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Research Article

Larvicidal activity of essential oil from *Citrus sinensis* and *Citrus paradisi* against *Anopheles gambiae*

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ABSTRACT: Malaria is a major health challenge in the developing world causing millions of death annually. Synthetic pesticides used in the vectors control are not environmentally safe and are expensive. We screened the essential oils of *Citrus sinensis* and *Citrus paradisi* peels at concentrations ranging between 40 and 400 ppm against late 3rd instar of *Anopheles gambiae* larvae. The effects of ethanol and methanol on the larvicidal activity of these oils were examined. The effects of combined oils formulations on larvicidal activity were also determined. The larvicidal concentration (LC_{50}) of the orange and grape oil was 73ppm and 76 ppm, respectively in methanol, and 121 ppm and 82 ppm in ethanol solution. The LC_{50} of the combined oil formulations ranged between 54 and 99ppm. The different oil combinations showed synergism except when combined at equal proportion in ethanol. Conclusively, orange and grape oils could be used as biopesticides against *A. gambiae* larvae.

KEYWORDS: *Anopheles gambiae; Citrus paradisi; Citrus sinensis*; essential oils; Mosquito larvae

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INTRODUCTION

Malaria is a life threatening disease caused by parasites that are transmitted to people through the bites of infected mosquitoes. According to the latest estimates released in December 2013, there were about 207 million cases of malaria in 2012 and an estimated 627 000 deaths (WHO, 2014). Key interventions to control malaria includes: prompt and effective treatment with artemisinin-based combination therapies; use of insecticidal nets by people at risk and indoor spraying with insecticides to control the vector mosquitoes (WHO, 2014). Several antimalarial synthetic drugs with single or combined chemically active agents are being developed, marketed and used in the treatment of malaria but the problem of drug resistant parasites still remains an issue that calls for integrated approach to reduce the incidence of malarial mortality.

Mosquitoes belong to the genera Anopheles, Aedes and Culex; they act not only as a vector for the malaria parasite but also for numerous parasitic diseases such as denge fever, yellow fever, filariasis, encephalitis etc. (Ghosh *et al*., 2012; Manimaran *et al*., 2013; Patil *et al*., 2014). Therefore, limiting the population of the vector is crucial to the management of not only malaria but many other diseases. Prevention by vector control involves the use of biocidals such as larvicides and insecticides to kill larva and adult mosquitoes respectively. The control of adult mosquitoes is seemingly becoming difficult due to insecticide resistance and mosquitoes behavioural changes like avoidance of residual insecticides (Nauen, 2007). It is much easier and more efficient to control mosquito larvae that are relatively static and more concentrated in their natural breeding sites (Intirach *et al*., 2012). However, there have been records of mosquito larva resistance to some WHO recommended conventional synthetic insecticides such as carbamates, pyrethroids, organophosphates and organochlorines (Intirach *et al*., 2012; Nauen, 2007). A promising approach to overcome this kind of resistance is the use of biopesticides of botanical origin (Burfield & Reekie, 2005; Shaalan, *et al*. 2005). Unlike single active agent synthetic biocides, resistance to botanical or whole plant extract is rare because they contain many bioactive compounds which exert different larvicidal effects through different numerous mode of action (Burfield & Reekie, 2005; Ghosh *et al*., 2012). Also, biopesticides of botanical origin are target specific, non-toxic to human, biodegradable and environmentally safe (Burfield & Reekie, 2005; Intirach *et al*., 2012; Senthil Nathan, 2007).

Currently, effort is being geared towards sourcing and use of biopesticides of botanical origin for mosquito control (Burfield & Reekie, 2005; Ghosh *et al*., 2012; Kishore *et al*., 2011; Shaalan *et al*., 2005). Essential oils from neem, camphor, lemon, cinnamon, citronella, pine, mint and several plants have been shown to posses mosquito larvicidal properties (Ghosh *et al*., 2012; Intirach *et al*., 2012; Kishore *et al*., 2011; Manimaran *et al*., 2013; Shaalan *et al*., 2005). Efficacy of biopesticides is known to be enhanced in mixed formulations of botanical biocides because of an increased available number of active secondary metabolites with inherent synergistic effects and multiple mechanisms of action (Manimaran *et al*., 2013).

