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Laboratory evaluation of extract from peels and seeds of some *Citrus* species against *Anopheles* mosquitoes (Diptera: Culicidae)

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Abstract

Mosquitoes are the most important group of insects known for their public health importance. Efforts to eradicate this disease involve elimination of the vector with natural base insecticides which are safer when compared to synthetic insecticides which are harmful to human. Toxicity effect of extracts from peels and seeds of *Citrus sinensis*, *Citrus aurantifolia* and *Citrus limonum* were evaluated against adult *Anopheles* mosquitoes at different concentrations and time of exposure; 20, 40 and 60 minutes. The peels and seeds were air dried for 15 days before pulverization. The pulverized peels and seeds were subjected to extraction using soxhlet apparatus and ethanol as solvent. The stock solutions were prepared by adding 0.1 mL of extract to 9.9 mL of ethanol to make 1% concentration. The following different concentrations 5, 10, 15, 20, and 25% of the extract were prepared as followed; 0.5, 1.0, 1.5, 2.0 and 2.5 mL of the extract were dissolved in 9.5, 9.0, 8.5, 8.0 and 7.5 mL of the solvent (Ethanol) respectively with three (3) replicates for each concentration, while 10 mL of ethanol was used as the control (0%). The qualitative and quantitative analysis of the phytochemical constituents were analysed. The efficacy of the extracted oils were tested on adult female *Anopheles* mosquitoes. At 25% concentration, *C. aurantifolia* peel extract recorded 62.22% mortality at 60 minutes while the seed extract recorded 50% mortality at the same concentration and time. The toxicity increased in respect to time of exposure, the toxicity estimation of LC₅₀ was 22.05% from the extract of *C. aurantifolia* peels and LC₉₅ was 112.27% for *C. limonum* peels extract after 60 minutes of application. The potency of the *Citrus* peels and seeds extracts may be as a result of the phytochemical constituents present in them. These results suggest that the *Citrus* peel and seed extracts have the potential to be used as ideally in eco-friendly approach for the control of the vector control programmes.

Keywords: *Anopheles*, *Citrus sinensis*, *Citrus aurantifolia*, *Citrus limonum*, Phytochemicals

1. Introduction

Female *Anopheles* mosquito is one of the most important disease vectors in the insect world, being the vector of Malaria. This vector is of a significant threat to public health because of their ability to transmit parasitic pathogens which afflict millions of people worldwide. Malaria killed over one million people each year, in which the most vulnerable are Children and pregnant women [1, 2] reported that about 3.3 billion people are at risk of malaria, out of which 1.2 billion are at high risk.

Approach to combat these diseases largely relies on interruption of the disease transmission cycle by either targeting on the mosquito larvae at breeding sites through spraying stagnant water or by killing/repelling the adult mosquitoes using insecticides [3, 4]. Synthetic chemical insecticides had been favourable so far due to their quick action and simple application, but this has resulted to insecticide resistance in medically important vectors. Insecticide resistance is increasingly becoming a problem for many vector control programs [5, 6]. The frequent use of chemical insecticides to manage insect has also leads to destabilization of the ecosystem [7]. Number of compounds and materials known today as effective insecticides and pesticides are numerous. Most of these compounds and materials are not without their shortcomings. To alleviate these problems, major emphasis has been on the use of natural plant based products as larvicides and adulticides which can be a safe alternate to synthetic insecticides [8].

Citrus seeds and peels contain certain compounds with varied level of bitterness and these compounds have been tested against insects and proved to be effective [9]. The dried peels of most *Citrus* fruits have been used in various ways in controlling pests of storage importance by

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using it in powered form. Recently, attention has been channelled towards the usage of oil extracted from the leaves, peels and seeds of various species of *Citrus* fruits in controlling vectors of public health importance most especially vector of parasitic diseases. *Citrus* essential oils are made up mainly of hydrocarbons designated as terpenes and of a smaller amount of sesquiterpenes; these two components serve as carrier for more important class of oxygenated compounds which are usually the bearers of the characteristic odour of the oil in which they are contained [10].

