Original Article

Avicenna Journal of Medical Biotechnology
Volume 17, Issue 1, 56-63
https://doi.org/10.18502/ajmb.v17i1.17678

Check for updates

Repellency Effects, Cytotoxicity Assessment and Chemical Compositions of *Citrus limon* Seeds Extracts and Fractions Against *Anopheles gambiae sl* Mosquitoes

Mukaram Akintunde Adeniyi-Akee [1,2*], Kingsley Adibe Mbachu [3], Kolade Ibrahim [4], Paul Matthew Osamudiamen [3,5], Olapeju Oluyemisi Aiyelaagbe [3], Sunday Olakunle Idowu [2], and Olajire Aremu Adegoke [2]

1. Department of Pharmaceutical Chemistry, College of Pharmacy, Igbinedion University, Nigeria
2. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ibadan, Oyo State, Nigeria
3. Department of Chemistry, Faculty of Science, University of Ibadan, Oyo State, Nigeria
4. Department of Zoology, Faculty of Science, University of Ibadan, Oyo State, Nigeria
5. Department of Chemical Sciences, Bells University of Technology, Ota, Nigeria

Abstract

Background: *Citrus limon* seeds have limited exploration, compared to other parts. The increasing prevalence of mosquito menace prompted the investigation into the repellent effects of the seed n-hexane extract and fractions of *Citrus limon* (Rutaceae) against adult *Anopheles gambiae sl* mosquitoes. The cytotoxicity and chemical constituents of the 100% n-Hexane Fraction (HF) were also evaluated.

Methods: Successive extraction was done with cold-maceration using, n-hexane, Ethyl Acetate (EA) and methanol. The three solvent extracts were screened against *Anopheles gambiae sl* mosquitos, using human bait method, at doses, 1.5, 2.5 and 5.0 *mg/ml* and with Deet and acetone used as positive and negative controls respectively. The n-hexane extract was further fractionated with column chromatography. Ten fractions (100% n-hexane, 10, 20, 30, 40, 60, 80% EA in n-hexane, 100% EA, 50% methanol in EA, and 100% methanol) were collected, and all tested at a single optimized dose of 5 *mg/ml* for 2 *hr*. The cytotoxicity and the chemical constituents of HF were determined using brine shrimp lethality test and gas chromatography-mass spectrometry respectively.

Results: N-hexane extract and HF, both at 5 *mg/ml*, showed the most protective effect of 97.52 and 89.40% respectively, compared with standard (Odomos Deet) 100% activity, with no significant difference ($p>0.05$) between the three. HF was toxic with LC_{50} values of 29.2354 *ppm*. Thirty compounds representing 79.25% of HF were identified. Major compounds present in the fraction were heptadecanamine (8.31%), N-(3-methylbutyl) acetamide (7.89%), 2-ethyl- 1-hexanamine (6.65%).

Conclusion: This promising repellent activity further justified the traditional usage of *Citrus limon* as mosquito repellent.

Keywords: *Anopheles*, *Citrus*, Gas chromatography-mass spectrometry, Insect repellents

To cite this article: Adeniyi-Akee MA, Mbachu KA, Ibrahim K, Osamudiamen PM, Aiyelaagbe OO, Idowu SO, et al. Repellency Effects, Cytotoxicity Assessment and Chemical Compositions of *Citrus limon* Seeds Extracts and Fractions Against *Anopheles gambiae sl* Mosquitoes. Avicenna J Med Biotech 2025;17(1):56-63.

*** Corresponding author:**
Mukaram Akintunde Adeniyi-Akee, Ph.D., Department of Pharmaceutical Chemistry, Igbinedion University, Nigeria
Tel: +234 7087662856
E-mail: mukkyakee@yahoo.com
Received: 1 May 2024
Accepted: 23 Sept 2024

Introduction

Global mortalities and illnesses are majorly cause by disease transmission from insects, with transmission from mosquitoes alone reaching several millions annually [1]. The causative agent of Malaria in Africa is *Anopheles gambiae*, and studies have shown that there are five hundred million Malaria infections and about four million Malaria death annually [2]. Also, in Nigeria, the level of mosquito population is massive, which make majority of the people to be exposed to infections from mosquitoes. The fact that ailments from insects cause immense health chaos and mortalities in tropical climate, does not guarantee safety for the rest of the world from the scourge [1]. In West Africa, Nigeria alone has about 76% of the population living in high Malaria transmission areas and based on the 2020 World Malaria Report, the most Malaria cases in 2019, which led to the greatest number of Malaria mortality originated from Nigeria [3]. Malaria, being a global public health disease has caused about 25% of death in children and pregnant women; it has also caused miscarriages and abnormal births [4]. Female anopheles' mosquitoes are the causative agents of Malaria, and their successful control will automatically control Malaria.

Mosquito repellent is an intervention method used to control mosquito, which prevents contact between the host and the vector, and automatically reduce the disease transmission rate [5]. Plant extracts could be a natural source of repellents [6,7]. Repellents are good replacement to the use of insecticides, because bites from insects could be better prevented with repellents and eradicating infestation [1]. In many instances, the only easy way to prevent mosquito bites is the usage of repellents on the skin. It may be used, both, to prevent insects from stored products and food packaging, and also to deter ticks, mites and mosquitoes from biting individuals [1]. Volatile oils from plants have been reported to exhibit insecticidal and repellent effects [8].

