Basic Sciences of Medicine 2020, 9(1): 1-7
DOI: 10.5923/j.medicine.20200901.01

Repellent Properties of Compounds and Blends from *Nigella sativa* Seeds Against *Anopheles gambiae*

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Abstract Malaria, transmitted to humans by the bites of infected *Anopheles* mosquitoes is a public health concern. Some available repellents against the insect have adverse effects on humans and environment. This study determined *Anopheles gambiae* repellent compounds and blends from *Nigella sativa* L. seed. From GC-EAD analysis, repellent compounds were 1,2,3,4,5-pentamethylcyclopentane, α-thujene, α-pinene, β-pinene, tetradecane, *p*-cymene, α-longipinene and longifolene. Repellency of blend 1 consisting of six GC-EAD active compounds was lower than that of essential oil and DEET. Subtraction of *p*-cymene and (-)-α-pinene from blends 2 and 6 respectively significantly increased repellency while subtraction of (+)-β-pinene, (-)-β-pinene, (+)-α-pinene, tetradecane or 1,2,3,4,5-pentamethylcyclopentane significant decreased repellency of the blends. Blends 2 and 6 repelled 100% of mosquitos at 0.1g/ml while (+)-α-pinene repelled 99.38%. These findings confirm that essential oil of *N. sativa* L. seeds contain compounds that repel *An. gambiae*. The positive enantiomer of α-pinene was the most repellent which confirms the compound to be the main active ingredient the oil.

Keywords *Nigella sativa*, Essential oil, *Anopheles gambiae*, Repellence, GC-EAD

1. Introduction

Malaria, a vector-borne disease transmitted to humans by the bites of infected *Anopheles* mosquitoes, is a public health concern especially in Africa [1]. Malaria occurs mostly in poor tropical and subtropical areas of the world. In many of the countries affected by malaria, it is a leading cause of illness and death. In 2016, malaria caused an estimated 216 million clinical episodes and 445,000 deaths worldwide with about 90% of deaths occurring in African [2]. One of the best ways to deal with this global enemy is to prevent mosquito bites using physical and chemical barriers, treatment of fabric with toxicants, and the use of topical (skin) repellents [3]. *N*,*N*-dimethyl-3-methylbenzamide (DEET) has been the mainstay among repellents approved for use on human skin [4] because it has broad-spectrum activity and effectively repels mosquitoes as well as other insects including flies, chiggers, fleas, and ticks [4]. Due to side effects and the adverse effects on the environment and food chain [5-7], the use some of the synthetic chemicals has been banned. Previous studies have shown that in plants there are bioactive compounds that have gained increasing interest as potential therapeutic agents [8-11]. Extracts from numerous plants species have been shown to possess repellent and toxic effects against insects [12-18]. Several plants have been studied as possible mosquito repellents revealing the existence of natural repellents with good efficacy [19-21]. Insect repellents from plant origin are preferred since they are more friendly to both the user and the environment.

Nigella sativa L. (also called black cumin, or black seed) is an annual herb of the Ranunculaceae family and is cultivated in various parts of the globe [22]. *N. sativa* L. seeds have been used traditionally to treat of asthma, fever, cough, eczema, headache, rheumatism, influenza and bronchitis [23]. Previous studies have shown that extracts and oils from *N. sativa* L. seed to have anti-inflammatory, antimicrobial and antioxidant activities [24,25]. Extracts from the plant also exhibited insecticidal and insect repelling activity against *Amblyomma americanum*, *Tribolium castaneum*, *Tuta absoluta* and Mosquito [26,27]. Most researchers have concentrated more of determining the bioactivity of the crude extracts and oils from the plant on the plant. The main components of *N. sativa* seed essential oil are *p*-cymene, thymol, thymoquinone and 9-eicosyne [24,28]. The objective of the present study was to determine the mosquito repellence efficacy of compounds and blends from *Nigella sativa* seed essential oil.

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Published online at http://journal.sapub.org/medicine

2. Materials and Methods

2.1. Plant Material

Dry *N. sativa* seeds were obtained from the Kenyan local market. The seeds were authenticated at the Department of Botany, Kenyatta University in Kenya. The sample was cleaned and freed from dust and foreign material. A weight of 125 g of *N. sativa* seeds were ground using an electric house-hold spice grinder.