Recently, we reported the secondary metabolites and the antimicrobial effect of essential oil of *C. paradisi* (Okunowo *et al*., 2013). Similarly, we had characterized the active constituents of *C. sinensis*. Hence, this study was carried out to investigate the effect of *C. paradisi* and *C. sinensis* essential oils on mosquito larva and possible adjuvant action.

MATERIALS AND METHODS

Plants material

Fresh *C. paradisi* and *C. sinensis* fruits were purchased from Idi-araba and Mushin fruit market, Lagos, Nigeria. The voucher specimen of the fruits were deposited in the herbarium section of the Department of Botany, Faculty of Science, University of Lagos, Lagos, Nigeria, with herbarium specimen numbers LUH5248 for *Citrus paradisi* and LUH 5249 for *Citrus sinesis.*

Extraction of essential oil

The rinds from fresh mature *C. paradisi* and *C. sinensis* fruits were removed and kept in separate containers. A 300 g fresh rind was ground to puree in a Malex Exceller blender containing 1 litre of water. This was subjected to hydrodistillation in a 2 L round bottom flask adapted to a Clevenger-type apparatus to obtain the essential oil. The extracted oils were dried over anhydrous sodium sulphate (Na_2SO_4) and stored in air-tight amber-coloured bottle at 4°C (Okunowo *et al*., 2013).

Sourcing and identification of test organism

Wild type mosquito larvae were collected from a natural breeding sites at Bariga, Lekki and Oko-baba (ebute-metta) metropolis of Lagos, Nigeria and were transported to the laboratory where the larvae were introduced to clean non-chlorinated water and sorted for identification. The test organism was identified as *Anopheles gambiae* larvae at the insectary of the Nigerian Institute of Medical Research, Yaba by Dr Adedayo Oduola. Further Identification of the mosquito species was also done by Mr Emmanuel Adebayo Fatan at the Department of Zoology, University of Lagos, Akoka, Lagos, Nigeria.

Table 1. Larvicidal activity of essential oil of orange and grape peels against late third instar larvae of *Anopheles gambiae*

Concentration of essential oil (ppm)	Mortality % (Mean ± SEM)			
	OE	OM	GE	GM
40	11 ± 1.7	33 ± 3.9	16 ± 2.2	30 ± 4.3
120	46 ± 3.6	57 ± 4.2	64 ± 5.8	53 ± 4.6
200	85 ± 3.5	87 ± 3.8	88 ± 6.3	87 ± 5.3
280	87 ± 3.9	97 ± 2.1	96 ± 1.1	97 ± 2.1
360	98 ± 1.4	100 ± 0.0	100 ± 0.0	99 ± 0.7
400	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
LC_{50} (95% CI, ppm)	121(99 – 142)^a	73(58 – 88)^b	82(67 -97)^b	76(64 -89)^b

Values for mortality percent are mean ± SEM (n = 4), LC_{50} = the mean values of four replicates determined by probit analysis, while the values in parenthesis are lower 95% CI and upper 95% CI, LC_{50} values with the same superscript on each row are not significantly different (P > 0.05) when subjected to ANOVA followed by Tukey's multiple comparism test. OE = Orange oil in ethanol, OM= Orange essential oil in methanol, GE = Grape essential oil in ethanol, GM = Grape essential oil in methanol, ppm = part per million.

Mosquito larva and adult maintenance

Wooden entomological cages of 30x30x30 cm dimensions with WHO recommended mosquito net covering were constructed. The wild type mosquito larvae were segregated from the contaminant, cleaned and transferred to distilled water and allowed to adapt for 12 h. The larvae were maintained under sterile condition at 27±2°C and 70-80% relative humidity under a photoperiod of 12 h light and dark cycle in the cages (Lyons *et al*., 2012). The larvae were fed with dog biscuit and powdered yeast (ratio 1:3) and allowed to mature into pupae (Manimaran *et al*., 2013). After the pupae has emerged to adult mosquitoes, they were transferred with the aid of a WHO recommended aspirator to new cages and fed with 10% sucrose solution and 5% multivitamin syrup soaked in cotton wool (Intirach *et al*., 2012). To allow for oviposition, adult female mosquitoes were allowed to feed on rat (Intirach *et al*., 2012) and their eggs were harvested in Petri dishes with moistened filter paper for hatching. Larvae were also fed with dried fish powder and separated from the molted pupae prior to use for the larvicidal experiment.