Citrus limon (L.) (*C. limon*) Osbeck is an evergreen plant with dynamic growth, belonging to rutacea family and is native to Asia [9], but its availability has spread to other continents like Africa. It has been reported to exhibit numerous pharmacological activities [10]. Traditionally, dry peels from citrus plants are burnt in the living room at night to deter the efficiency of mosquitoes [11]. The oil of the peel of lemon is used as a non-toxic insecticide treatment [12]. Also, in China, *C. limon* and *Citrus sinensis* oils tested as the most toxic fumigant among 5 citruses. Citral was shown to be responsible for lethality [13]. Some parts of the plant like seeds and peels which can produce valuable by-products are thrown as wastes, whereas some parts of plant species are medicinally valuable and are as well used in toiletry, confectionary and in making perfume [14]. The seeds of lemon have limited exploration, compared to other parts. *C. limon* seeds is a by-product of lemon, just like the peels and pulps of lemon. It is usually disposed as waste, which could lead to ecological problem [15]. The extraction of oil from lemon seeds is a common practice and the de-oiled seeds which are thrown away as waste still have an important use as plant processing residue [16]. The juice is applied locally as an astringent and to reduce the scourge of sore throats. Lemon juice also has bactericidal effect; it is a good antiperiodic and can be used as antimalarial and in treating other fevers when substituted for quinine [17]. The fruit is useful in the treatment of rheumatic conditions and it contains bioflavonoids that help to strengthen the inner lining of blood vessels, especially veins and capillaries, thus help to counter varicose veins and easy bruising [17].

Some of the recent applications of the plants are sources of anti-oxidants and chemical exfoliants in specialized cosmetics. The medicinal, dietary and economic values of the oil of citrus seeds have resulted in increased research on the lipids composition of the seed oil of various species of citrus plants [18]. Some of the works have measured the oil content of citrus seeds [19].

This study therefore is aimed at investigating the mosquito repellent effects of the seed n-hexane extract and fractions of *C. limon* against adult *Anopheles gambiae sl* mosquitoes. The cytotoxic effect and the chemical constituents of the most active fraction would be investigated.

Materials and Methods

Plant collection and authentication

Seeds of *C. limon* were purchased from juice seller at Oje market in Ibadan, Nigeria and identified at Forest Research Institute of Nigeria (FRIN), Ibadan, Nigeria, where samples with voucher number: FHL112112 was deposited.

Sample preparation of the seed extract

The seeds were dried under shade for a fortnight and ground. The air-dried and pulverized seeds of *C. limon* (800 *g*) were taken through successive and exhaustive, cold maceration extraction using three different solvents of different polarity (n-hexane, ethyl acetate and methanol) for three days. Rotary evaporator (Buchi R215, USA) was used to remove the solvent after extraction, and were further kept in the vacuum desiccators at 40*°C* prior to further analysis.

Fractionation of the n-hexane extract of C. limon

The n-hexane extract (18 *g*) of the seeds of *C. limon* was adsorbed on 30 *g* of silica gel (200-400 mesh) and introduced into a column packed with the same adsorbent (150 *g*) in n-hexane. The column was eluted using a step gradient of n-hexane, ethyl acetate, and methanol, starting with 100% n-hexane stepped to 10, 20, 30, 40, 60, 80% ethyl acetate in n-hexane, then 100% ethyl acetate, 50% methanol in ethyl acetate, and later 100% methanol [20]. 100 *ml* of each fraction was collected. The procedure was repeated three times to build up the yield. The fractions obtained were coded as CLE1-CLE10 and were all screened for their repellent activities. All fractions with percentage repellencies of greater than or equal to 70% were considered for further purification and isolation of the active compounds.

Ethical approval for repellent assay using human volunteers

Ethical approval was obtained from the College of medicine, University of Ibadan, Nigeria's Institute for Advanced Medical Research and Training (IAMRAT), with registration number NHREC/05/01/2008a and Ethics Board assigned number UI/EC/17/0308.

Human volunteer's recruitment

Recruitment was done from two areas for this study; inside and around University of Ibadan, Oyo State of Nigeria. Permission for ethics was obtained from college of Medicine, University of Ibadan, Nigeria's Institute for Advanced Medical Research and Training (IAMRAT), with an application that contains the full proposal about the study. The permission, in addition with the thoughtful speech by the ethical group, on the danger of Malaria and the importance of its control, assist in winning the co-operation of the elders and the majority of the people in the study areas. Furthermore, the importance of the research in the control of Malaria and the sources of the plant extracts to be evaluated were discussed in English and in native language. The people, (adult males and females only) were then allowed to declare individually, their consent and willingness to participate as volunteers. Again, these chosen volunteers were still given the opportunity to opt out at any time they wished.

