TECHNICAL SESSION V

Mosquito Repellent Compounds from the Seeds of *Nigella sativa* L (Black cumin)

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Abstract:

Isolation and characterization of Mosquito repellent compounds from the seeds of Nigella sativa *L (Black cumin) is presented. The volatile oil of the seeds was isolated by steam distillation followed by extraction with diethyl ether. Mosquito repellent activity of oils was investigated by carrying out bioassays with mosquitoes. Seed oil of N.sativa showed significant ability to repel mosquitoes. The compounds responsible for the repellent activity were isolated and characterized by chemical and spectroscopic methods. These compounds were identified as terpenoids containing carbonyl and hydroxyl functionalities.*
Key words: *N. sativa*, seed oil, bioassay, mosquito repellent

Introduction

Recently we have reported the presence of cytotoxic compounds in the seeds of *N. sativa* (Bulugahapitiya, 2006). This work was further elaborated to investigate the mosquito repellent ability of the seed oil. Although some ointment are available in the market as mosquito repellent, finding the potential compounds or potential plant extracts which can either repel or destroy the mosquitoes are still a great need in this era.

N. sativa L (Family: Ranunculaceae) is an annual flowering plant with the height of 20 –30 cm. The fruits are large and inflated capsule composed of 3 – 7 follicles each containing numerous seeds. The seeds are tiny (1 – 2 mm long) and black in colour (Jayaweera, 1982). The seeds give strong spicy taste. *N. sativa* L is native to Southwest Asia. It has been cultivated in India but not grown in Sri Lanka. The seeds are available in the market in Sri Lanka.

Many parts of the plant of *N. sativa* L has been used for many purposes including health care purposes worldwide. Knowledge of traditional uses of this plant may lead to find many potential compounds from the plant. Some parts of the plant has been used mainly for lung complaints, coughs, jaundice, hydrophobia, tertiary fever, paralysis, rheumatism and related inflammatory diseases and eye sores. The oil of the seeds of Black cumin has been used as a radiant, for smooth skin and as an hair promoter. The seeds are also used as spices and as insect repellent. Some of products of *N. sativa* are available in the market as oils, cream, shampoo, capsules and powder.

Many reports are available in the literature about *N. sativa* L. Some compounds from the seeds have been isolated and characterized *ie* thimoquinone and dithymoquinone. These compounds have been identified to have anti-inflammatory, anti-oxidative and cytotoxic activity (Worthen *et al,* 1998). The objective of this work was establishing the mosquito repellent activity of the seed oils of *N. sativa* L and the isolation and characterization of active compounds responsible for the mosquito repellent activity.

Materials and method

Preparation of plant material: Seeds of *N. sativa* L were purchased from the market and it was confirmed by Ayurvedic practitioners. The impurities were removed from the seeds by sinking them in cold water. The pure seeds were dried at room temperature for two days.

Extraction of oil: Dried seeds (150 g) were crushed and subjected to steam distillation. The steam distillate was extracted with diethyl ether. Ether was evaporated under vacuum and the crude oil was obtained.

Determination of mosquito repellent activity of crude seed oil (bioassay):
The bioassays were carried out using mosquitoes. Mosquitoes were trapped during the day and night. At night they were trapped by using trapping equipment (Figure 1). During day time they were caught directly when they came out from their pupae stage for the very first time in their life cycle. Bioassays were carried out using three different methods: Cylinder Glass Tank method, Cage method and Bottle method. These methods were developed by us with the knowledge of literature methods.

Cylinder glass tank method:

A two feet long, one foot diameter cylinder tank was used for the assay (Figure 2). Assays were done via two ways. At first method, covered mosquito contained container was put at the covered end of the Glass Cylinder. The inside of the lid of the Glass Cylinder was applied with crude seed oil. Simultaneously, the lid of the mosquito container was opened and the cylinder was closed with the lid which contained seed oil. The behaviour of the mosquitoes was observed.

