively and quantitatively determined using appropriate standard methods. Five different concentrations were prepared (volume for volume), 5%, 10%, 15%, 20% and 25% from each extract stock and tested on live and active mosquito larvae and pupae, respectively, to determine their minimum lethal concentrations (MLSc). The results were matched with each other, and with those of the control, Altosid Liquid Larvicide, (a commercial water-soluble larvicide) using Chi-square test. The observed variations in minimum lethal concentrations (MLCs) exhibited by the different extracts were statistically significant ($P < 0.05$). These differential MLCs of the extracts may be due to the corresponding variations in their active ingredients contents. There was also a significant variation ($P < 0.05$) between the 5% MLCs of the extracts from peels and the control larvicide (Altosid Liquid Larvicide). This implies that the phytochemical extracts have proved more efficacious in killing mosquito larvae and pupae than the control larvicide used in this study.

INTRODUCTION

The discovery of resistance to chemical insectidices (like DDT) between 1946 – 1947 (Herringway and Ranson, 2000), and the recent report that over 500 species of arthropods are resisting various insecticides (Shelton, *et al*., 2007) had been the motivating factor behind the global on-going search for phytochemicals as the best possible alternative to synthetic insecticides in the nearest future. Phytochemicals are relatively safe, inexpensive, environmentally friendly, and are readily available through out the world (ICMR, 2003).

It has also been suggested that the screening of locally available medicinal plants for mosquito control would not only generate local employment, but also reduce dependence on expensive imported products and stimulate local efforts to enhance public health (Bowers *et al*., 1995). The use of citrus fruits as source of phytochemicals in the sustainable control of insect vectors would help effectively to clear the unutilized bulk and heaps of citrus fruits dumped as waste in our indigenous markets and citrus plantations.

A review of literature on control of different species of mosquito showed that evaluation of different phytochemicals from various plants have been carried out by a number of researchers on vector control (Sukumar *et al*., 1991), but with very minimal assessment of insecticidal potentials of various species of citrus fruits. *Ageratina adenophora* (Spreng) have been sufficiently exploited against mosquito species of *Aedes aegypti* and *Culex quinquafasciatus* (Rajmohan and Ramaswamy, 2007), *Ocimum sanctum* Linn. have shown larvicidal and repellent activities against *A. aegypti*, and Neem seed kernel extracts have been used as larvicides against *A. aegypti* (Green *et al*., 1991; Palsson and Janeson, 1999). Also, a detailed laboratory study on extracts of fruits of *Piper nigrum* Linn. against larvae of *Culex pipines*, *A. aegypti* and *A. togoi* have also been reported (Park *et al.*, 2002).

It is therefore in this regard that this collaborative work was undertaken to add to the knowledge of efficacy of active compound from citrus species in sustainable control of mosquitoes, the vectors of malaria.

MATERIALS AND METHOD

Sources of Citrus Fruits

Citrus fruits obtained from indigenous markets in Calabar, Cross River State.

Extract Preparation

The fruits were first washed clean under tap water to remove sand and other possible contaminants. The epicarp was peeled off as thinly as possible unto clean white sheets of cardboard paper. The entire endocarp (all pulp) of each peeled fruit was removed and the juice in it was exhaustively expressed out, and the moist chaffs were spread on another sets of white sheets of cardboard papers. The seeds too were removed, washed and spread similarly. The cardboard sheets carrying the peels, pulp and seeds were left on the laboratory benches for 21 days to air-dry. Maximum care was taken to avoid contamination from the laboratory environment. At the end of the three weeks the air-dried peels, pulp and seeds were ground into powdered materials using adequately sterilized Corona manual grinding machine and stored separately in air-tight glass bottles ready for oil extraction. The volatile oils were exhaustively extracted from the materials by means of Soxhlet extractor using diethyl ether as solvent at temperature range of between 60^0C and 80^0C for 6 hours. The extracts were thereafter left overnight at room temperature of $28.5^0C \pm 3$ for the remaining ether in it to evaporate.

