EFFECTS OF GRAPEFRUIT (*CITRUS PARADISI* MACF) (RUTACEAE) PEEL OIL AGAINST DEVELOPMENTAL STAGES OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

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Abstract. Laboratory bioassay of the essential oil extracted from the grapefruit (*Citrus paradisi*) peel by steam distillation was carried out against the developmental stages of the yellow fever vector *Aedes aegypti* to evaluate its toxicity, and ovicidal and larvicidal potency. Volatile oil components isolated and characterized by coupled gas chromatography/mass spectrometry included varying levels of monoterpene aldehydes, alcohols, and esters. Test results of the essential oil showed that egg hatching was completely inhibited at 400 ppm, while further development of 1st to 2nd larval stage was inhibited at 100 ppm. Regression analysis results also indicated that the peel essential oil significantly ($p<0.01$) reduced the viability of the test eggs and inhibited the development of 1st larval stage to 2nd larval instar. The LC_{50} and LC_{90} values obtained for 2nd instars (180.460, 334.629 ppm, respectively); and for 4th instars (210.937, 349.489 ppm, respectively) after 24-hour exposure were time but not dose dependent, as each LC value was a product of an inverse relationship between the oil concentration and exposure time. The results indicated that the peel oil could be a potent persistent larvicide.

Keywords: *Aedes aegypti*, *Citrus paradisi*, larvicide, ovicide, peel oil

INTRODUCTION

Anthropod-borne infections remain major sources of illness and death globally. Mosquitoes alone transmit diseases such as yellow fever, malaria, and dengue to more than 700 million persons annually, while malaria fever kills 3 million individuals each year including one child every 30 seconds (WHO, 2005). Dengue (break-bone fever), including its more severe form, dengue hemorrhagic fever, and yellow fever constitute major arthropod-transmitted diseases vectored principally by *Aedes aegypti*: a day-biting, peridomestic mosquito species with a cosmopolitan range extending from latitude 40° N to 40° S (Toma *et al,* 2011). Dengue and its variant, dengue hemorrhagic fever, exert great impact on affected individuals and communities in terms of morbidity and mortality (Gubler, 2002; Deen *et al,* 2006).

Yellow fever is the prototypical viral hemorrhagic fever caused by a flavivirus closely related to the dengue virus. The clustering of jaundice cases with fatality rates of 10-50% typically highlights the public health importance of yellow fever

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infection (The Dawn Media Group, 2012). Between 1987 and early 1990, Nigeria sustained a series of sylvatic (jungle) and urban outbreaks of yellow fever with a case-fatality rate exceeding 50% (Jentes *et al,* 2011). In addition, between 1987 and 1991, 18,735 yellow fever cases and 4,522 deaths were reported to WHO, mostly from Africa, representing the highest number of yellow fever activity for any 5-year period since 1948 (Robertson *et al,* 1996).

Control of *Ae. aegypti* can be achieved by eliminating or clearing water-holding containers that serve as larval habitats, by application of chemical insecticides, biological control such as using *Bacillus thuringiensis* subspecies *israelensis*, or by using chemical repellents (Eldridge, 2008).

Mosquito control measures are continuously being revised and modified, partly as a result of insecticide resistance acquired by the parasites due to indiscriminate application of synthetic insecticides, and partly due to environmental concerns about toxic affects on non-target organisms (Eldridge, 2008). In Nigeria, vector control programs have relied on the chemical approach with the application of insecticides in artificial containers on the main breeding sites of *Ae. aegypti* mosquitoes (Self and Pant, 1966). Elsewhere, studies on *Ae. aegypti* susceptibility profiles have shown resistance to organophosphates and carbamates (Rawlins, 1998; Vaughan *et al,* 1998; Lima *et al,* 2003; Braga *et al,* 2004).

To avoid mosquito resistance to chemical/synthetic insecticides and to protect the environment and public health, many studies have been conducted on insecticidal plants (Barnard, 1990; Dakhil and Morsy, 1999; Calvacanti *et al*, 2004; Cheng *et al*, 2004; Ivoke and Odii, 2010). Because they constitute a rich source of bioactive chemicals and are commonly employed as fragrances and flavoring agent for foods and beverages, plant essential oils have been suggested as an alternative source of material for insect control (Isman, 2000).