The composition varies considerably with the variety of fruit and with the location where the fruit is grown which is based on analyses of juice from different varieties and from different production areas. Terpenoid, one of the major components of *Citrus* oil has higher level of D-Limonene which serves as a natural insect repellent for *Citrus* fruits. Direct contact with D-Limonene can act as an organic insecticide. The essential oils extracted from *Citrus* genus have been proved to contain phytochemicals which includes Saponin, Terpenoid, Cardiac glycoside, Tannin, Alkaloid and Flavonoid. The presence and abundance of these phytoconstituents depends on varieties of the *Citrus* species [11, 12, 13].

Materials and methods

Study Area

This study was carried out at Environmental Biology and Public Health research laboratory, Federal University of Technology, Akure, Ondo State. The State lies between latitudes 5°45' and 7° 52'N and longitudes 4°20' and 6°05'E. Its land area is about 15,500 square kilometers.

Source of Citrus Fruits and their Classification: Freshly harvested species of *Citrus* fruits (*Citrus sinensis*, *Citrus limonum* and *Citrus aurantifolia*) were brought from local market in Ogbese, Ondo State, Nigeria. The *Citrus* fruits were brought to the Public Health Research Laboratory, Federal University of Technology Akure, Ondo State.

Preparation of Extracts

Freshly harvested oranges were washed with distilled water before peeling. The peels of the oranges were removed by using blade. The seeds of each *Citrus* fruits were separated manually. The fresh orange peels and seeds of citrus fruits were air dried separately at room temperature (28±3 °C) for 15 - 17 days. The powdered materials were obtained by grinding the dry peels and seeds into a fine powdered separately using commercial electrical blender. The grounded material was loaded into soxhlet apparatus for solvent extraction of oil by steam distillation method using ethanol as solvent. One and half litres (1/2 L) of ethanol was used sequentially with 500g of powered *Citrus* materials and was subjected to extraction using soxhlet apparatus [14].

Determination of Phytochemical Active Constituents

Qualitative and Quantitative Phytochemical screening was carried out in Biochemistry Postgraduate Research Laboratory, Federal University of Technology, Akure using established protocols as described by [15, 16]. A stock solution of the extracts with a concentration of 1mg/mL was prepared and used for the screening.

Source and Collection of Anopheles Mosquitoes

Eggs of Female *Anopheles* mosquitoes were collected from a

slow flowing stream and allowed to hatch in the container used for collection in the laboratory. The larvae were reared in the laboratory to 4th instar stage by feeding them with baker yeast and dog biscuits before the emergence of the adults. The pupal stages were transferred into a rearing cage where the young mosquitoes emerged. The adults were provided with 10% sucrose solution.

Identification of Adult Anopheles Mosquitoes

The *Anopheles* Mosquitoes were identified using identifying keys by [17].

Preparation of Stock Solution

The stock solutions were prepared by adding 0.1mL of extract to 9.9mL of ethanol to make 1% concentration. The following different concentrations viz 5, 10, 15, 20, and 25% concentrations of the extract were prepared as followed; 0.5, 1.0, 1.5, 2.0 and 2.5mL of the extract were dissolved in 9.5, 9.0, 8.5, 8.0 and 7.5mL of the solvent (Ethanol) respectively with three (3) replicates for each concentration, while 10mL of ethanol was used as the control (0%).

Application of Extracts

Three boxes were used as screen cage (40cm x 40 cm x 40cm). The window screen is made up of 0.25 mesh size 8.5 by 8.5 cm. The prepared extract concentrate was sprayed through the window screen by the means of an insecticide sprayer. Mortality count was taken after 20, 40 and 60 minutes of application. Lethal time (LT) was recorded from the average of three replicates [18].

Analysis of Data

The data was analyzed using one way Analysis of Variance (ANOVA) followed by Duncan's multiple range test (DMRT) to compare and separate the mean mortality at 5% level of significance. Probit Analysis was used to estimate the toxicity of the extracts.