In the second method, seed oil was diluted with acetone (1:1) and was applied at the covered end of the Glass Cylinder. A covered mosquito container was placed at the middle of the cylinder. Simultaneously, the lid of the mosquito contained container was opened and the cylinder was closed with a lid. The behaviour of the mosquitoes was observed. The method was repeated three times.

Cage method: Carried out under different conditions (A, B, C, D and E):

A: Large cage (1.5 Ft x 1.5 Ft x 1.5 Ft) prepared and all six sides were covered with mosquito net and then the seed oil applied glass plate (15 x 12 cm) was set into the inside of the cage (Figure 3). About 15 mosquitoes were released into the cage and the behavior of mosquitoes was observed.
B: All six sides of the cage were covered with mosquito net and then small container containing seed oil (about 0.25 mL) was put into inside. Then about 15 mosquitoes were released into the cage and the behavior of mosquitoes was observed.
C: One side of the cage was covered with mosquito net and the other five sides were covered with polythene papers (Figure 4). Then a small container containing seed oil was set inside of the cage and about 15 mosquitoes were released into the cage. The behaviour of mosquitoes was observed.
D: All sides of the cage were covered with polythene papers except one side. It was covered with mosquito net (Figure 5). Then a small container with seed oil was kept inside the cage and about 15 mosquitoes were released into the cage. The behaviour of mosquitoes was observed.
E: All sides of the cage were covered with polythene papers with small cavities (Figure 6). Then pure seed oil compound 01 and pure seed oil compound 02 applied glass plate (12 x 15 cm) were kept inside the cage (as a separate experiment) and about 10 mosquitoes were released into the cage for each experiment. The behaviour of mosquitoes was observed.

All the above methods were repeated three times.

The control test was carried out with pure acetone. Fifteen mosquitoes were used for the assay.

