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# Research Article
# Analysis of Black Seed Effect on *Aedes aegyptii*

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## Abstract
**Background and Objective:** Insect vectors, especially mosquitoes are responsible for spreading serious human diseases like malaria, Japanese encephalitis, yellow fever, dengue and filariasis. The various synthetic products and devices designed to combat such vectors are not successful because of increased resistance developed by various mosquito species. The usage of plant extracts is one of the possible methods of pollution free method in insect control. The objective of this study was to find new method for controlling adult of *Aedes aegyptii* using extract seeds of *Nigella sativa*. **Materials and Methods:** The study was conducted from July, 2016-August, 2017 at Laboratory of Toxicology, University of Malaya, Kuala Lumpur, Malaysia. The crude extract was obtained by powdered seed of *Nigella sativa* and extracted by n-hexane solution for 72 h. Bioassays was conducted using both Y-Olfactometer and wind tunnel condition. Identification of *Nigella sativa* has been conducted by injecting crude extract into GC-MS. **Results:** Dual choice bioassay showed that 64% females mosquito significantly ($p<0.001$) avoided black seed within 3.2 min whereas the longest time was 10.29 min for female mosquito to avoid the black seed at concentration 5000 ppm. In the wind tunnel, single mosquito was tested against 250-5000 ppm for 100 cm in wind or no wind conditions. In wind tunnel assays the threshold for single mosquito to exhibit avoidance behavior was 1000 and 5000 ppm in the presence of wind within 100 cm whereas it was 5000 ppm in the presence of no wind condition within 100 cm. The result of this study showed that the compounds of styrol, thymoquinone and p-cymene in the black seed were confirmed to be effective disturbing the behavior of mosquito. **Conclusion:** The black seed *Nigella sativa* has a potential to managing behavior of mosquito *Aedes agyptii* and can be used in term of integrated management system of pest control.

Key words: *Nigella sativa*, *Aedes agyptii*, Y-olfactometer, wind tunnel, managing behavior

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The mosquitoes *Aedes aegyptii* are vectors of several globally important arbovirus, such as; dengue virus, yellow fever virus and chikungunya virus[1-4]. Dengue fever is a mosquito-borne disease of major global public health concern. It is endemic to tropical and sub-tropical countries, especially in the urban and sub-urban areas[5]. *Aedes* sp. was initially considered an endemic species in south east Asia, however; its geographical range has dramatically increased in the last century due to trade and climate changes which opened new ecological niches in temperate areas[6]. Dengue fever and DHF remain serious health risks in urban and rural populations of Indonesia, its leading causes of excess mortality and hospitalization among children in the country. Indonesia witnessed the largest epidemic on record with 72,133 reported cases of DF/DHF and 1,414 dengue-attributable deaths[7,8].

Insect vectors, especially mosquitoes are responsible for spreading serious human diseases like malaria, Japanese encephalitis, yellow fever, dengue and filariasis. The various synthetic products and devices designed to combat such vectors are not successful, because of increased resistance developed by various mosquito species. Most of the mosquito control programmes target the larval stage in their breeding sites with larvicides, because adulticides may only reduce the adult population temporarily[9,10].

Protection against mosquito bites has been reported from the plant genus *Cymbopogon* sp.[11], *Pellargonium citrosum*[12], *Lantana camara*[13], *Tagetes* sp.[14] and *Ocimum* sp.[15]. Other essential oils from plants like *Myrtus comunis*, *Origanum syriacum*, *Lavandula stoechos* and the pure compounds like thymol, carvacrol and a-pienene have been documented for larvicidal activities towards *Culex pipiens* mollestus[16].

*Nigella sativa* or black seed (Family: Ranunculaceae) is a flowering herb native to the Mediterranean and central Asia. It is known for its pungent black seeds, which figure prominently in traditional Indian and Middle Eastern cuisine and medicine. Previous study reported that *N. sativa* essential oils are repellant and toxic to growing larvae and adults of *T. castaneum*. These repel the adult beetles significantly even at very low concentration[17].

Biologically active plant extracts have been, therefore studied for their potential efficacy to minimize the extent of population and reduce the cost[18]. Use of plant extracts is one of the possible methods of pollution free method in insect control. Promising results have been achieved towards attaining this goal by treating eggs, nymphs and adult insects with extract of whole plants, leaves, roots fruits and seeds of various species of plants[19]. The usage of plant extract is one of the possible methods of pollution free method in insect control. However, there has been no report on the managing behavior of mosquito using extract of *Nigella sativa*. This study was conducted to evaluate the adult of *Aedes aegyptii* behavior against *Nigella sativa*.

