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Chemical Composition and Antimicrobial Activity Against Food Poisoning of Alcoholic Extract of *Nigella Sativa L*

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Abstract: Nowadays using natural sources for treating disease is one of the most favorable methods because of fewer side effects. Reporting antimicrobial properties of a new natural source can be useful for designing new natural medicines antimicrobial effect of *Nigella Sativa L.* seeds n-butanol extract will be useful for treating or preventing for many infections. In our study we report the first GC-MS analysis of n-butanol extract of *Nigella Sativa L.* seed and also its antimicrobial activity against some food poisoning and nosocomial infection causing microorganisms. *Nigella Sativa L.* seed powder was subjected to n-butanol with ratio 1:10 in a flask and then kept on shaker. After filtering, the extract has been analyzed by GC-MS. After that, the extract was tested against bacteria and fungi by three method, disc diffusion, well diffusion, and micro dilution methods. The GC-MS analysis revealed that fatty acids and terpenoids are the major constituents. The result showed that *pseudomonas aureoginosa, klebsiella pneumoniae, acinetobacter baumanii, Yersinia enterocolitica, Candida albicans, Candida parapsilosis* and *Candida krusei* were the sensitive microorganisms. According to great antimicrobial activity and having nourishing components (fatty acids and terpenoids) adding this plant to a daily food diet is a good way for having a healthy life and this plant can be a new source for therapeutic uses.

Keywords: *Nigella Sativa L*; GC-MS analysis; antimicrobial; food diet.

1. Introduction

Nigella Sativa L. is an annual flowering plant in the family Ranunculaceae. It's native to Turkey, Pakistan and Iran [1]. *Nigella Sativa* seeds, a medicinal herb frequently known as black seed and its oil has been used for their medicinal, aromatic or flavoring properties since ancient times in different civilizations[2]. Traditionally, *Nigella Sativa L.* seeds extract has several beneficial biological effects including diuretic, diaphoretic, stomachic, liver tonic and digestive. It's also had been used in leucoderma, alopecia, eczema, freckles and pimples and also had been used as antibacterial and antioxidant [3]. Previous studies on *Nigella sativa L.* premiered 6% moisture, 4% ash, 32% fat 20% crude protein. Also it has been showing some minerals like Magnesium, Calcium, potassium, sodium and Iron [4]. *Nigella Sativa* seed essential oil that has been extracted by Clevenger apparatus, Microwave and steam distillation method, revealed the essential oil contains lots of the bioactive compound as the terpenoids that the main compound is Thymoquinone, a monoterpenoid ketone that has magnificent properties [5-7]. Oil composition of *Nigella Sativa L.* seed showed both saturated and unsaturated fatty acids, that two kinds of necessary fatty acids α-linolenic acid (ALA) – an omega 3 fatty acid and linoleic acid (LA) - an omega 6 fatty *acid* are the main ones [8-9].

Different solvent extracts of *Nigella Sativa L.* seed contain both terpenoids and fatty acids [9-10]. Food poisoning (Foodborne disease) that is defined as a disease of infectious caused by food, is one of the major concerns worldwide. Food borne infections are caused by many different disease-causing pathogens that can contaminate foods, while food borne poisoning is caused by poisonous chemicals or harmful substance that are present in food. Some of the major food poisoning organisms are *Salmonella typhimurium, Staphylococcus aureus, bacillus cereus, clostridium botulinum and Escherichia Coli* [11]. *Yersinia enterocolitica* is one of the gram negative bacteria that is belonging to Enterobacteriaceae and recovered from animal and environmental reservoirs [12-13]. This organism acquired by insufficiently cooked meat or contaminated milk, water, fish and meat [14]. *Yesinia enterocolitica* is thought to be a significant food-borne pathogen, recently have been reported that is one of the food poisoning organism in Japan [14]. Hospital-acquired infections (HAI) or nosocomial infections encompass almost all clinically evident infections that do not originate from patients original admitting diagnosis. Nosocomial infections are one of the leading causes of death and increased recovery and force additional cost to health care systems [16]. Causative agents of nosocomial infections can be (by order of importance) different types of bacteria, fungi, viruses and parasites. Among the bacteria, *Escherichia coli, Staphylococcus aureus ,Klebsiella pneumoniae* and *Acenitobacter baumannii* are the most common and antibiotic resistance bacteria[17]. *Pseudomonas aeruginosa* as the cause of infection in burn centers are used in the present study [18]. Among fungi *Candida albicans, Candida tropicalis, Candida krusei, Candida glabrata* and *Candida parapsilosis* can cause nosocomial infections in patients who are somehow immunocompromised [19]. Due to drug resistance of agents causing nosocomial infections and many side effects of conventional synthetic antibiotics [20-21], nowadays many researchers work on finding new natural compounds as alternatives to synthetic drugs, since using natural and herbal drugs have fewer side effects and will enhance the quality of treatment [22]. In previous studies about solvent extracts of *Nigella Sativa L.* seeds, there was no report of GC-MS analysis of n-butanol extracts. There were just two qualitative reports about categories of chemical compounds in the extracts and there was nothing about the details of these compounds [23-24]. In this study the compounds of n-butanol extract of *Nigella Sativa L.* seeds by GC-MS analysis and also its antimicrobial properties against some food poisoning and nosocomial infection causing microorganisms in order to introduce this new plant as a rich source of nutritional components and new natural medicine for treating disease. Also, scientific evidence of traditional uses has been investigated.