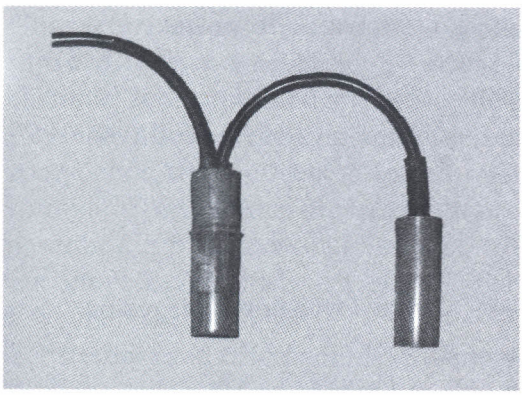

Figure 1. Insect trapping equipment

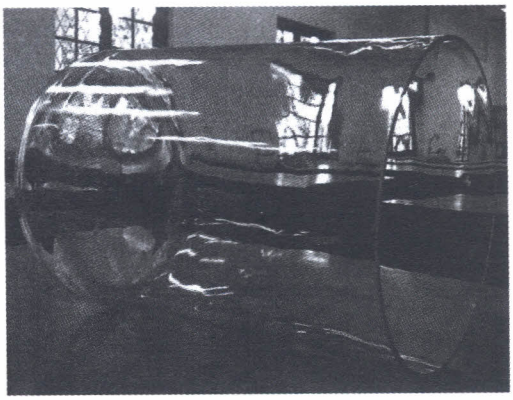

Figure 2. Glass cylinder tank

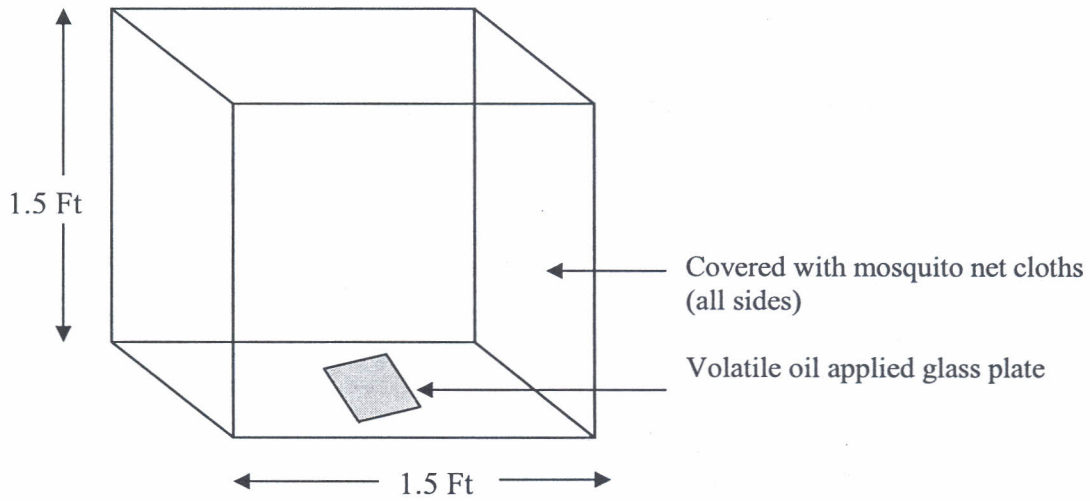

Figure 3. Mosquito cage

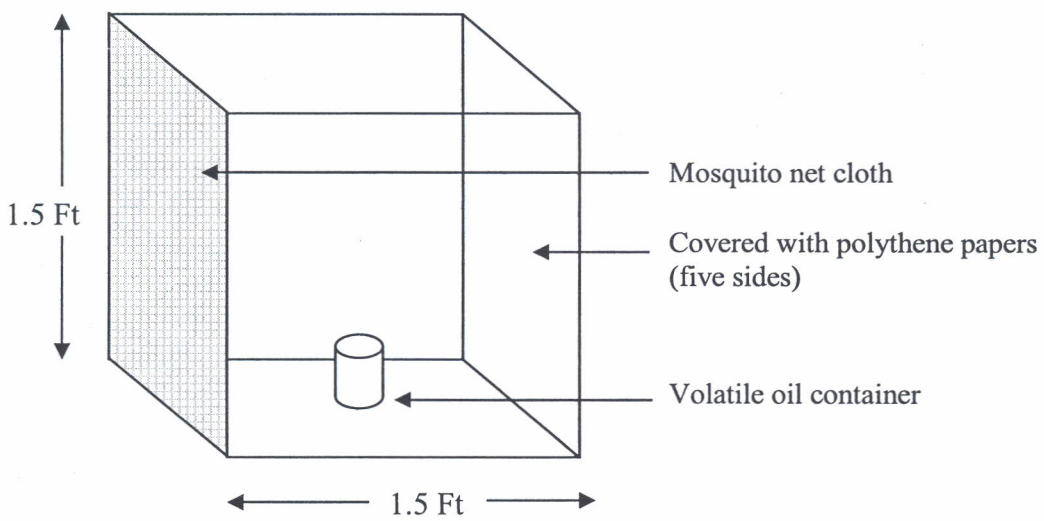

Figure 4. Mosquito cage with volatile oil container

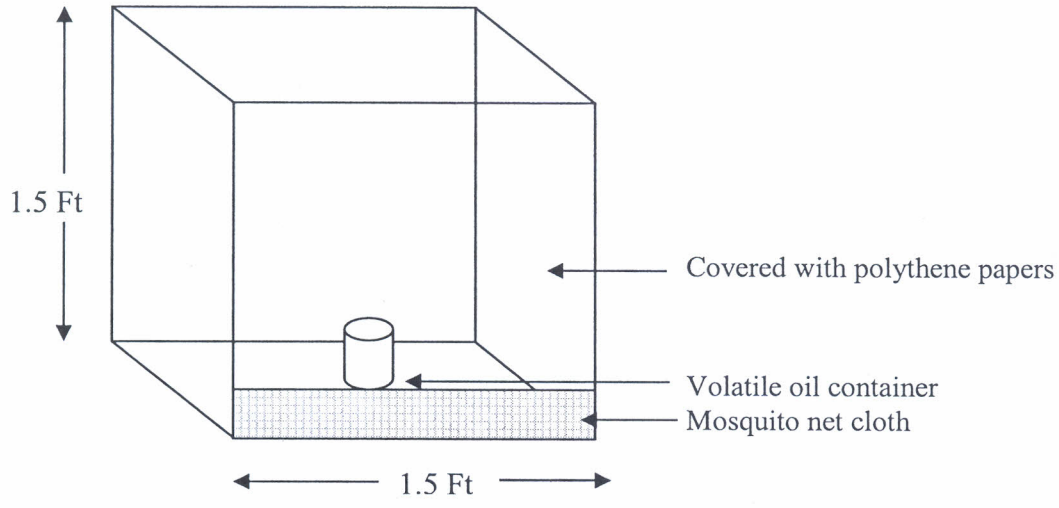

Figure 5. Mosquito cage with volatile oil container

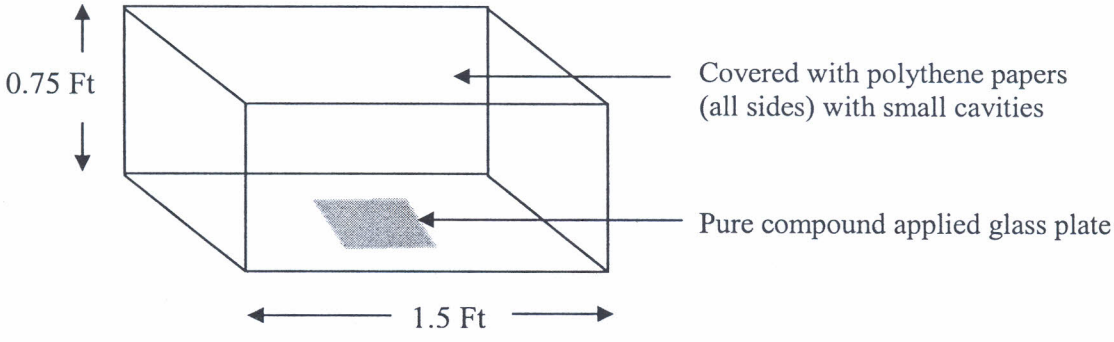

Figure 6. Mosquito cage with volatile oil applied glass plate (for pure compounds)

Bottle method: A large polythene bag and a large clean transparent bottle were used for the assay. The mosquitoes were trapped by insect trapping equipment and they were put into the large polythene bag. The inside of the large clean bottle was applied with crude seed oil and then the mouth of the bottle was fitted to the mouth of the polyethylene bag containing mosquitoes. The whole system was kept in a parallel manner and the behaviour of mosquitoes was observed. The test was carried out three times.

Thin layer chromatographic (TLC) analyses of the crude seed oil: The crude seed oil was analysed by TLC (one dimensional and two dimensional). Hexane: dichloromethane 3:2 was used as the solvent system and spots were visualized by using UV, spraying of I_2, phosphomolybdic acid and anisaldehyde reagents.

Separation of the components present in crude seed oil by column chromatography: Crude seed oil (1.5 g) of *N. sativa* was subjected to column chromatography with 60 g of silica gel and with 3:2 of hexane: dichloromethane as the eluent.

Mosquito repellent assays with pure seed oil components which were previously identified having cytotoxic ability: Bioassays were carried out with pure compounds isolated from crude seed oil of *N. sativa* using the same procedure as described in 2.4 above.

Characterization of mosquito repellent compounds isolated from seed oil of *Nigella. sativa* L: This was done by using chemical methods (phytochemical screening and chemical screening) and spectroscopic methods (Infra-Red spectroscopy).

Phytochemical screening tests for secondary metabolites: The crude seed oils and pure compounds were subjected to the following tests in order to determine the nature of the secondary metabolites present in them. The tests were carried out according to the methods described in the literature (Harbone, 1984).

