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Evaluation of the Kill Times of Minimum Concentrations of Extracts from Citrus Fruit Peels, Pulp and Seeds Tested Against Mosquito Larvae and Pupae

Effiom, O. E.
Ph.D (Corresponding Author)
Department of Biological Sciences, Veritas University (The Catholic University of Nigeria)
Abuja, Federal Capital Territory, Nigeria

Abstract:- The kill times of the minimum lethal concentrations (MLCs) of extracts from peels, pulp and seeds of five species of citrus fruits tested against mosquito larvae were evaluated. To obtain the phytochemicals from these citrus fruit parts Soxchlet extraction using Diethyl Ether as solvent was carried out at a temperature range between 60^{o}C and 80^{o}C for 6hours. Five concentration grades of 5%, 10%, 15%, 20% and 25% were prepared, and 100ml of each was poured into a transparent plastic container holding 20 live active mosquito larvae in 500ml of clean water. A control was set up with the same concentrations of Altosid liquid larvicide. The time each concentration was added was noted; the interval between the time of application of extract to the container of water holding the larvae and the time all the 20 larvae in any of the concentrations died were recorded. The peel extracts of *C. sinensis, C. limonum, C. aurantifolia,* and pulp extract of *C. reticulata* showed minimum lethal concentrations (MLCs) of 5% each, with respective kill times (KTs) of 2, 3, 2 and 1 minute, compared with control in which MLC was 10% and KT of 4 minutes. The extracts were more effective than the control. Besides, Pearson correlation test to ascertain relationship between KT and quantitative composition of active ingredients in the phytochemical extracts proved significant (P < 0.01).

Keywords:- *Citrus Fruits, Phytochemical Extracts, Minimum Lethal Concentration, Kill Time, Mosquito Larvae, Altosid Liquid Larvicide.*

I. INTRODUCTION

There is a global concern arising from emergence of resistant sibling species of malaria vectors to chemical insectidices (like DDT) between1946–1947 (13), and especially recent reports that more than 500 species of arthropods have develop resistance against the various types of insecticides (21). Therefore, the current trend in the search for phytochemical alternatives to replace synthetic insecticides in the nearest future is a welcome development. Phytochemicals generally are relatively safe, inexpensive, environment friendly, and are readily available throughout the world (5, 1, 17).

Previous studies had reported that the screening of local plants of medicinal importance for mosquito control would beside providing local employment, but also limit dependence on expensive imported products as well as enhancing public health (12). One of the significance of exploiting citrus fruits for sustainable control of the developmental stages of insect vectors is that would help in recycling of the bulks of unutilized citrus fruits which are often found littered in our various market environments and citrus plantations.

Even though various plants have been screened for insect vectors control, little or no consideration had been given to the exploration of insecticidal and larvicidal properties of citrus fruits. (22). A study by Park *et al*., (19) revealed the exploitation of *Ageratina adenophora* (Spreng) against of *Aedes aegypti* and *Culex quinquafasciatus* while *Ocimum Sanctum* Linn. extracts were said to have shown larvicidal and repellent activity against *A. aegypti*. In earlier research Neem seed kernel extract was used as larvicides against *A. aegypti* (5) and this finding was confirmed in later years by (3). Similarly, a laboratory study with extracts of fruits of *Piper nigrum* Linn., demonstrated efficacy against larvae of *Culex pipines*, *A. aegypti* and *A. togoi* (18).

It has also been found that in spite of the high global interest and concentration in developing herbal alternatives to the currently easily resisted synthetic insecticides, pesticides, fungicides, etc., little or no attention had been given to the evaluation of the kill time (KT) of the different concentrations of the various herbal products so far developed. The importance of KT in the assessment of the efficacy or activity of a given concentration of a phytochemical extract against any biological agent cannot be overemphasized.

It is therefore this commonly neglected aspect of knowledge that this work was undertaken to address, and with a view to adding to knowledge of efficacy, kill time factor as exhibited by the different concentrations of extracts from the different citrus fruit parts in the control of mosquito in Calabar, Cross River State, Nigeria.

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II. METHODS

➢ *Source of the Citrus Fruits*

Citrus fruits were obtained in October, 2015, from Ika Ika Oqua, Mbukpa and Watt markets in Calabar, Cross River State. They were identified into species using the identification keys recommended by Swingle and Reece (25); Hodgson (28); Wardowski *et al.* (24); Morton (26); Cottin (29); Koskinen (27).

