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Preliminary study on cytotoxic compounds from the seeds of *Nigella sativa* L (Black cumin)

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Isolation and characterization of cytotoxic compounds from the seeds of *Nigella sativa* L is presented. The oil present in seeds was isolated by steam distillation followed by extraction with Diethyl ether. The cytotoxic activity of the oil was investigated by carrying out bioassays with bacteria (*Erwinia carotovora*) and MosquitoL larvae. The seed oils of *N. sativa* L showed significant cytotoxic effect towards the *Erwinia carotovora* and mosquito larvae. The cytotoxic compounds were separated by column chromatography and characterized by chemical and spectroscopic methods. These cytotoxic compounds were identified as terpenoids containing carbonyl and hydroxyl functionalities.

Key words: *Nigella sativa*, Volatile oil, Bioassay, Cytotoxicity

1. Introduction

Nigella 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) and black in colour. The flowers are delicate. The seeds are used as a spice. *N. sativa* L is native to Southwest Asia. It has been cultivated in India (Jayaweera 1982). The seeds are available in the market of Sri Lanka.

N. sativa L commonly known as black cumin or black seeds are used in folk medicine all over the world for treatment and prevention of a number of diseases including allergies, asthma and treating immune disorders, lung complains, cough, jaundice, hydrophobia, rheumatism and related inflammatory diseases (Chopra et al. 1986), Brown 1995, Chevallier 1996. The recent research has shown that *N. sativa* increases the number of mammary cells in laboratory animals. Great research has been done on *N. sativa* in regards to its anti-cancer properties especially breast cancer with promising results (Ali and Gerald 2003). The pharmacological action of the crude extract of seeds has been reported to include protection against nephrotoxicity and hepatotoxicity induced by either disease or chemicals (Awad 2005).

It has been reported that seeds contain both fixed and essential oil, proteins alkaloids and saponins. Many of the compounds present in seeds of *N. sativa* L have been characterized. The main components of seed oil of *N. sativa* L were *p*–cymene,

thymol with only small amount of thymoquinone (Motetti *et al* 2004, Worthen *et al* 1998, Chen *et al* 1996, Ur-Rahman *et al* 1992).

This is a preliminary study on the cytotoxic activity of seed oil of *N. sativa*.

2. Experimental

2.1. General

Hexane (Fluka), Ethyl acetate (Fluka), and Methanol (BDH) solvents were purified by distillation (electro thermal). The solvents were dried with anhydrous Na_2SO_4 (Merk). Extracts were concentrated using Rotary Evaporator (Heidolph WB 2000 Automated Rotary Evaporator). UV lamp (German) was used to visualize the spots of Thin Layer Chromatographic analyses (G_6F_{254} 13% $CaSO_4$, BDH). Infra red spectra were obtained by using Hitachi 270-50 IR Spectrophotometer (270-50 data processor). All bacterial strains were maintained in nutrient agar [NA (peptone 5 g, beef extract 3 g, NaCl 8 g, agar 18 g, de-ionized water 1000 mL)] at 4 ^{0}C. Microbial plates were incubated at room temperature overnight. Size of inhibition zone was measured using a metric ruler.

2.2. Preparation of plant material

N. sativa L seeds were purchased from the market and it was confirmed by Ayurvedic practitioners. The impurities of the seeds were removed by sinking in cold water. The pure seeds were dried at room temperature for 48 hours.

2.3. Extraction of oil

150 grams of air dried seeds were crushed and subjected to steam distillation. The steam distillate was extracted with diethyl ether. Ether was evaporated under vacuum and the crude seed oil was obtained (Satyajit *et al* 2006).

2.4. Determination of Cytotoxic activity of crude volatile oil (Bioassay)

The assays for the cytotoxicity of the seed oil were carried out with bacteria species (*E. carotovora*) and with mosquito larvae. The standard methods described in the literature were used for the bacterial assays (Kivak 2001, Stepanovic et al. 2003, Erdogrul 2002, Ates and Ozlem 2003), Ferdous et al. 1992) and the methods developed by us with the previous knowledge were used for the mosquito larval assays.

