Ethanolic extract of *Nigella sativa* protects Fe(II) induced lipid peroxidation in rat's brain, kidney and liver homogenates

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Abstract: The study describes the effect of ethanolic extract of *Nigella sativa* against Fe(II) induced lipid peroxidation. Basal and Fe(II) induced thiobarbituric acid reactive species (TBARS) production was significantly inhibited by the ethanolic extract of *Nigella sativa* at 25-200µg/ml. Our data revealed that the extract has high DPPH radical scavenging activity at highest tested concentrations. The extract significantly chelated Fe(II) and scavenged hydroxyl (OH●) radical at 25-200µg/ml concentration. The nutritional analysis was performed and carbohydrate, fats, fiber, protein, moisture and ash content were measured in the studied extract. The phytochemical analysis confirmed the presence of alkaloid, carbohydrate & sugar, glycosides, phenolic compounds, flavonoids, protein and amino acid, phytosterols, tannins, gum and mucilage. The extract also showed significant antimicrobial activities against 10 bacterial strains i.e. *Salmonella typhi, Bacillus subtilis, Bacillus cereus, Klebsiella pneumonia, Escheria coli, Xanthomonas, Salmonella heidelberg, Staphylococcus aureus, Clostridium* and *Escheria coli* (human) and 5 fungal strains i.e. *Aspergillus niger, Entomola, Aspergillus flavus, Alternaria alternata* and *Penicillium.* This study confirms the potential antioxidant and antimicrobial activities of ethanolic extract of *Nigella sativa* which can be considered not only as a diet supplement but can be used against a variety of free radical induced damage diseases.

Keywords: *Nigella sativa*, lipid peroxidation and antimicrobial activities.

INTRODUCTION

Reactive oxygen species (ROS) have important physiological functions in living organisms and are actively involved in cellular signal transduction, cell proliferation and even apoptosis (Ames *et al*., 1993; Bland, 1995). The ROS production is counter balanced by antioxidant defense systems which include both reducing molecules and various enzymes. The biological situation where the imbalance between pro-oxidants and antioxidant is disrupted in favor of the former can lead to a process known as oxidative stress (Halliwell & Gutteridge, 1990). ROS, which are potent oxidant, can damage various bio-significant molecules ranging from DNA damage to protein carbonylation and lipid peroxidation. The involvement of ROS has been implicated in a variety of pathological manifestations like cancer, neurodegenerative diseases and aging etc. (Collin, 1999; Floyd, 1998). The area of antioxidant therapy is exploding in the literature and various classes of chemical compounds have been explored for potential antioxidant therapy. However, very less success has been achieved with the later because of the complex pharmacological and toxicological processes (Amarowicz *et al*., 2000; Aruoma *et al*., 1992). One of the most important strategies could be the use of dietary antioxidant intake; which may inhibit or delay the oxidation of susceptible cellular substrates and ultimately can help in reducing the risk of various diseases. In this regard flavonoids, phenolic acids, tannins and tocopherols have received attention for their high antioxidant activity (Rice-Evans *et al*., 1998) and less toxicity problems which may arise from the use of synthetic antioxidants (Amarowicz *et al*., 2000; Aruoma *et al*., 1992). It could be summarized that plants have many Phytochemicals which are potential sources of natural antioxidants and can be useful against a variety of oxidative stress related diseases.

Nigella sativa Linn sometimes known as black seed or black cumin belongs to botanical family of Ranunculaceae. The seeds of this plant have been used in the Southeast Asia and Middle East for a long time against a variety of diseases like asthma, hypertension, diabetes, inflammation, arthritis, tumor and gastrointestinal disturbances (Ali and Blunden, 2003; El-Din *et al*., 2006; Ramadan, 2007). The plant is widely used as spice and can be added to hot beverages. It has been reported that the seed of *Nigella sativa has* over 100 different chemical components including various organic acids, inorganic acids, reducing sugars, alkaloids, flavonoids, sterols, tannins and saponins. The most putative pharmacologically active constituents of *Nigella sativa* are thymoquinone (30-48%), p-cymene (7-15%), carvacrol (6-12%), 4-terpineol (2-7%), t-anethole (1-4%) and a sesquiterpene longifolene (1-8%) (Burits and Bucar,

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2000) thymoquinone derivatives like dithymoquinone, thymohydroquinone and thymol (Padhye *et al*., 2008).