➢ *Processing of the Citrus Fruits for Phytochemical Extraction*

The fruits were prepared for phytochemical extraction by first washing them thoroughly in running tap water to remove sand other contaminants. The epicarp of the washed fruits was carefully peeled off as thinly as possible unto clean white sheets of cardboard paper. The entire endocarp (or pulp) of each peeled fruit was removed and after the juice in it has been exhaustively expressed out, and the moist chaffs were spread on another set of white cardboard papers. The seeds were gathered together, washed thoroughly and spread similarly. The cardboard sheets carrying the processed fruit parts were left on the laboratory benches for 21 days to air-dry. Adequate precautions were taken to avoid contamination from the laboratory environment. After the 3 weeks air-drying they were ground into powder using adequately sterilized Corona manual grinding machine, and stored separately in air-tight glass bottles ready for phytochemical extraction. The volatile oils were exhaustively extracted from the materials by Soxchlet extraction method 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 laboratory temperature of 28 ± 05^0C to enable the remaining Ether in it to evaporate.

➢ *Cultivation of Mosquito Larvae and Pupae*

The test organisms (mosquito larvae) were cultivated using 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.

➢ *Characterization of Phytochemicals*

The active substances in the phytochemical extracts were determined using the various methods described by Harborne (13) for alkaloids and phenolics, Van Burden and Robinson (14) for tannins, Obadoni and Ochuko (4) for saponins, and Boham and Kocipai (17) for flavonoids.

➢ *Determination of Minimum Lethal Concentrations (MLCs) and Kill Times (KTs)*

Minimum lethal concentrations (MLC) and Kill time (KT) were verified by preparing five (5) different concentrations, 5%, 10%, 15%, 20% and 25%, v/v from the 3 respective extract stocks in distilled water. Five corresponding sets of transparent plastic containers were arranged in the laboratory bench and labeled 5%, 10%, 15%, 20% and 25%, and filled with 500mls of water. Twenty (20) live and active mosquito larvae and pupae caught from a mosquito culture by means of a dropper were transferred into the containers of water. 100ml of each concentration of the extracts were poured respectively into the containers carrying the test organisms. 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 were introduced into the containers as before. However, in this latter setup, five different concentrations of the control water-soluble larvicide (a commercial Altosid Liquid Larvicide) were prepared and poured into the respective containers. The setups were allowed to stand for 30 minutes and curiously observed for larval and pupal death. The least concentration that killed all the larvae and pupae in its container together with the time taken for all the larvae and pupae to die, were noted as the minimum lethal concentration (MLC) and the kill time (KT) of that extract, respectively. The exercise was repeated two more times and the average was computed and plotted.

➢ *Determination of pH of the Extracts*

The pH-meter was used to measure the pH values of the extracts and recorded accordingly.

➢ *Data Analysis*

The relationship between KTs of MLCs and the proportions of active compounds found in the extracts was verified using Pearson Correlation Test.

III. RESULTS

The minimum lethal concentrations of the extracts and the respective kill times are represented in Figure 1. The figure shows that the MLCs of the extracts from peels and seeds *C. aurantifolia* (lime) were 5% and 15% with corresponding KTs of 3minutes and 10minutes. Pulp extracts of *C. aurantifolia* and *C. limonum* did not show any MLC. Extracts obtained from peels and seeds of *C. limonum* recorded MLCs of 5% and 10% with respective KTs of 2minutes and 7minutes. The MLCs and the KTs of phytochemical extracts from the peels, pulp and seeds of *C. reticulata* were 15%, 5% and 25%, and 8minutes, 1minute and 4minutes, respectively. Extracts from the three parts of *C. vitis* all showed very high MLCs and KTs (Figure 1).