Mosquito collection and culture

Adult female *Anopheles gambiae* mosquitoes (5-6 days old) which have not been blood-fed for 24 *hr* were used for the repellency assay [21]. The positive and negative controls were DEET and acetone, respectively. Mosquito larvae were collected from various habitats in Ibadan metropolis, Nigeria, and were authenticated at the Department of Zoology of the Faculty of Science, University of Ibadan, Nigeria. They were left to hatch into adults inside the (30×30×30 *cm*) netted cage, at the Insectary of the Department of Zoology, University of Ibadan, Nigeria. Full-grown female mosquitoes were maintained on 10% sugar solution at 27±2°*C* and 68±2% Relative humidity.

Repellency bioassay for the extracts and fractions

Human bait technique (Dose-percentage protection relationship) [22], which simulates skin condition to which repellents would be eventually applied was used. 5-6 days old, 50 blood starved mosquitoes were kept in (30×30×30 *cm*) cage for the test. The untreated forearm of the volunteer was inserted to confirm mosquito readiness to bite (when about 10 mosquitoes were observed landing within 30 *s*), and the hand was removed. The arm was then covered with a glove, with only about 35 cm^2 dorsal side exposed. Acetone (Control) was applied to the exposed skin first, dried for 1 *min*, and dipped inside the cage. The number of mosquitoes that landed (shaken off), over 5 *min* period, at every 15 *min* break was observed and noted. Each extract at 1.5, 2.5, and 5 *mg/ml* was then applied separately, starting with the lowest concentration. The repellency effect for the various seed fractions obtained from the extracts were also evaluated as described for the extracts. The number of mosquitoes that landed (shaken off), over 5 *min* period, at every 15 *min* break was observed for 2 *hr* and noted. Each fraction was applied separately at an optimized dose of 5 *mg/ml* [23]. Three replicates for the extracts and fractions were done for each test. Tests were conducted at room temperature and normal atmospheric pressure. Percentage repellency was calculated using a formula as shown below [24]:

$$\% \, Repellency = \frac{Ta - Tb}{Ta} \times 100$$

Ta=Number of mosquitoes in the control, Tb=Number of mosquitoes in the treated

Brine shrimp lethality test

The method of Mann *et al* [25] was used to assess the lethality of the brine shrimps. This test is a preliminary cytotoxicity assessment and was evaluated for the 100% n-Hexane Fraction (HF), which was the most active fraction of the *C. limon* seeds n-hexane extract.

Hatching of eggs

The seawater collected from the bar beach, Lagos was transferred into a soap case that has been divided, using a slide, to make a dam which was half covered. Brine shrimp eggs were added into the covered (dark) side and the whole case was exposed to light. The hatching of eggs to larvae occurred after 2 days and the larvae moved to the open or exposed side of the case, they were attracted to light (phototaxis).

Preparation of samples

The 100% HF was prepared in concentrations of 1000, 100 and 10 *µg/ml*. The stock solution was prepared by weighing 0.002 *g* (2 *mg*) of the sample and dissolving it in 2 *ml* of dimethyl sulphoxide in a test tube (labelled as test tube A) to give a 1 *mg/ml* (1000 *ppm*) solution. From this, 0.5 *ml* was taken and added to 3 separate test tubes. To each of the test tubes, 10 naupli (Artemia salina) and 4.5 *ml* of seawater was added using a micropipette. From the stock solution (test tube A), 0.2 *ml* was taken and 1.8 *ml* of sea water was added into a test tube (labeled as test tube B) to prepare 100 *ppm* concentration. From this, 0.5 *ml* was taken and added to 3 separate test tubes. To each of the test tubes, 10 naupli and 4.5 *ml* of seawater was added using a micropipette. From test tube B, 0.2 *ml* was taken and 1.8 *ml* of sea water was added into a test tube (labeled as test tube C) to prepare 10 *ppm* concentration. From this, 0.5 *ml* was taken and added to 3 separate test tubes. To each of the test tubes, 10 naupli and 4.5 *ml* of seawater were added using a micropipette. For the control, 10 naupli and 5.0 *ml* of seawater were added to 3 separate test tubes.

Assessment of brine shrimp for mortality

After 24 *hr* incubation at room temperature, all the set-ups were assessed and the dead and surviving larvae were counted and noted, by pouring the content into petri dishes and observed under light. The values were used for % mortality estimations. The LC_{50} values were also calculated using Finney's Probit Test Analysis for Quantal Data [26].

The gas chromatography-mass spectrometry analysis of 100% HF

The 100% HF of *C. limon* seed was analysed using Gas Chromatography-Mass Spectrometry, (GC-MS). The machine is an Agilent 7809 A Gas Chromatography together with Agilent Mass Detector. The detector had split/splitless injector coupled to mass selective detector functioning at 70 *eV*. The temperature of the ion source was 200°*C* and the mass spectral range was *m/z* 50-700 operating at 1428 *amu/sec* scan rate. The GC column was HP-5MS, 30 *m* length dimension, internal diameter 0.25 *mm*, film thickness of 0.25 *μm* and the stationary phase was 5% phenyl methyl silox. The programming of the oven temperature was done at 80°*C* initially for 2 *min* and then raised at 10°*C/min* to 240°*C* for 6 *min*. The carrier gas; Helium, was operated at 1 *ml/min* flow rate. Pressure, linear velocity and injection volume were operated at 56.2 *KPa*, 362 *cm/s* and 1.0 *μl*, respectively. The temperature of the oven was set at 60°*C* for 1 *min*, then increased at 10°*C/min* onto 180°*C* for 3 *min*. Then an increment of 10°*C/min* until final temperature of 280°*C* for 2 *min*. Both the injector and detector temperatures were fixed at 250°*C* [27].