The present work was designed to explore the antioxidant activity of the ethanolic extract of *Nigella sativa* using *in-vitro* methods. To get a deeper insight into the therapeutic use of this plant we have determined its protective effect against either basal or Fe(II) induced lipid peroxidation in rat's brain, liver and kidney homogenate. Several other biochemical tests were performed which will give us an idea to explain the possible mechanism of action of this extract.

MATERIAL AND METHODS

Chemicals

Thiobarbituric acid (TBA), malonaldehyde (MDA), bis-dimethyl acetal, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and phenanthroline were purchased from Sigma (St. Louis, MO, USA). Iron (II) sulphate from Reagen (Rio de Janeiro, RJ, Brazil). All the chemicals used were analytical grade and were obtained from standard commercial supplier, while the water was glass distilled.

Sample collection and identification

The seeds of *Nigella sativa* were purchased from local market of Peshawar in February 2012. The dried plant seeds were grinded using pestle and mortar and packed in polythene bags and placed in a dried place for further extractions. The plant species were identified by experts in Pakistan Council of Scientific and Industrial Research (PCSIR), Peshawar.

Ethanolic extract preparation

The extract of the *Nigella sativa* was prepared in ethanol; briefly, about 1 g of the *Nigella sativa* was soaked in 100 ml ethanol for 5 min. Thereafter, the mixture was centrifuged at 2,000 rpm for 10 min. The supernatant was used for the determination of antioxidant activity (reducing power, Fe(II) chelating ability and OH radical scavenging ability) and lipid peroxidation bioassay.

Proximate or Nutritional analysis

The proximate analysis (carbohydrate, fats, protein, moisture and ash) of *Nigella sativa* was determined by using the Association of Official Analytical Chemists (AOAC) methods 1990 (AOAC, 1990). Carbohydrate value was determined by difference (100- (moisture + ash + protein + fat)). The nitrogen content was determined by Kjeldahl method and multiplied to factor 6.25 to find the protein content. The weight difference method was used to find moisture and ash content. Nutrient contents were valued in percentage.

Phytochemical screening

Several chemical tests were carried out to evaluate the presence of secondary active metabolites like alkaloid, saponins, carbohydrate and sugar, glycosides, phenolic compounds, flavonoids, protein and amino acid, phytosterols, tannins and gum and mucilage in the selected plant seeds using standard procedures described by Sofowora (1993) and Trease and Evans (2000).

DPPH radical-scavenging

Scavenging of the stable radical, DPPH was assayed *in vitro* (Hatano *et al*., 1998). The extract (0-50 mg/ml) was added to a 0.5 ml solution of DPPH (0.25 mM in 95% ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm in a spectrophotometer. Percent inhibition was calculated from the control. Vitamin C was used as a standard compound in the DPPH assay.

Fe(II) chelation assay

The ability of the extract to chelate Fe(II) was determined using a modified method with a slight modification as described by Puntel *et al*., 2005. Freshly prepared 500 µM $FeSO_4$ (150 µl) was added to a reaction mixture containing 168 µl 0.1 M Tris–HCl (pH 7.4), 218 µl saline, and the ethanolic extract of the *Nigella sativa* (0-25µl). The reaction mixture was incubated for 5 min before the addition of 13 µl 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer.

OH radical scavenging ability

The ability of the *Nigella sativa* extract to prevent Fe^{2+}/H_2O_2-induced decomposition of deoxyribose was carried out using the method of Halliwell and Gutteridge (1989). Briefly, freshly prepared ethanol extract (0–100 µl) was added to a reaction mixture containing 120 µl 20 mM deoxyribose, 400µl 0.1 M phosphate buffer, 40 µl 20 mM hydrogen peroxide, and 40µl 500 µM $FeSO_4$ and the volume were made to 800µl with distilled water. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 0.5ml of 2.8% trichloroacetic acid; this was followed by the addition of 0.4ml of 0.6% thiobarbituric acid (TBA) solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm in a spectrophotometer.

Animals

Adult male wistar rats from our own breeding colony (250-350 g) were maintained in an air-conditioned room (22-25°C) under natural lighting conditions, with water and food (Guabi, RS, Brazil) ad libitum. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

Preparation of homogenates

The rats were decapitated under mild diethyl ether anesthesia and brain, liver and kidney were quickly

removed, placed on ice and weighed. The tissues were subsequently homogenized in cold saline (1/10 w/v) with about ten up-and-down strokes at approximately 1,200 rpm in a Teflon glass homogenizer. The homogenates were centrifuged for 10 min at 3,000 × g to yield a pellet that was discarded and a low-speed supernatant (S1) containing mainly water, proteins, lipids (cholesterol, galactolipid, individual phospholipids, gangliosides), DNA and RNA that was kept for lipid peroxidation assay.

