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Research Article

Ethnomedicinal Plants: Study on the Chemical Composition and Antibacterial Activity of the *Nigella sativa* (Black Seed) Oil's

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ABSTRACT
Medicinal plants are considered new resources for producing agents that could act as alternatives to antibiotics in treatment of antibiotic-resistant bacteria. The aim of the study was to evaluate the chemical composition and antibacterial activity of the oil of *Nigella sativa* (Black Seed) against *Escherichia coli O157:H7, Staphylococcus aureus*, and *Bacillus subtilis*. Gas chromatography mass spectrometry was run to specify their chemical composition. As a screen test to detect antibacterial properties of the oil, agar disk and agar well diffusion methods were employed. Macrobroth tube test was performed to determinate MIC. Presence of phellandrene, α-pinene, β-pinene, p-cymene, Cis-carveol, Trans-anethole, Thymoquinone, Thymol, α-Longipinene and Longifolene were identified in composition of the oil of *N. sativa*. The MIC values was 0.031 g/ml for oil except in case of *B. subtilis* which was 0.015 g/ml. Moreover, MBC was resulted in 0.031 g/ml for the oil in all test bacteria. Thus, the research represents the antibacterial effects of the medical herb on *E. coli*, *S. aureus*, and *B. subtilis*. We believe that the article provide support to the antibacterial properties of the oil. The results indicate the fact that the oil of the plant can be useful as medicinal or preservatives composition. Fractionation and characterization of active molecules will be the future work to investigate.

Keywords: *Nigella sativa*, Antibacterial activity, GC/MS

INTRODUCTION

Infectious diseases pose serious problems to health and they are main cause of morbidity and mortality worldwide[1]. Nowadays, multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in treatment of infectious diseases[2]. Herbs and spices are invaluable resources useful in daily life as food additives, flavors, fragrances, pharmaceuticals, colors or directly in medicine. These plants contain medicinal properties which make them potent to cure or prevent diseases[3]. According to World Health Organization (WHO), more than 80% of world's population relies on traditional medicine for their healthcare needs. The uses of herbs in treatment of animal and human diseases have long been established. Most plant extracts have been shown to possess antimicrobial agents active against microorganisms in vitro. Some medicinal plants used in traditional Iranian medicine are effective in treating various ailments caused by bacterial and oxidative stress[4]. Studies have shown that the phenolic compounds play an important role in the antimicrobial properties of plants. These compounds spoil microorganisms through destroying the cell walls and proteins, interfering in the work of membrane enzymes and affecting DNA and RNA replication. Aromatic oils are used in many industries including food preservation, pharmacy and medicine[5-7]. They are expected to form new sources of antimicrobial drugs especially against bacteria[8]. The antibacterial effectiveness of aromatic oils has been divided into a good, medium or bad[9,10]. These oils can also produce some defense products against several natural enemies[11]. In addition, and in order to continue their natural growth and development, aromatic oils may produce some secondary metabolites in response to some external stress[12]. *N. sativa* also known as *nigella* or *kalonji*) often called black cumin is an annual flowering plant in the family *Ranunculaceae*. In fact, *N. sativa* is an annual herb of the *Ranunculaceae* family grows in countries bordering the Mediterranean Sea, Pakistan, India and Iran[13]. The historical tradition of *N. sativa* use in medicine is substantial. *N. sativa* is known to have beneficial effects on a wide range of diseases, antimalarial[14], antiasthmatic[15], antitumor[16], antiviral[17], antimicrobial[18], anti-inflammatory[19], gastroprotective[20], antihypertensive[21], antidiabetic[22], anti-atherosclerotic[23], protective and antioxidant[24], nutritional[25] and anti-cholesterol[26]. Thymoquinone, the main constituent of the essential oil of *N. sativa* seeds, was capable to also exert beneficial effects on acute gastric ulcer[27]. In addition,

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thymoquinone and its reduced product thymohydroquinone have been reported to have an antimicrobial activity and beneficial interaction with some antibiotics[28]. The aim of the study was to screen the in vitro antibacterial activity of the plant oil on some bacteria including *E. coli*, *S. aureus*, and *B. subtilis.*

MATERIALS AND METHODS

Plant sample collection

In this empirical-experimental study, medicine plant collected from Kermanshah. The sample was cleaned from any strange, plants, dust, or any other contaminants.

Oil extraction of N. sativa by Steam Distillation:

The hot steam helps to release the aromatic molecules from the plant material since the steam forces open the pockets in which the oil is kept in the plant material. The molecules of the volatile oil then escape from the *N. sativa* material and evaporate into the steam. The temperature of the steam needs to be carefully controlled just enough to force the *N. sativa* material to let go of the oil, yet not too hot as to burn the *N. sativa* material or the oil. The steam which then contains the oil is passed through a cooling system to condense the steam, which forms a liquid from which the essential oil and water is then separated. The steam is produced at greater pressure than the atmosphere and therefore boils at above 100 degrees Celsius which facilitates the removal of the oil from the plant material at a faster rate and in so doing prevents damage to the oil.