## MATERIALS AND METHODS

**Crude extract of *N. sativa*:** The study was conducted from July, 2016-August, 2017 at Laboratory of Toxicology, University of Malaya, Kuala Lumpur, Malaysia. The dry of *N. sativa* seeds were obtained from the Kuala Lumpur local market imported from Saudi Arabia. About 125 g of *N. sativa* seeds were weighed and crushed using a grinder, then transferred into a clean conical flask and 500 mL of n-hexane was added. After 72 h the extract was filtered and concentrated using rotary evaporator at 69°C to 150 mL as stock extract. The crude extract was diluted with pure acetone to make a concentration of 10% and stored in amber-colored vial at -4°C for analysis and bio-assays. This assays was carried out according WHO protocols[20].

**Mosquito:** The *Aedes aegyptii* mosquitoes were obtained from colonies reared in the insectaries at IMR (Institute Medical Research) Kuala Lumpur, Malaysia and reared according to the WHO protocol[21]. The mosquitoes were initially obtained from the field in Seremban area. Mosquito eggs were hatched by simultaneously flooding the moist filter paper platforms. Rearing was carried out in the insectary maintained at 27-28°C and approximately 80% humidity on a 12 h/12 h 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. The total number of mosquitoes used for this study was 1,500 adult of *Aedes aegyptii*.

**Y-tube olfactometer:** Bioassays were conducted by using methodology adopted from Blackmer *et al.*[22]. Y-tube olfactometer designed with a 40 mm diameter×36 cm long glass and had 50° inside angle. Incoming air was filtered through activated charcoal and humidified with doubly distilled, deionized water. The filtered air was split between two, 2 L holding chambers; 1 chamber served as a control (clean air) and the other chamber held the test material. From each holding chamber, the air passed into the respective arms of the Y-tube and then through a series of screens before entering the main tube of the olfactometer. Airflow through the system was maintained at 4.8 L min$^{-1}$ (= 3.8 m min$^{-1}$ inside the tube) by an inline flowmeter. A smoke test demonstrated laminar airflow in both arms and throughout the olfactometer. A 60 cm long, wide-spectrum fluorescent lamp (GE, F20T12-PL/AQ) was positioned 22 cm above the arms of the Y-tube. Before each trial, light intensity over each arm was measured with a light meter and the tube was adjusted until intensity was the same in both arms. The Y-tube setup was surrounded by a 50×70×60 cm black fabric enclosure and the holding chambers containing the treatments were placed outside the enclosure to eliminate visual cues. Holding chambers were illuminated by 40 W incandescent bulbs, which provided approximately 3000 lux to the black seed extract during the trials.

A live female mosquito *Aedes agyptii* was placed in vertical arm (C) acrylic Y-tube (3.0 cm diameter×10.0 cm length) covered with a wire net at both ends arm. The crude extract *Nigella sativa* was placed at right horizontal arms (B) whereas the left horizontal arm~~s~~ was empty as control (A) (Fig. 1). It was observed for 10 min. Adult mosquito movement and time taken to avoid crude extract were recorded. A positive result was scored when the mosquito avoided crude extract and negative result was obtained when mosquito moved towards crude extract. The experiments were replicated 25 times and in order to prevent contamination, the Y-tube olfactometer was wiped with ethanol before and after the experiment. Crude extract *Nigella sativa* was placed on whatman filter paper cut into 4×4 cm and spotted using Hamilton syringe.

**Wind tunnel bioassay:** A wind tunnel (30.0 cm diameter× 100 cm length) was constructed for the behavioral bioassay studies using a transparent cylinder made of acrylic resin. One end was permanently covered with a plastic net and the other end closed using a cover made from similar netting. A fan was used to blow air through the tunnel. The speed of airflow in the wind tunnel was maintained at 0.2 m sec$^{-1}$. Activated charcoal was placed in front of the fan to ensure that only clean air passed through the wind tunnel. The tunnel was marked in 10 cm segments using masking tape. Data were recorded using conversion number of mosquito responses (Table 1).