For alkaloids: Mayer's test and Wagner's test.

For saponins: Froth test

For steroids and triterpenoids: Libermanne - Burchardt tests

For flavanoids: coloured test

For cardiac glycosides: Kedd test and Keller - Killiani test.

Chemical tests: Brady`s test, Fehling`s test, sodiumnitroprasside test and diazotization test were carried out for active compounds isolated from the seed oil of *N. sativa*.

Infra Red Spectroscopic analysis: The active compounds isolated from the seed oil were analysed by IR spectroscopy. The liquid films of the compound were prepared by dissolving pure compounds in anhydrous dichloromethane.

Results and discussion.

The results of bioassay:

The results of the mosquito repellent activity under glass cylinder tank method are as follows: The mosquitoes moved inside the tank just at the beginning and they did not come towards the seed oil applied surface. After about 5 minutes, the whole area of the tank was saturated with seed oil and then after 10 minutes all the mosquitoes became unconscious and fell down. Similar results were observed with pure seed oil components and seed oil diluted with the acetone method. After 12 minutes mosquitoes became unconscious and fell down.

The results of the mosquito repulsion activity under the cage method are as follows:

Method A: Mosquitoes did not come towards the surface applied with seed oil. Within one hours time mosquitoes did not become unconscious but they showed some difficulties in flying.

Method B: Mosquitoes did not come towards the seed oil container. They always tried to move away and settle on the surface and keeping a maximum distance from the oil container. Within one hours time the mosquitoes did not become unconscious.

Method C: The same situation as in B.

Method D: Mosquitoes did not come towards the oil container. They always tried to move away and settle on the surface and keeping a maximum distance from the oil container. After about thirty minutes, one mosquito became unconscious and fell down and after one and half hours time, three mosquitoes became unconscious and fell down. All of the mosquitoes showed flying difficulties within this time period. After the disturbance, they settled on the surface quickly, keeping a maximum distance from the seed oil container.

Method E: Mosquitoes did not come towards the pure compound applied on the glass plate (Both pure compounds). They always tried to move away and settle on the surface, keeping a maximum distance from the oil container. With pure compound 01, one mosquito became unconscious within 10 minutes. After one hour all the mosquitoes showed difficulties, but they did not become completely unconscious. With pure compound 03, after three minutes one mosquito became unconscious and fell down and after 45 minutes, three mosquitoes had fallen down. All the mosquitoes showed flying difficulties.

The results of the mosquito repellent activity under the bottle method: In the situation of the bottle and the polyethylene bag kept together, all mosquitoes became unconscious and fell down within one minute. In the control test without any substance and with pure acetone, no mosquitoes became unconscious. After one and half hours even their flying behaviour did not change.

The results of chromatography: The results of one dimensional and two dimensional TLC analysis of crude seed oil extracted from the seeds of *N. sativa* L are given in Figures 7 and 8. According to these results, the oil in the seed is a mixture of about ten compounds. Using column chromatography, eight components were separated. These are shown in Table 1.