Lipid peroxidation and thiobarbituric acid reactions

The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al*. (1979). S1 fraction (100µl) was mixed with a reaction mixture containing 30µl of 0.1M Sodium Phosphate buffer, extract (0-200 µg/ml), and 30µl of the pro-oxidant solution (250 µM freshly prepared/$FeSO_4$). The volume was made up to 300µl with water before incubation at 37°C for 1h. The color reaction was developed by adding 300µl 8.1% sodium dodecyl sulfate to the reaction mixture containing S1; this was subsequently followed by the addition of 600µl of acetic acid/HCl (pH 3.4) and 600µl 0.8% TBA. This mixture was incubated at 100°C for 1h. The thiobarbituric acid reactive species (TBARS) produced were measured at 532nm.

Antimicrobial assay

Microorganism collection and maintenance

For antimicrobial activity 10 bacterial strains i.e. *Salmonella typhi, Bacillus subtilis, Bacillus cereus, Klebsiella pneumonia, Escheria coli, Xanthomonas, Salmonella heidelberg, Staphylococcus aureus, Clostridium and Escheria coli* (human) and 5 fungal strains i.e. *Aspergillus niger, Entomola, Aspergillus flavus, Alternaria alternata* and *Penicillium* were taken from Food Technology Center, Pakistan Council of Scientific and Industrial Research (PCSIR) Labs Complex, Peshawar, Pakistan. All the strains, before the subculture were kept in Muller-Hinton Agar (MHA) at 4-C^o in the refrigerator.

Antibacterial assay of the Nigella sativa against selected bacterial species

The tests for susceptibility were performed using modified agar well diffusion method (Okeke *et al*., 2001) to examine the antibacterial activity of the *Nigella sativa*. The MHA was used as medium. The incubation temperature was fixed AT 37-C^o and the culture in triplicate was kept for 24 to 72 hours. In a Petri-dish the broth culture (0.6ml) of the test organism was taken. To this culture 20ml of the sterile molten MHA was mixed. Wells were jaded and added to the medium using *Nigella sativa* extract (0.2ml). Inoculation was performed for one hour to ensure the strong diffusion of the antimicrobial agent to the medium. The inoculation plates were incubated at 37-C^o for 24 hours. The zone of inhibition of microbial growth was measured in millimeters.

Antifungal assay of the Nigella sativa against selected fungal species

The antifungal activity was determined by the agar well diffusion method (Okeke *et al*, 2001). The crude extract was dissolved in DMSO (50/mg/5ml). Sterile Sabouraud's dextrose agar medium (5ml) was placed in a test tube and inoculated with the sample solution (400µg/ml) kept in slanting position at room temperature overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 29°C and growth inhibition was observed and percentage growth inhibition was calculated.

RESULTS

The nutritional analysis (carbohydrate, fats, fiber, protein, moisture and ash etc.) of *Nigella sativa* is shown in table 1. Moisture, fiber and ash contents were found in the range of 3.8 to 4.8%. Fat (4.7%), crude protein (20.5%) and total fats (4.7%) were estimated in the extract. Total soluble solids (0.8%), total acidity (0.72%) and vitamin C (11.7%) were measured in the extract. The pH of the extract was slightly acidic with 6.07 values. The phytochemical analysis indicated that the extract is rich in phyto-nutrients (table 2) and confirmed the presence of alkaloid, carbohydrate and sugars, phenolic compounds, flavonoids, protein & amino acid, phytosterols, tannins and gum and mucilage. The DPPH radical scavenging activity (table 3) of the extract also revealed high antioxidant activity. As shown in table 3, the extract significantly chelated Fe(II) at 25-200µg/ml concentration. Our data also revealed that extracts had high $OH^{\bullet}$ scavenging ability at all tested concentrations i.e. (25-200 µg/ml). It is apparent from fig.1 that the Fe(II) caused a significant increase in TBARS production in brain, kidney and liver respectively and the ethanolic extract of *Nigella sativa* inhibited the lipid peroxidation process at higher tested concentrations. The extract also showed significant antimicrobial activities against 10 bacterial strains i.e. *Salmonella typhi, Bacillus subtilis, Bacillus cereus, Klebsiella pneumonia, Escheria coli, Xanthomonas, Salmonella heidelberg, Staphylococcus aureus, Clostridium* and *Escheria coli* (human) and 5 fungal strains i.e. *Aspergillus niger, Entomola, Aspergillus flavus, Alternaria alternata* and *Penicillium*.

