Polyphenolic Profile and Antioxidant Activities of *Nigella Sativa* Seed Extracts *In Vitro* and *In Vivo*

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Abstract—*Nigella sativa* L. is an aromatic plant belonging to the family Ranunculaceae. It has been used traditionally, especially in the middle East and India, for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema. Several biological activities have been reported in *Nigella sativa* seeds, including antioxidant. In this context we tried to estimate the antioxidant activity of various extracts prepared from *Nigella sativa* seeds, methanolic extract (ME), chloroformic extract (CE), hexanic extract (HE : fixed oil), ethyl acetate extract (EAE) water extract (WE). The Folin-Ciocalteu assay showed that CE and EAE contained high level of phenolic compounds 81.31 and 72.43µg GAE/mg of extract respectively. Similarly, the CE and EAE exhibited the highest DPPH radical scavenging activity, with IC_{50} values of 106.56µg/ml and 121.62µg/ml respectively. In addition, CE and HE showed the most scavenging activity against superoxide radical generated in the PMS-NADH-NBT system with respective IC_{50} values of 361.86 µg/ml and 371.80 µg/ml, which is comparable to the activity of the standard antioxidant BHT (344.59 µg/ml). Ferrous ion chelating capacity assay showed that WE, EAE and ME are the most active with 40.57, 39.70 and 22.02 mg EDTA-E/g of extract. The inhibition of linoleic acid/ß-carotene coupled oxidation was estimated by ß-carotene bleaching assay, this showed a highest relative antioxidant activity with CE and EAE (69.82% of inhibition). The antioxidant activities of the methanolic extract and the fixed oil are confirmed by an *in vivo* assay in mice, the daily oral administration of methanolic extract (500 and 800 mg/kg/day) and fixed oil (2 and 4 ml/kg/day) during 21 days, resulted in a significant enhancement of the blood total antioxidant capacity (measured by KRL test) and the plasmatic antioxidant capacity towards DPPH radical.

Keywords—Antioxidant Capacity, Chelating, Phenolic Compounds, *Nigella Sativa*, Scavenger

I. INTRODUCTION

IT is commonly recognized that reactive oxygen species (ROS) are involved in a variety of physiological processes, including cellular signal transduction, cell proliferation, differentiation and apoptosis [1]-[2]. In healthy individuals, ROS production is continuously balanced by natural antioxidative defence systems. Oxidative stress is a process where the physiological balance between pro-oxidants and antioxidant is disrupted in favor of the former, ensuing in potential damage for the organism [3]. ROS production can induce DNA damage, protein carbonylation, and lipid peroxidation, leading to a variety of chronic health problems, such as cancer, aging, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis [4]-[5].

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Dietary antioxidant intake may be an important strategy for inhibiting or delaying the oxidation of susceptible cellular substrates, and is thus relevant to disease prevention in many paradigms. Phenolic compounds such as flavonoids, phenolic acids and tannins have received attention for their high antioxidative activity [6]. There has been an increasing interest in the use of natural antioxidants, such as tocopherols, flavonoids and plant extracts for the preservation of food materials in recent years, because these natural antioxidants avoid the toxicity problems which may arise from the use of synthetic antioxidants, such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and propyl gallate (PG) [7]-[8]. Plants, including herbs and spices, have many phytochemicals which are potential sources of natural antioxidants, *Nigella sativa L.* is a spices plant belonging to the family Ranunculaceae. It has been used traditionally, especially in the Middle East and India, for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema [9].

The objective of the present study was to evaluate the antioxidant activity of *Nigella sativa* seed extracts *in vitro* and *in vivo*.

II. MATERIAL AND METHODS

A. Materials

1. Chemicals

PMS (Phenazine-Methosulphate), ß-NADH (ß-Nicotinamide-Adenine-Dinucleotide), NBT (Nitroblue-Tetrazolium), Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonicacid)-1,2,4-triazine), $FeCl_2$, Tween40, *t*-BHP (tert-butyl hydroperoxide), Gallic acid, Quercetin, BHT (butylated hydroxytoluen), α-tocophérol, and Folin–Ciocalteau reagent were purchased from de Sigma. DPPH (1,1-diphenyl-2-picryl-hydrazyl), linoleic acid and ß-carotene were obtained from Fluka. All other chemicals and solvents used were of analytical grade

2. Animals

Swiss albino mice weighing 20–25 g were purchased from Pasteur Institute of Algiers, Algeria. The mice were divided into different groups each consists of 6-9 animals, and were allowed to acclimatize to the animal room conditions for one week and had free access to food and water *ad libitum*.