Identification of components

Chemical constituents were identified based on comparison of mass spectral fragmentation patterns (NIST database 14.L/chemstation data system) with the data previously reported in the literature [28].

Statistical analysis

Statistical analysis of the experimental data was performed using the computer software SPSS 14 version and MS EXCEL 2003. One-way ANOVA in SPSS was used to calculate and compared the percentage repellency of the extracts at each concentration on plant basis. Results were also calculated as mean±standard deviation.

Results and Discussion

Repellent effects of the n-hexane, ethyl acetate and methanol extracts of C. limon seed

Repellent effects of the plant extracts were demonstrated using human volunteers. Activities varied according to solvent extracts and their concentrations. Only the n-hexane extract at different concentration showed a dose-dependent increase in activities, compared to ethyl acetate and methanol extracts. The effects were more pronounced at higher concentrations (5 *mg/ml*), of which the n-hexane and methanol extracts of the *C. limon* seeds, both at 5 *mg/ml*, showed the highest repellent effects of, 97.52 and 86.77%, respectively, when compared with the positive standard, DEET, 100.00% activity (Table 1). This variations in the repellent effects among different solvent suggest that, the activities depend both on the doses of the extracts and the polarity of the solvent. The least active is the ethyl acetate extract with 0.00% effect at 2.5 *mg/ml*. This high repellent effect is in-line with Sutanto *et al* who reported the potential of *C. limon* as repellent by testing the lethality of its peel extract on *Aedes eagypti* (*A. eagypti*) mosquito specie [29]. The most potent activity recorded at 30% dose was 97.3%, while the least repellent effect was 77.1% at 25% dose. Also, Hue *et al* reported effective activity against *A. eagypti* mosquito at a dose of 0.01 *ml*, with one *hr* ten *min* protection time and 0.9% biting percentage when compared to negative control ($p<0.05$) [30]. The result of the present study on the seeds of *C. limon* corresponds with this result on its peel, which brings future expectation and hope of developing new mosquito repellents from *C. limon*.

Repellent effects of the fractions from the most active n-hexane extract of C. limon seeds

C. limon n-hexane extract which were subjected to open chromatography in an attempt to separate the active constituents showed ten fractions and were obtained with percentage repellent effects (Table 2). The 100% n-hexane and 10% ethyl acetate in HF showed the most potent repellent effects of 89.47% and 71.07%, respectively compared with the positive standard, DEET, 100.00% activity (Table 2). No significant difference was observed between the activity of HF and that of the standard drug (DEET), at $p\geq0.05$. These fractionation results indicate that the most active fractions are obtained using 100% non-polar solvent (n-hexane) and also 10% ethyl acetate (intermediate polarity) in 90% n-hexane (non-polar) solvent mixture.

Table 1. Repellency of the *C. limon* seeds n-hexane, ethyl acetate, and methanol extracts

Plants	Extracts	Conc. (*mg/ml*)	Mean landed mosquitoes (±SD)			% Repellency
			20 *min*	40 *min*	1 *hr*	
	+ve control	Deet (20%)	0.00	0.00	0.00	100.00 [a]
C. limon	N-hexane	1.5	3.00±0	6.66±0.58	14.33±0.58	7.69
		2.5	2.33±0.58	4.33±1.53	9.00±1	35.05
		5.0	0.33±0.58	0.33±0.58	0.33±0.58	97.52 [a]
	-ve Control	Acetone	2.33±1.16	7.66±1.53	13.33±3.06	0.00
	E. acetate	1.5	0.33±0.58	1.33±0.58	5.00±1	68.07
		2.5	0.00±0	5.67±0.58	15.33±1.53	0.00
		5.0	3.00±0	4.33±0.58	9.00±0	29.757
	-ve control	Acetone	3.33±0.58	8.33±0.58	15.33±0.58	0.00
	Methanol	1.5	1.00±0	2.33±0.58	9.67±0.58	58.84
		2.5	4.00±0	7.00±1	11.33±0.58	51.48
		5.0	0.33±0.58	1.33±0.58	3.00±1	86.77 [a]
	-ve control	Acetone	7.00±1	13.67±1.53	22.67±2.52	0.00

No significant difference between values with superscript a at $p\leq0.05$.