2.2. Extraction of Essential Oil

The ground *N. sativa* seeds (125 g) were transferred into a clean flask and 500 ml of distilled water was added. 10 g of sodium chloride was added to the mixture to reduce the foaming during boiling. Oil extraction was done by hydro-distillation method using the Clevenger-type apparatus [29]. The apparatus were set-up and the mixture were heated at 100 ℃ for three hours. The oil was separated from the aqueous phase and diluted with pure acetone to make a concentration of 10%, which was stored in amber-colored vial at -4 ℃ for phytochemical analysis and bio-assays.

2.3. Mosquito Rearing

Anopheles gambiae s.s. were obtained from colonies reared in the insectaries at International Centre of Insect Physiology and Ecology (ICIPE, Nairobi, Kenya) and were reared according to the WHO protocol [30]. Mosquito eggs were hatched by simultaneously flooding the moist filter paper platforms. Rearing was carried out in the insectary maintained at 27-28 ℃ and approximately 80% humidity on a 12h/12h light and darkness cycle and maintained at optimal larval concentrations to avoid possible effects of competition. Mosquito larvae were fed on ground baby fish food while adults were offered a fresh 10% (w/v) sucrose solution meal daily and on hamsters as a source of blood meals when required to produce eggs.

2.4. Chemicals

Standards were purchased from Sigma-Aldrich namely (+)-β-pinene, (-)-β-pinene, (+)-α-pinene, (-)-α-pinene, p-cymene, α-longipinene, tetradecane and 1,2,3,4,5-pentamethylcyclopentane. The purchased chemicals were of analytical grade purity.

2.5. Gas Chromatography with Electroantennographic Detector (GC-EAD)

GC-EAD analysis was used to isolate EAD-active components from *N. sativa* seeds essential oil. GC-EAD tests were performed on HP 5890 series II gas chromatograph equipped with a flame ionization detector (FID) and HP Ultra 1 (cross-linked methyl silicon gum) capillary column (50 m × 0.2 mm × 0.33 μm) using nitrogen at a flow rate of 0.8 ml/min as the carrier gas. The oven temperature was programmed from 60 ℃ for 5 min and then 5 ℃ /min to 280 ℃, where it was held for 15 min. Antennae of thirty (30) laboratory reared 5-7 days old females of *An. gambiae* were used. A glass micro-pipette containing Beadle-Ephrussi saline was inserted through the head of the insect. The fine tip of the micro-pipette was pushed through the head between the thorax and the head of the insect. The other end of the micro-pipette was sheathed over a silver wire, the recording electrode, which was connected to the input of a universal AC/DC UN-05 amplifier (Syntech, The Netherlands). To complete the circuit, the distal end of the antenna was nipped off with a scalpel and the open end inserted into similar glass micro-pipette containing the saline and was sheathed over a silver wire electrode that was grounded.

The effluent from the capillary column was split in a ratio of 1:1 into two 50 cm long de-activated silica columns, one connected to the FID and the other connected to a stainless-steel tube (5mm i.d.) that was focused onto the antennal preparation. A make-up gas (40 ml/min) was added just before the split point to accelerate the effluent through deactivated columns. The deactivated transfer line carrying the effluent over the antennal preparation was maintained at 150 ℃ by a THC-3 temperature control unit (Syntech, The Netherlands). Aliquots (8-10 μl) of *N. sativa* seeds essential at concentration of 0.1% by mass were analyzed by the GC-EAD and GC signals were monitored synchronously using a program on a GC/EAD interface card (Syntech, The Netherlands) installed in a PC (Harvard Professional computer, American Megatrends Inc.).