Result

Phytochemical Analysis of Extract

The qualitative phytochemical screening of oil extracted from *Citrus* fruits peels and seeds are presented on Table 1. The screening shows that Alkaloids and Steroid are absent in all the *Citrus* peels and seeds, while Tannins, Cardiac glycosides, Saponins, Flavonoids and Terpenoid are present. Table 2 shows the quantitative phytochemical screening of extract from peels and seeds of *Citrus* fruits. High percentage of saponins was found in the *Citrus* fruits peel and seed extracts with *C. sinensis* peels having the highest percentage (26.38%). *C. aurantifolia* peels contained the highest terpenoid (3.23%) and tannins (0.24%). Extract of *C. limonum* peels had 2.72% of terpenoid, while *C. limonum* and *C. sinensis* peels and seeds had the same quantities of tannins 1.85% and 1.6% respectively.

Insecticidal Effect of Extract from Citrus Fruits Peel and Seed on Mortality of Anopheles Mosquitoes

Table 3 shows the insecticidal effect of *Citrus* peel extracts on adult *Anopheles* mosquitoes at different concentrations and periods of exposure, the percentage mortality of the mosquitoes varied with the period of exposure, with extracts from peels of *C. aurantifolia* at 25% concentration had the highest mortality (62.22%), *C. limonum* (55.55%) and *C.*

sinesis (41.11%) respectively at 60 minutes of application. However, there was a significant difference ($p < 0.05$) in the mortality rate recorded.

Table 4 shows the insecticidal effect of the seed extracts, at 25% concentration for 60 minutes, the highest mortality of 51.11% was recorded in *C. aurantifolia* seed extracts while the lowest mortality of 38.88% was recorded in *C. sinensis* extracts at the same time.

Toxicity Effect of Citrus Extracts against Adult Anopheles Mosquitoes at L50

At 20 minutes of L50, *C. limonum* seed extract had the highest toxicity 96.10 while the peels recorded the least toxicity of 43.04. At 60 minutes of L50, *C. sinensis* peel extracts recorded the highest mortality of 39.56 with the least of 22.05 in *C. aurantifolia* peel extracts. (Table 5).

The impact of the citrus extracts at different concentrations after 40 minutes of exposure to the mosquitoes showed

different level of toxicity (Table 6). Lower percentage mortality of the mosquitoes were recorded from 5 and 10% concentrations of the *Citrus* fruits extracts. At Concentration 15, 20 and 25%, *Citrus* species extracts eliminated more than 40% of the adult mosquitoes. *C. aurantifolia* peels had the high toxicity of 47.47% at LC₅₀, while *C. limonum* had estimation of 509% in eliminating 95% (LC₉₅) sample population. High percentage mortality of 62.22, 51.11 and 50% is seen to have occurred by the impact from 25% concentration of *C. aurantifolia* peels, seeds and *C. limonum* seeds respectively. The highest percentage mortality (62.22%) of mosquitoes was recorded 25% concentration of *C. aurantifolia* peels. The toxicity estimation of lethal concentration 50% (LC₅₀) of the sample population was shown from the effect of *C. aurantifolia* peel (LC₅₀ of 22.05%), which is more toxic than other *Citrus* fruits used in quick elimination of the 50% sample population.

Table 1: Qualitative Phytochemical Analysis of the Extracted oil from Peels and Seeds of *Citrus* fruits

Citrus fruits Species	Constituents						
	Alkaloid	Tannins	Cardiac glycosides	Saponins	Steroid	Flavonoid	Terpernoid
<i>C. aurantifolia</i> Peels	-	+	+	-	+	+	+
<i>C. sinensis</i> Peels	-	+	+	-	+	+	+
<i>C. limonum</i> Peels	-	+	+	-	+	+	+
<i>C. aurantifolia</i> Seeds	-	+	+	-	+	+	+
<i>C. sinensis</i> Seeds	-	+	+	-	+	+	+
<i>C. limonum</i> Seeds	-	+	+	-	+	+	+