Larvicidal test of essential oils at diagnostic dosage

The assay for larvicidal activity of the *C. paradisi* and *C. sinensis* essential oils was done according to the standard WHO protocol for larval susceptibility test (WHO, 2005). One gram oil extract was dissolves in 100 ml ethanol or methanol as 1% stock solutions. The stock solutions were further diluted (1 ml 1% stock solution in 25ml organic solvents) to diagnostic dosage of 400ppm. Groups of 25 late 3rd instar larvae of *A. gambiae* were exposed to the test solutions containing 249 ml of distilled-deionized water and 1ml 400ppm oil solution in a 500ml container. Four replicate experiments were maintained for the individual test oil solution, the control and the untreated groups. A control group received either 1ml ethanol or methanol-distilled water, while the untreated group was maintained in distilled water only. Mortalities of treated larvae (test) and control were determined after an exposure period of 24 hours. The percentage mortalities were calculated using Abbott's formula: [(% test mortality - % control mortality)/ (100 - % control mortality)] x 100.

Dose-response bioassay of essential oil

To establish the baseline susceptibility of the larvae population to the oils, the 1% stock solutions of the oil-ethanol mixture and oil-methanol mixture were further diluted in the appropriate organic solvents to desired concentrations ranging from 40, 120, 200, 280 and 360 ppm, respectively. The experiment was conducted as previously described above by exposing the larvae to a test solution containing 1ml desired oil concentration and 249ml distilled-deionized water, and the percentage mortalities were determined.

Essential oils mixed-formulation and larvicidal property

The *C. paradisi* and *C. sinensis* oils combined effect was determined by preparing the most potent concentration of grape or orange oil solution as obtained from the dose response assay above. These were combined in different mixing ratio (75:25%v/v, 50:50%v/v and 25:75%v/v), diluted to different test concentrations (40, 120, 200, 280 and 360 ppm) and evaluated on mosquito larvae according to WHO susceptibility assay protocol (WHO, 2005). The cotoxicity coefficient (CTC) of the mixed oil formulation was calculated (Intirach *et al*., 2012). And when CTC value is 100, it shows probability of similar (additive) effect of the components oil in the mixture. A CTC greater than 100 indicates synergism while CTC lower than 100 indicate antagonistic effect of the component oils in the mixture.

Statistical analysis

The values for mortality percent were expressed as mean ± standard error of mean (SEM) for four replicate experiments. The mean lethal concentration (LC_{50}) of the oil and its 95% confidence limit of lower and upper confidence levels were calculated from mortality values using probit analysis. Larvicidal activity was reported as LC_{50} values and was compare between oils and oils combination using the One Way Analyses of Variance (ANOVA) followed by Tukey's multiple comparism test. Values were considered significantly different between treatments at $P \leq 0.05$. The analyses were done using the GraphPad Prism version 5 for windows (GraphPad Software, San Diego California USA).

RESULTS AND DISCUSSION

In this study *A. gambiae* was the only species of mosquito obtained from the different breeding sites in Lagos, Nigeria. Similarly, a survey study of the bionomics and distribution of the malaria vectors in Lagos State, Nigeria showed only Anopheles mosquito species as predominant to this environment (Oyewole & Awolola, 2006). The incidence was highest (39.6%) with *A. gambiae*, followed by *Anopheles arabiensis* (29.0%), *Anopheles funestus* (20.3%) and *Anopheles rivulorum* (13.8%) (Oyewole and Awolola, 2006).

Table 2. Larvicidal activity of combined essential oil of orange and grape peels against late third instar *Anopheles gambiae* larvae

Concentration of essential oil combinations (ppm)	Mortality % (Mean ± SEM) in Ethanol			Mortality % (Mean ± SEM) in Methanol		
	OG(25:75%)	OG(50:50%)	OG(75:25%)	OG(25:75%)	OG(50:50%)	OG(75:25%)
40	28 ± 4.9	34 ± 4.4	38 ± 4.7	46 ± 6.2	43 ± 5.1	33 ± 4.9
120	55 ± 5.4	51 ±5.3	83 ± 5.1	61 ± 6.0	57 ± 4.8	79 ± 6.1
200	70 ± 6.1	68 ± 6.0	99 ± 0.4	84 ± 4.7	80 ± 4.9	80 ± 4.9
280	99 ± 0.7	96 ± 3.4	100 ± 0.0	98 ± 1.4	100 ± 0.0	97 ± 2.7
360	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
400	10 0± 0.0	100 ±0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
LC_{50} (95% CI, ppm)	84(74-93)^a	99(87- 110)^b	54(48-61)^c	65(57-73)^c	63(55-71)^c	63(57-69)^c

Values for percent mortality are mean ± SEM (n = 4), LC_{50} = the mean values of four replicates determined by probit analysis, while the values in parenthesis are lower 95% CI and upper 95% CI, LC_{50} values with the same superscript on each row are not significantly different ($P > 0.05$) when subjected to ANOVA followed by Tukey's multiple comparism test. OG = Orange:Grape, ppm = part per million.