Preparation of Mosquito Culture

The mosquito larvae used in this study were cultivated. Three 5-litre transparent plastic buckets were filled with clean tap water and placed at slightly exposed and undisturbed places behind the students' hostels in the Calabar Campus of Cross River University of Technology. They were allowed to stay for 2 weeks for mosquitoes to oviposit and breed sufficiently in them after which they were then taken to the laboratory for mosquito larvae harvest.

Determination of Active Components in Fruit Extracts

The active ingredients of the fruit samples were determined using appropriate standard methods as described by Harborne (1998) for alkaloids and phenolics, Van Burden and Robinson (1981) for tannins, Obadoni and Ochuko (2001) for saponins, and Boham and Kocipai (1974) for flavonoids.

Determination of Minimum Lethal Concentration

Five different concentrations (5%, 10%, 15%, 20% and 25%) were prepared v/v from the respective extract stocks of peels, pulp and seeds. Correspondingly, five sets of transparent plastic containers labeled 5%, 10%, 15%, 20% and 25% were filled with 500mls of water. 20 live and active mosquito larvae and pupae caught from a mosquito culture by means of a dropper were transferred into each container of water thereafter. 100ml of each extract concentration was poured accordingly into the containers. The time the extracts were added was noted.

Another set of five containers of the same type, size and volume, holding the same quantity of water (500ml) were arranged on another bench. The same number of active mosquito larvae and pupae from the same mosquito culture source was introduced into the containers as before. In this latter setup, five different concentrations of the control larvicide (a commercial Altosid Liquid Larvicide) were prepared and poured into the respective containers. The preparations were allowed to stand for 30 minutes for larval and pupal death. The least concentration that killed all the larvae and pupae in its container was noted as the minimum lethal concentration (MLC) of that extract. The exercise was repeated and the averages were computed and plotted. The experiments were conducted under normal laboratory temperature.

Analysis of Data

The data obtained in this study were subjected to statistical analysis using the Chi-square test (of SPSS Model 15), to ascertain the significance of the observed variations in MLCs of the different phytochemical extracts compared, and with those of the control Altosid liquid larvicide.

RESULTS

The results show that for *Citrus sinensis,* only volatile oil extracts from peels and pulp exhibited minimum lethal concentrations (MLCs) (Fig. 1).