Gas chromatography mass spectrometry (GC/MS)

To analyze oil of *N. sativa* by GC-MS, fused silica DB-5 column with 0.25 µm thickness film was used. The oven temperature was kept at 500°C for 5 minutes and then programmed from 50-2800°C for 40 minutes. Helium flow rate was maintained at 2 ml/min, with the split ratio of 1:3. Sample injection of 1µl and ionization voltage of MS-analysis was run by EI technique at 70ev. The volatile oil constituents were identified by matching their MS and retention index data with those of the standards spectra and by matching their fragmentation pattern in Mass Spectra[29]. NIST standard reference database (AMDIA version 2.70) was used to interpret the mass spectral data.

Source of microorganisms

Three bacterial species namely *E. coli O157:H7* (ATCC No. 25922), *S. aureus* (ATCC No. 25923) and *B. subtilis* (ATCC No. 21332) were procured from Iranian Research Organization for Science and Technology as lyophilized. Each bacterial strain was activated on Tryptic Soy broth, constant at 37°C for 18 h. Then 60 µl of the broth was transferred to Nutrient agar and incubated at 37°C for another 24 h; cell concentration was then adjusted to obtain final concentration of 10^8 cfu/ml using Muller Hinton broth.

Culture media

Mueller-Hinton Agar (Müller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing) was prepared according to the manufacturer's instruction, autoclaved and dispensed at 20 ml per plate in 12 x 12cm Petri dishes. Set plates were incubated overnight to ensure sterility before use.

Evaluation of antimicrobial activities

Agar disk and agar well diffusion methods were used as screen tests to evaluate antibacterial property of the oil of *N. sativa*. Based on standard protocol. The solution of the plant was yielded in 1g/ml from which six fold serial dilutions (v/v) were prepared. 60 µl of each dilution was poured on each disk and well in order. After a period of 24 hours' incubation, the diameters of growth inhibition zones around the disks and wells were measured. DMSO was used as negative control whereas kanamycin and cephalexin were used as positive controls in case of *E. coli* and *B. subtilis*/ *S. aureus*, respectively. Minimum inhibitory concentration (MIC) means the lowest concentration of the probable antimicrobial agent which prevents growing of bacteria (regardless of killing the bacteria or stopping the growth of them). The lowest dilution which no gross microbial growth has been seen indicates MIC. Minimum bactericidal concentration (MBC) means the lowest concentration of the agent which causes death to test bacteria. The last can be revealed by pouring 60 µl of MIC tube and six dilutions before contents on agar plate. In this case, after incubation period, the lowest concentration which makes no growth indicates MBC. For determination of MIC value, macrobroth dilution method was applied. Interpretation of the results was done due to national accepted letter[30].

Statistical Analysis

Antibacterial effect was determined by One-way variance analysis (ANOVA), using the SPSS 18 software package. Data were considered statistically significant at $p \leq 0.05$.

RESULTS

Chemical composition

In examining the chemical composition of the oil of *N. sativa* using Gas chromatography mass spectrometry (GC/MS), the presence of α-hellandrene, α-pinene, β-pinene, p-cymene, Cis-carveol, Trans-anethole, Thymoquinone, Thymol, α-Longipinene and Longifolene in this herb were specified.

Agar disk diffusion test

All test bacteria were sensitive to undiluted oil of *N. sativa*. Growth inhibition zones due to different dilutions are listed in table 1. No inhibition zone was observed due to DMSO.

Agar well diffusion test

In regard to *N. sativa* oil, the widest zone was seen in 0.125 g/ml, due to *S. aureus* and *B. Subtilis* (11 mm). It was no growth inhibition in 0.007 g/ml and less for all bacteria. The data are discoverable in table 2.

MIC and MBC ascertaining

The most and the least values for MIC were acquired in 0.031 g/ml for *E. coli*/*S. aureus* and 0.015 g/ml for *B. subtilis.* Toward oil of *N. sativa*, MBC was 0.031 g/ml for all bacteria (table 3).

As the table shows, the oils of *N. sativa* have prevented the growth of *E. coli*, *S. aureus* and *B. subtilis*. Also, by increasing the concentrations of the oil of *N. sativa*, the inhibition zone increased ($p \leq 0.001$). The results determined that in tested bacteria, there was a significant difference ($p \leq 0.001$) in terms of sensitivity to the oil. In other words, the most sensitivity was observed in *S. aureus*.

Table 1: The diameters of growth inhibition zones in agar disk diffusion test in different dilutions of the oil from *N. sativa*.

Dilution(g/ml)	Inhibition zone (mm) in disk diffusion		
Microorganism	*E. Coli*	*S. aureus*	*B. Subtilis*
Positive control	22	26	22
1/8 (0.125)	11	19	14
1/16 (0.062)	10	12	10
1/32 (0.031)	9	10	9
1/64 (0.015)	9	9	8
1/128 (0.007)	0	0	0
1/256 (0.003)	0	0	0
Negative control	0	0	0

Table 2: The diameters of growth inhibition zones in agar well diffusion test in different dilutions of the oil from *N. sativa*.