2.4.1. Preparation of bacterial broth Nutrient agar containing peptone 5 g, beef extract 3 g, NaCl 8 g, distilled water 1000 mL was prepared under sterilized condition. It was then inoculated with stock culture of *E. carotovora* species and shaken overnight. This culture was poured into sterilized petri dishes (143 mm diameter) and about 15 mL of melted (at 50^{o}C) nutrient agar was added to above dishes and mixed well to make it homogeneous and then allowed to solidify.

2.4.2. Cytotoxic assay with bacteria culture Prepared *E. carotovora* (Gram negative) bacteria strains were used for cytotoxic assay. Assays were carried out via two methods: Agar Diffusion method and Air Diffusion method (James and William 2000, Perez et al. 1990, Nair et al. 2005, Salie et al. 1996 and Indian Pharmacopoeia 1985).

Agar diffusion method

The cavities (wells) were prepared in the bacterial plates using cork borer. The cavities were filled with 20 μL of crude seed oil. Control test was carried out with 20 μL of diethyl ether. The plates were incubated at room temperature overnight. Size of the inhibition zone was measured using a metric ruler from the edge of the cavity to the edge of the inhibition zone.

Air diffusion method

Half concentrated agar plates with the strain of *E. carotovora* were used in this method. The plates were prepared as described under 2.4.1. An opened volatile oil containers with 20 μL of volatile oil were kept at the middle of the above plates and the inhibition of the growth of bacteria was monitored.

2.4.3. Cytotoxic assay with mosquito larvae Post mosquito larval stages grown in clear water were selected for the assays. The larvae were caught without any damage and transferred into small container. The ethanolic solution of crude volatile oil was prepared by dissolving 0.8 mL of volatile oil in 25 mL of 95% Ethanol. 2 mL, 4 mL and 6 mL of the above solution was poured into beakers containing 100 mL of aerated distilled water and shaken well. About 15 mosquito larvae were added into the each of the above solution and the time taken for making unconscious or killing of mosquito larvae was measured. The control test was carried out only using 2 mL, 4 mL and 6 mL of 95% ethanol respectively. The procedure was repeated three times using similar mosquito larval stages.

2.5. Thin layer chromatographic (TLC) analysis of the crude volatile oil

The crude volatile oil was analysed by Thin Layer Chromatography (one dimensional and two dimensional) using 3:2 ratio of hexane:dichloromethane as the solvent system. The spots were visualized by different ways i.e. visualizing under Ultraviolet–visible radiations, spraying of Iodine, spraying of phosphomolybdic acid and spraying of anisaldehyde.

2.6. Separation of the components present in crude volatile oil

The components of crude seed oil of *N. sativa* L were separated by column chromatography (1.5 g of crude seed oil, 60 g of silica gel and 3:2 ratio of hexane:dichloromethane as the solvent system were used).

2.7. Cytotoxic assays with separated compounds from the seed oil

The cytotoxic assays were repeated with pure compounds isolated from the crude volatile oil of *N. sativa* L according to the procedure described in 2.4.2 and 2.4.3 with bacteria and mosquito larvae respectively.

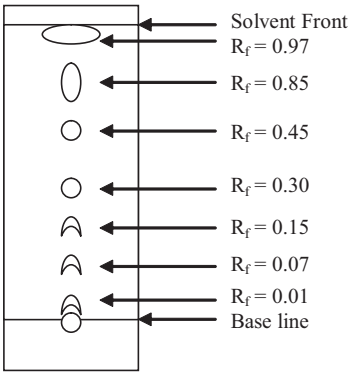

Figure 1 Onedimensional TLC analysis of the seed oil of N. *sativa* L.

2.8. Characterization of the compounds responsible for the cytotoxic activity in volatile oils of *N. sativa* L

The compounds responsible for the cytotoxic activity towards bacteria and mosquito larvae were characterized by chemical methods (phytochemical screening and chemical screening) and by spectroscopic methods (Infra red spectroscopy).

2.8.1. Phytochemical screening tests The crude volatile oil and the active compounds were subjected to phytochemical screening in order to determine the nature of the secondary metabolites. Screenings were carried out according to the methods described in the literature Harbone (1984).

2.8.2. Chemical tests The functional nature of the active compounds isolated from seed oil were analysed by carrying out the standard chemical tests for the identification of functional groups.