DISCUSSION

The nutritional analysis of the ethanolic extract of *Nigella sativa* are presented in table 1 which revealed (in % age) the presence of crude fat (4.7), fiber (4.2), protein (20.5), moisture (3.8), ash (4.8), total soluble solids (0.8), total acidity (0.72) and 11.7 mg/100ml vitamin C. Our data is in agreement with the findings of Sultan *et al*. (2009), which reported 6.46% moisture, ash 4.20%, protein 22.80%, fiber 6.03% and fat 31.16%. The reduced levels of crude fiber obtained for the studied extract is not

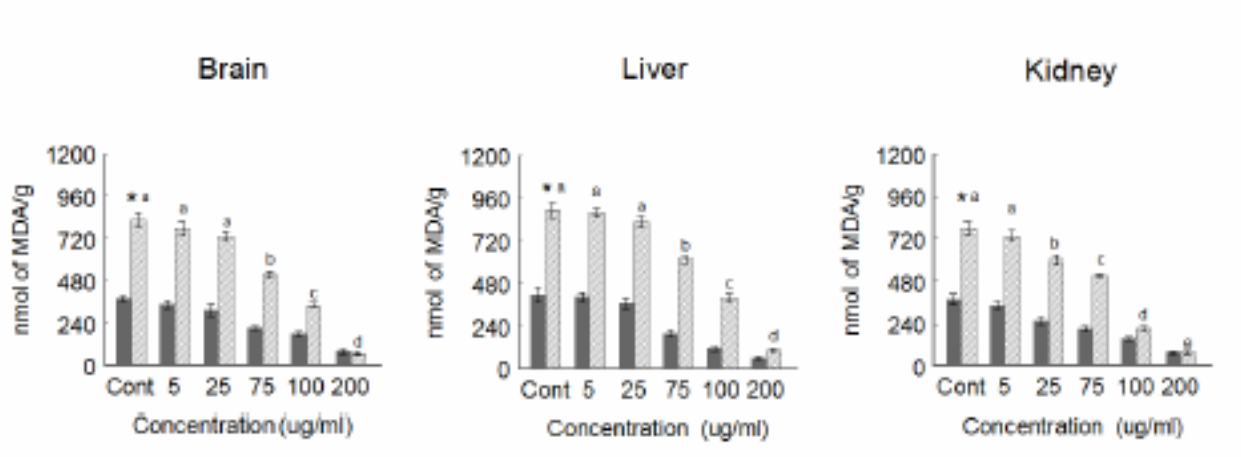

Fig. 1: Effect of ethanolic extract of *Nigella sativa* on basal (Shaded) or Fe (II)-induced TBARS (Line Bars) production in supernatants of homogenates from brain, liver and kidney of rats. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n=5–7). Asteric shows main effect Fe (II) while different letters shows main effect of extract at $p<0.05$.