2. Materials and Methods

2.1. Collection of Nigella Sativa L. Seeds.

Nigella Sativa seeds were purchased from local herb shop in Tehran, Iran. The plant species was confirmed and a voucher was deposited at herbarium of faculty of pharmacy, Tehran University of medical science (pmp-747).

2.2. Extract preparation.

The *Nigella Sativa* seeds were dried at ambient temperature and the seed were grinded into fine powder by electric grinder. *Nigella Sativa L.* seed powder was subjected to n-butanol with ratio 1:10 in a flask and then kept on shaker at room temperature on continuous shaking for one week. The extract then filtered through whatman filter paper 1. The filtrated extract

then dried in a rotary evaporator until all the solvent gets evaporated. Then the extract was stored at 4 ºC for further research uses [24-25].

2.3. GC-MS analysis.

The chemical composition of the extracted seed was analyzed by GC-MS using an Agilent network system (GC: 6890 N; MSD: 5973 N). MS source temperature was 230ºC and MS quad temperature was 150 ºC . The chromatographic column for the analysis was a TRB-5 capillary column (60 m × 320 µm id, film thickness of 0.5µm). Helium was used as carrier gas at a flow rate of 1 ml/min. The injection was performed in split 1:1 mode at 280 ºC. The extract was injected and analyzed with the column held initially at 40 ºC for 5 minutes and then increased to 280 ºC with a 10 ºC/min heating ramp and subsequently kept at 280 ºC for 5 minutes.

2.4. Microbial Studies.

2.4.1. Collection of strains.

The antimicrobial activities, nosocomial infection causing were studied using selected bacteria: *Pseudomonas aeruginosa* (ATCC 27853), Klebsiella pneumoniae (ATCC BAA-1706), *Acinetobacter Baunmannii* (ATCC BAA-747), food poisoning bacteria *Escherichia Coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Yersinia Enterocolitica* (ATCC 9610) and nosocomial infection causing fungi *Candida albicans* (PFCC 89-1078), *Candida tropicalis* (PFCC 90-797), *Candida krusei* (PFCC 89-840), *Candida glabrata* (PFCC 80-3033) and *Candida parapsilosis* (PFCC 90-1248). These Microorganismswere obtained from Pasteur institute of Iran

2.4.2. Preparing microorganism, extract's samples and antibiotics.

To prepare pure colonies of bacteria, each of them was passaged in nutrient agar separately and the plates were incubating for 24 hours at 35 ºC. For preparing a pure colony of fungi, a passage from each type of them was performed in Sabouraud agar and the plates were incubated at 35 ºC for 48 hours. The test samples were prepared by dissolving 1.5, 2, and 2.5 µl of extract in 1 ml of dimethyl sulfoxide (DMSO) solvent (Merck number: 317275).

Two antibiotics; Gentamicin and Amphotericin B were considered in bacteria and fungi plates as positive control respectively. Using DMSO solvent and adding the powder of antibiotics, concentration of Gentamicin was adjusted to 10µg/ml and for Amphotericin B concentration was adjusted to 50µg/ml.