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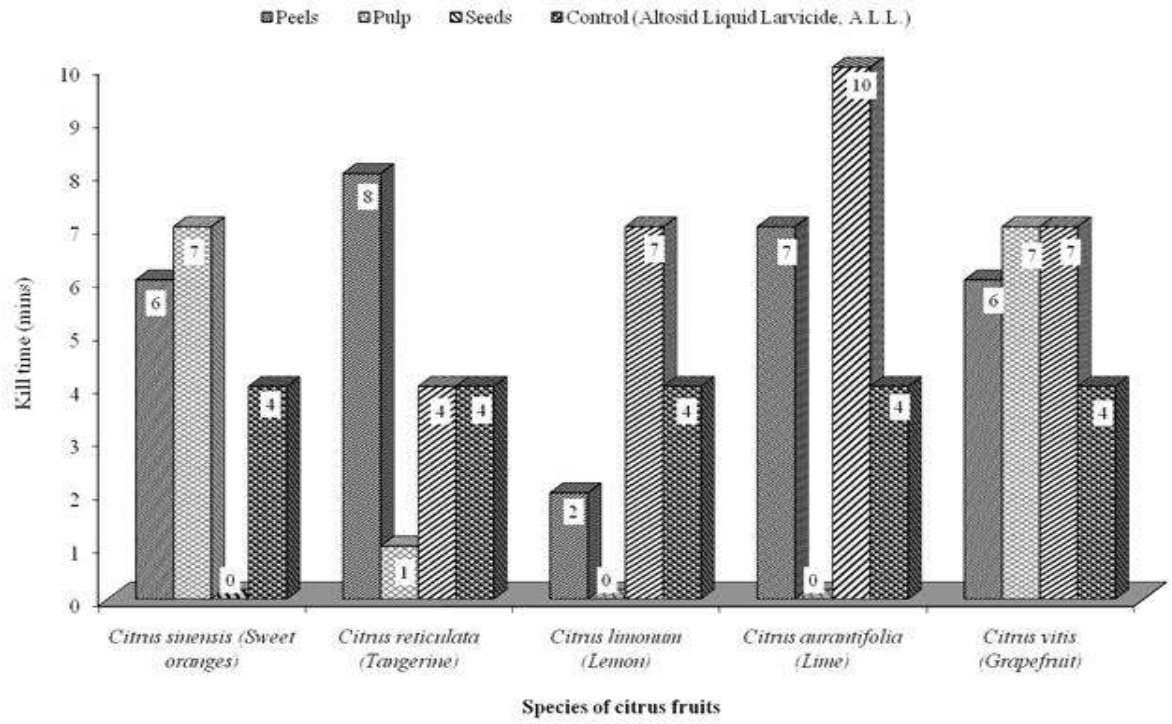

Fig 1:- Kill Time (kt) of Volatile Extracts from Peels, Pulps and Seeds of Citrus Fruit Species

The control (Altosid Larvicide) showed a MLC of 10% and a KT of 4minutes. Comparison of the results of the phytochemical extracts with those of the control revealed that some extracts had proved more efficient killers of mosquito larvae and pupae since even at very low concentrations they still killed faster than the commercial liquid larvicide (Altosid). The quantity of active compounds in the 3 extracts were as recorded in Table 1.

Active Compound	Citrus fruit species (mg/l)	Quantity of Active Compounds in the extract		
		Peels	Pulps	Seeds
Alkaliods	*C. sinensis* (Sweet orange)	0.005	0.005	0.010
	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
Flavonoids	*C. sinensis* (Sweet orange)	0.040	0.020	0.010
	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
Saponins	*C. sinensis* (Sweet orange)	0.030	0.030	0.035
	C. reticulata (Tangerine)	0.050	0.070	0.040
	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
Phenolics	*C. sinensis* (Sweet orange)	0.350	0.435	0.049
	C. reticulata (Tangerine)	0.330	0.351	0.072
	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
Tannins	*C. sinensis* (Sweet orange)	0.65	0.045	0.100
	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

. Table 1:- The Active Compounds Found in the Peels, Pulp and Seeds Extracts of Citrus Fruits Species
Significance: ($P < 0.05$)

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Table 2 reveals the respective pH values of extracts, and it shows that the extracts were all acidic though of different degrees. The peels and pulp extracts of *sinensis, reticulata,* were more acidic, while the moderately acidic ones were those from the *limonium, aurantifolia, vitis*, and pulp of *aurantifolia* and seeds of *C. vitis.* Peel extracts of vitis, limonium and seeds of sinensis, limonium C. vitis as well as pulp of limoniun which was found to be weakly acidic.

Species of Citrus fruit	pH values of the extracts		
	Peels	Pulp	Seeds
C. Sinensis (Sweet orange)	3.99	3.76	5.94
C. reticulata (Tangerine)	3.74	3.90	6.27
C. Limonum (Lemon)	4.24	6.90	5.05
C.aurantifolia (Lime)	4.46	4.10	4.61
C.vitis (Grape fruits)	5.72	3.97	5.33

Table 2:- The Result of pH Measurements of These Volatile Phytochemical Extracts from Peels, Pulp and Seeds of the Five Species of Citrus Fruits, Using the pH–Meter

The result of the data analysis to verify the correlation between KT and the amount of active compounds in the phytochemical extracts gave a significant result ($P < 0.01$).