Table 3. Cotoxicity effects of orange/grape oil combinations on late third instar *Anopheles gambiae* larvae

Essential oil	Organic solvent used	Combination of essential Oil (%)	LC_{50} (ppm)	Cotoxicity Coefficient (CTC)	Effect
Orange peel (O)	Ethanol	100	121	____	___
Grape peel (G)		100	82	____	___
O	Methanol	100	73	____	___
G		100	76	____	___
O + G	Ethanol	75:25	54	200	Synergism
O + G	Ethanol	50:50	99	98	Antagonism
O + G	Ethanol	25:75	84	106	Synergism
O + G	Methanol	75:25	63	123	Synergism
O + G	Methanol	50:50	63	118	Synergism
O + G	Methanol	25:75	65	116	Synergism

There are reports of *A. gambiae* which is endemic to Nigeria and some parts of Africa, developing multiple insecticides resistant mechanisms such as the over-expression of metabolic genes of the cytochrome P_{450} (*CYP6P3* and *CYP6M2)*; which encode permethrin detoxifying enzymes, (Djouaka *et al*., 2008) and the over-expression of the knock-down resistant (kdr) genes for pyrethroid (Awolola *et al*., 2009; N'Guessan, Corbel, Akogbéto, & Rowland, 2007). However, this problem of resistance may be overcome with the use of botanical pesticides with their inherent multiple modes of actions.

In this work, essential oil of indigenous orange and grape peels showed larvicidal activities against *A. gambiae* at the diagnostic dosage (400 ppm) with 100% larvae mortality (Table 1) thus, showing the oils as promising biocides for *A. gambiae*, and the lethal effect of the oils increased with increasing oil concentrations. Unlike the mosquito genera, Aedes and Culex, there are limited published reports of larvicidal botanical extracts against the genera Anopheles particularly, *A.gambiae.* (Aina *et al*., 2009; Amer & Mehlhorn, 2006; Dua *et al*., 2009; Kweka *et al*., 2012; Ndung'u *et al*., 2004; Okumu *et al*., 2007; Shaalan *et al*., 2005). Although, a few number of plants have been shown to possess insecticidal and repellant activity against adult *A.gambiae* (Bossou *et al*., 2013; Innocent *et al*., 2010; Nonviho *et al*., 2010). The larvicidal concentrations (LC_{50}) of each oil type differ according to the dissolution solvent used. This was lower in methanol with each type of oil (Table 1). This suggests that methanol is a better solvent for the dissolution of the oils. The difference in the larvicidal activity of the oils in the two organic solvent may also be due to the difference in the solubility and properties of various bioactive compounds present in the oils. Similar study has shown that organic solvent affects the solubility and lethal activity of components of plant extract on malaria vectors (Rahuman *et al*., 2009).

We have not found any published report on the larvicidal activity of indigenous orange and grape peels essential oils against the strains of *A.gambiae* in Nigeria.

However, the larvicidal activities of the citrus peel oils reported in this paper are different from those reported on some other malaria vectors; *Anopheles stephensi*, Aedes and Culux (Din *et al*., 2011; Giatropoulos *et al*., 2012; Mansour *et al*., 2004; Murugan *et al*., 2012). The difference may be due to the origin and species of orange and grape used or the

difference in the species or genera of mosquitoes employed in the larvicidal screening assays. The larvicidal activities of some indigenous plants such as the ethanolic and aqueous extracts of the fruits of *Xylopia aethiopica* (Dunal) A. Rich (Annonaceae); *Physalis angulata* L.(Solanaceae) and seeds of Thonn (Piperaceae); *Jatropha curcas* Linn. (Euphorbiaceae) and *Piper guineense* Schum against the species of *A.gambiae* in Nigeria have been reported (Aina *et al*., 2009). Interestingly, much higher larvicidal activity was recorded with only *Piper guineense* seed ethanolic extract (LC_{50} = 0.028 mg/ml) (Aina *et al*., 2009). Although, all the plant extracts tested (Aina *et al*., 2009) were against the 2nd instar larvae of *A. gambiae* as against the late 3rd instar larvae reported in this study. The other plants had much lower larvicidal activity compared to those reported in this paper for both orange and grape peel oils. Also a previous report has shown that the larvae of *A.gambia* was sensitive to essential oil extracted from Indian borage (*Plectranthus amboinicus*), the LC_{50} value of 55.2 ppm was recorded after 24 hours exposure to the oil (Kweka *et al*., 2012).