2.4.3. Antimicrobial properties.

Agar well diffusion method: For studying antimicrobial properties, from pure colony of microorganisms a suspension was prepared in the vials containing sterile normal saline and turbidity of the suspension was compared with 0.5 McFarland standard by visual comparison (a 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dehydrate with 9.95 ml of 1% sulfuric acid). In this way, the cell density is about 1x108 CFU/ml per vial. Mueller-Hinton medium is a standard environment for antibiogram. Therefore, each suspension was inoculated on plate (10 cm in diameter) containing Mueller-Hinton agar for bacteria and Mueller-Hinton agar with 2% glucose for fungi by sterile swab, and

microorganisms spread throughout the media. 5 wells, each with 5mm diameter, 5mm depth and 2.5mm apart, were made with a puncher and to close the bottom of wells, 25 µl molten Mueller-Hinton agar was poured at a temperature of 50°C In liquid form. After cooling, depth of wells is closed with a thickness of 1mm Mueller-Hinton agar .in wells no. 1 to no. 3, 100µl of the extract at concentrations 1500, 2000 and 2500 ppm (1.5,2 and 2.5µl/ml) was added respectively. In well no. 4 of each plate, 100µl of antibiotic was added as positive control (Gentamicin for bacteria and Amphotericin B for fungi) and in well no. 5, 100 µl DMSO was added as negative control. Bacteria plates were incubated for 24 hours and fungi plates for 48 hours in 37ºC. For each organism the process was repeated three times and the means and standard deviations of inhibition zone diameter were calculated.

Disk diffusion method: Sterilized Petri dishes (10 cm diameter) already poured with nutrient agar media were inoculated with 0.01 mL of nutrient agar media (105 - 106 bacteria per ml). Discs injected with extract of different concentrations 1500, 2000 and 2500 ppm (1.5, 2 and 2.5µl/ml) and were applied on the solid agar medium by pressing tightly. One disc of antibiotic (10µg Gentamicin disc and 50µ Amphotericin B disc) was used as positive control and one disc is injected with DMSO as negative control. The treated Petri dishes were incubated at 37°C for 24 hours for bacteria and 48 hours for fungi. The process was repeated three times for each microorganisms. At the end of the period, the antimicrobial activity was expressed as the mean diameter of the inhibition zone (mm) produced by plant extracts.

Micro dilution method: In addition to good diffusion and disc diffusion technique, minimal inhibitory concentration (MIC) for bacteria and fungi was calculated. Micro dilution method was used for this study so that 7 columns in 96-well plate were chosen for seven microorganisms that show sensitivity to the compounds in the extract. 1µl of Muller-Hinton agar broth was added to all wells, and then 1µl of suspension containing 8 µl of the extract dissolved in 1ml of DMSO after being filtered by a 0.2 µm diameter microbiological filter, was added to all wells in the first row. With this account, concentration of the extract in well no. 1 of each microorganism was regulated as 4 µl/ml (4000ppm).6 serial dilutions were made from wells no. 1 to 6 (4, 2, 1, 0.5, 0.25, 0.125 µl/ml). Each well was inoculated with 20 µl of the microorganism suspension similar to 0.5 McFarland standards. For positive control for each microorganism, a well containing the microorganism and the culture was made in a 96 well microliter plate and incubated at 35 ºC. After 24 hours for bacteria and 48 hours for yeasts, the MIC was read as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after incubation

3. Results and Discussion

3.1. GC-MS analysis.

The n-butanol extract of *Nigella Sativa* seeds was identified by comparing their retention time with those of a computer library (Wiley 7n.l). The result of GC-MS analysis is presented in (table 1).

The major component of fatty acids was Linoleic acid (26.156%) followed by α-linolenic acid (21.268%) and Linolelaidic acid (6.538%) and the major components of terpenoids were o-cymene (7.452%) and Thymoquinone (6.753%). In a previous study about compounds of n-butanol extract of *Nigella Sativa L.* seeds, the extract's qualitative analysis of secondary metabolites showed that terpenoids are the main compounds [24]. Also in this study among the secondary metabolites, terpenoids are the main ones. Compared to other solvent