Table 2. Repellency of the 100% HF of *Citrus limon*

Fractions	Conc. (*mg/ml*)	Mean landed mosquitoes (Triplicate) Mean±SD				% Rep
		25 *min*	**45 *min***	**80 *min***	**120 *min***	**mean±SEM**
Odomos (+ve Control)	Deet (20%)	0.00	0.00	0.00	0.00	100.00 [a]
-ve Control	Acetone	11±1	18.33±1.15	28±1.73	34.67±0.58	0.00
100% Hex	5.00	0.33±0.06	1.33±0.58	2±0	3.67±0.58	89.47±1.74 [a]
10% E/H	5.0	4.33±3.21	6±2.65	7.33±1.53	10±2	71.07±6.20 [b]
-ve Control	Acetone	9.67±0.577	14.33±0.58	20±1	29.33±1.53	0.00
20% E/H	5.00	2.33±0.58	6.33±1.16	9.67±1.53	13±2	55.67±8.63 [bc]
30% E/H	5.00	3.33±0.577	6.67±0.58	10.33±0.58	15.33±1.53	47.73±5.15 [c]
-ve Control	Acetone	10.67±1.53	16±1.73	22.33±2.08	33±2	0.00
40% E/H	5.00	5.33±1.155	9±1.73	12±2	17.33±2.08	47.48±6.25 [c]
60% E/H	5.00	11±1	15.67±1.53	22±1.73	28.67±2.52	13.12±5.72
-ve Control	Acetone	11.33±1.16	17.67±1.16	24±1.73	35.33±0.58	0.00
80% E/H	5.00	7±1	11.33±1.53	17.33±1.55	24.67±1.53	30.17±8.99
100% EA	5.00	8±1.732	12.67±2.08	20±2	28.67±2.52	18.85±15.08
-ve Control	Acetone	9.33±1.53	14.33±0.58	19.33±1.16	31.67±1.53	0.00
50% M/E	5.00	16.33±1.53	23±2	30.67±2.52	39±3.61	-21.12±4.15 [e]
100% M	5.00	16.33±1.53	23.67±2.08	32.33±2.52	40.67±3.05	-27.03±9.81 [e]

No significant difference between values with superscript a at $p \geq 0.05$, Superscript a are similar values, superscript b are similar values, superscript c are similar values, superscript e are similar values, E/H: Ethyl acetate in n-hexane, EA: Ethyl acetate, M/E: Methanol in ethyl acetate, M: Methanol, n-Hexane Fraction (HF).

The high repellent activity of HF must be due to the presence of some important bioactive phytoconstituents.

C. limon dry peels have been reported to be burnt, locally, in the living room at night to deter mosquitoes from biting or entering the house [11]. Also, the oil of the peel of lemon and orange are used as a non-toxic insecticide treatment [12]. The mechanism of action could be related or in line with that of Girgenti and Suss [31], in their work on the evaluation of the repellent effect of plant extracts on *A. aegypti* mosquitoes. The mosquitoes were not attracted, probably because the chemical constituent from the extract has inhibited the receptor cells of the lactic acid that attracts the mosquitoes [32]. The mosquitoes could therefore not make the contact to feed on blood, because they have failed to recognize the human skin as meal source. This suspected action is that the extract's active ingredient evaporated together with the carbon dioxide (CO_2) from the skin and change signature to that of the plant extract instead of the human carbon dioxide signature, which the mosquitoes desire [33,34]. The sneezing and itching reactions by volunteers were short-lived, so they were viewed as allergy.

Cytotoxicity evaluation of the most active 100% HF

The 100% HF was assayed for its brine shrimp lethality. The assay showed that the fraction had high level of toxicity (LC_{50} value <100 *µg/ml*), with LC_{50} values of 29.24 *µg/ml* [35]. Preliminary assessment of plants and their isolated compounds for their cytotoxicity can be carried out using brine shrimp lethality assay. This can be done to assess their safety to biological cells prior to human consumptions. The assay can also be used to detect medicinal properties such as antimicrobial and antitumor activities of plants and their compounds, based on the LC_{50} values they exhibited [35]. The HF showed cytotoxic activity on the larva. The observed mortality was found to be directly proportional to the concentration. The high toxicity level shown by HF, (LC_{50}=29.24 *µg/ml*) could be an indication of the existence of some cytotoxic compounds in the fraction [35] and also an indication that the fraction cannot be potentially recommended as a safe repellant.

Chemical composition of the 100% HF

The yield of the 100% HF of the volatile oil had mean (±standard deviation) of 0.356±0.03% with a pale yellow colouration. A total of thirty compounds representing 79.25% of HF were identified. Major compounds present in the fraction were heptadecanemine (8.31%), N-(3-methylbutyl) acetamide (7.89%), 2-ethyl-1-hexanamine (6.65%), 5-methyl-2-hexanamine (5.33%), octodrine (5.15%), propenamide (4.32%), tetradecanamine (4.15%). Heptadecanamine and its derivatives; polyacryl-dimethyl-heptadecanamine-mullite, were found useful as a promising sorbent for VO^{2+} and Cr^{3+} ions [36]. Another derivative, Hexadecyl trimethyl ammonia bromide has been found useful in the synthesis of schwertmannite applied in the removal of ROX (Roxarsone, a toxic non-organic arsenic) pollution in waste water [37]. 2-ethyl-1-hexanamine is applied in the manufacturing of pharmaceutical products and also in production of n-alkyl carbamates used in medicine [38] (Table 3).