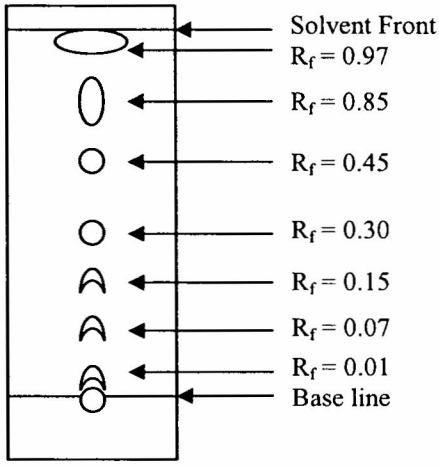

Figure 7. One dimensional TLC analysis

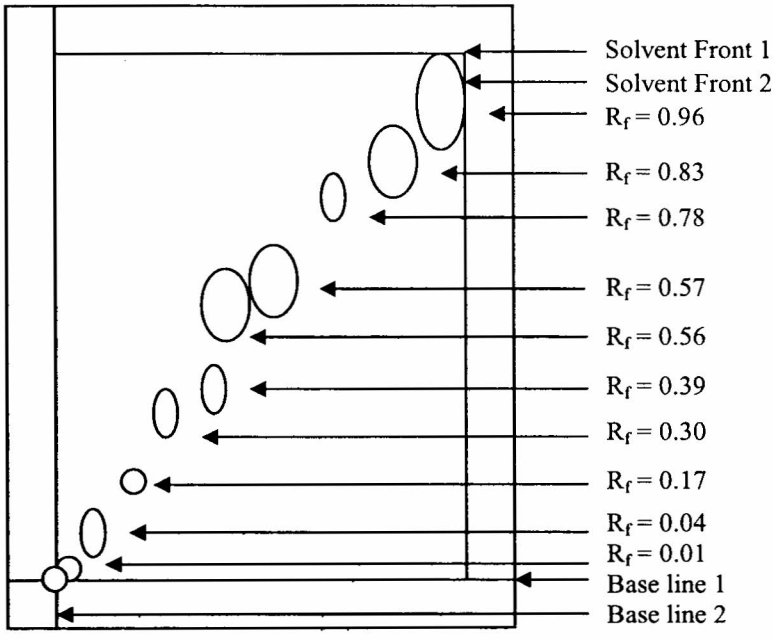

Figure 8. Two dimensional TLC Analysis

Table 1. Results of column chromatography

Extract	Amount of silica	Eluant	Number of fractions
Volatile oil	60 g	Hexane: CH_2Cl_2 3:2	8

The results of phytochemical screening and chemical tests: The results of photochemical screening for the two active compounds are given in the Table 2. According to these results, the two active compounds are mainly terpenes. The results of the chemical tests for the active compounds are given in the Table 3. According to these results one of the active compounds contains carbonyl functional group and the other active compound contains both carbonyl and hydroxyl functional groups.

Table 2. Results of phytochemical screening of active compounds in volatile oil

Fraction of Volatile Oil	Alkaloid		Confirm test, # of Spots	Quaternary Alkaloid		Steroid / Terpinoid		Cardiac Glycosides	Flevanoids	Saponin
	Mayer	Wagner		Mayer	Wagner	Libermann - Berchardt	Salkowski			
Compound 01	-	-	-	-	-	√	√	-	-	-
Compound 03	-	-	1	-	-	√	√	-	-	-

Table 3: Results of chemical tests of active compounds in volatile oil

Fraction of Volatile Oil	Brady's test	Felhing's test	Sodium nitropraside test	Diazotization test	$FeCl_3$ test
Fraction 01	√√	-	-	-	-
Fraction 03	√√	-	-	√√	√

The results of IR spectroscophy: The results of the Infra-red spectroscopic analysis for the active compounds isolated from the seeds of *N. sativa* L are given in the Table 4. The IR spectra of active compounds showed that one compound contains carbonyl functional groups and the other compound contains both carbonyl and the hydroxyl functionalities. These results confirm the results obtained by chemical tests.

Table 4. Results of spectroscopic analysis of active compounds in volatile oil

Stretching frequency υ /cm^{-1}	Group	Cytotoxic compounds of Volatile oil	
		Compound 01	Compound 03
3200-4000	N-H	-	-
3525-3250	O-H	-	√
2900-3050	Sp^3 C-H	√	√
1600-1700	C=O	√	√
1390-1440	C-H bend	√	√

Conclusion

The seed oil of *N. sativa* L is a mixture of more than eight compounds Of these compounds mainly two compounds have sigificant mosquito repellent activity and these two compounds are present in higher percentage in the seed. These two active compounds are characterized as terpenoids which contain carbonyl and hydroxyl functionalities.

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