

Original Article

Bioassay Screening of the Essential Oil and Various Extracts of *Nigella sativa* L. Seeds Using Brine Shrimp Toxicity Assay

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Abstract

Background and Aim: Since cytotoxicity screening is the first step necessary for any new drug development, this study was designed to find out and compare the cytotoxicity effects of the essential oil and various extracts of *Nigella sativa* L. seeds using Brine Shrimp Lethality (BSL) assay.

Materials and Methods: Essential oils and various extracts of *N. sativa* were assessed by two methods of disk and solution of BSL. Data analysis was carried out using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, 250 USA). Data were processed in probit-analysis program to estimate LC₅₀ values.

Results: All of the tested fractions demonstrated more cytotoxicity in the solution method. Petroleum ether and chloroform extract of *N. sativa* showed the most cytotoxicity with LC₅₀ values 7 and 21 µg/ml respectively; while aqueous and ethanolic had no significant cytotoxicity. Moreover, the GC/MS analysis of the essential oil of *N. sativa* showed the *p*-cymene (48.1%), α -thujone (14.38%) and dihydro carveol (9.11%) as the main compounds.

Conclusion: These results suggest some limitation for using this spice in diet. Furthermore, this plant could be considered as a source of cytotoxic compounds which should be studied in details.

Keywords: *Nigella sativa*, Black cumin, *Artemia salina*, Cytotoxicity, Essential oil

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Introduction

Since last centuries, plant extracts and plant-derived compounds due to having fewer side effects, low cost and high availability are a successful approach to treat a wide range of diseases such as cancer, diabetes, cardiovascular diseases and oxidative dysfunction (1). *Nigella sativa* L. (Family Ranunculaceae) is commonly known as black cumin seed grown in the Middle East, Eastern Europe,

Western and Middle Asia which is traditionally used as a natural treatment for a number of diseases and conditions including asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness and influenza (2). Various pharmacological effects such as antioxidant, anti-inflammatory, anticancer and antimicrobial have been reported for *N. sativa* essential oil or its active principles which include thymoquinone, carvacrol, *p*-cymene and thymol (3-7). A general bioassay that

appears to have a capacity of detecting a broad spectrum of bioactivity present in the crude extracts is the brine shrimp lethality test (BSLT) (8). The eggs of the brine shrimp have been used in a simple bench top bioassay which has yielded good results. *Artemia salina*, with same purine metabolism as that of mammalian cells, has been shown to have a good correlation with antitumor activity, although drugs that require metabolic activation in the liver may not be detected by *Artemia*. Furthermore, the DNA-dependent RNA polymerases of *A. salina* are also similar to the mammalian type (9). Considering that cytotoxicity screening is the first step necessary for any new drug development, the aim of the study was to find out and compare the cytotoxicity effects of the essential oil and various extracts of *N. sativa* seeds using BSLT.

Materials and Methods

Collection of plant materials

The seeds of *N. sativa* were collected from rural regions of Kerman district of Kerman province, in June 2012. The identity was confirmed by Dr. Mirtajaldin, a botanist at department of botany, Shahid Bahonar University of Kerman, Kerman, Iran. A voucher specimen of the plant materials was deposited at the herbarium belong to the department of pharmacognosy, faculty of pharmacy, Kerman University of Medical Science, Kerman, Iran (KF1356).

Isolation of the essential oil

Crushed seeds of *N. sativa* (100 g) were subjected to hydro-distillation for 3 h using an all-glass cleverger-type apparatus. The obtained essential oil was dried over anhydrous sodium sulfate and stored in darkness at 4°C in airtight glass vials closed under nitrogen gas until testing (10).