Crude extract source to be tested was spotted on a 4×4 cm No. 1 Whatman filter paper and then left to dry for 1-2 min before being hung in the center at the upwind end of the wind tunnel. One *Aedes aegyptii* was released at the down wind end at a distance of 100 cm away from the bait. Mosquito movements, behavior and duration of each behavior in response to the pheromone source were recorded for a period of 15 min. This test was carried out in 20 replicates with a new *Aedes aegyptii* adult tested each time.

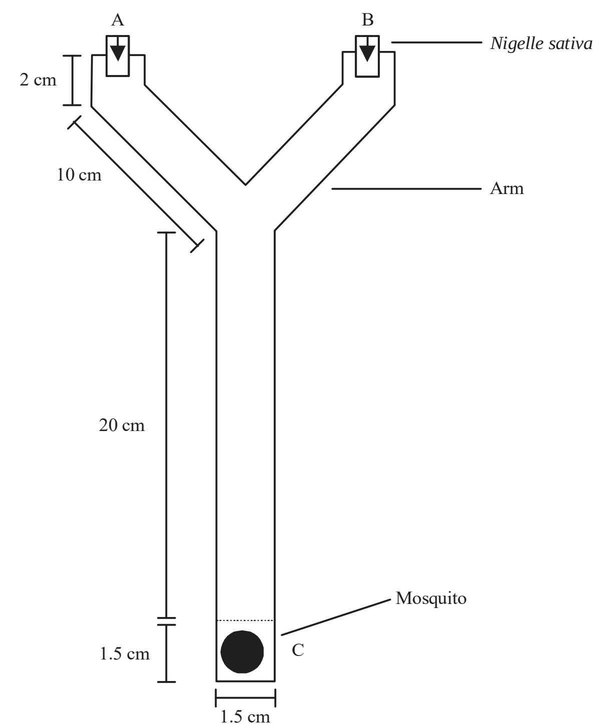


Fig. 1. Y-tube olfactometer

Table 1: Mosquito response was scored using the following conversion number

| Score | Description |
|---|---|
| 5 | Strong positive: Flew upwind in less than 5 min<br>Then flew avoiding or stay not more than 30 cm from release point, wings and antenna showed disturbing behavior |
| 4 | Medium positive: Flew upwind in more than 5 min but less than 10 min<br>Then flew avoiding or stay not more than 30 cm from release point, wings and antenna showed disturbing behavior |
| 3 | Slight positive: Flew upwind in more than 10 min<br>Then flew avoiding or stay more than 30 cm from release point, wings and antenna showed disturbing behavior |
| 2 | Negative: Approached extract by flying upwind within vicinity 0-10 cm<br>from extract in more than 5 min, but less than 10 min, normal behavior of wings and antenna |
| 1 | Strong negative: Approached extract by flying upwind within vicinity 0-10 cm<br>from extract in less than 5 min, normal behavior of wings and antenna |

**Statistical analysis:** The data were subjected to statistical analysis for comparison analyzed by using statistical software (Sigma Stat V2.03). The values for behavior and mean were analyzed using one-way ANOVA for determination of variance ratio and least significant difference (LSD).

**Gas Chromatography Mass Spectrometry (GCMS):** GC-MS followed the protocol developed by Villas-Bôas *et al.*[23]. The sample for identification was injected into oven of GC-MS (Qp5050A) and the temperature was kept at 40°C for 5 min and then increased to 280°C at 10 min. This step was maintained at 280°C at 10 min. Helium gas was used as the carrier gas of Gas Chromatography. The helium gas was adjusted to 1.3 mL $min^{-1}$ for flow rate in mobile phase. The stationary phase was the BP5 capillary column. The BP5 column has the length of 29.0 m and diameter of 0.25 mm. The mass range at 80 and above was done for scanning. The combination of gas chromatography and mass spectrometer can further identified the compounds by checking the retention time of each detected compound and run again in Mass Spectrometer to determine the mass spectra.

## RESULTS

**Behavior of *Aedes agyptii* towards *Nigella sativa* source in dual choice olfactometer:** The results of the bioassays using a dual-choice Y-shaped olfactometer to determine *Aedes agyptii* behavior for different concentration of *Nigella sativa* sources were summarized in Table 2. The test mosquito released at the vertical end (C) immediately flew (in less than 5 sec) avoiding the extract source at the end of the olfactometer arm (A). The test was replicated 25 times using a new mosquito each time. Table 2 showed that mosquito significantly avoiding the extract of black seed ($\chi^2$; p<0.001) to controls. Meanwhile, other extract of black seed with different concentration also had significant different from the control.