Table 3. Chemical constituents of 100% HF of *C. limon*

S/No	Retention time (*min*)	Compounds	Molecular formular	Molecular weight (*g/mol*)	% Area
1	5.55	N-butylethylenediamine	$C_6H_{16}N_2$	116.20	1.88
2	5.70	4-methyl -2-pentanamine	$C_6H_{15}N$	101.19	0.84
3	5.80	4-florohistamine	$C_5H_9FN_3$	130.14	1.21
4	5.84	Methyldodecylamine	$C_{13}H_{29}N$	199.38	0.67
5	5.88	N-methyl- 1-octadecanamine	$C_{19}H_{41}N$	283.54	2.43
6	6.28	5-methyl-2-hexanamine	$C_7H_{17}N$	115.22	5.33
7	6.77	Tetradecanamine	$C_{14}H_{31}N$	213.40	4.15
8	7.18	2-ethyl- 1-hexanamine	$C_8H_{19}N$	129.24	6.65
9	7.35	Octodrine	$C_8H_{19}N$	129.24	5.15
10	8.44	N-(1-methylpropyl)- acetamide	$C_6H_{13}NO$	115.17	0.76
11	8.51	3,3—dimethyl-4-methylamino-butan-2-one	$C_7H_{15}NO$	129.12	0.55
12	8.81	N-(1-cyclohexylethyl)-propanamide	$C_{11}H_{21}NO$	183.29	0.86
13	9.25	Propanamide	C_3H_7NO	73.09	4.32
14	9.39	Heptadecanamine	$C_{17}H_{37}N$	255.48	8.31
15	10.02	N,N-dimethyl-guanidine	$C_3H_9N_3$	87.12	2.38
16	10.09	hexahydro-(1,3,5)-trimethyl-(1,3,5)-Triazine	$C_6H_{15}N_3$	129.20	1.47
17	10.13	Di (pent-4-enyl) amine	$C_{10}H_{19}N$	153.26	0.86
18	10.25	Dodecanamine	$C_{12}H_{27}N$	185.35	2.15
19	10.36	1,2,3,4-butanetetrol	$C_4H_{10}O_4$	122.12	1.41
20	10.51	2-cyano-acetamide	$C_3H_4N_2O$	84.08	2.58
21	10.56	N,N-dimethyl-1,8-octanediamine	$C_{10}H_{24}N_2$	172.31	0.70
22	10.95	N-methyl-N-(2-cyanoethyl)-2-mercapto propylamine	$C_7H_{14}N_2S$	158.26	2.91
23	11.16	2-oxo-3-methyl-cis-perhydro-1,3-benzoxazine	$C_9H_{15}NO_2$	169.22	2.12
24	11.21	N-methyl-1,3-propanediamine	$C_4H_{12}N_2$	88.15	1.00
25	11.39	2-(methylaminomethyl) cyclohexanol	$C_8H_{17}NO$	143.23	0.77
26	11.69	N-(3-methylbutyl)acetamide	$C_7H_{15}NO$	129.20	7.89
27	12.35	5-hydroxy-1H-(1,2,4)-Triazole-3-carboxylic acid	$C_3H_3N_3O_3$	129.07	3.18
28	16.08	Allantoin	$C_4H_6N_4O_3$	158.16	2.81
29	16.70	dl-alanyl-dl-asparagine	$C_7H_{13}N_3O_4$	203.20	2.35
30	38.23	Pentadecylamine	$C_{15}H_{33}N$	227.43	1.56
Total					79.25

n-Hexane Fraction (HF).

N-(3-methylbutyl) acetamide (MBA); the major volatile compound in the vespine wasps' venom, *Vespula germanica* (*V. germanica*) had been shown to be fast in producing recruitment and attack behaviour. Its presence in the venom of numerous vespids and the attraction of male to the venom of some specific females led to the hypothesis that MBA has the function of an attractant that aids in maintaining the mating of the wasps [39]. Methylhexaneamine (MHA) also known as geranamine is originally a stimulant, used as supplements [40], but its safety was a controversy [41], due to numerous side effects. It was re-introduced in 2005 for various functions, like supplement for muscle enhancer and weight loss [40]. Octodrine also known as dimethylhexylamine (DMHA) was a stimulant for the central nervous system. It was used as a respiratory aid during the 1950's. Presently, it sold as supplement for fat-burner and workout [42]. These identified compounds could be responsible for the repellent and toxic effects of the fraction. Hence, this promising repellent activity further justified the traditional usage of *C. limon* as mosquito repellent.

Conclusion

C. limon extracts and fractions from seeds have demonstrated effectiveness as mosquito repellents at meaningful doses. The chemical constituents of the most effective fraction for mosquito repellency indicated compounds which, through their synergistic effect, could contribute to the observed activity. These constituents could be utilized in synergy with the existing synthetic and plant-based repellents against mosquito bite. The active fraction and the volatile constituents could also be used in further studies, such as agricultural pests and other insects. Isolation, identification and purification of the active molecules from the active fractions and evaluation of their respective repellent and cytotoxic effects would be investigated with view of formulating novel mosquito repellent agents. The application of the results could also be beneficial in vector control, mostly in local communities, where poor socio-economic circumstances may prevent protection against insect vectors using modern techniques. Repellents of plant background are human-friendly, eco-friendly and very cheap to acquire. The repellent activities of the volatile constituents are being reported for the first time and their incorporation in combined vector control would go a long way to reduce the scourge of mosquito bites in the environment. The results of the brine shrimp lethality test gave further information concerning the cytotoxicity of the volatile compounds in the active fraction and that the fraction cannot be potentially safe with the high level of cytotoxic effect (LC_{50} <100 *μg/ml*).