The oils of certain plants have been found to demonstrate potential for controlling larvae of many mosquito species (Cheng *et al,* 2004; Tare *et al,* 2004; Ivoke and Odii, 2010). Barnard (1990) and Cheng and others (2004) have also demonstrated that several plant essential oils not only possess reproduction inhibitory effect against termites and fungi, but also show strong mosquito repellency and larvicidal activities. The toxicity of citrus oils to several stored-product insects have been reported (Su *et al,* 1972) while the larvicidal activities of peel oils of different citrus fruits against mosquitoes have also been demonstrated (Mwaiko, 1992; Mwaiko and Savaeli, 1994; Dakhil and Morsy, 1999; Ezeonu *et al,* 2001).

Grapefruit (*Citrus paradisi* Macf) is a subtropical and tropical citrus tree grown for its bitter fruit. The tree reaches 4.5-6.0 m tall with spreading branches. The leaves are evergreen, ovate (7.5-15.0 cm long, and 4.5-7.5 cm wide), and dark green. The white, four-petal flowers are 4.5-5.0 cm across and are borne singly or in clusters in the leaf axil. The fruit is nearly round or oblate, with smooth, finely dotted peel up to 1 cm thick; pale lemon outside and white spongy and bitter inside (Morton, 1987). The peel is commonly employed in soft-drink flavoring, while the seed can be used for soil conditioning after extracting the oil. An essence prepared from the flowers of grapefruit tree is taken to overcome insomnia, and the pulp is considered an effective aid in the treatment of urinary disorders (Morton, 1987).

In this article we report the effect of

Citrus paradisi Macf peel essential oil extract against the developmental stages of laboratory-reared Nigerian strain of *Ae. aegypti* (L). *Ae. aegypti* was selected because it is a commonly used model Culicine species, and, as a vector of human yellow fever and dengue, it remains a prime target for research on mosquito control.

MATERIALS AND METHODS

Plant materials

Fresh mature grapefruits (*Citrus paradisi*) were harvested from the orchard at St. Teresa's Church premises, Nsukka and taken to the Plant Anatomy Laboratory, Department of Botany, University of Nigeria, Nsukka for taxonomic identification. A voucher specimen was deposited at the herbarium unit of the Department. Each fruit was washed thoroughly with distilled water to remove any adhering sand and grit, stored in cold conditions (10ºC), and processed one week later. Peels of the fruit were obtained by manually, gently pressing halved grapefruits in a juicer, Santos-10 (Santos SAS; Vaulx-en-Velin Cedex, France) and ensuring that no portions of the mesocarp came out with the peels. The peels were subsequently sliced into small pieces to facilitate absorption by the solvent. A total weight of 1,175 g of grapefruit peel was utilized.

Oil extraction

The grapefruit peel essential oil extraction was carried out in the Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka, by steam distillation as described by Cho and others (2002). Briefly, peels (1,175 g) were added to distilled water (500 ml), thoroughly mixed at high speed for 3 minutes in a Waring Pro blender, MX1000R, (Waring Consumer Products, East Windsor, NJ), and submitted for 4 hours to hydrodistillation in an essential oil determination apparatus (Clevenger Type), G11340/1, (Edutek Instrumentation; Ambala Cantt, Haryana, India) to extract the oil. The solvent (n-Pentane) was of analytical grade and was obtained from Aldrich Laboratories (St Louis, MO). After extraction, the solvent was removed by means of rotary evaporator, yielding the desired essential oil of 20%.

Characterization of volatile compounds

One hundred gram of grapefruit peel were pulverized in a Freezer/Mill, 6770 (SPEX SampleI SamplePrep LLC, Metuchen, NJ) for 3 minutes in the Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka; 0.5 g was stirred into 30 ml of 2:1 mixture of pentane/dichloromethane. Phase separation was achieved by centrifugation at 9,000*g* for 5 minutes. Upper organic phase was recovered, dried over anhydrous sodium sulphate and finally concentrated at 37ºC with a 25 cm distillation column to a volume of 2 ml. Solvent extract was diluted (1/10 v/v) in the pentane/dichloromethane mixture. Gas chromatography in the same faculty was used for the analysis of the volatile components. A Varian gas chromatograph, model CX-3400® (Varian, Lexington, MA) was used under the following conditions: carrier gas hydrogen injector (detector flame ionization detector, FID), temperature, 220ºC, and 225ºC, respectively, capillary column (Supelcowax®; Sigma Aldrich Corp, St Louis, MO) ~10, 30 m x 0.32 mm film thickness 0.25/ m); and temperature programmed from 2 minutes at 40-270ºC at 5ºC/minute. The area percentage was obtained on Varian 4400 integrator. The identity of the component was assigned by comparing their linear retention indices with those of authentic samples.