The treatments of empty vs. empty, control vs. empty, 250 vs. 500 ppm and 5000 and 10000 ppm shows no significant different. Meanwhile, the Table 2 showed that mosquito significant avoiding the extract of black seed on control vs. 250 ppm, control vs. 500 ppm, control vs. 5000 ppm and control vs. 10000 ppm at p<0.001.

**Mosquito response towards different concentrations of extracts in different wind conditions in wind tunnel bioassay:** In the control experiments, the mosquito upon release flew randomly without showing any specific flying pattern. The mosquito including replicates (N = 20), stayed at the downwind end near the point of release until the end of observation. The antennae usually were held in a U-shaped pattern while resting. When using the extract of black seed, mosquito showed a positive response to the extract would fly in a different shaped pattern twice before avoiding and landed far from extract spot of the filter paper (Fig. 2).

The difference response of mosquito towards different concentrations in wind and no wind condition was shown in Table 3. The mosquito showed significant different at concentration 250 ppm (1.68±2.12[a]) toward control (4.80±3.21[c]), whereas 5000 ppm (4.36±3.62[c]) showed not significant difference toward control under wind condition. Under no win condition the mosquito showed significant different at concentration 250 ppm (1.12±1.25[a]), toward control (3.80±3.11[d]) whereas 5000 ppm (3.92±3.39[d]) showed not significant difference toward control.

The percentage of different behavior components exhibited by mosquitoes tested using different concentrations of black seed extract which released from a distance of

Table 2: Response of *Aedes agyptii* behavior towards *Nigella sativa* sources in dual choice bioassay tested using chi-square test (p<0.001)

| Sources | Treatments | Female (No.) | $\chi^2$ value | p-value |
|---|---|---|---|---|
| A | Empty | 12 | 0.03 | NS |
| B | Empty | 13 | | |
| A | Control | 14 | 0.17 | NS |
| B | Empty | 11 | | |
| A | Control | 8 | 4.92 | p<0.001 |
| B | 250 ppm | 17 | | |
| A | Control | 4 | 11.97 | p<0.001 |
| B | 500 ppm | 21 | | |
| A | Control | 3 | 9.11 | p<0.001 |
| B | 1000 ppm | 22 | | |
| A | 250 ppm | 15 | 1.07 | NS |
| B | 500 ppm | 10 | | |
| A | Control | 4 | 14.28 | p<0.001 |
| B | 5000 ppm | 23 | | |
| A | Control | 3 | 9.11 | p<0.001 |
| B | 10000 ppm | 22 | | |
| A | 5000 ppm | 14 | 0.17 | NS |
| B | 10000 ppm | 11 | | |

Table 3: Response of mosquitoes *Aedes agyptii* towards different treatments under wind and no wind conditions at a distance of 100 cm in the wind tunnel using Dunnett test (p<0.05)

| | Condition | |
|---|---|---|
| Treatments (ppm) | Wind±SE | No wind±SE |
| Control | 4.80±3.21[c] | 3.80±3.11[d] |
| 250 | 1.68±2.12[a] | 1.12±1.25[a] |
| 500 | 2.00±2.83[a] | 1.32±1.77[a] |
| 1000 | 2.20±3.59[a] | 1.76±3.39[b] |
| 5000 | 4.36±3.62[c] | 3.92±3.39[d] |
| 10000 | 3.36±3.22[b] | 2.16±3.47[c] |

*Alphabets following numbers shows significant different according Dunnett test

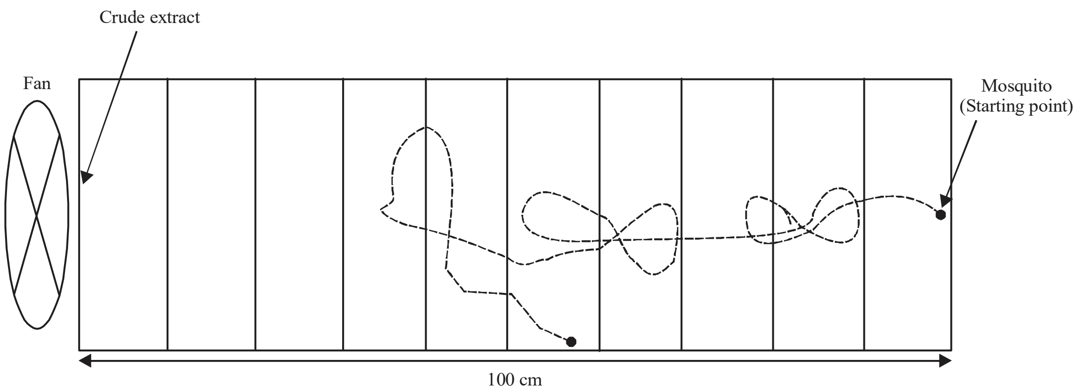


Fig. 2: Positive response of *Aedes agyptii* towards crude extract *Nigella sativa* source on filter paper in wind tunnel

