



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

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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* s/ 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* s/ 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 (Odorous 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

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Introduction

Global mortalities and illnesses are majorly caused 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 deaths 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

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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 toiletary, 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 assay²¹. 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 cm² 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/ml²³. 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 below²⁴:

$$\% \text{ Repellency} = \frac{T_a - T_b}{T_a} \times 100$$

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 LC₅₀ values were also calculated using Finney's Probit Test Analysis for Quantal Data²⁶.

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°C²⁷.

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 literature²⁸.

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 $\pm$ 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 ef-

fects 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 egypti* (*A. egypti*) mosquito specie²⁹. 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. egypti* 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 $p \geq 0.05$. 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 ($\pm$ SD)			% Repellency
			20 min	40 min	1 hr	
<i>C. limon</i>	+ve control	Deet (20%)	0.00	0.00	0.00	100.00 ^a
	N-hexane	1.5	3.00 $\pm$ 0	6.66 $\pm$ 0.58	14.33 $\pm$ 0.58	7.69
		2.5	2.33 $\pm$ 0.58	4.33 $\pm$ 1.53	9.00 $\pm$ 1	35.05
		5.0	0.33 $\pm$ 0.58	0.33 $\pm$ 0.58	0.33 $\pm$ 0.58	97.52 ^a
	-ve Control	Acetone	2.33 $\pm$ 1.16	7.66 $\pm$ 1.53	13.33 $\pm$ 3.06	0.00
	E. acetate	1.5	0.33 $\pm$ 0.58	1.33 $\pm$ 0.58	5.00 $\pm$ 1	68.07
		2.5	0.00 $\pm$ 0	5.67 $\pm$ 0.58	15.33 $\pm$ 1.53	0.00
		5.0	3.00 $\pm$ 0	4.33 $\pm$ 0.58	9.00 $\pm$ 0	29.757
	-ve control	Acetone	3.33 $\pm$ 0.58	8.33 $\pm$ 0.58	15.33 $\pm$ 0.58	0.00
	Methanol	1.5	1.00 $\pm$ 0	2.33 $\pm$ 0.58	9.67 $\pm$ 0.58	58.84
		2.5	4.00 $\pm$ 0	7.00 $\pm$ 1	11.33 $\pm$ 0.58	51.48
		5.0	0.33 $\pm$ 0.58	1.33 $\pm$ 0.58	3.00 $\pm$ 1	86.77 ^a
	-ve control	Acetone	7.00 $\pm$ 1	13.67 $\pm$ 1.53	22.67 $\pm$ 2.52	0.00

No significant difference between values with superscript a at $p \leq 0.05$.

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

Fractions	Conc. (mg/ml)	Mean landed mosquitoes (Triplicate) Mean±SD				% Rep mean±SEM
		25 min	45 min	80 min	120 min	
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 house¹¹. Also, the oil of the peel of lemon and orange are used as a non-toxic insecticide treatment¹². The mechanism of action could be related or in line with that of Girgenti and Suss³¹, 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 mosquitoes³². 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 (CO₂) 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 (LC₅₀ value <100 µg/ml), with LC₅₀ values of 29.24 µg/ml³⁵. 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 LC₅₀ values they exhibited³⁵. 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, (LC₅₀=29.24 µg/ml) could be an indication of the existence of some cytotoxic compounds in the fraction³⁵ and also an indication that the fraction cannot be potentially recommended as a safe repellent.

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 heptadecanamine (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-mulite, were found useful as a promising sorbent for VO²⁺ and Cr³⁺ ions³⁶. 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 water³⁷. 2-ethyl-1-hexanamine is applied in the manufacturing of pharmaceutical products and also in production of n-alkyl carbamates used in medicine³⁸ (Table 3).

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

S/No	Retention time (min)	Compounds	Molecular formula	Molecular weight (g/mol)	% Area
1	5.55	N-butylethylenediamine	C ₆ H ₁₆ N ₂	116.20	1.88
2	5.70	4-methyl -2-pentanamine	C ₆ H ₁₅ N	101.19	0.84
3	5.80	4-florohistamine	C ₅ H ₉ FN 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 ₇ H 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 ₈ H ₁₉ N	129.24	6.65
9	7.35	Octodrine	C ₈ H ₁₉ N	129.24	5.15
10	8.44	N-(1-methylpropyl)- acetamide	C ₆ H ₁₃ NO	115.17	0.76
11	8.51	3,3—dimethyl-4-methylamino-butan-2-one	C ₇ H 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 ₃ H ₇ NO	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 ₃ H ₉ N ₃	87.12	2.38
16	10.09	hexahydro-(1,3,5)-trimethyl-(1,3,5)-Triazine	C ₆ H 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 ₄ H ₁₀ O ₄	122.12	1.41
20	10.51	2-cyano-acetamide	C ₃ H ₄ N ₂ O	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 ₇ H ₁₄ N ₂ S	158.26	2.91
23	11.16	2-oxo-3-methyl-cis-perhydro-1,3-benzoxazine	C ₉ H ₁₅ NO ₂	169.22	2.12
24	11.21	N-methyl-1,3-propanediamine	C ₄ H ₁₂ N ₂	88.15	1.00
25	11.39	2-(methylaminomethyl) cyclohexanol	C ₈ H ₁₇ NO	143.23	0.77
26	11.69	N-(3-methylbutyl)acetamide	C ₇ H ₁₅ NO	129.20	7.89
27	12.35	5-hydroxy-1H-(1,2,4)-Triazole-3-carboxylic acid	C ₃ H ₃ N ₃ O ₃	129.07	3.18
28	16.08	Allantoin	C ₄ H ₆ N ₄ O ₃	158.16	2.81
29	16.70	dl-alanyl-dl-asparagine	C ₇ H ₁₃ N ₃ O 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, *Vespa 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 wasps³⁹. Methylhexanamine (MHA) also known as geranamine is originally a stimulant, used as supplements⁴⁰, but its safety was a controversy⁴¹, due to numerous side effects. It was re-introduced in 2005 for various functions, like supplement for muscle enhancer and weight loss⁴⁰. 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 workout⁴². 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 ($LC_{50} < 100 \mu\text{g/ml}$).

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Conflict of Interest

The authors declare there is no conflict of interest.

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