2.8.3. Infra red spectroscopic analysis of pure seed oil The active compounds isolated from the seed oil were further analysed by Infra red spectroscopy (Colthup *et al* 1975). Liquid films were used and active compounds were prepared by dissolving pure compounds in anhydrous dichloromethane.

3. Results and Discussion

The results of one-dimensional TLC analysis and two-dimensional TLC analysis of crude volatile oil extracted from the seeds of *N. sativa* L are given in Figure 1 and 2 respectively. According to these results, the seed oil is a mixture of about ten volatile compounds. Eight components were separated from the crude seed oil by using column chromatography. Out of these eight compounds, two compounds were present in relatively higher percentages. The results given in the Figures 3 and 7 show that the seed oil of *N. sativa* L has ability of inhibition of the growth of bacteria to the some extent. This is further supported by the graph given in Figure 4, in where the inhibition ability of the growth of bacteria is higher in seed oil than the other solvent extracts of the seeds. The results of the cytotoxic assays with

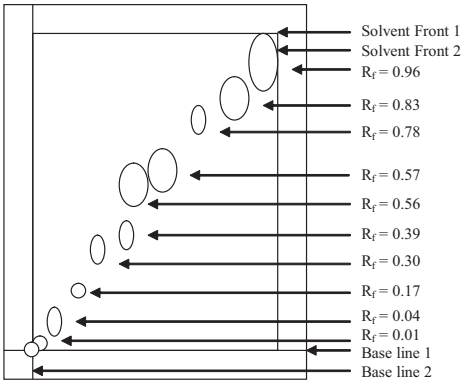

Figure 2 Two-dimensional TLC analysis of the seed oil of *N. sativa*. L

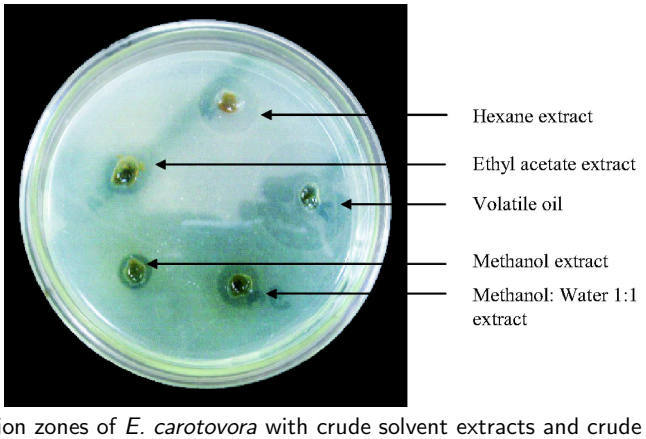

Figure 3 Inhibition zones of *E. carotovora* with crude solvent extracts and crude oil of the seeds of *N. sativa* L by Agar diffusion method.

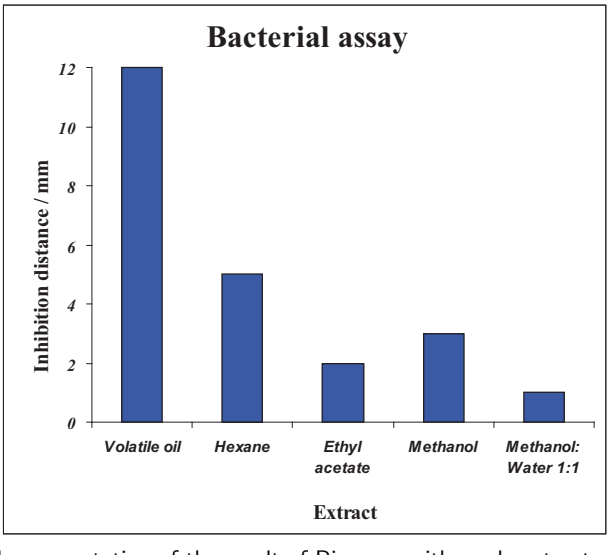

Figure 4 Graphical representation of the result of Bioassay with crude extracts seeds of *N. sativa* L.

Table 1 The results of the Mosquito larval assay with crude seed oil of *N. sativa* L.