3. Plant material

Nigella sativa seeds were harvested in June 2007 from Bordj Bou Arréridj, Algeria. The plant material was identified by Dr. Houssine Laouar, University of Setif. The voucher specimen was deposited at the laboratory of botanic in the

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University of Sétif, Algeria. The fixed oil of *Nigella sativa* seed was purchased from a local herbstore.

B. Methods

1. Preparation of Nigella Sativa Seeds Extracts

100 g of seeds were ground to powder and soaked in 70% aqueous-methanol (1/10, w/v) for 24 hours with frequent agitation. After filtration and solvent evaporation in a rotary evaporator, the methanolic extract (ME) was further fractionated through solvent–solvent partitioning to obtain different fractions according to the operation flowchart given in "Fig. 1" The solvents used to achieve low to high polarity for solvent–solvent partitioning were hexane, chloroform, and ethyl acetate. After solvent evaporation, The ME and its four fractions of hexane (HE) chloroform (CE) ethyl acetate (EAE) and water (WE).Were stored in darkness at 4 C°.
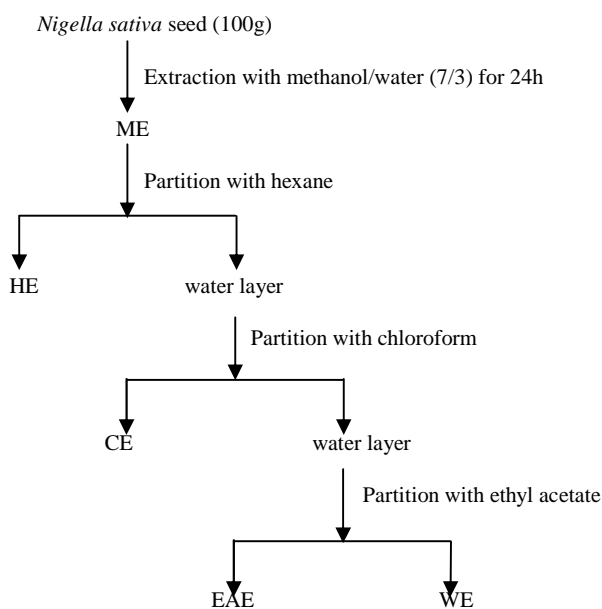
Nigella sativa seed (100g)

Extraction with methanol/water (7/3) for 24h

ME

Partition with hexane

HE   water layer

Partition with chloroform

CE   water layer

Partition with ethyl acetate

EAE   WE

Fig. 1 Preparation of *Nigella sativa* seed extracts

2. Determination of Total Phenolics

Total phenolic content was estimated by the Folin–Ciocalteu method [10]. Two hundred microlitres of diluted sample were added to 1 ml of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 µl of saturated sodium carbonate (75 g/l) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (0–160 ùg/ml) was used for the standard calibration curve. The results were expressed as Gallic acid equivalent (GAE)/g extracts.

3. Determination of Total Flavonoids

The total flavonoid content was determined according as the aluminum chloride colorimetric method described by Bahorun *et al*. [11]. Briefly, 1 ml of 2% aluminium trichloride (AlCl₃) in methanol was mixed with the same volume of extracts, Absorption readings at 430 nm were taken after 10 min against a blank sample consisting of a 1 mL extract solution with 1 ml

methanol without AlCl₃. Quercetin was used for the standard calibration curve. The data were expressed as milligram Quercetin equivalents (QE)/g extract.

4. Antioxidant Activity In Vitro
i. DPPH Free Radical Scavenging Assay

The radical scavenging assay was conducted as described by Mansouri *et al*. [12]. The DPPH solution was prepared by dissolving 2.5 mg DPPH in 100 ml of methanol. 25µl of extract or standard antioxidant (quercetin, BHT) were added to 975µL of DPPH solution. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature and the decreases in the absorbance values were measured at 517 nm. The percentage of DPPH scavenging activity was calculated using the following equation.

$$\%DPPH\ scavenging\ activity = 100(A_{control} - A_{Sample}/A_{control})$$

Where $A_{control}$ is the absorbance of the control reaction mixture without the test compounds, and A_{sample} is the absorbance of the test compounds. IC_{50} values, which represented the concentration of the extract that caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentages against concentration.

ii. Superoxide Anion Scavenging Assay

The superoxide scavenging ability of *Nigella sativa* seed extract was assessed according to the method of Ani *et al*. [13] Superoxide anions were generated in samples that contained 100 µl each of 1.0mM NBT, 3.0mMNADH and 0.3mMPMS and the final volume was adjusted to 1ml with 0.1 M phosphate buffer (pH 7.8) at ambient temperature. The reaction mixture (NBT and NADH) was incubated with or without extract at ambient temperature for 2 min. Quercetin and BHT were used as standard antioxidants. The reaction was started by adding PMS. The absorbance at 560 nm was measured against blank samples after 3 min. Decrease in absorbance in the presence of extracts indicated superoxide anion scavenging activity. The percent inhibition was calculated by using the following equation.