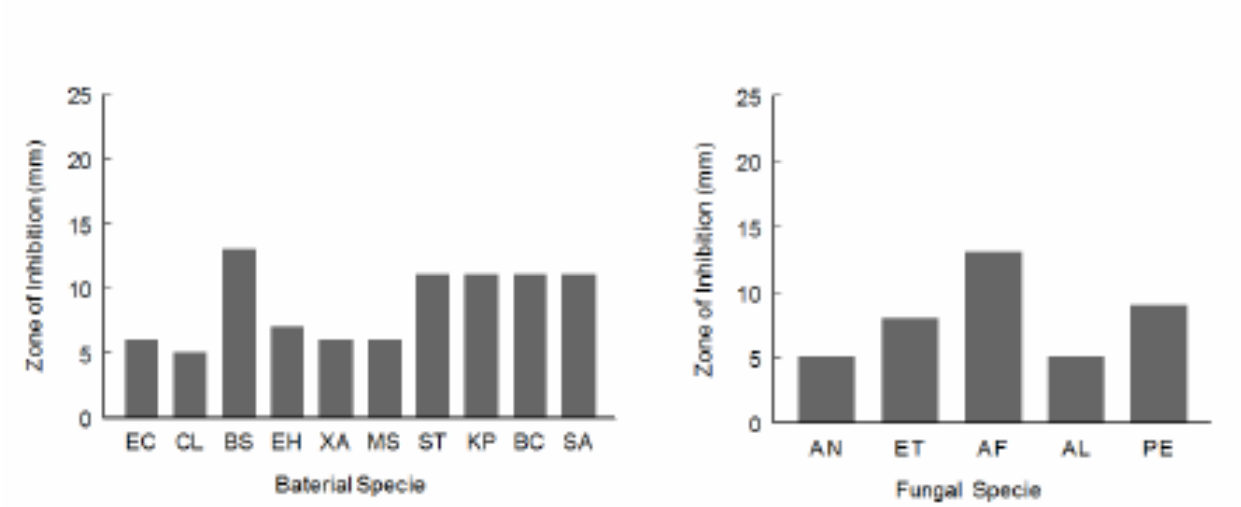

Fig. 2: Antimicrobial activities of ethanolic extract of *Nigella sativa* against different bacterial and fungal strains. Zone of inhibition is reported in mm.

harmful as they are not usually consumed in isolation but as adjuncts or additives to other foods. Hence, their low fiber contents serves as a boost to the total dietary fiber of the dishes in which they are used. We have shown from our data (table 2) that the ethanolic extract contains various secondary metabolites. These may contribute not only to the antioxidant activity but may be able to exert antimicrobial effects as well. In this context it has been reported that flavonoids have antibacterial, anti inflammatory, antioxidant and antiviral activity (Alan and Miller, 1996). Steroids are of much importance in pharmacy and have reported role is sex hormones variations (Okwu, 2001). Alkaloids play significant role for fitness of survival to plant species. Roger and Wink *et al*., (Roger and Wink, 1998) has reported that alkaloids have insecticidal properties and can work as an important component of certain medicines and recreational drugs. While, saponins are produced by plants as a deference mechanism to stop attacks by foreign pathogens (Mandal, 2005).

It is apparent from the fig.1 that Fe(II) induced TBARS formation as compared to the control; suggesting a possible damage of tissues with iron treatment. Houglum *et al*. have reported that Iron overload results in the formation of lipid peroxidation products in the liver and kidneys (Houglum *et al*., 1990). It has been reported that free iron in the cytosol and mitochondria can cause considerable oxidative damage by increasing superoxide production, which can react with Fe (III) to regenerate Fe(II) that participates in the Fenton reaction (Fraga and Oteiza, 2002).

The Fenton reaction can be written as follow;

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-1} + OH^{\bullet}$
Similarly; Fe(II) can oxidized to Fe (III) in a basal or auto oxidation process in the presence of O_2.
$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -1}$

It is apparent from above reactions that Fe(II) can generate various free radicals. The superoxide produced in above reactions may react with H_2O_2 or any lipid peroxide (LOOH) in a metal catalyzed (Haber–Weiss reaction) to produce the extremely reactive hydroxyl radical, which may then abstract hydrogen atoms from polyunsaturated fatty acids and can triggers the lipid peroxidation process. Thus it is not surprising to see that Fe(II) at a concentration of 30 µm significantly enhanced TBARS formation in all tested tissue homogenates.

Table 1: Proximate values of *Nigella sativa* in percent (%.) unless specified.

S. No.	Constituent	Quantity
1.	Crude fat	4.7±0.19
2.	Crude fiber	4.2±0.1
3.	Crude protein	20.5±0.2
4.	Moisture	3.8±0.1
5.	Ash	4.8±1.9
6.	Total sugar	-
7.	Total soluble solids	0.8±0.01
8.	Total acidity	0.72±0.01
9.	pH	6.07±1.5
10.	Vitamin C (mg/100ml)	11.7±2.0

Ethanolic extract of *Nigella sativa* significantly protected against lipid peroxidation at 25-200µg/ml in all tested homogenates. In order to explore the possible mechanism of action of the ethanolic extract of *Nigella sativa,* we measured the Fe(II) chelation ability of the extract. Fe(II) has been reported to be involved in the initiation or propagation of Fe(II) induced tissue damages (Fenton Reaction). The use of iron chelation as therapy for the management of Fe(II) associated oxidative stress would solve the problem to some extent. As shown in table 3, the extract significantly chelated Fe(II) at 25-200µg/ml concentration. There is considerable amount of literature which has proved that some iron-oxygen complexes such as the ferryl ion (Pederson *et al*., 1973), perferryl ion (Bucher *et al*., 1983) and Fe^{+2}-$O_2^{\bullet}$-Fe^{+3} complexes (Bors *et al*., 1979) are involved in the initiation of Fe(II)-dependent lipid peroxidation processes. A plausible mechanism by which the extract is conferring protective action against Fe(II)-induced lipid peroxidation in these homogenate is that extract could be interacting directly (possibly chelating) with Fe(II) or its oxidized forms. This high ability of extract to form complexes with Fe(II) may have caused a significant reduction in the available Fe(II) that will be required to catalyze the production of free radicals which is the principal cause of oxidation. This high Fe(II) chelating ability may have contributed immensely to the ability of the extract to prevent Fe(II)-induced lipid peroxidation in the rat's brain, kidney and liver homogenates.