Time / Minutes	**Percentage mortality**					
	Volatile oil + Absolute Ethanol + 100 mL Water mixture			**Control test**		
	2 mL	**4 mL**	**6 mL**	**2 mL**	**4 mL**	**6 mL**
5	0	0	0	0	0	0
10	0	0	4.5	0	0	0
15	0	15.8	36.4	0	0	0
20	13.8	21.1	54.6	0	0	0
25	25.0	42.1	68.2	0	0	0
30	43.8	47.4	77.3	0	0	0
35	43.8	47.4	90.9	0	0	0
40	50.0	52.6	100.0	0	0	6.25
45	56.2	52.6	100.0	0	0	18.75
50	62.5	94.7	100.0	0	22.22	37.50

Table 2

The Results of the phytochemical screening of pure compounds 1 and 3 of seed oil of *N. sativa* L.

Fraction of Volatile Oil	Alkaloid		Confirm test, # of Spots	Quaternary Alkaloid		Steroid / Terpenoids		Cardiac Glycosides	Flevanoids	Saponin
	Mayer	Wagner		Mayer	Wagner	Lib-Bech.	Sal.			
Compound 01	-	-	-	-	-	√	√	-	-	-
Compound 03	-	-	-	-	-	√	√	-	-	-

Table 3 The Results of the Chemical test of pure compounds 1 and 3 of seed oil of *N. sativa* L.

Fraction of Volatile oil	**Brady's test**	**Fehling's test**	**Sodium nitropraside test**	**Diazotization test**	**$FeCl_3$ test**
Compound 01	√√	-	-	-	-
Compound 03	√√	-	-	√√	√

Table 4 Result of the IR spectroscopic analysis for the pure compounds 1 and 3 of seed oil of *N. sativa* L

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

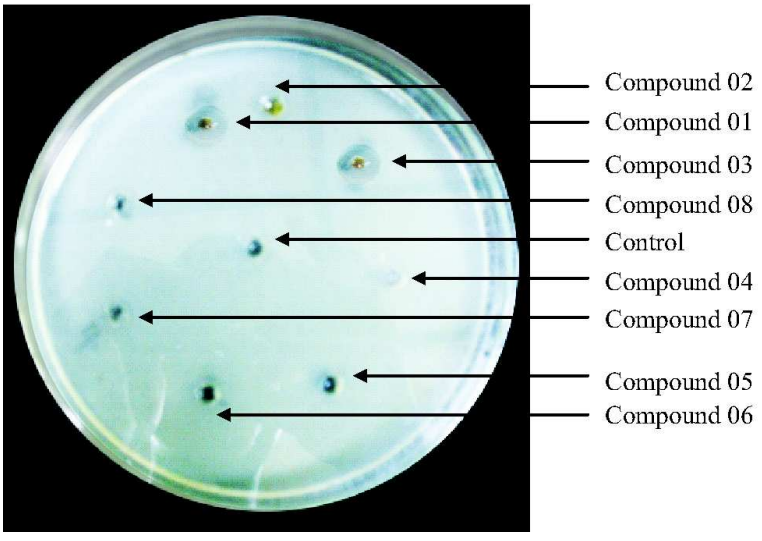

Figure 5 Inhibition zones of *E. carotovora* with separated components of seed oil of *N. sativa* L by Agar diffusion method .

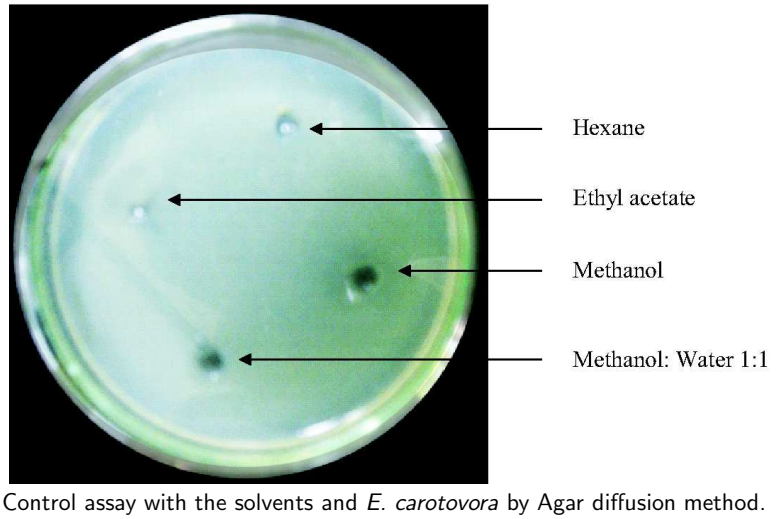

Figure 6 Control assay with the solvents and *E. carotovora* by Agar diffusion method.