The larvicidal concentrations (LC_{50}) of the combined formulations of essential oils of the orange and grape were mostly lower than those of the individual oils (in Table 1) except at mixing ratios 25:75% and 50:50% (orange:grape) in ethanol (Table 2). This indicates that larvicidal activity of the essential oil of orange or grape against *A. gambiae* is increased and more favoured by oil combinations. And the cotoxicity coefficient of the orange and grape oil combinations showed that the larvicidal effect of the formulations were synergistic except for a mixture of 50:50% (orange: grape oil) combination in ethanol which was antagonistic (Table 3). Reports have shown that combined phytoxical formulations do not only improve activity but also reduce the dose of individual phytochemical needed for the integrated resistance management of disease vectors (Intirach *et al*., 2012; Manimaran *et al*., 2013).

Formulations with different plant essential oils contains more bioactive components that act synergistically with different modes of action (Intirach *et al*., 2012; Manimaran *et al*., 2013).

This study has demonstrated that orange and grape peel essential oils possess larvicidal properties on *A. gambiae*, these properties may be due to the presence of some mosquito larvicidal agents such as β-phellandrene, D-limonene, linalool, caryophyllene, β-caryophyllene and oleic acid that we have previously identified in grape oils (Okunowo *et al*., 2013) and *p*-cymene, β-phellandrene, D-limonene, linalool, Terpinen 4-ol and α- terpineol identified in orange peel oil (Okunowo *et al*., 2015). For example, *α*-phellandrene, limonene, *p*-cymene, γ-terpinene, terpinolene and α-terpinene isolated from leaves of *Eucalyptus camaldulensis* possess significant larvicidal activity against fourth-instar larvae of *A. aegypti* and *A. Albopictus* (Kishore *et al*., 2011).

The larvicidal activity against *A. aegypti* larvae was highest (LC_{50} = 14.7 µg/mL) with α-terpinene in 24 hours, followed by the compounds *α*-phellandrene (LC_{50} = 16.6 µg/mL, LC_{90} = 36.9 µg/mL), limonene (LC_{50} = 18.1 µg/mL, LC_{90} = 41.0 µg/mL), *p*-cymene (LC_{50} = 19.2 µg/mL, LC_{90} = 41.3 µg/ mL), terpinolene (LC_{50} = 28.4 µg/mL, LC_{90} = 46.0 µg/mL) and γ-terpinene (LC_{50} = 30.7 µg/mL, LC_{90} > 50.0 µg/mL) (Kishore *et al*., 2011).

Similarly, β-caryophyllene and linalool isolated from leaves of different *Cinnamomum osmophloeum* showed strong larvicidal activity (LC_{50} = 50 µg/mL) against *A. Aegypti.* (Kishore *et al*., 2011). Also, the fatty acid components oleic and linoleic acids isolated from *Dirca palustris* have been reported to exert a larvicidal activity (LC_{50} = 100 µg/mL) against fourth instar *A aegypti.* (Kishore *et al*., 2011). These fatty acids have also been reported in *Citrullus colocynthis* (Linn.) Schrad and have been shown to be highly potent against fourth instar larvae of *A aegypti* . (LC_{50} 8.8, 18.2 and LC_{90} 35.4, 96.3 ppm), *Anopheles stephensi* Liston (LC_{50} 9.8, 11.5 and LC_{90} 37.4, 47.4 ppm), and *Culex quinquefasciatus* Say (LC_{50} 7.7, 27.2 and LC_{90} 30.7, 70.4 ppm) (Rahuman *et al*., 2009).

In conclusion, this study shows the efficacy of the essential oils from agrowastes; *C. sinensis* and *C. paradisi* as strong larvicidal agents and potential biopesticides for use against *A. gambiae* in the integrated management of malaria vectors to reduce the high level of mortality associated with malaria in the tropical regions.

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