Acknowledgement

We are highly thankful to Mr. Ibrahim and the volunteers from the Zoology Department of the University of Ibadan, for their support in the Mosquito repellency assays. We also acknowledge Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan for providing the ethical approval (registration number: NHREC/05/01/2008a and Ethics Board number: UI/EC/17/0308) for this work.

Conflict of Interest

The authors declare there is no conflict of interest.

References

1. Adeniran OI, Fabiyi E. A cream formulation of an effective mosquito repellent: a topical product from lemongrass oil (Cymbopogon citratus) Stapf.
2. World Health Organization (1999). Malarial fact sheet. No. 94. Geneva (accessed May 3, 2002 at http:www.who.int/inf.Fs/en/fact 094.html).
3. World Health Organisation (2019). World Malaria Report 2019.
4. World Health Organisation. (1996). Report of the WHO informal consultation on the evaluation and testing of insecticides CTD/WHO PES/IC/96.1. Geneva 69.
5. Toure Y. (2002). Malaria vector control strategies and challenges in Africa, Mali. In: Epidemiology of Parasitic diseases. University of Mali Press.
6. Thorsell W, Mikiver A, Malander I, Tunón H. Efficacy of plant extracts and oils as mosquito repellents. Phytomedicine 1998 Aug;5(4):311-23.
7. Govere J, Durrheim DN, Du Toit N, Hunt RH, Coetzee M. Local plants as repellents against Anopheles arabiensis, in Mpumalanga Province, South Africa. Cent Afr J Med 2000 Aug;46(8):213-6.
8. Isman MB, Miresmailli S, Machial C. Commercial opportunities for pesticides based on plant essential oils in agriculture, industry and consumer products. Phytochemistry Reviews 2011 Jun;10:197-204.
9. Ahmad MM, Iqbal Z, Anjum FM, Sultan JI. Genetic variability to essential oil composition in four citrus fruit species. Pakistan Journal of Botany 2006 Feb;38(2):319.
10. Tomotake H, Koga T, Yamato M, Kassu A, Ota F. Antibacterial activity of citrus fruit juices against Vibrio species. Journal of Nutritional Science and Vitaminology 2006;52(2):157-60.
11. Anyanwu GI, Amaefule EC, Ngurukwem C. Larvicidal effects of lemon peels on mosquito larvae. Journal of Aquatic Sciences 2001;16(2):111-4.
12. Hofrichter M. Industrial applications of lemon. Chemistry and Biochemistry 2010;6:224.
13. Yang P, Ma Y, Zheng S. Adulticidal activity of five essential oils against Culex pipiens quinquefasciatus. Journal of Pesticide Science. 2005;30(2):84-9.
14. El-Adawy TA, Rahma EH, El-Bedawy AA, Gafar AM. Properties of some citrus seeds. Part 1. Physico-chemical characteristics of proteins. Food/Nahrung. 1999 Dec 1; 43(6):374-8.
15. Sharma K, Mahato N, Cho MH, Lee YR. Converting citrus wastes into value-added products: Economic and environmently friendly approaches. Nutrition 2017 Feb;34:29-46.
16. Yilmaz E, Güneşer BA. Cold pressed versus solvent extracted lemon (Citrus limon L.) seed oils: yield and properties. J Food Sci Technol 2017 Jun;54(7):1891-1900.
17. Penniston KL, Nakada SY, Holmes RP, Assimos DG. Quantitative assessment of citric acid in lemon juice, lime juice, and commercially-available fruit juice products. J Endourol 2008 Mar;22(3):567-70.
18. Anwar F, Naseer R, Bhanger MI, Ashraf S, Talpur FN, Aladedunye FA. Physico-chemical characteristics of citrus seeds and seed oils from Pakistan. Journal of the American Oil Chemists' Society 2008 Apr;85(4):321-30.
19. Reda SY, Leal ES, Batista EA, Barana AC, Schnitzel E, Carneiro PI. Characterization of rangpur lime (Citrus limonia Osbeck) and" sicilian" lemon (Citrus limon) seed oils, an agroindustrial waste. Food Science and Technology. 2005;25:672-6.
20. Zhang Y, Liu C, Zhang Z, Qi Y, Wu G, Li S. Solvent gradient elution for comprehensive separation of constituents with wide range of polarity in Apocynum venetum leaves by high-speed counter-current chromatography. J Sep Sci 2010 Sep;33(17-18):2743-8.
21. Dua VK, Gupta NC, Pandey AC, Sharma VP. Repellency of Lantana camara (Verbenaceae) flowers against Aedes mosquitoes. J Am Mosq Control Assoc 1996 Sep; 12(3 Pt 1):406-8.
22. Murugan K, Vahitha R, Baruah I, Das SC. Integration of botanical and microbial pesticides for the control of filarial vector, Culex quinquefasciatus Say (Diptera: Culicidae).
23. Venkatachalam MR, Jebanesan A. Repellent activity of Ferronia elephantum Corr. (Rutaceae) leaf extract against Aedes aegypti (L.). Bioresour Technol 2001 Feb;76(3): 287-8.