Insect culture

Eggs, larvae, and adult stages of *Ae. aegypti* were obtained from the Nigerian strain maintained at ambient rearing conditions in the entomology laboratory insectary of the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. Pieces of filter paper with singly laid *Ae. aegypti* eggs were placed in plastic trays (50 x 25 x 7 cm) containing 2 liters tap water, and powdered dry yeast and dog biscuit in the ratio of 3:1 as larval food. The larvae were reared to the 3rd and 4th instars and allowed to pupate in the trays. The pupae were collected from the trays and transferred into plastic cups containing tap water and placed in screen cages (23 x 30 x 23 cm) where the adults emerged. Using an aspirator 20 males and 30 females that emerged were introduced into oviposition cages.

Adult males were maintained on 10% aqueous sucrose solution while females were fed blood from an anesthetized albino rat. Oviposition cages were covered with cloth to maintain constant humidity (80.0 ± 5.0%). One bowl containing dechlorinated water was also kept inside the cages to facilitate oviposition by females.

Bioassay

Aliquots of 0.0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 ml of the grapefruit peel essential oil from the stock were diluted with 2 ml Tween® 80 (Sigma Aldrich Corp, St Louis, MO), a surfactant, in 250 ml beakers and further dissolved in 1,000 ml distilled water to obtain 0, 50, 100, 150, 200, 250, 300, 350, and 400 ppm.

Ovicidal activity

Altogether, 320 eggs were obtained from the laboratory-bred mosquito culture. Twenty eggs were assigned to eight treatments of 50, 100, 150, 200, 250, 300, 350, and 400 ppm of the essential oil. Treatments were replicated four times. Controls (0 ppm) received only 2 ml Tween-80. The eggs were observed for hatching and subsequent survival into larval instars. The number of eggs, at each concentration, that hatched into 1st larval instars was counted. Percentage of egg viability/ hatchability was calculated by dividing the number of larva that emerged from the treated eggs 7 days post-treatment exposure, by the total number of eggs deposited. All bioassays were conducted at 30.0 ± 3.0ºC, 76.0 ± 5% relative humidity (RH), and 14:10 (Light:Dark) photoperiod using the method of Tripathi and others (2004).

Larvicidal activity

Larvae of two instars (2nd and 4th) were collected with a Pasteur pipette (selected for good movement) and transferred, 20 per test, with a fine brush to disposable plastic cups (250 ml) containing the test essential peel oil at concentrations of 50, 100, 150, 200, 250, 300, and 400 ppm on WHO (1981) protocols. Eight lots of 20 larvae from each instar per concentration and 4 concentrations per test were used. Controls received only 2 ml Tween 80 solution in 0 ppm of dechlorinated tap water. Treatment and control larvae were held at the same conditions as described above. The percentage of larval mortality was determined at 6-, 12-, and 24- hours after oil incubation at the conditions described earlier. Larvae were considered dead when probing with a fine needle produced no larval response.

Data analysis

The dose-response data of the essential oil were subjected to probit analysis using Polo-Plus® (LeOra Software, Petaluma, CA). Lethal concentrations ($LC_{50,}$ $LC_{90,}$ and LC_{95}) in ppm were interpolated by probit analysis using this software.

Differences among mortalities were detected using Turkey's multiple comparison test. In all cases, the significant level was $p<0.05$.

RESULTS

From the data provided in Table 1, the essential oil extracted from the grapefruit peel is a highly volatile, fairly transparent, viscous yellow liquid with a persistent and fairly pleasant odor. Analysis of the peel oil by gas chromatography/mass spectrometry indicated the presence of abundant levels of numerous volatile constituents, including monoterpene aldehydes with ocimene as the major volatile constituent, along with pinene (4.36%), sabinene (1.42%), and limonene (0.33%) as minor constituents. Monoterpene alcohols, especially linalool (2.06%), were also detected with low traces of monoterpene ester (Table 2).