2.6. Gas Chromatography - Mass Spectrometry (GC-MS)

The antennal active peaks from the essential oil of *N. sativa* seeds were identified by GC-MS on a GC-MS HP 8060 series II GC coupled with a VG Platform II mass Spectrometer. The MS was operated in the EI mode at 70 eV and an emission of 200 μA, with the temperature of the source held at 180 ℃ multiplier voltage at 300 V. The MS had a scan cycle of 1.5 s (scan cycle of 1 s and inter-delay of 0.5 s) and scan ranges *m/z* 38-650. Helium was used as the carrier gas and the column temperature, 50 ℃ for 5 min, rising to 90 ℃ at 5 ℃ min^{-1}, then 200 ℃ at 2 ℃ min^{-1}, and then rising 280 ℃ at 20 ℃ min^{-1} held for 20 min. The compounds were identified by analysis of their mass spectra and direct comparison with the Institute of Standards Technology libraries 98.1 (NIST) and Wiley Registry of Mass Spectral Data, 8^{th} edition database of library of mass spectra, on the GC-MS equipment.

2.7. Repellency Bioassay

Tests were done according to WHO protocol [30] on *An. Gambiae* s.s. Repellency assays were done with 5-7 days old females of *An. gambiae* that had been starved for 18 hours but previously fed on 6% glucose solution [31]. Subtractive bioassays were done to identify constituents that contributed significantly to the repellence of the blend of EAG-active compounds. The essential oil from *N. sativa* seeds,

GC-EAG-active compounds and blends of the GC-EAG-active compounds were tested at concentrations of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1}. The tests were performed on 6 human adults (18yrs ≤ age ≤ 50yrs). Participants had no contact with lotions, perfumes oil or perfumed soaps on the day of the experiment. An insect bite cream was provided to the participants in case of any minor bites and associated irritations. A total of 18 cages each measuring 50×50×50 centimeters were used [31]. Test solutions (1.0 ml) were dispensed on one of the forearms of a volunteer from wrist to the elbow covering an area of 500 cm^2. The rest of the hand was covered with a glove. Acetone was dispensed on the other forearm to serve as control. The control and treated arms were interchanged regularly to eliminate bias [31]. The control arm was first introduced into the cage immediately after releasing the 50 experimental mosquitoes and kept there for 3 minutes. The number of insects that landed on control arm during the test was recorded. The treated arm was then introduced into the cage for the same period of time and the number of landing insects recorded. The different concentrations of the sample and DEET were tested starting with lowest concentrations. For each test, six pairs of arms were used. Percentage protective efficacy (PE) was calculated using the formula PE = (C-T/C) ×100% where C and T are the mean numbers of mosquitoes that landed on the control and the test arm respectively [32]. Dose-response relationship was determined using probit analysis and repellent doses at RD_{50} and RD_{75} values obtained from regression model [32].

2.8. Ethical Issues

Ethical approval for this study was given by Kenyatta University Ethics Review Committee, Kenya with the reference number of KU/R/COMM/51/16. The approval was given after submitting the detailed proposal of the study to the Committee for thorough review. The volunteers were given written consent forms which they were taken through before signing in presence of a witness who was not a participant in study participant.

2.9. Data Analysis

The data obtained was subjected to analysis of variance (ANOVA) and means ranked using Student-Newman-Keuls (SNK) at the 5% significance level.

3. Results and Discussion

3.1. Gas Chromatography/Electro-Antennographic Detection Using *An. gambiae*

Hydro-distillation of the seeds of *N. sativa* gave a dark yellow colored oil (0.68 g) with a characteristic odor. The GC-EAD analysis of the revealed that eight compounds consistently elicited electrophysiological responses from the antennae of the female *An. gambiae* (Figure 1). The compounds were identified by GC-MS by comparing their fragmentation patterns using Institute of Standards Technology libraries 98.1 (NIST) and Wiley Registry of Mass Spectral Data, 8th edition (Figure 2). The compounds were identified as 1,2,3,4,5-pentamethylcyclopentane (**1**, Rt 9.13 min), α-thujene (**2**, Rt 9.38 min), α-pinene (**3**, Rt 9.49 min), β-pinene (**4**, Rt 10.41 min), tetradecane (**5**, Rt 10.88 min), *p*-cymene (**6**, Rt 11.42 min), α-longipinene (**7**, Rt 16.57 min) and longifolene (**8**, Rt 17.33 min) and the structures are given in Figure 3. The results are in agreement with previous studies which reported the insecticidal and insect repellent activities of the compounds against various insects [33]. Both isomers of compound **3** [(-)-α-pinene and -(+)-α-pinene] repelled house fly, *Musca domestica*, [33] while in another studies the compounds also showed larvicidal activity against *Anapheles subpictus*, *Aedes albopictus*, *Culex tritaeniorhynchus* [34]. *p*-Cymene (**6**) showed repellent activity against *Amblyomma americanum* L. and *Aedes aegypti* L. [35]. Longifolene (**8**) showed termiticidal and antifeedant activities against *Reticulitermes speratus* Kolbe [36].