the separated compounds from seed oil are given in the Figure 5. It is clear that the compounds 1 and 3 have the potent inhibition ability for the bacterial growth. This is further supported by the results given in Figure 7, which shows clearly two inhibition zones in the bacterial plate. These two inhibition zones are due to two potent cytotoxic compounds present in the seed oil. The results shown in Figure 6 as the control test with solvents indicate that, the solvents have no effect on destroying bacterial growth.

Results of the mosquito larval assays are given in the Table 1 and Figure 8. According to the Table 1 when the amount of seed oil was increased from 2 mL to 6 mL all the larvae died within 40 minutes. In contrast to this no larvae died in

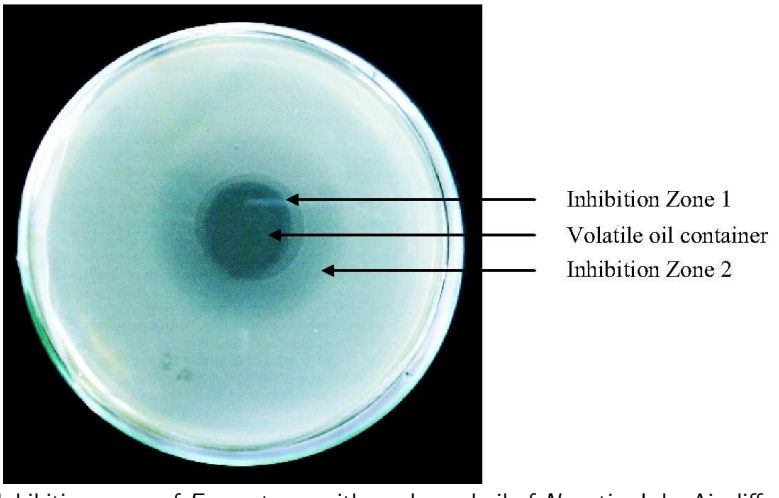

Figure 7 Inhibition zones of *E. carotovora*with crude seed oil of *N. sativa* L by Air diffusion method.

the presence of solvent during 40 minutes. These results can be further explained using the graph given in Figure 8. It was observed that the seed oil had no effect on mosquito larvae when it was present in low concentration. However, it showed toxicity to mosquito larvae when it was present in somewhat higher concentration.

The results of phytochemical screening of the two cytotoxic compounds 1 and 3 of seed oil are given in Table 2. It reveals that, these active compounds are terpenoids. The results of functional group analysis of 1 and 3 are given in Table 3 indicates that, the compound 1 contains only a carbonyl group whereas the compound 2 contains both carbonyl and hydroxyl groups. These results were further confirmed by the Infrared spectroscopic analysis given in Table 4.

Cytotoxic activity was visualized as a clear zone of bacterial growth on nutrient agar containing petri plates. Same concentration of bacterial inoculums were used (optical density = 0.9) to compare the cytotoxic activity of tested bacterial strains against different crude extracts. During the bioassay, the concentration of nutrient agar medium had to be diluted to half the concentration, to limit the bacterial growth on the medium for clear observation of the inhibition zone.

4. Conclusion

From the results of the study on the volatile oil isolated from the seeds of *N. sativa* L, the following conclusions can be made. The volatile oil present in the seeds of *N. sativa* L is a mixture of about ten volatile compounds. Two of these compounds showed potent cytotoxic activity towards the bacteria (bacteriocidal) and mosquito larvae. These two compounds were found to be terpenoids, one of which showed presence of carbonyl functionality whereas the other showed the presence of both carbonyl and hydroxyl functionalities.

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