Gas chromatography/mass spectrometry (GC/MS) analysis of essential oil

GC analysis

In this study, GC analysis was carried out by a Shimadzu QP 5000 gas with a HP-5MS column (30m × 0.25mm, film thickness 0.25 mm). The column temperature was maintained at 60°C for 3 min and programmed to 220°C at a rate of 5°C per min, and kept constant at 220°C for 5 min. Injector and

interface temperatures were 220°C and 290°C, respectively. The flow rate of Helium as carrier gas was 1mL/min C.F. The percentages were calculated by electronic integration of FID peak areas without the use of response factors correction. Linear retention indices for all components were determined by coinjection of the samples with a solution containing homologous series of C8–C20 n-alkanes.

GC/MS analysis

GC–MS analysis was performed using a Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (30m × 0.25 mm, film thickness 0.25 mm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 220°C and 250°C, respectively. Mass range was from 40 to 400u. Oven temperature program was the same given above for the GC.

Identification of the essential oil components

The components of the essential oil were identified by comparison of their relative retention time and mass spectra with those of standards Wiley 2001 library data of the GC–MS system or with those of reported in the literature data (11).

Preparation of the various extracts

Air-dried seeds of *N. sativa* (200g) were ground and extracted through percolation method by petroleum ether, ether, methanol, chloroform and water consequently for 72 h at room temperature. The extracts were passed through filter paper (Whatman No. 3, Sigma, Germany) to remove plant debris, then concentrated in vacuum at 50°C using a rotary evaporator (Heidolph, Germany) and stored at -20°C, until testing (12).

Phytochemical screening

Seeds of *N. sativa* were subjected to phytochemical studies for searching the flavonoids, alkaloids, tannins and saponins as described elsewhere (13).

Cytotoxicity testing against the brine shrimp

Hatching shrimp

Brine shrimp eggs (*A. salina* leach) were prepared from the fishery center of Hormozgan province and were hatched in artificial sea water which prepared by dissolving 38g of sea salt (Sigma chemicals Co., UK) in 1 L of distilled water. The two glass compartments chamber with several holes on the divider was used for

hatching. One compartment was illuminated. After 48 hours incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the lighted side, whereas their shells were left in the dark side (14).

Brine shrimp assay

Brine shrimp cytotoxicity assay was performed based on method described by Meyer et al, (1982) with some modifications (15). The collected nauplii were treated with various concentrations (10, 100 and 1000 µg/ml) of extracts and essential oil of *N. sativa*. Potassium dichromate was used as positive control. This bioassay was done in two modified methods of disk and solution. In the disk method, various concentrations of the extracts were loaded on paper disks (d=0.5cm), air dried and placed in test tubes. A volume of five ml of artificial sea water was added to the tubes and were shaken to give homogenous solution. In the next step, 10 active larvae were placed to each tube and subjected under light. Survivors were counted after 24 h and the percentage of deaths were determined. In solution method, various concentrations of each fraction were dissolved in dimethyl sulfoxide (DMSO) 1%, placed in test tubes. Then, 5 ml of artificial sea water was added and 10 active larvae was placed to the tubes and subjected under light. Similar to the disk method, survivors were counted after 24 h and the percentage of deaths were determined.

Statistical analysis

Data analysis was carried out by use of SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, 250 USA). The 50% lethal concentration (LC₅₀ value) at 95% confidence interval was calculated for each fraction using the probit analysis method described by Finney (16).

Results and Discussion

GC/MS analysis of *N. sativa* essential oil

In the present study, air-dried seeds of *N. sativa* were subjected to hydro distillation using a Clevenger apparatus and the yellow-colored essential oil was obtained (yield 0.58% v/w). Table 1 shows the identified compounds and percentage obtained by GC/MS. The main components were p-cymene (48.1%), α-thujone (14.38%) and dihydro carveol (9.11%).

Table 1: Essential oil composition of *N. sativa* seeds identified by GC-MS.