Table 3 summarizes the toxic (ovicidal) effects of varying concentrations (ppm) of the grapefruit peel essential oil on the hatchability of laboratory-reared eggs of *Ae. aegypti*. At a relatively low concentration of 50 ppm, a mean of 78.7% of test eggs hatched into 1st larval stage, and correspondingly, 21.3% of the eggs were inhibited from hatching compared to the control. Therefore, 21.3% of the test eggs were rendered unviable at this concentration. Increased concentration of the peel essential oil resulted in a gradual decrease in the rate of egg hatching, to the extent that at dose of 400 ppm the hatching process of eggs was completely inhibited by the essential oil. While 72.9% of the 1st larval instars were able to develop to the 2nd instars at 50 ppm, further development into the 2nd larval stage was completely inhibited at 100 ppm of the test essential peel oil. Linear regression analysis prepared on the data showed that grapefruit peel essential oil significantly reduced the viability of *Ae. aegypti* test eggs and also significantly ($p<0.01$) inhibited further development of 1st instars, as evidenced from the percentage of 2nd instar survival values.

Table 1
Physical characteristics of essential oil extracted from grapefruit peel.

Features	Status
Color	Golden yellow
Volatility	Highly volatile
Viscosity	Very viscous
Transparency	Fairly transparent
Odor	Strong, persistent, fairly pleasant
Form	Liquid

Table 2
Relative abundance of some volatile components in essential oil extract from grapefruit peel.

Compound	%
Monoterpene aldehydes	92.50
Ocimene	52.04
Limonene	0.33
Sabinene	1.42
Pinene	4.36
Monoterpene alcohols	4.12
Linalool	2.06
Octanal	0.82
Monoterpene esters	0.22

Lethal concentration values of the grapefruit peel oil against the larval instars of *Ae. aegypti* are shown in Table 4. The effect of the oil on the larvae appeared to be time- and dose-dependent as indicated by the LC_{50} and LC_{90} values that were, for the 2nd instars post-6 hour exposure, 208.89 ppm, and 361.42 ppm, respectively. Similar effects were obtained

Table 3
Effects of grapefruit peel essential oil on hatchability of eggs of *Ae. aegypti.*

Peel oil concentration (ppm)	Total eggs per test	Egg hatching Mean (%)	Survival (2nd instar) Mean (%)
Control	20	100.00 ± 0.00	100.00 ± 0.00
50	20	78.86 ± 0.98	72.90 ± 1.32
100	20	48.04 ± 0.79	0.00 ± 0.00
150	20	25.76 ± 1.20	0.00 ± 0.00
200	20	14.27 ± 1.04	0.00 ± 0.00
250	20	11.03 ± 1.00	0.00 ± 0.00
300	20	8.10 ± 0.80	0.00 ± 0.00
350	20	2.70 ± 0.52	0.00 ± 0.00
400	20	0.00 ± 0.00	0.00 ± 0.00

Regression analysis of data: percent egg hatch: Y=90.49-1.75X, df=8, R^2=0.93, F=522.35, p<0.01; percent larval survival, Y=70.14-1.54, df=8, R^2=0.64, F=246, p<0.01.

Table 4
Effects[a] of grapefruit peel essential oil on larvae of *Ae. aegypti* after varying periods of exposure.

Larval instar	Percent larval mortality	LC (ppm)		
		6 h	12 h	24 h
Second	50	208.89 (183-233)	189.73 (162-214)	180.46 (156-202)
	90	361.42 (326-414)	349.08 (313-402)	334.63 (303-379)
	95	404.65 (362-469)	394.25 (351-460)	378.33 (340-433)
Fourth	50	239.04 (215-265)	221.23 (196-247)	210.94 (188-233)
	90	376.12 (338-432)	364.43 (326-421)	349.49 (316-397)
	95	453.32 (403-532)	478.98 (421-570)	406.72 (364-469)

[a]Effect is presented as mean ± SD (95 % confidence limit).

against the 4th larval stage at 239.04 ppm and 376.12 ppm, respectively. After 24-hour exposure, the LC_{50} and LC_{90} values of the peel essential oil against 2nd and 4th instars showed similar trends in larval mortality as the respective differences only differed by a factor of 1.1. Generally, the 2nd larval stage appeared to be more susceptible to the toxic effect of the grapefruit peel oil than the 4th instar as indicated in the relatively lower LC_{50} and LC_{90} corresponding values.