extracts and essential oil of *Nigella Sativa L.* seeds, Thymoquinone was one of the main components. Among the terpenoids of n-butanol extract, unlike other extracts, o-Cymene (7.452%) was more than Thymoquinone (6.753%) [1, 5, 10]. Among the fatty acids that are found in other extracts and essential oil, Linoleic acid (omega 6) and α-linolenic acid ((ALA) (omega 3) were the main unsaturated fatty acids and also have been observed in n-butanol extract as the main ones [4, 9-10]. In n-butanol extract of *Nigella Sativa L.* seed one unsaturated fatty acid, Linolelaidic acid, has been observed that had not been reported from other extracts and essential oil before. Linolelaidic acid (LA) is a geometric isomer of Linoleic acid. They both are an omega 6 fatty acid but linoleic acid is *cis*-omega 6 and linolelaidic acid is *trans*-omega 6 fatty acid (Figure 1). Previous studies on Linolelaidic acid revealed that this unsaturated fatty acid has anti-inflammatory and cancer protective activity [26]. Also, it's been revealed that *Daniella Oliveri* seed oil that contains 56.57% Linolelaidic acid showed great antibacterial activity against Klebsiella granulomatis [27].

Table 1. Chemical composition of n-butanol extract of *N. sativa* analyzed by GC–MS.

Compound	Retention time (min)	Area (%)	RI
α-thujene	13.47	0.332%	931
Sabinene	14.52	0.183%	976
Butanoic acid	14.72	2.064%	1002
o-cymene	15.37	7.452%	1027
γ-terpinene	15.88	0.801%	1062
Terpinen-4-ol	16.96	0.900%	1177
Thymoquinone	19.06	6.753%	1249
Carvacrol	19.77	3.350%	1298
α-longipinene	20.66	0.195%	1343
Isolongifolene	21.53	2.360%	1402
2,4-ditert-butylphenol	23.17	4.310%	1512
2,3-dihydrofarnesol	24.61	0.194%	1604
γ-eudesmol	26.02	2.034%	1646
Lauric acid	27.52	4.980%	1786
Palmitic acid	28.45	1.278%	1884
Myristic acid	28.78	3.572%	1977
Sandaracopimaradiene	29.24	5.280%	1980
Linolelaidic acid	29.40	6.538%	1982
Linoleic acid	31.16	26.156%	2076
α-linolenic acid	33.73	21.268%	2116

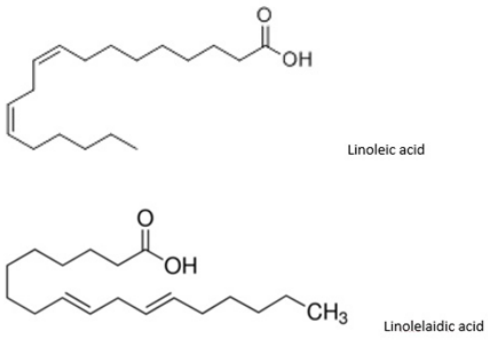

Figure 1. Cis-linoleic acid and trans-linolelaidic acid.

According to this research it can be concluded that the presence of Linolelaidic acid might be the reason for amazing antimicrobial activity of n-butanol extract of *Nigella Sativa L.* seed. Unsaturated fatty acids help to lower levels of total cholesterol and LDL cholesterol in the blood [28]. *Nigella Sativa L.* is a rich source of unsaturated fatty acids (according to table 1 about 54% of unsaturated fatty acids). So, *Nigella Sativa L.* n-butanol extract can be used for controlling total and LDL cholesterol in the blood. Interest in medicinal plants has grown due

to increased efficiency of new plant-derived drugs and the interest in natural products [29]. Plants were long used for curing infections also several plant products were documented to inhibit growth of pathogenic bacteria [30].

3.2. Antimicrobial properties.

Examining the plates after incubation in agar well diffusion and disc diffusion method, confirmed the inhibitory effect of the extract in all three concentrations on bacteria *Pseudomonas aeruginosa , Klebsiella pneumoniae, Acinetobacter Baunmannii and Yersinia Enterocolitica* and also fungi *Candida albicans, Candida krusei and Candida parapsilosis* (table 2 and table 3). No inhibition of growth was observed for other microorganisms. Also for controls, No inhibition zone around wells containing DMSO and the disc that is injected with DMSO (negative control) was observed and when using antibiotics (Gentamicin for bacteria and Amphotericin B for fungi as positive control) inhibition zones 10-13mm for bacteria and 5-10 mm for fungi were observed.