No	Compound	Percentage
1.	Camphene	0.07
2.	t-Anethole	0.3
3.	β-Pinene	0.06
4.	α-Pinene	0.05
5.	γ-Terpinene	0.4
6.	α-Terpinene	0.01
7.	Limonene	0.8
8.	Sabinene	1
9.	ρ-Cymene	48.1
10.	α-Terpinolene	0.05
11.	ρ-Cymene-8-ol	0.4
12.	Carvacrol	3.3
13.	Longipinene	0.4
14.	Camphor	1.1
15.	Linaloolcis	0.21
16.	Sabinenehydrate	0.2
17.	Longifolene	1.1
18.	Dihydro carveol	9.11
19.	Thymol	2.3
20.	4-Terpineol	2.1
21.	Carvone	0.5
22.	Thymoquinone	7.6
23.	α-thujone	14.38
24.	Thujone	1.5
25.	Unknown peak	3.1
Total		98.2

Phytochemical screening

The findings of primary phytochemical screening of *N. sativa* indicate the presence of high amounts of alkaloid and lacking the flavonoids in this plant. In addition, *N. sativa* was found to possess very low content of tannins and saponins.

The results of extraction

The yield of extraction of *N. sativa* seeds with chloroform and petroleum ether extracts were 20.88% and 20.36% w/w respectively. Moreover, extraction of *N. sativa* seed using percolation method resulted low percentage of extract with methanol (8.16% w/w), ether (6.87% w/w) and water (4.12% w/w).

Brine shrimp lethality assay

The cytotoxicity effects of the essential oil and various extracts of *N. sativa* seed against brine shrimp in two methods of disk and solution were shown in Figures 1 and 2. The cytotoxicity values (LC₅₀) ranged from 7 to 216µg/ml in the solution method and 225 to 4147µg/ml in the disk method. The upper and lower LC₅₀ in two methods are also considerable. In the disk method, the methanolic and ether extract of *N. sativa*

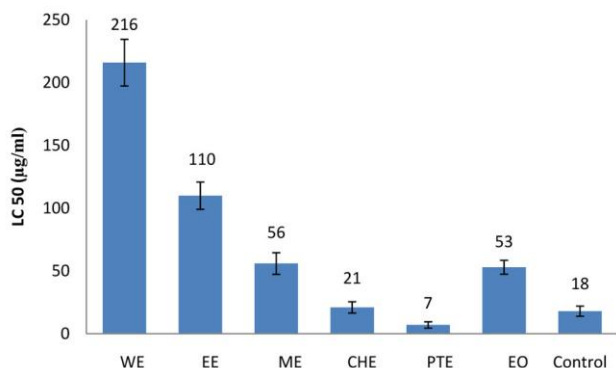


Figure 1. The mean LC₅₀ (µg/ml) confidence interval for the various extracts (PTE: Petroleum ether ex.; CHE: Chloroform ex.; EE: Ether ex.; ME: Methanol ex.; WE: Water ex.) of *N. sativa* screened using BSL assay- disk method. The 50% lethal concentration (LC₅₀ value) at 95% confidence interval was calculated for each extract, using the probit analysis method described by finney.

showed upper and lower cytotoxicity against brine shrimp larvae with LC₅₀ values 225 and 4147 µg/ml, respectively in comparison with potassium dichromate with LC₅₀ value of 22 µg/ml. However, in the solution method, petroleum ether and water extract demonstrated upper and lower cytotoxicity against brine shrimp larvae with LC₅₀ values 7 and 216µg/ml, respectively in comparison with potassium dichromate with LC₅₀ value of 18 µg/ml. Moreover, DMSO 1% had no cytotoxicity on brine shrimp larvae.