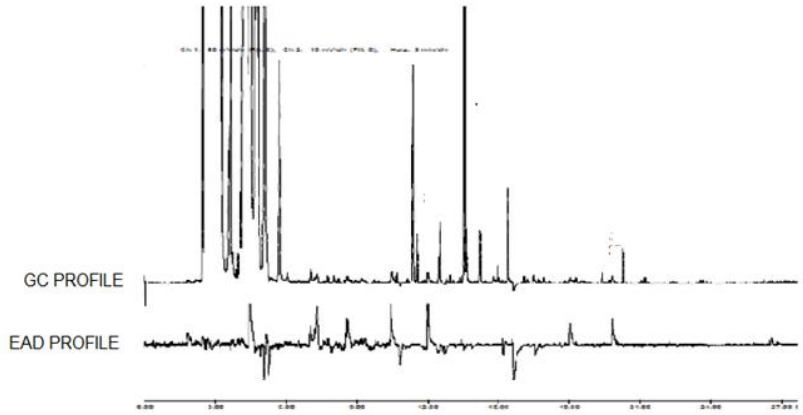

Figure 1. Gas chromatography-electroantennographic detection (GC-EAD) using the antennae of *Anopheles gambiae* females in response to *Nigella sativa* L. seed essential oil

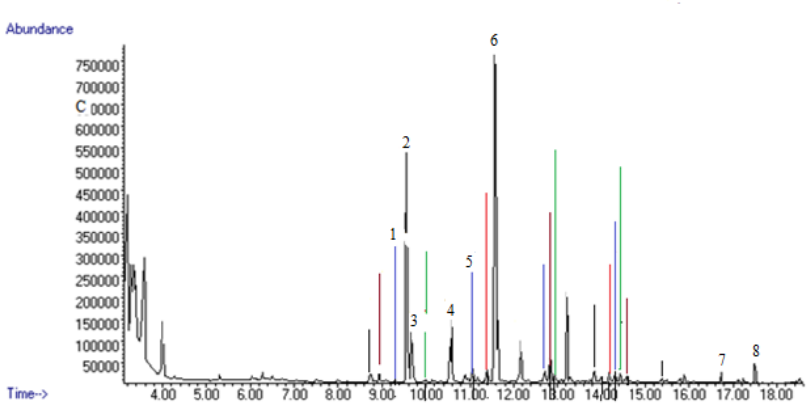

Figure 2. GC-MS Chromatogram of essential oil from *N. sativa* seeds

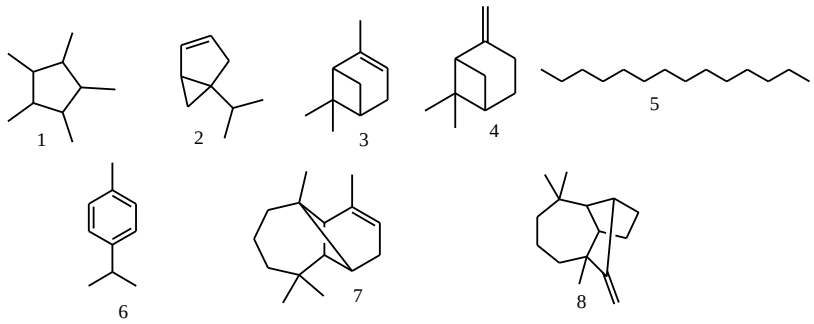

Figure 3. GC-EAD active compounds from *Nigella sativa* essential oil

Table 1. Mean percentage repellency (±SE) of blends and standards at different concentrations