Note: + present, - absent

Table 2: Quantitative Phytochemical Analysis of the Extracted oil from Peels and Seeds of *Citrus* fruits

Citrus fruits Species	Compound composition (%)				
	Tannins	Cardiac glycosides	Saponins	Flavonoid	Terpernoid
<i>C. aurantifolia</i> Peels	0.2350	0.1045	19.909	11.5939	3.2287
<i>C. sinensis</i> Peels	0.1850	0.7684	26.3812	0.7671	1.8471
<i>C. limonum</i> Peels	0.1849	0.4389	12.7909	1.1627	1.9718
<i>C. aurantifolia</i> Seeds	0.1633	0.0562	8.8172	0.4327	1.4711
<i>C. sinensis</i> Seeds	0.1601	0.5573	9.0371	0.0892	1.0230
<i>C. limonum</i> Seeds	0.1627	0.2190	9.3111	0.2137	1.4018
	0.2350	0.1045	19.909	11.5939	3.2287

Table 3: Mortality of Adult Anopheles Mosquitoes on Application of Citrus Peel Extracts

Citrus Fruits Species	% Mortality (Mean ±S.D)			
	Conc. (%)	20mins	40mins	60mins
<i>C. limonum</i> Peel	5	1.11±1.92 ^{ab}	4.44±1.93 ^{ab}	7.78±1.92 ^{ab}
	10	3.33±3.34 ^{ab}	8.89±1.92 ^{bc}	11.11±1.92 ^{abc}
	15	11.11±1.92 ^c	13.33±3.34 ^{cd}	16.67±0.00 ^{bc}
	20	15.55±3.85 ^c	17.78±3.85 ^{de}	35.56±16.44 ^d
	25	22.22±5.09 ^d	29.99±5.77 ^{gh}	55.55±6.94 ^e
	Control	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
<i>C. aurantifolia</i> Peel	5	5.56±1.92 ^b	17.78±5.09 ^{de}	34.45±10.72 ^d
	10	12.22±1.92 ^c	27.78±8.39 ^{fg}	34.45±1.93 ^d
	15	23.33±3.33 ^d	35.56±1.93 ^{hi}	36.67±3.34 ^d
	20	24.44±1.92 ^d	36.67±3.34 ^{hi}	42.22±6.94 ^d
	25	25.56±1.92 ^d	40.00±3.34 ⁱ	62.22±10.18 ^e
	Control	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
<i>C. sinensis</i> Peel	5	0.00±0.00 ^a	8.89±1.92 ^{bc}	12.22±1.92 ^{bc}
	10	11.11±1.92 ^c	12.22±1.92 ^{cd}	18.89±1.92 ^{bc}
	15	11.11±1.92 ^c	15.56±5.09 ^{cd}	21.11±3.85 ^c
	20	14.45±3.85 ^c	21.11±1.92 ^{ef}	36.67±3.33 ^d
	25	15.55±3.85 ^c	26.67±3.34 ^{fg}	41.11±8.39 ^d
	Control	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a

Note: Means followed by the same letter in column are not significantly different ($p > 0.05$) from one another using ANOVA followed by Duncan's New Multiple Range Test (DNMRT).

Table 4: Mortality of Adult Anopheles Mosquitoes on Application of Citrus Seed Extract