DISCUSSION

A considerable number of plant essential oils have been known to demonstrate potential toxicity, ovicidal, repellent, and insecticidal activities against various insect species (Saxena, 1989; Isman, 2000). Furthermore, volatile compounds of many plant essential oils have also been shown to consist of alcohols, aldehydes, and terpenoids, and to exhibit fumigant potentials (Coats *et al*, 1991; Kim and Ahn, 2001).

The result of gas chromatograph (GC) and mass spectrometer (MS) analyses of the grapefruit peel essential oil, conducted in our current study indicated the presence of varying proportions of volatile constituents, particularly monoterpene hydrocarbons (92.5%) with ocimene (52.04%) as its major volatile component.

The results of our study are consistent with the findings of Prat *et al* (2001) who demonstrated the presence of some of these constituents in a comparative analysis of essential oils from lemon, sweet orange, and mandarin; and those of Jaenson *et al* (2006) who isolated some compounds and characterized the volatile constituents as potentially insecticidal in nature.

The result of the ovicidal assay suggested that the tested essential oil not only significantly ($p<0.01$) inhibited the hatching of the mosquito eggs at 400 ppm, but also delayed the development of the 1st larval instars to the 2nd larval stage. Mosleh and Abou El-Ela (2011) demonstrated that two natural toxin microcystin and nodularin for the control of *Anopheles multicolor* caused delay in the development of the 4th larval instars. Furthermore, Pathak and Tiwari (2012) reported that neem seed oil extract caused delayed larval development of *Corcyra cephalonica*, thus causing larval pupal intermediates, deformed adults, and poor emergence.

The findings of the present study are in agreement with the report of Kumar and Babu (1998) that Azal-T/S (1% azadirachtin) and Neem Azal-F (5% azadirachtin) have adverse effects on fecundity and delayed larval development of *Henosepilachna vigintiocpunctata.* The ovicidal potency of the peel essential oil was therefore dose-dependent, varying exponentially with the concentration. In this connection, the tested oil appeared to be a good candidate as a naturally occurring mosquito control agent.

In this study, the observed LC_{50} to LC_{95} for 2nd and 4th larval instars were concentration-dependent as larval mortalities increased with increasing concentration; indicating that increasing concentrations of the essential oil exacerbated mortality. However, it was observed that, at the same exposure period, the LC_{50} and LC_{90} were dose-dependent as shown by the differences in the LC values.

Furthermore, the results appear to indicate that the LC_{50} and LC_{90} values were inversely proportional to the time of exposure. The observed LC_{50} value of 180.6 ppm post 24-hour larval exposure using *C. paradisi* peel oil, contrasts with results of studies by Calvacenti and others (2004) in which LC_{50} values of 519 ppm was recorded for adult *Ae. aegypti* post 24-hour exposure using *C. limonia* and 538 ppm using *C. sinensis* peel oil. Differences in the insecticidal effects among essential oils from *Citrus* species have been reported (Choi *et al,* 2003). For example, *Citrus aurantifolia soringel* oil was highly effective against adult *Trialeurodes vaporariorum*, whereas the other *Citrus* oils were almost ineffective. These findings, in addition to our own results appear to suggest that qualitative and/or quantitative chemical composition among *Citrus* species may be different.

In conclusion, the results of this study indicate that grapefruit peel essential oil induces a toxic effect on the hatching of treated *Ae. aegypti* eggs and emergence of larval instars. It is thus potent ovicide and larvicide and may be useful for the control of *Ae. aegypti.* The oil might form a new source for managing various mosquito larvae in field ecosystem, although

its effects on non-target organisms remain unclear. However, because the grapefruit peel oil is commonly employed in soft-drink flavoring (Morton, 1987) its use as larvicidal agent will be safer. It is therefore suggested that the dissemination of the oil in the breeding water may be beneficial in the control program of *Ae. aegypti*. However, for the practical use of this oil as a naturally occurring larvicide further research is needed on formulations for improving the ovicidal and larvicidal potency and stability.

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