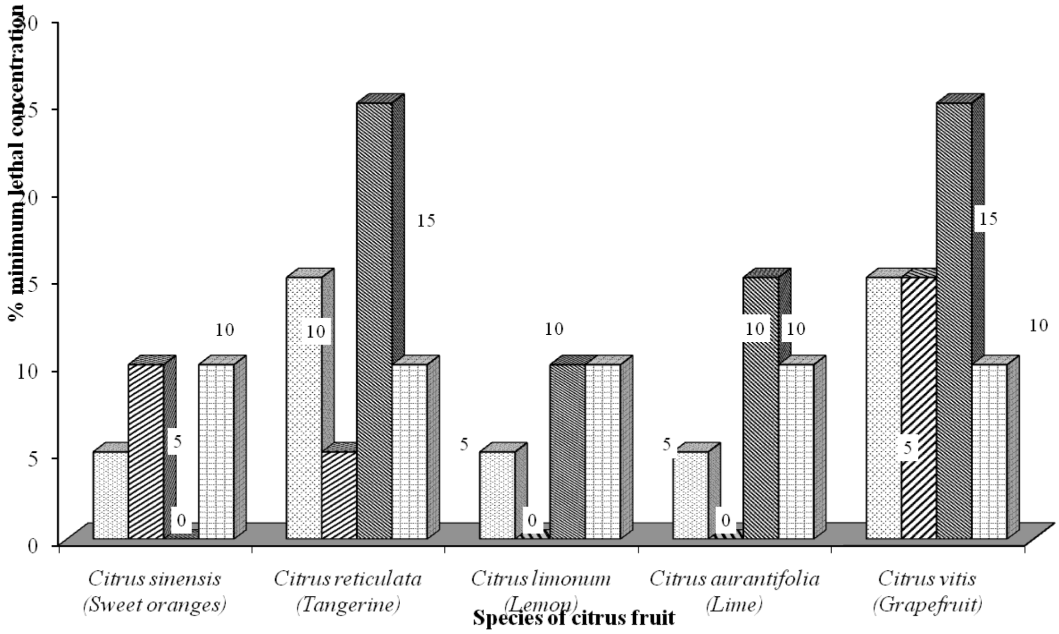

Fig. 1: Minimum lethal concentrations of the volatile oil as shown by susceptibility of the mosquito larvae

The concentrations from seeds of *C. sinensis* did not show any lethal effect on the larvae and pupae as these test organisms were found wriggling and swimming actively in all their containers. Statistical comparison of the MLCs of the extracts showed a significant difference in action of the fruit extracts ($P < 0.05$). Figure 1 depicts that extracts from peels, pulp and seeds of *Citrus reticulata* (tangerine) produced lethal effect with minimum lethal concentrations (MLCs) at 15%, 5% and 25% extract concentration. Those from *Citrus limonum* (lemon) showed minimum lethal concentrations (MLCs) when 5% of peels extract was used, none for pulp and 10% for seeds on the test organisms. For *Citrus aurantifolia* (lime), the MLCs were obtained at 5% for peels, none for pulp and 15% for seeds. Extracts from peels, pulp and seeds of *Citrus vitis* (grapes) showed MLCs of 15%, 15% and 25%, respectively. The control experiments exhibited MLC at 10% level of concentration..

Similarly, a statistical match of the MLCs of the citrus larvicide extracts with each other, and with those of the control Altosid liquid larvicide using Chi-square test, revealed a significant difference ($P < 0.05$)

The analysis of the phytochemical extracts for active ingredients revealed the presence, in varying quantities, of alkaloid, saponin, tannin, phenolic and flavonoid compounds (Tables 1 and 2)

Table 1: Qualitative determination of the active compounds present in the phytochemical extracts from peels, pulp and seeds of the five species of citrus fruits.

Chemical Compound Determined	Species of Citrus fruit	Sources of phytochemical extracts/quality of active compound. Peels	Pulps	Seeds
	C. sinensis (Sweet orange)	+ve	+ve	-ve
	C. reticulata (Tangerline)	+ve	+ve	-ve
Alkaloids	*C. limonum* (Lemon)	+ve	+ve	-ve
	C. aurantifolia (Lime)	+ve	+ve	+ve
	C. vitis (Grape fruits)	+ve	+ve	-ve
	C. sinensis (Sweet orange	+ve	+ve	-ve
	C. reticulata (Tangerine)	+ve	-ve	+ve
Flavonoids	*C. limonum* (Lemon)	+ve	-ve	+ve
	C. aurantifolia (Lime)	+ve	-ve	+ve
	C. vitis (Grape fruits)	+ve	-ve	+ve
	C. sinensis (Sweet orange)	-ve	-ve	+ve
	C. reticulata (Tangerine)	-ve	+ve	-ve
Saponins	*C. limonum* (Lemon)	+ve	-ve	+ve
	C. aurantifolia (Lime)	+ve	-ve	+ve
	C. vitis (Grape fruits)	-ve	-ve	-ve
	C. sinensis (Sweet orange)	+ve	+ve	+ve
	C. reticulata (Tangerine)	-ve	-ve	-ve
Tannins	*C. limonum* (Lemon)	+ve	+ve	-ve
	C. aurantifolia (Lime)	+ve	+ve	+ve
	C. vitis (Grape fruits)	-ve	-ve	+ve
	C. sinensis (Sweet orange)	+ve	-ve	+ve
	C. reticulata (Tangerine)	+ve	+ve	-ve
Phenolics	*C. limonum* (Lemon)	+ve	-ve	+ve
	C. aurantifolia (Lime)	+ve	+ve	+ve
	C. vitis (Grape fruits)	+ve	+ve	+ve

Key: +ve means present; -ve means absent.