Table 2: Qualitative analysis of the phytochemicals of medicinal seeds of *Nigella sativa*

S. No.	Phytoconstituents	*Nigella sativa*
1.	Alkaloid	+
2.	Saponins	-
3.	Carbohydrate & sugar	+
4.	Glycosides	-
5.	Phenolic compound	+
6.	Flavonoids	+
7.	Protein & amino acid	+
8.	Phytosterols	+
9.	Tannins	+
10.	Gum and mucilage	+

It is worthy to note that antioxidants present in *Nigella sativa* seeds include selenium, tocopherol, all-trans retinol, thymoquinone and thymol etc. (Badary *et al*., 2003). There are reports that thymoquinone, a main constituent of the of *Nigella sativa* protected against Fe-dependent microsomal lipid peroxidation. The safety of the compound was evident from the fact that it did not cause DNA damage (Al-Naggar *et al*., 2003).

A classical model to evaluate the interaction between different agents or extract (in this case) and *in vitro* $OH^{\bullet}$ formed via Fe(II) + H_2O_2 reaction is the deoxyribose degradation assay (Halliwell and Gutteridge, 1981; Gutteridge, 1981). The ability of the ethanolic extract to inhibit Fe(II)/H_2O_2- induced decomposition of deoxyribose as an index for hydroxyl radical ($OH^{\bullet}$) scavenging ability is presented in table 3. The results revealed that extracts had high $OH^{\bullet}$ scavenging ability at the concentration of the extract tested (25-200µg/ml). The added extract to the reaction mixture removed the hydroxyl radicals and prevented it from degradation. The results suggest that the extract is capable for scavenging free radicals and thus may be able to prevent the initiation of free radical-mediated chain reactions by stabilizing reactive species before they can participate in deleterious reactions (e.g., the abstraction of hydrogen from susceptible polyunsaturated fatty acids). The radical scavenging ability of the extract can also be proved from DPPH assay which is often used to evaluate the ability of antioxidant to scavenge free radicals. Table 3 demonstrates DPPH scavenging activity, expressed in percents, caused by different concentrations of extract. The DPPH radical scavenging activity of the extract also revealed high antioxidant activity. It is apparent from fig. 2 that *Nigella sativa* has antibacterial and antifungal obtained with (BS) and least (1.95 cm) with (MS) in case

Table 3: DPPH, Fe (II) chelating and $OH^{\bullet}$ scavenging ability (%) of ethanolic extract of *Nigella sativa*. Data are mean ± S.E.M of (n=5-7) experiments. Different letters show significant difference from control and among the groups at $p<0.05$

Concentration (µg/ml)	DPPH Radical Scavenging Ability (%)	OH● Scavenging Ability (%)	Fe (II) Chelating Ability (%)
Control	15±1	34±7	41±2
5	18±3	39±6	45±3
25	24±2	41±9	46±5
75	31±3	49±3	51±2
100	37±4	50±3	53±3
200	69±5	71±5	59±4

of bacterial strain while 13mm for (AF) and 5mm for (AN) and (Al) in case of fungal strains. Our findings are in accordance with previous reports where the methanolic extract of *Nigella sativa* seeds showed significantly better antimicrobial activity using different methods. Reports have suggested that *Nigella sativa* has antibacterial activity against 188 bacterial isolates of Gram-positive and Gram-negative belonging to 11 different genera (Masood *et al*., 2006).

CONCLUSION

Nigella sativa can serve as an important constituent of human diet supplying the body with sufficient amount of proteins, carbohydrates and energy. We have proved that the presence of biologically active metabolites can contributes to nutritional value of this plant. Moreover our in-vitro results have demonstrated that the ethanolic extract of *Nigella sativa* exhibited higher antioxidant potential. It is reasonable to conclude that the antioxidant effect of the extract could be attributed to the significantly higher Fe(II) chelation ability and radical scavenging ability (as shown in DPPH and Deoxyribose degradation assay). The study also supports and provided the anti-microbial potential of *Nigella sativa* which can be used as diet supplement in variety of free radical induced diseases. However more work is needed to identify the exact chemical compounds responsible for the observed antioxidant and antimicrobial activity.

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