	Dose in g/mL						
Sample	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**	**RD_{50}**	**RD_{75}**
DEET	51.11 ±13.32^{C,a}	86.22 ±4.51^{B,a}	94.29 ±3.69^{AB,a}	100.00 ±0.00^{A,a}	100 ±0.00^{A,a}	1.36	1.59
Essential oil	36.97 ±1.81^{D,b}	50.41 ±2.87^{C,b}	84.17 ±0.78^{B,b}	98.81 ±1.19^{A,a}	100.00 ±0.00^{A,a}	1.93	2.24
Blend 1	29.21 ±1.02^{E,d}	40.49 ±1.01^{D,c}	46.71 ±2.63^{C,d}	62.36 ±1.43^{B,de}	86.03 ±1.32^{A,b}	4.30	5.80
Blend 2 (Blend 1 minus *p*-cymene)	32.32 ±2.25^{E,bc}	41.96 ±0.95^{D,c}	60.04 ±3.61^{C,c}	80.53 ±1.13^{B,b}	100.00 ±0.00^{A,a}	2.84	3.50
Blend 3 (Blend 1 minus (-)-β-pinene)	7.96 ±2.03^{E,e}	25.80 ±0.72^{D,e}	39.82 ±0.85^{C,de}	51.67 ±2.49^{B,g}	63.96 ±1.04^{A,d}	5.930	7.48
Blend 4 (Blend 1 minus (+)-β-pinene)	28.36 ±0.84^{D,d}	37.78 ±1.20^{C,cd}	47.13 ±1.33^{B,d}	55.82 ±2.29^{A,fg}	58.21 ±2.18^{A,d}	8.84	14.44
Blend 5 (Blend 1 minus (+)-α-pinene)	19.21 ±1.38^{D,bcde}	31.77 ±1.81^{C,d}	39.63 ±6.66^{C,de}	51.09 ±1.56^{B,g}	64.24 ±2.15^{A,d}	7.20	10.11
Blend 6 (Blend 1 minus (-)-α-pinene)	11.51 ±3.95^{E,de}	30.54 ±2.03^{D,de}	55.46 ±2.16^{C,c}	74.04 ±1.73^{B,c}	100.00 ±0.00^{A,a}	3.29	3.81
Blend 7 (Blend 1 minus α-longipinene)	20.18 ±0.75^{E,bcde}	33.02 ±1.22^{D,de}	39.21 ±0.63^{C,de}	58.11 ±1.72^{B,ef}	78.89 ±1.92^{A,c}	5.12	6.70
Blend 8 (Blend 1 minus tetradecane)	20.80 ±6.63^{B,bcde}	31.51 ±2.38^{B,de}	45.20 ±2.08^{A,d}	49.78 ±2.04^{A,g}	58.01 ±3.83^{A,d}	8.42	12.49
Blend 9 (Blend 1 minus 1,2,3,4,5-pentamethylcyclo pentane)	21.67 ±1.14^{C,bcde}	23.16 ±1.57^{C,f}	27.12 ±1.16^{C,f}	43.20 ±3.12^{B,h}	52.48 ±1.80^{A,e}	15.37	24.59
(+)-β-pinene	16.51 ±0.39^{E,cde}	23.73 ±1.03^{D,f}	39.79 ±1.33^{C,de}	50.79 ±1.03^{B,g}	78.96 ±2.19^{A,c}	5.40	6.90
(-)-β-pinene	9.44 ±1.19^{E,e}	23.40 ±1.33^{D,fg}	42.01 ±1.98^{C,d}	58.72 ±1.21^{B,ef}	73.74 ±1.21^{A,c}	4.95	6.06
(+)-α-pinene	14.76 ±0.58^{E,cde}	29.39 ±0.53^{D,ef}	35.94 ±1.20^{C,de}	64.70 ±1.15^{B,d}	99.38 ±0.62^{A,a}	3.86	4.60
(-)-α-pinene	3.74 ±1.44^{E,e}	20.25 ±0.91^{D,g}	30.94 ±0.94^{C,ef}	39.41 ±0.72^{B,h}	59.12 ±0.58^{A,d}	6.92	8.59