Treatment of live and active larvae and pupae with different concentrations of these phytochemical extracts from citrus fruit species revealed significance difference ($P < 0.05$) in their minimum lethal concentrations (MLCs). Only extracts from peels of *C. sinensis, C. limonum, C. aurantifolia,* and pulp of *C. reticulata* recorded 5% MLCs, meaning that they are more efficacious or potent because the extracts were able to kill all the test organisms of low concentrations. A match of the MLCs of the extracts with that of the control larvicide (Altosid Liquid Larvicide) showed that the extracts from the peels of *C. sinensis, C. linonum and C. aurantifolia,* and the pulp of *C. reticulata,* proved efficaciously more promising ($P > 0.05$) than the commercial larvicide (Altosid) used as control, with MLC as 10%.

Table 2: Quantitative determination of the active compounds present in the phytochemical extracts from peels, pulp and seeds of the five species of citrus fruits.

Chemical Compound Determined	Species of Citrus fruit(mg/l)	Sources of phytochemical extracts and quantity of active compound		
		Peels	Pulps	Seeds
	C. sinensis (Sweet orange)	0.005	0.005	0.010
Alkaliods	*C. reticulata* (Tangerine)	0.010	0.010	0.005
	C. limonum Lemon)	0.010	0.015	0.015
	C. aurantifolia (Lime)	0.005	0.005	0.010
	C. vitis (Grape fruits)	0.010	0.005	0.040
	C. sinensis (Sweet orange)	0.040	0.020	0.010
Flavonoids	*C. reticulata* (Tangerine)	0.040	0.005	0.015
	C. limonum (Lemon)	0.020	0.010	0.025
	C. aurantifolia (Lime)	0.025	0.030	0.010
	C. vitis (Grape fruits)	0.050	0.030	0.010
	C. sinensis (Sweet orange)	0.030	0.030	0.035
	C. reticulata (Tangerine)	0.050	0.070	0.040
Saponins	*C. limonum* (Lemon)	0.055	0.045	0.035
	C. aurantifolia (Lime)	0.050	0.055	0.045
	C. vitis (Grape fruits)	0.050	0.050	0.020
	C. sinensis (Sweet orange)	0.350	0.435	0.049
	C. reticulata (Tangerine)	0.330	0.351	0.072
Phenolics	*C. limonum* (Lemon)	0.046	0.229	0.061
	C. auarantifolia (Lime)	0.078	0.361	0.036
	C. vitis (Grape fruits)	0.240	0.351	0.033
	C. sinensis (Sweet orange)	0.65	0.045	0.100
Tannins	*C. reticulata* (Tangerine)	0.49	0.160	0.015
	C. limonum (Lemon)	1.21	0.400	0.020
	C. aurantifolia (Lime)	1.30	0.015	0.005
	C. vitis (Grape fruits)	0.75	0.010	0.005

Significance: ($P < 0.05$)