Dilution(g/ml)	Inhibition zone (mm) in well diffusion		
Microorganism	*E. Coli*	*S. aureus*	*B. Subtilis*
1/8 (0.125)	10	11	11
1/16 (0.062)	9	9	9
1/32 (0.031)	8	8	8
1/64 (0.015)	0	8	0
1/128 (0.007)	0	0	0
1/256 (0.003)	0	0	0
Negative control	0	0	0

Table 3: MIC and MBC of the oil of *N. sativa*.

The oil of *N. sativa*			
Microorganism	*E. Coli*	*S. aureus*	*B. Subtilis*
MIC	1/32 (0.031)	1/32 (0.031)	1/64 (0.015)
MBC	1/32 (0.031)	1/32 (0.031)	1/32 (0.031)

DISCUSSION

In spite of the current interest in drug discovery by molecular modelling, combinatorial chemistry and other synthetic chemistry methods, plant-derived compounds are still substantiating to be an important source of medicines for human being. The significance and uses of plants in modern drug discovery has been recounted in recent reports[31,32]. Plant oils have been used for many thousands of years[33], in food preservation, pharmaceuticals, alternative medicine and natural therapies[34,35]. Oils are potential sources of novel antimicrobial compounds, especially against bacterial pathogens[36]. *In vitro* studies in this work showed that the oils inhibited bacterial growth but their effectiveness varied. The antimicrobial activities of many oils has been previously reviewed and classified as strong, medium or weak[37]. *N. sativa*, is an annual flowering plant that grows to 20-30 cm tall, is native to Asia and the Middle East. The flowers of this plant are very delicate and pale colored and white. The seeds are used in Middle Eastern cooking, such as in their local breads. *N. sativa* is also used by thousands for their natural healing abilities. Concerning the method of oil and preventing from using high temperature to decrease the rate of destruction of effective herbal compound. Several compounds such as sterols and phenolic constituents were found in *N. sativa*. Presence of phellandrene, α-pinene, β-pinene, p-cymene, Cis-carveol, Trans-anethole, Thymoquinone, Thymol, α-Longipinene and Longifolene were identified in the composition of the obtained oil of *N. sativa.* Thymoquinone or thymohydroquinone (2-isopropyl-5-methyl-1,4-benzoquinone)[38] are active components of the oil of *N. sativa* that have different pharmacological activities such as anti-inflammatory, antioxidant and antihypertensive effects[39]. In addition, a great antibacterial action of Thymoquinone against *Paenibacillus larvae* was observed (MIC values ranging from 8 to 16 mg/ml)[40]. Alkharfy et al. reported that Thymoquinone treatment reduced mortality in mice following Lipopolysaccharid and live *E. coli* challenge by 80-90%[41]. There is a partial difference between these results and the similar studies. Many components of *N. sativa* were characterized by Burits et al.[42] and Ali et al.[43] using GC-MS, but the major ones were thymoquinone, p-cymene and carvacrol. All of these compounds had antibacterial effects. The results indicated that *N. sativa* oil with concentration about 0.031 g/ml has prevented the growth of *E. coli*, *S. aureus* and *B. subtilis*. Thus, the research suggests the antibacterial effects of the medical herb on Gram-negative and Gram-positive pathogenic bacteria. A number of authors have mentioned the antimicrobial activity of *N. sativa*. In a study done by Shahidi et al. also investigated *N. sativa* products indicated a good effect on the standard *S. aurous*[44]. Morsi et al.[45] had proven that both the crude alkaloid extract and the water extract of the *N. sativa* were effective against some tested microorganisms like *staphylococcus* despite their resistance to other antibiotics. In a study conducted by Niakan et al., the antimicrobial effect of oil extract of *N. sativa* against *S. aureus* in laboratory was studied. They concluded that the anti-microbial effect of *N. sativa* oil extract is comparable with antibiotics such as Ceftazidime, Cefaclor, Cefamandole and Cefuroxime. They recommend experimental use of *N. sativa* to control *S. aurous* infections[46]. Farrag et al. found that the fixed oil of black cumin had an inhibitory effect against Gram-positive such as *S. aureus* and *B. cereus*. The essential oil showed antibacterial activity against *B. cereus* and *S. aureus*[47]. Finally, our results are in agreement with others who showed that *N. sativa* oils produce antimicrobial activity against a broad range of microbes and especially against multiple-antibiotic resistant bacteria. From the study it can be concluded that the oil of *N. sativa* possess antibacterial activity. Our results support the use of the plant in traditional medicine and suggest that *N. sativa* oil possess compounds with good antibacterial properties. It can be used as an antibacterial supplement in the developing countries towards the development of new therapeutic agent. Additional *in vivo* studies and clinical trials would

be needed to justify and further evaluate the potential of it as an antibacterial agent in topical or oral applications.

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