Citrus Fruits Species	% Mortality (Mean ±S.D)			
	Conc. (%)	20mins	40mins	60mins
<i>C. limonum</i> Seed	5	4.45±3.85 ^{ab}	14.44±1.93 ^{bcd}	21.11±1.92 ^{de}
	10	12.22±1.92 ^{cd}	16.67±3.33 ^{cde}	32.22±1.92 ^{fg}
	15	17.78±3.85 ^{de}	25.56±1.93 ^{fg}	33.33±3.34 ^{ef}
	20	18.89±1.92 ^{de}	31.11±1.92 ^{gh}	46.47±3.34 ^{ij}
	25	21.11±1.92 ^e	33.33±3.34 ^h	50.00±6.67 ^j
	Control	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
<i>C. aurantifolia</i> Seed	5	4.44±1.93 ^{ab}	13.33±3.34 ^{bcd}	15.56±1.93 ^{bc}
	10	10.00±5.77 ^{bc}	17.78±1.92 ^{cde}	34.44±1.93 ^{ef}
	15	12.22±5.09 ^{cd}	21.11±3.85 ^{ef}	37.78±5.09 ^{fh}
	20	20.00±3.33 ^e	31.11±3.85 ^{gh}	41.11±6.94 ^{hi}
	25	23.33±3.34 ^e	32.22±1.92 ^h	51.11±3.80 ^j
	Control	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
<i>C. sinensis</i> Seed	5	4.44±5.05 ^{ab}	10.00±5.77 ^b	10.00±5.77 ^b
	10	10.00±0.00 ^{bc}	12.22±3.85 ^{bc}	24.44±1.93 ^{de}
	15	11.11±5.09 ^{bc}	18.89±3.85 ^{de}	28.89±1.92 ^{ef}
	20	18.89±1.92 ^{de}	28.89±5.09 ^{gh}	32.22±1.92 ^{fg}
	25	18.89±1.92 ^{de}	31.11±1.92 ^{gh}	38.89±3.85 ^{gh}
	Control	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a

Note: Means followed by the same letter in column are not significantly different (p > 0.05) from one another using ANOVA followed by Duncan’s New Multiple Range Test (DNMRT)

Table 5: Toxicity Effect of Citrus Materials Extract against Anopheles Mosquitoes at L95

Citrus species	LC ₅₀ (LB – UB)		
	20min	40min	60min
<i>C. limonum</i> Peels	43.04 (29.19 – 269.57)	58.66 (32.02 – 172.29)	26.44 (20.83 – 43.21)
<i>C. limonum</i> Seeds	96.10 (37.19 – 404.2)	64.69 (28.75 – 548.05)	26.18 (16.92204.29)
<i>C. aurantifolia</i> Peels	71.42 (32.36 – 455.7)	47.47 (23.35 – 88.17)	22.05 (13.13 – 15127)
<i>C. aurantifolia</i> Seed	74.44 (35.28 – 170.7)	73.64 (30.55 – 823.2)	27.16 (17.72 – 176.01)
<i>C. sinensis</i> Peel	77.86 (36.21 – 411.9)	97.58 (36.89 – 416.2)	39.562 (23.72 – 469.29)
<i>C. sinensis</i> Seed	92.67 (37.88 – 564.66)	73.62 (32.04 – 757.23)	37.79 (23.09 – 389.34)

LC₅₀ = Lethal concentration at which 50% population response

LB = Lower bound

UB = Upper bound

Table 6: Toxicity Effect of Citrus Materials Extract against Anopheles Mosquitoes

Citrus species	LC ₉₅ (LB – UB)		
	20min	40min	60min
<i>C. limonum</i> Peels	156.68 (64.74 – 144.8)	509 (116.90 – 350.76)	112.27 (59.95 – 559.43)
<i>C. limonum</i> Seeds	1899.13 (182.29 – 2280)	2387.07 (201.45 – 4368)	652.26 (121.76 – 5476.13)
<i>C. aurantifolia</i> Peels	1430.03 (171.6 – 1320)	2435.4 (193.76 – 6948)	1445.40 (147.21 – 2126)
<i>C. aurantifolia</i> Seed	989.14 (147.59 – 3785)	2917.78 (215.62 – 1220.56)	572.66 (117 – 795.95)
<i>C. sinensis</i> Peel	686.08 (121.33 – 515.7)	2383.8 (201.59 – 9166)	631.95 (129.38 – 4659.74)
<i>C. sinensis</i> Seed	1334 (161.87 – 3122)	1897.8 (190.23 – 1727)	580.88 (123.69 – 3177.38)