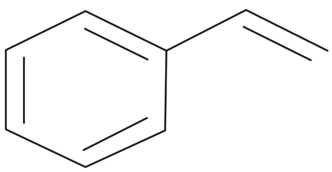

Fig. 3: Chemical structure of styrol ($C_8H_8$)

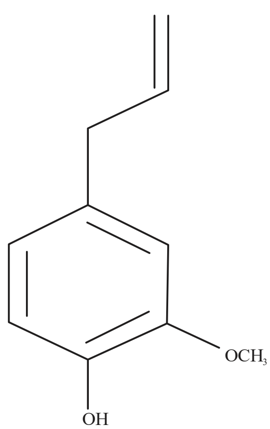


Fig. 4: Chemical structure of thymoquinone ($C_{10}H_{12}O_2$)

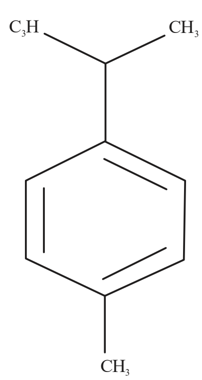


Fig. 5: Chemical structure of p-cymene ($C_{10}H_{14}O$)

100 cm were shown in Fig. 2. Positive response of mosquito shown when its flew avoiding the crude extract source at the upwind end and landed far from the filter paper extract at concentration 250 ppm. The intensity of the positive response increased as the concentration of the crude extract rose until 5000 ppm, but the mosquitoes showed no significant different at concentration of 10000 ppm. Data in Fig. 2 showed that 67% of mosquito landing far more than 50 cm from crude extract, 11% of mosquito flying around the crude extract (less than 5 cm from crude extract or more than 50 cm from crude extract) and 23% flew and landing to crude extract. The behavior of mosquito also recorded when it's avoiding the crude extract, such as the antennae were closed up wide.

**Analysis *Nigella sativa* using gas chromatography mass spectrometry:** By using GC-MS with authentic standard, components were eventually recognized in the *Nigella sativa* extract. Comparing retention times with those compounds that coincided in retention checked the nature of the compounds. The retention time was reported in minutes.

There were several compounds being identified to present in the crude extract by using GC-MS. The compounds identified included styrol, thymoquinone and p-cymene. Those compounds have potential to manipulate behavior of *Aedes agyptii*.

## DISCUSSION

The result obtained from the bioassays showed that when the treatment with different concentration were applied, the behavior of mosquito increased as the concentration of treatment increased. This was supported by statistical analysis, Chi-square. The analysis data showed that the calculated F-value was greater than critical F-value at certain time after treatment. For the larvae *A. aegypti*, the calculated F-value and the critical F-value were 1.274 and 0.019, respectively. In this case, the F calculated value is greater than critical F-value, the null hypothesis, $H_0$ is rejected. This indicated that at least one of the means is significantly different from the others, it indicated the behavior of mosquito *A. Aegypti* with different treatment concentration is significantly different. The p-value indicated the probability of getting a mean difference between the groups were high as what is observed by chance. The lower the p-value is, the more significant the difference between the groups. The p-value obtained from the statistical analysis was zero. Hence, the difference in behavior of mosquito with different concentration is strongly supported.

The behavior of the mosquito in wind tunnel, flying avoiding the crude extract were influenced by the concentration of the crude extract. As the crude extract dosage increased from 250-10000 ppm, mosquito flew upwind at a progressively higher speed. Some mosquito landed on crude extract, flew around or landed far from to the bait (30 cm away from the bait). This study found that the inhibitory threshold was reached for crude extract of black seed, where there was a reduction in response at the higher crude extract concentration tested.

The first peak in chromatogram with retention time 5.025 min and abundance 0.09% was identified as styrol with molecular weight 104 g mol$^{-1}$ and chemical formula $C_8H_8$. It is also known as styrene with chemical structure as below stated in Fig. 3.