Table 2. Antimicrobial activity of *Nigella Sativa L.* n-butanol extract by well diffusion method.

Microorganisms	Concentration of extract			DMSO (negative control) (mm)	Antibiotics[a] (positive control) (mm)
	1500 ppm (1.5 µl/ml) (mm)	2000 ppm (2µl/ml) (mm)	2500 ppm (2.5 µl/ml) (mm)		
pseudomonas aeruginosa	41±0.3	46±0.1	50±0.2	0	11±0
Klebsiella	20±0.2	21±0.1	23±0.2	0	13±0
Pneumonia	31±0.1	33±0.2	35±0.2	0	12±0
Acinetobacter Baunmannii	-	-	-	0	10±0
Escherichia Coli	-	-	-	0	12±0
Staphylococcus aureus	28±0.2	32±0.2	35±0.2	0	13±0
Yersinia Enterocolitica	30±0.4	37±0.3	41±0.6	0	9 ±0
Candida albicans	-	-	-	0	7±0
Candida tropicalis	20±0.3	22±0.1	24±0.2	0	5±0
Candida krusei	-	-	-	0	10±0
Candida glabrata	22±0.3	25±0.3	26±0.4	0	7±0

a. Gentamicin (10µg/ml) for bacteria and Amphotericin B (50µg/ml) for fungi

Table 3. Antimicrobial activity of *Nigella Sativa L.* n-butanol extract by disc diffusion method.

Microorganisms	Concentration of extract			DMSO (negative control) (mm)	Antibiotics[a] (positive control) (mm)
	1500 ppm (1.5 µl/ml) (mm)	2000 ppm (2µl/ml) (mm)	2500 ppm (2.5 µl/ml) (mm)		
Pseudomonas aeruginosa	60±0.2	40±0.3	40±0.1	0	11±0
Klebsiella pneumonia	50±0.2	46±0.3	46±0.2	0	13±0
Acinetobacter Baunmannii	48±0.1	50±0.2	47±0.4	0	12±0
Escherichia Coli	-	-	-	0	10±0
Staphylococcus aureus	-	-	-	0	12±0
Yersinia Enterocolitica	40±0.2	39±0.3	38±0.1	0	13±0
Candida albicans	42±0.3	27±0.2	22±0.2	0	9 ±0
Candida tropicalis	-	-	-	0	7±0
Candida krusei	10±0.1	15±0.2	11±0.1	0	5±0
Candida glabrata	-	-	-	0	10±0
Candida parapsilosis	30±0.2	25±0.1	30±0.3	0	7±0

a. Gentamaicin for bacteria and amphotericin B for fungi

The results of MIC for sensitive microorganisms are shown in (table 4). Compared to other sensitive microorganisms, *Candida albicans and Yersinia Enterocolitica* have higher sensitivity in smaller doses.

Table 4. Minimum inhibitory concentration (MIC) of microorganisms sensitive to n-butanol extract of *Nigella Sativa L.* seeds.

Microorganism	**MIC µl/ml (ppm)**
pseudomonas aeruginosa	0.5 µl/ml (500 ppm)
Klebsiella pneumoniae	0.25 µl/ml (250 ppm)
Acinetobacter Baunmannii	1 µl/ml (1000 ppm)
Yersinia Enterocolitica	0.125 µl/ml (125 ppm)
Candida albicans	0.125 µl/ml (125 ppm)
Candida krusei	0.25 µl/ml (250 ppm)
Candida parapsilosis	0.5 µl/ml (500 ppm)