LC₉₅ = Lethal concentration at which 95% population response

LB = Lower bound

UB = Upper bound

Discussion

Plant products had been reported to have different phytoconstituent compounds and these depend on the plant

varieties and the time of harvest. These compounds are used by the plant as defense against disease agents. The phytochemical compounds found in *Citrus* peels and seeds

must have been the reason behind the bioactivities of the extracts as previously reported by ^[9]. Tannins (Tannoid) are acidic in nature and the acidic reaction is attributed to the presence of phenolics or carboxylic group. They are used as antiseptic and this activity is due to presence of the phenolic group. Tannins were found more in *C. aurantifolia* peel (0.24%), *C. limonum* peel and *C. sinensis* peel contained 0.18%, while all the seed extracts contained 0.16% of tannin. This agreed with the findings of ^[19]. The presence of Tannins may be the reason for bitter and astringent taste of *Citrus* fruits particularly in Lime and Lemons.

Flavonoids are important group of polyphenols, they are aromatic compounds and numerous reports support their use as antioxidants or free radical scavengers. Quercetin, Kaempferol and Quercetin are common flavonoids present in plants and Quercetin is known for ability to relieve hay fever and eczema. All the *Citrus species* extracts contained Saponins which are extremely poisonous, as they cause hemolysis of blood cells and are known to cause cattle poisoning. They possess a bitter and acrid taste, besides causing irritation to mucous membranes ^[20]. Terpenoid were found in all the extracts. Terpene acts as irritants when applied externally and when consumed internally their action resembles that of gastrointestinal tract irritant. The triterpenes include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity.

The ethanolic extracts of *Citrus* fruit peels and seeds revealed their insecticidal potential against adult *Anopheles* mosquitoes as previously reported by ^[21, 22] reported the use of hexane and petroleum ether as solvent in extracting plant products against *Aedes aegypti* and *Anopheles stephensis* which were found to be effective. Ethanolic extracts from *Citrus sinensis* peels and *Piper guineense* (seeds and leaves) has also been found to be effective as reported by ^[23].

Of the different concentrations, 25% concentration recorded the highest mortality rate at lethal time 60 (LT60). This result is similar to the work reported by ^[24]. Extracts from the seeds of *C. limonum* and *C. aurantifolia* showed higher mortality rate on the vectors (50%) at LT60. However, the effectiveness of all the seeds varied at LT20, LT40 and LT60 respectively.

All the extracts used showed different levels of potency against the adult *Anopheles* mosquitoes but the most effective peel extract was from *Citrus aurantifolia* (62.22%) at 25% concentration after 60 minutes of application. This corroborates the work of ^[9, 25] who reported that *Citrus limonum* contain abundant terpene (D - limonene) which is known to have natural insecticide repellent.

Percentage mortality recorded from all the seed extracts at LT20 were the same, the lethal effect of *C. aurantifolia* and *C. limonum* seeds gave 50% mortality, which is similar to the result of adulticidal activities of *Citrus* seeds Oil reported by ^[26]. *C. aurantifolia* peels and seeds have been identified to contain 7% typical volatile essential oil, where the main components are limonene, β -pinene and fenchone. The major component of *C. aurantifolia* oil is limonene which has been described by ^[27] to have insecticidal activities. Limonene was also identified as organic insecticide ^[28].

^[29] reported that the extracts from *Citrus aurantifolia* and *Citrus sinensis* peels had better insecticidal activity after 60 minutes of used as indoor spray insecticide, with *C. sinensis* having greater potency on cockroach. However, this did not correlate with the result of this work, where *C. sinensis* showed

low efficacy against the vectors. This may be due to the different vectors involved in both research works. Adulticidal activities of extracts from *Citrus reticulata* and *Citrus sinensis* (peels and seeds) was also reported by ^[30]. The potential ability of all the *Citrus* extracts maybe due to their phytochemical properties which are secondary metabolites and may have interfered with the insect nervous system.

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