Figure 4 showed that the peaks in chromatogram with retention time 10.800 and abundance 3.00% was identified as thymoquinone, $C_{10}H_{12}O_2$ with molecular weight 164 g mol$^{-1}$. The next peak with retention time 11.442 min was identified as p-cymene, $C_{10}H_{14}O$. Its molecular weight is 150 g mol$^{-1}$ with abundance 0.73% and the chemical structure was shown as in Fig. 5.

The extraction of plants with hexane is an extraction method which is commonly used by scientists to carry out their study[24,25]. The pharmacological investigations of the seed extracts revealed a broad spectrum of activity including immunopotention and antihistaminic, antidiabetic, anti-hypertensive, anti-inflammatory and anti-antimicrobial activities.

The *N. sativa* has been employed for thousands of years as a spice and food preserve, as well as a protective and curative remedy for numerous disorders. Traditionally, there is a common Islamic belief that black seed is a panacea for all ailments, but cannot prevent aging or death. Blackseed is also identified as the curative black cumin in the Holy Bible and is described as Malathion by Hippocrates and Dioscorides and as Gith by Pliny[24].

There were other research on *N. sativa* Linn. which belong to family Ranunculaceae and genus *Nigella* showed that it contains >30% of fixed oil and 0/4-0/45% (wt/wt) of volatile oil. The volatile is known to contain 18.4-245 thymoquinone (TQ) and 46% of monoterpenes such as; p-cymene and α-piene[26]. Hence, hexane is used as the solvent to carry out the extraction of seeds of *N. sativa*. By hexane extraction, compounds in the seeds can be effectively extracted out.

Previous study showed that thymoquinone had a potential of the immune-modulation towards dengue fever vector. Furthermore, thymoquinone has been shown to exert immune-enhasive effects on the both the humoral and melanization responses of *A. Aegypti* using biological and non-biological agents[27]. Hence, thymoquinone that was identified in the GC-MS of crude extract of *N. sativa* may be active compound that caused the disturbing behavior of *Aedes agyptii*. Another chemical compounds in *N. sativa* is styrol or known as styrene, $C_8H_8$. In natural condition, it can be found from a Turkish tree, the Oriental sweet gum (*Liquid ambarorientalis*) from which it was first isolated and not for the tropical styrax trees from which benzoin resin is produced. Low levels of styrene occur naturally in many kinds of plants, *N. sativa* is one of it as well as a variety of foods such as fruits, vegetables, nuts, beverages and meats. Styrene is regarded as a "hazardous chemical", especially in case of eye contact but also in case of skin contact, of ingestion and of inhalation, according to source[28].

The chemicals derived from plants have been projected as weapons in future mosquito control programmed as they are shown to function as general toxicant, growth and reproductive inhibitors, repellents and oviposition-deterrent[29]. Many authors worldwide started large screening activity for using extracts of medicinal and herbaceous plants to control mosquitoes[30]. Pyrethrin based products have been widely used to protect people from mosquito bites through their repellent and killing effects. Many other material products of botanical origin especially, essential oils hold significant promise in insect vector management[31]. Plant essential oils in general have been recognized as an important natural resource of insecticides[32]. Environmental safety is considered to be of paramount importance. An insecticide does not need to cause high mortality on target organisms in order to be acceptable, but should be eco-friendly in nature. Phytochemicals may served as these are relatively safe, inexpensive and readily available in many parts of the world. Several plants were used in traditional medicines for the mosquito larvicidal activities in many parts of the world. The screening of locally available medicinal plants for mosquito control would generate local employment, reduce dependence on expensive and imported products and stimulate local efforts to enhance the public health system. The ethno-pharmacological approaches used in the search of new bioactive toxins from plants appear to be predictive compared to the random screening approach[33].

## CONCLUSION

The result of this study showed that the compounds of black seed *Nigella sativa* such as; styrol, thymoquinone and p-cymene were confirmed to be effective disturbing the behavior of mosquito. It also has potential to managing behavior of mosquito *Aedes agyptii* and can be used in term of integrated management system.

## SIGNIFICANCE STATEMENT

This study had developed new isolation techniques and chemical characterization through different types of spectroscopy and chromatography together with new pharmacological testing have led to an interest in plants as the source of new adulticide compounds. Synergistic approaches such as application of mosquito with botanical blends and microbial pesticides will provide a better effect in reducing the vector population and the magnitude of epidemiology.

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