Although the modern medicine is well developed in most countries of the world, large sections of the populations in developing countries still rely on medicinal plants and herbal medicines in primary health care. In industrialized countries also, the clinical, pharmaceutical and economical value of herbal medicine in natural therapies has greatly increased (17). The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxicity and anti-tumor properties (4). In the present study, considering that cytotoxicity study is the first step necessary for any new drug development, the essential oils and various extracts of *N. sativa* seed against brine shrimp larvae were subjected for toxicity studies using the procedure of Meyer et al (1982) in two methods of disk and solution (15). Our findings revealed that petroleum ether and

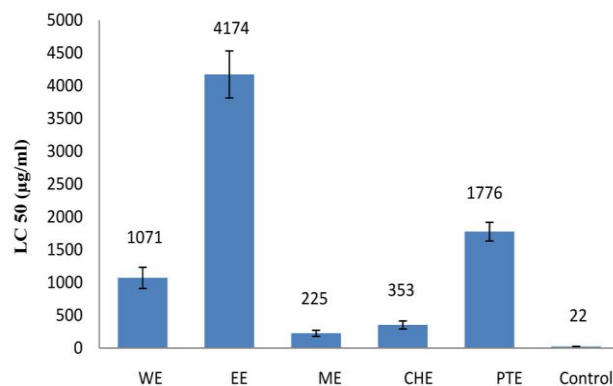


Figure 2. The mean LC₅₀ (µg/ml) confidence interval for the essential oil and various extracts (PTE: Petroleum ether ex.; CHE: Chloroform ex.; EE: Ether ex.; ME: Methanol ex.; WE: Water ex.; EO: Essential oil) of *N. sativa* screened using BSL assay- solution method. The 50% lethal concentration (LC₅₀ value) at 95% confidence interval was calculated for each extract, using the probit analysis method described by finney.

chloroform extract have shown the most cytotoxicity against brine shrimp larvae with LC₅₀ values 7 and 21µg/ml, respectively. In contrast, almost all extracts of *N. sativa* have indicated low cytotoxicity with LC₅₀ values > 200µg/ml in disk method. Totally, among tested extracts, petroleum ether extract of *N. sativa* has shown the highest cytotoxicity activity. However, various studies have shown that administration of *N. sativa* seed extract and its components as oral or intraperitoneally represent low level of cytotoxicity in rats and mice (18-21). Furthermore, cisplatin (a widely used chemotherapeutic drug) is toxic to the kidney. Administration of *N. sativa* can reduced the cisplatin-toxic side effects in rats including nephrotoxicity (21). Among the different extracts, the petroleum ether extract constitutes the highest percentage of *N. sativa*, which indicates the high cytotoxicity of the plant. Phytochemical screening of *N. sativa* indicated the presence of alkaloids, fatty acids and alcoholic terpens. Due to the solubility of these compounds in solvents such as petroleum ether and chloroform, they would be responsible for the high cytotoxicity of these extracts. Moreover, the low cytotoxicity of this plant in disk method may be attributed to aforementioned compounds. These observed differences between disk and solution methods might be due to high distribution and complete solubility extracts in the solution method. The results drawn from the present study exhibited that solution method is the best method for evaluation of cytotoxicity against brine shrimp larvae,

although, in various studies have been demonstrated that the disk method is suitable for this assay (22). In this study, it was found that main components of *N. sativa* essential oil are p-cymene (48.1%), α -thujone (14.38%) and dihydro carveol (9.11%) when being analyzed by GC/MS, whereas in some investigations, the main constituents of *N. sativa* essential oil are thymoquinone (27.8%–57.0%), p-cymene (7.1%–15.5%) and carvacrol (5.8%–11.6%) (2). These variations in composition of the essential oils from the same species would be due to different factors such as geographic region of production, the season of harvest and the method of isolation (23).

Conclusion

The findings of the present study indicated a considerable cytotoxicity of *N. sativa* seeds by BSL assay. The greatest toxicity of the plant is related to its petroleum ether and chloroform extracts. However, for determining the limits of safety of this plant, extensive *in vitro* and *in vivo* toxicological studies and animal assays should be undertaken. The results of this research suggested that the use of this plant, as spices in a food or drug for humans or as nutraceutical, should be treated cautiously and caution should be applied in the use of the other herbal spices preparations until exhaustive phytochemical and bioassay-guided fractionation of the components are achieved. It is also probable to access some compounds with cytotoxicity effect from these herbal sources, however more investigations are needed for logical conclusion.

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Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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