Means (±SE) with different (i) capital letters in a row, and (ii) small letters within a column are significantly different at α = 0.05 (Student-Newman-Keuls test)

3.2. Repellency of Compounds and Blends from *Nigella sativa*

The repellence activity of essential oil, selected standards [(+)-β-pinene, (-)-β-pinene, (+)-α-pinene, (-)-α-pinene, p-cymene, α-longipinene, tetradecane and 1,2,3,4,5-pentamethylcyclopentane] and blends of the standards were determined. Mean percent repellency at different doses and RD_{50} and RD_{75} values are given in Table 1. All the tested drugs exhibited concentration dependent activities against *An. gambiae*. At higher doses the percent repellence of the essential oil was comparable to that of DEET. The essential showed 98.81% repellence at 0.01g/ml while at 0.1g/ml it gave 100% repellence against the mosquito.

The repellence activity of Blend 1 which contained six GC-EAD active compounds [(+ & -)-β-pinene, (+&-)-α-pinene, *p*-cymene, α-longipinene, tetradecane and 1,2,3,4,5-pentamethylcyclopentane] was significantly lower than that of essential oil, implying that the other two EAG-active constituents (longifolene and α-thujene) which were not available for testing, could be contributing significantly to the repellence of essential oil. Subtraction of (+)-β-pinene, (-)-β-pinene, (+)-α-pinene, tetradecane, or 1,2,3,4,5-pentamethylcyclopentane from blend 3, 4, 5, 8 and 9 respectively led to significant decrease in the repellent activities of the resulting blends, suggesting that the compounds contributed significantly to the repellent activity of the essential oil. On the other hand, subtraction of *p*-cymene and (-)-α-pinene from blend 2 and 6 respectively led to significant rise in the repellence of the resulting blends, suggesting that the compounds contributed negatively to the repellent activity of the essential oil. The difference in the activities of the two optical isomers of α-pinene was confirmed by comparing the individual repellence of each isomer. The repellence of (+)-α-pinene was higher than that of (-)-α-pinene. Interestingly, in another study only positive enantiomers of pinene were found to have microbial activities against *C. albicans*, *C. neoformans* and *R. oryzae* [37]. The two optical isomers of β-pinene or isomeric mixture of the two did not show any significant difference in repellence activity. Blend 2 was the most repellent with RD_{50} and RD_{75} values of 2.84 and 3.50 g/ml respectively followed by blend 6 which had RD_{50} and RD_{75} values of 3.29 and 3.81 respectively. Both blends 2 and 6 repelled 100% of the mosquitos at 0.1g/ml while (+)-α-pinene repelled 99.38% of the insects at the same concentration. Previous studies have focused largely on the bioactivities of crude extracts and oil of the plant. Extracts from the plant exhibited insecticidal and insect repelling activity against *Amblyomma americanum*, *Tribolium castaneum*, Tuta absoluta and Mosquito [26,27].

Use of drug combinations (bends) in the correct proportions can increase the beneficial effects using lower doses of the active compounds due to selective synergism against a particular target. This can help reduce the adverse effects associated with a particular drug and also minimize the induction of drug resistance [38]. Combined therapies are widely used for the treatment of the most dreadful diseases, such as cancer and AIDS [39,40]. Out of all the pure compound tested, (+)-α-pinene was the most repellent which confirms the compound to be the main active ingredient in *N. sativa* L. seed oil.

4. Conclusions

Essential oil of *N. sativa* seeds contains compounds that repel *An. gambiae*. The repellency of the oil and those of blends 2 and 6 were comparable to the positive standard. The results from the present study lays down groundwork for downstream development of appropriate blends for personal and space protection against *An. gambiae.*

ACKNOWLEDGEMENTS

The authors acknowledge ICIPE for allowing us to perform GC-MS, GC-EAD, mosquitoes repellency assays and providing us mosquitoes for bio-assay in their institution. We also acknowledge Mr. Xavier Cheseto (ICIPE) for running GC-FID and GC-MS, Mr. Vincent Odhiambo (ICIPE) who assisted in GC-EAD and Mr. Richard Ochieng (ICIPE) who assisted in carrying out repellence assays. We also thank the volunteers for their assistance in repellent tests.

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