24. Yoon JK, Kim KC, Cho YD, Cho HS, Lee YW, Choi BK, et al. Development and evaluation of a semifield test for repellent efficacy testing. J Med Entomol 2014 Jan; 51(1):182-8.
25. Mann A, Ibrahim K, Oyewale AO, Amupitan JO, Fatope MO, Okogun JI. Brine shrimp toxicity evaluation of the root bark extract of Terminalia avicennioides. Afr J Sci Res 2011;4(1):221-8.
26. Aboaba SA, Aiyelaagbe OO, Ekundayo O. Chemical composition, toxicity and larvicidal activity of the essential oil from the whole plant of Acalypha segetalis from south-west Nigeria. Nat Prod Commun 2010 Mar;5(3): 481-3.
27. Mbachu KA, Ibok MG, Adeniyi-Akee MA, Ajala OE. Chemical compositions and antioxidant activity of leaf and stem essential oils of Bryophyllum pinnatum (lam.) Kurz. GSC Biological and Pharmaceutical Sciences 2019 Nov 30;9(2):057-64.
28. Adams RP, 2007. Identification of Essential Oil Components by GC-MS. Allured Publication Corp., Carlo Stream, II. USA
29. Zen S, Sutanto A, Rohmah A. The Effect of Lemon (Citrus Limon L.) Extract on Mortality of Aedes Sp. and Potential As Repellent. PENBIOS: JURNAL PENDIDIKAN BIOLOGI DAN SAINS 2020 Nov 12;5(02):39-45.
30. Hue PT, Van Loc T, Vien LT, Hieu HV, Khoa PT. Optimization of citrus limon peel extraction, determination of main chemical components and effectiveness in repellency against aedes mosquito dengue fever vector. Vietnam Journal of Biotechnology 2022 Jun 30;20(2):329-38.
31. Girgenti P, Suss L. Attività repellente nei confronti di Aedes aegypti (L.) di formulati a base di estratti vegetali naturali o principi attivi di sintesi [Repellent activity against Aedes aegypti (L.) of formulas based on natural vegetable extracts or synthetic active agents]. Ann Ig. 2002 May-Jun;14(3):205-10. Italian.
32. Effiom OE, Avoaja DA, Ohaeri CC. Mosquito repellent activity of phytochemical extracts from peels of citrus fruit species. Global Journal of Science Frontier Research. 2012;12(1):1-8.
33. Jacobson M. Plant derived Insect Deterents glossary. CRC Press, Inc., Boca Raton, Florida; (1990).
34. Foster S, Duke JA. Naptalactose, a mild sedative compound in Catnip, also possesses herbicidal and insect-repellent properties. Peterson Field Guides, Eastern/Central Medicinal plants. Houghton Mifflin Co. New York. 1990.
35. Aboaba SA, Oladosu IA, Ekundayo O. Chemical composition, acetyl cholinesterase inhibitory properties and brine shrimp toxicity of essential oils from Ficus capensis Thunb from Nigeria. International Journal of Biological and Chemical Sciences 2010;4(5).
36. Eliwa AA, Mubark AE, Dakroury GA, El-Shazly EA, El-Azony KM. Polyacryl-dimethyl-heptadecanamine-mullite as a promising sorbent for chromium and vanadium sorption from ilmenite. Journal of Environmental Chemical Engineering 2022 Dec 1;10(6):108886.
37. Li B, Zang S, Zhou H, An S, Qiu H. Study on the Removal of ROX from Wastewater by Modified Schwertmannite with Hexadecyl Trimethyl Ammonia Bromide. Russian Journal of Physical Chemistry A 2023 Apr;97 (4):672-8.
38. Jager P, Rentzea CN, Kieczka H. Carbamates and carbamoyl chloride. Ullmann's Encyclopedia of industrial Chemistry Weinheim: Wiley-VCH. 2000.
39. Elmquist DC, Landolt PJ, Cooper WR, Reed H, Foutz J, Clepper T, et al. The Venom Compound N-(3-methylbutyl) acetamide Attracts Several Polistes (Fuscopolistes) Species (Hymenoptera: Vespidae). J Econ Entomol 2020 Jun 6;113(3):1073-1079.
40. Lopez-Avila V, Zorio M. Identification of methylhexaneamine by GC high-resolution TOFMS and soft ionization. Forensic Sci Int 2013 Sep 10;231(1-3):113-9.
41. Eliason MJ, Eichner A, Cancio A, Bestervelt L, Adams BD, Deuster PA. Case reports: Death of active duty soldiers following ingestion of dietary supplements containing 1,3-dimethylamylamine (DMAA). Mil Med 2012 Dec;177(12):1455-9.
42. Catalani V, Prilutskaya M, Al-Imam A, Marrinan S, Elgharably Y, Zloh M, et al. Octodrine: New Questions and Challenges in Sport Supplements. Brain Sci 2018 Feb 20;8(2):34.