Compared to previous study about antimicrobial activity of n-butanol extract of *Nigella Sativa L.* seeds, the antimicrobial activity of n-butanol extract have been studied against bacteria *Enterococcus faecalis, Staphylococuss aureus, Acinetobacter junii, Escherichia coli, Proteus mirabilis, Serratia marcescens and Enterobacter cloacae* [24]. The n-butanol extract of *Nigella Sativa* seeds have not been tested against *Klebsiella pneumoniae, Acenitobacter baumannii, Pseudomonas aeruginosa, Yersinia enterocolitica* and fungi *Candida albicans, Candida tropicalis, Candida krusei, Candida glabrata* and *Candida parapsilosis* for antimicrobial activity, so this research is the first report of antimicrobial activity against these microorganism for n-butanol extract of *Nigella Sativa L.* seeds. According to previous studies of other extracts and essential oil of *Nigella Sativa L.* seeds, essential oil of *Nigella Sativa L.* seeds showed antimicrobial activity against *Staphylococuss aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneeumoniae and Candida albicans* [5,8]. Ethanol extract showed antimicrobial activity against Escherichia coli, Staphylococcus aureus, Candida albicans [25-26]. Methanol extract showed antimicrobial activity against *Staphylococuss aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneeumoniae* and *Candida albicans* [24, 31-32]. Aqueous, chloroform and acetone extract showed antibacterial activity against bacteria *Staphylococuss aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and also acetone extract showed antifungal activity against Candida albicans [24, 25, 31-32]. For other microorganisms that have been studied in this research, previous studies have shown that essential oil of Nigella Sativa seed has antibacterial activity against Yersinia *enterocolitica* [33] but against Acinetobacter baumannii the essential oil was insensitive [34]. Against fungi *Candida krusei, Candida tropicalis* the SFE extracted volatile oil of *Nigella Sativa L.* seed showed antifungal activity [35] and also it has been reported that *Candida glabrata, Candida tropicalis* and *Candida parapsilosis* were sensitive to *Nigella Sativa* seed essential oil [36].

Yersinia enterocolitica is one of the most important food poisoning causing organisms around the world. This organism is responsible for milk, meat, pork, seafood, water, vegetables poisoning. It's been reported that this organism can survive at low temperature [37-38]. So finding a natural source to control this organism is an amazing achievement. *Nigella Sativa L.* seed n-butanol extract showed a great antibacterial effect against *Yersinia enterocolitica* (28 to 40 mm inhibition zone). According to this result, using this plant's extract can be effective for restraining the infections that are caused by *Yersinia enterocolitica*.

Given the prevalence of nosocomial infections among patients in hospitals [39], especially in immunocompromised patients [40], identification of suitable herbal antimicrobial compounds can make the course of treatment more favorable. Acinetobacter genus commonly causes nosocomial infections [41]. Acinetobacter *baumannii* is one of the most common and multidrug resistant microorganism [42] and the leading organism of infection in almost every

ICU, especially in emergency, neurosurgery ICU's and respiratory tract infections [43-44]. *Klebsiella pneumoniae* is the other multidrug resistant [45], blood stream infection and surgical site infection causing organism [43]. *Pseudomonas aeruginosa* is the other organism that causes bloodstream infection and respiratory tract infections [43]. Candida is a genus of yeast and is the most common cause of fungal infection around the world [46]. *Candida spp.* Can cause lots of fungal nosocomial infection, such as urinary tract infection, especially Candida albicans [43, 47]. *Candida krusei* is associated with hematological unit infections and it has a high risk of mortality [48]. It has been found that *Candida parapsilosis* is the most common organism in onychomycosis (nail infection) and *Candidemia* in hospitals [49-50]. According to the results obtained in present study *Nigella Sativa L.* seed n-butanol extract displayed magnificent inhibition against these nosocomial infection causing bacteria and fungi. So it can be a new source to control these infections for patients in hospitals during their treatment. Since the plant-derived compounds cause fewer side effects [51-52], they can be a good alternative to synthetic antibiotics. Having great antimicrobial activity of this plant can be evidence for traditional uses of this plant as antibacterial agent.

4. Conclusions

Antimicrobial effect of n-butanol extract of *Nigella Sativa L.* seed on nosocomial infections causing organisms and food poisoning causing organism is quite interesting. Since the extract has great antimicrobial activity, *Nigella Sativa L.* seed n-butanol extract can be used besides antibiotics to increase the treatment ability. Linolelaidic acid is one of the fatty acids that have been found in this extract and this compound has not been studied independently, therefore separation of Linolelaidic acid from fatty acids in the extract and further investigations are recommended. Also, it is recommended to study fatty acids and terpenoids of this extract independently, because it has been studied in a mixture and it is a question that they would show better biological activity independently or in a mixture. Also, it is suggested to use Nigella Sativa L. seed in a daily food diet for a more healthy life, because of having unsaturated fatty acids and terpenoids, it can help to improve body's health.

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Conflicts of Interest

The authors declare no conflict of interest.

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