The efficacy of these extracts against mosquito larvae and pupae might be attributed to the presence of some active ingredients such as alkaloid, saponin, tannin, phenolic and flavonoid compounds in them. Similarly, a Chi-square comparison of MLCs of the extracts with each other showed significant variations ($P < 0.05$). This implies that the observed variations may be due to the corresponding variations in amount of active ingredients in extracts. This findings has, however, confirmed previous reports in which the biological implications of active compounds in plant natural products were investigated (Okwu, 2004; Okwu and Omodamiro, 2005; Okwu and Morah, 2007a). In one of such studies, Khanna and Kannabiran, (2007), investigated *Hemidesmus indicus, Gymnema sylvestre* and *Eclipta prostrata*, and attributed the mode of action of the larvicidal activity of the phytochemical extracts from of the plants against mosquito larvae to the interaction of saponin molecules with the cuticle membrane of the larvae, which consequently alters or disorientates the membrane and thus leads to larval death. It is suggested that as this happened there may be deficiency of dissolved oxygen owing to the presence of the antioxidant (saponin molecules). Phenolic molecules have been reported to be excellent facilitators of larvicidal activity against mosquito larvae generally through complex fomation (David *et al.*, 2000; Joseph *et al.*, 2004). The phenolic compounds in citrus are known to exhibit antiseptic, antifungal and antipathogenic properties (Okwu and Morah, 2007b. Similarly, the alkaloids from various plants sources had demonstrated effectiveness against mosquito larvae (Massoud *et al.*, 2007, Lee, 2000; Francois *et al.*, 1996). Also phytochemicals from various plants such as *Ageratina adenophora* (Spreng) had, due to the presence of active ingredients (phenolics, alkaloids, saponin and tannin compounds) proved toxic against larvae of mosquito species of *Aedes aegypti* and *Culex quinquafaciatus* (Rajmohan and Ramaswamy, 2007). Similarly, *Albizia amara*, Boiv and *Ocimum sanctum* Linn equally showed larvicidal and repellent activities against *Aedes aegypti,* while Neem seed kernel extracts demonstrated higher larvicidal activity (Green *et al*., 1991; Palsson and Janeson 1999).

CONCLUSION

Therefore, the observed effect of MLCs of phytochemical extracts obtained from the different citrus fruit parts against the larvae and pupae of mosquitoes observed in this investigation are all due to the presence of active ingredients in the extracts. Similarly, the differential efficacies also observed among the different citrus phytochemical extracts may be a reflection of the corresponding quantitative variation in the active compounds contents of the citrus peels, pulp and seeds.

ACKNOWLEDGEMENTS

Our appreciation goes to the Head, Department of Pure and Applied Chemistry, University of Calabar, Calabar, Mr. Emmanuel Okon Effiom (Assistant Chief Technologist), Dr. Ikpeme, M. E., Department of Biological Sciences, Cross River University of Technology, Calabar, for their contributions during the practical demonstrations of the extracts.

REFERENCES

Bowers, W. S., Sener, B. Evans, P. H. and Erdogani, F. B. (1995). Activity of Turkish medicinal plants against mosquito *Aedes aegyoti and Anopheles gambiae. Insect Science Application.*16: 339-342

Boham, B. A. and Kocipai, A. C. (1974). Flavonoids and condensed tannins from leaves of *Hawaiia vaccinum vaticulatum* and *V. calyscinium. Pacific science.* 48: 458 – 463.

David., J. P., Rey, D., Meyran, J. C. and Marigo, G. (2000). Involvement of lignin-like compounds in toxicity of dietary alder leaf litter against mosquito larvae. *Journal of Chemical Ecology.* 27 (1): 161 – 174.

Francois., G. Van Looveren, M., Timperman, G., Chimanuka., B. Ake Asi, L., Holenz, J. and Bringmann, G. (1996). Lanicidal activity of Napthylisoquinoline alkaloid dioconphylline–A against the malaria vector *Anopheles Stephens. Journal of Ethnopharmacology.* 54 (2-3): 125 – 130.

Green., M. M., singer, J. M., Sutherland, D. J. and Hibbon, C.R. (1991). Larval activity of *Tagetes minuta* (Marigold) towards *Aedes aegypti. Journal of American Mosquito control Association.* 7:282 – 286.

Harborne., J. B. (1998). Phytochemical methods, a guide to modern techniques of plant Analysis. 3rd Edition. Chapman and Hill Ltd., London. 279 pp.