IV. DISCUSSION

Live and active mosquito larvae and pupae treated with different concentrations of volatile oil extracts showed that the different extracts have different minimum lethal concentrations corresponding with different kill times on the test organisms. It was also observed that volatile oil of peel extracts from *Citrus sinensis, Citrus limonum, Citrus aurantifolia* and the pulp extract from *Citrus reticulata* all exhibited equal least lethal concentrations of 5% but with different kill times of 2 minutes, 3 minutes, 2 minutes and 1minute, respectively. It means that only pulp extract from *Citrus reticulata* has proved most effective in killing the test organisms (larvae and pupae) than those from other citrus fruit species since only 5% minimum concentration of it could kill test organisms in this study. The application of 10% concentrations of extracts from pulp of *Citrus sinensis* and from seeds of *Citrus limonum* both killed the test organisms within 7minutes. Similarly, the 15% concentrations from peel extracts of *C. reticulate, C. vitis,* and seeds and pulp extracts of *C. aurantifolia* and *C. vitis,* respectively, differed in their mosquito larvae and pupae kill times, with KT values of 8minutes, 6minutes, 10minutes and 7minutes, respectively, suggesting that the peels extracts from *C. vitis* kill faster than the corresponding extracts obtained from *C. reticulata*, seeds of *C. aurantifolia* as well as those from pulp of *C. vitis*, even at the same concentrations. The observed immediate or fast killing of test organisms (i.e. within or in less than 1minute) by higher concentrations of the extracts from peels of *Citrus sinensis, Citrus aurantifolia* and *Citrus limonum* can be attributed to the relatively very high concentrations rather than to high potency.

Earlier researches have reported that KT and/or degree of potency of an insecticide are/is the product of many dependent variables. The pH of the liquid insecticide, specific stage of insect's life cycle particularly the immature stages (larvae and pupae of mosquitoes, for instance), the prevailing temperature and humidity, the presence and amount of active ingredients in the extracts, etc., are among such factors that have been identified by scholars of entomology (30, 11, 16). Kill times of a larvicide, according to Woodruff (15), varies with its pH changes even though such variations are sometimes negligible. He reported that under high pH conditions larvicides and insecticides in general, may begin to disintegrate over time, except that the process of such active ingredients breakdown is normally very slow and unnoticeable, thereby making the affected larvicide to still remain effective for some hours or even days. Woodruff further remarked that what may cause such pH change and the subsequent active ingredients disintegration is usually exposure of the larvicide, which may in turn lead to evaporation of the volatile active compounds, and consequently decrease the efficacy and KT of the larvicide. In this study, the relationship between pH values and KT of the volatile oil extracts being investigated was verified using Pearson Correlation coefficient test, and only pH values of extracts from *C. limonum* species indicated significant relationship with KT. All other extracts did not show any relationship with pH values so as to suspect that their kill times (KTs) can be influenced by changes in pH of the extracts. Similarly, Nault and Shelton (3) in their study to investigate factors that influence efficacy of a larvicide reported that in some cases the fast respond of test organisms to minimal concentrations of a larvicide applied on them could be due to their larval stages since they are small and defenceless. Therefore, the reason(s) for the effectiveness and minimal kill times of some of the citrus volatile oil extracts studied in this research may not be unconnected with the these findings of Killeen *et al.*, (2) and Nault and Shelton (3).

The variations in the quantitative and qualitative composition of active ingredients (phenolics, saponins, flavonoids, alkaloids and tannins) also observed among the extracts from the same species of citrus fruit, could equally mean that the amount of active ingredients present in the different parts (peels, pulp and seeds) of the same species of citrus fruits vary. The same findings were made about the distribution of active compounds in the five species of citrus fruits. For example, the levels of flavonoids (in mg/l) found in peels, pulp and seed extracts of *C. limonum* were 0.020, 0.010, and 0.25, respectively. While the amounts of alkaloids in those same parts of the fruits of *C. vitis* were 0.010, 0.005 and 0.040 milligrams of alkaloids per litre, respectively (Table 1).

The relationship between these active chemical compounds in the volatile oil extracts and the kill time of the extracts using Pearson Correlation coefficient test showed very clear disparity among the extracts. Alkaloids also showed varying Correlation coefficients (r) values of 0.082, 0.240, 0.73 and 0.381 in *C. reticulata, C. limonum, C. aurantifolia and C. vitis,* respectively, and significant correlation with their corresponding KTs ($P < 0.01$). Thus, the KT of a citrus volatile oil extract depends to some extent, on the type and amount of active ingredients it contains. Comparatively, this finding have confirmed the results of earlier studies which reported that active ingredients in phytochemical extracts are richly endowed with biological potentials (7, 8, 1, 4, 11, 17, 20).

V. CONCLUSION

Citrus volatile oil extracts are fast killers of mosquito larvae and pupae, and should be adopted in sustainable intervention method of control of mosquito vectors and the deadly diseases they transmit

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