Herringway, J. and Ranson, H. (2000). Insect resistance in insect vectors of human diseases. *Annual Review in Entomology.* 45: 371 – 391.

ICMR (2003). Prospects of using herbal products in the control of mosquito vectors. Indian Centre for Malaria research *Bulletin.* 33(1):1–10

Joseph., C. C., Ndoile., M. M., Malima., R. C. and Nkunya., M. H. (2004). Larvicidal and mosquitocidal extracts, a coumarin, isoflavonoids and pterocarpans from *Neorautan-eniamitis. Transanctions of the Royal Society of Tropical Medicine and Hygiene.* 98 (8):451- 455.

Khanna, V. and Kannabiran, K. (2007). Larvicidal effect of *Hernidesmus indicus, Gymnema sylvestre,* and *Eclipta prostrata* against *Culex quinquafasciatus* mosquito larvae. *African Journal of Biotechnology.* 6 (3): 307-311.

Lee, S.E., (2000). Mosquito Larvicidal activity of Pipernonaline, a alkaloid derived from long pepper *Piper longum Journal of American Mosquito control Association.* 16 (3): 245 – 247.

Massoud, A.M., Labib, I.M. and Rady, M. (2001). Biochemical changes of *Culex pipiens* larvae treated with oil and oleo-resin extracts of Myrrh Commiphora molmol. *Journal of Egyptian Society of Parasitology*. 31 (2): 517-529.

Obadoni, B. O. and Ochuko, P.O. (2001). Studies and comparative efficacy of the crude extracts of some Homostatic plants in Edo and Delta States of Nigeria. *Global Journal of Pure and Applied Science*, 8b: 203 –208.

Okwu, D.E. (2004). Phytochemicals and Vitamins content of indigenous plant species of South Eastern Nigeria, *Journal of Sustainable Agriculture and Environment.* 6:30-37.

Okwu, D. E. and Omodamiro, O. D. (2005). Effect of hexane extracts and phytochemical contents of *Xylopia aethiopica* and *Ocimum gratissimum* on the Uterus of Guinea Pig. *Bioresearch.* 3: 30-37.

Okwu, D. E. and Morah, F. N. I. (2007a). Isolation and characterization of flavonone glucoside 4,5,7-tridioxyflavonone rhamnoglucose from *Garcinia Kola* seed. *Journal of Applied Science.* 7(2):306-309.

Okwu, D.E. and Morah, F. N. I. (2007b). Antimicrobial and phytochemical evaluation of seed of *Garcinia Kola seed and Dennettia tripetala* fruits. *Journal of Medicinal and Aromatic Plant Science*. 29: 20-23.

Palsson, K. and Janeson, T. G. T. (1999). Plant products used as mosquito repellents in Guinea Bissu, West Africa, *Acta Tropica.* 72: 39-52

Park, I. K., Lee S. G., Shin, S. C., Park, J. A. and Alm Y.J. (2002). Larvicidal activity of isobutylalanine identified in *Piper nigrum* fruit against three mosquito species. *Journal of Agricultural Food Chemistry* 50: 1866-1870.

Rajmohan, D. and Ramaswamy, M. (2007). Evaluation of Larvicidal activity of the leaf extract of a weed plant, *Ageratina adenophora,* against two important species of mosquitoes, *Aedes aegypti* and *Culex quinqufasciatus*. *African Journal of Biotechnology.* 6 (5): 631-638.

Shelton, A. M., Roush, R. T., Wang. P. and Zhao, J. Z. (2007). Resistance to insect pathogens and strategies to manage resistance. An update. In: *Field Manual of Techniques in Invertebrate Pathology*. Lacey, L.A. and Kaya, H.K. (Editors). 2nd Edition. Ktuwar Academics, Dortrecht, The Netherlands., pp. 793-811.

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

Van Burden T. P. and Robinson, W. C. (1981). Formation of complexes between protein and tannin acid. *Journal of Agricultural Food Chemistry* 1:78.