$$\%superoxide\ scavenging\ activity = 100(A_{control} - A_{Sample}/A_{control})$$

Where $A_{control}$ is the absorbance of the control reaction mixture without the test compounds, and A_{sample} is the absorbance of the test compounds. IC_{50} values, which represented the concentration of the extract that caused 50% neutralization of superoxide radicals, were calculated from the plot of inhibition percentages against concentration.

iii. Ferrous Ion Chelating Activity

Ferrous ion chelating activity was measured by inhibition of the formation of iron (II)–ferrozine complex after treatment of test material with Fe^{2+} following the method of Le *et al* [14]. The reaction mixture contained 500 µl of *Nigella sativa* seed extracts or the standard chelator EDTA, 100 µl of FeCl2 (0.6

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mM in water) and 900 of µl methanol. The control contained all the reaction reagents except the extracts or EDTA. The mixture was shaken well and allowed to react at room temperature for 5 min. One hundred microliters of ferrozine (5 mM in methanol) was then added, the mixture shaken again, followed by further reaction at room temperature for 10 min to complex the residual Fe^{2+} ion. The absorbance of the Fe^{2+-} ferrozine complex was measured at 562 nm against a methanol blank. The chelating effect was calculated as a percentage, using the equation below.

$$\%chelating\ activity = 100(A_{control} - A_{sample}) / A_{control}$$

Where $A_{control}$ is the absorbance of the control reaction mixture without the test compounds, and A_{sample} is the absorbance of the test compounds. IC_{50} values, which represented the concentration of the extract that caused 50% of Fe^{2+} ion chelation, were calculated from the plot of chelating percentage against concentration.

iv. β-Carotene Bleaching Assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation according to the method of Kartal et al.[15]. A stock solution of β -carotene/linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform, and 25µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml distilled water, saturated with oxygen (30 min 100 ml/min), were added with vigorous shaking; 2.5 ml of this reaction mixture were dispersed into test tubes and 350 µl portions of the extracts prepared at 2 mg /m1 concentrations, were added ,Then emulsion was incubated for 48 h at room temperature and the absorbance (490 nm) was recorded at different time intervals .The same procedure was repeated with the synthetic antioxidant, BHT, α-tocopherol, and a blank (containing only methanol). The relative antioxidant capacity (RAC) was calculated as follow.

$$RAC = 100\ (A_{t=48h\ sample} / A_{t=48h\ BHT})$$

Where $A_{t=48h\ sample}$ is the absorbance of the test compounds after 48h and $A_{t=48h\ BHT}$ is the absorbance of BHT after 48 h.

5. Antioxidant Activity In Vivo
i. Animals Treatment and Blood Collection

After an adaptation period of 6 days, the mice were randomly divided into six groups of 6-animals.Three groups (ME1, ME2, ME3) were given orally 300, 500 et 800 mg/kg of Methanolic extract (ME). Two other groups (FO1, FO2) were given 2 and 4 ml/kg of Nigella sativa fixed oil (FO). The control group received the same volume of normal saline solution.

After twenty one days of daily treatment, blood (0.5-1 ml) was collected by cardiac puncture after diethyl-ether anesthesia. A subsample of whole blood 100 µl was immediately diluted in 2.4 mL of PBS (NaCl 125 mM, sodium phosphate 10 mM, pH 7.4). The remaining quantity of the blood was centrifuged (1500g/5min/4C°). The obtained plasma was used to assess plasma antioxidant capacity.

ii. Blood Total Antioxidant Capacity

The global potential of antioxidizing defense was measured by KRL test (kit of dosage of the Free Radicals) according to the protocol described by Girard et al.[16]. With some modifications by replacing the radical AAPH [2,2-'-azo-bis azo-bis (2-amidinopropane) HCl] by the tert-butyle hydroperoxide (t-BHP) [17]. The principle of the test is to submit whole blood to a thermo-controlled free radical Aggression. All families of antioxidant present in the blood are mobilized to fight off the oxidant attack and to protect the integrity of erythrocytes resulting in the delay of hemolysis.

Briefly, Aliquots of 80 µL of diluted blood was deposited in 96-well microplate with 136 µL of t-BHP (166µM in solution) and then incubated at 37°C. The kinetic of hemolysis was followed using a 96-well microplate reader by measuring optical density decrease at 630 nm. The blood resistance to free radical attack is expressed by the time needed to hemolyze 50% of the red blood cells (half-Hemolysis Time, HT_{50} in min)

iii. Plasma Antioxidant Capacity

The capacity of the plasma to trap the DPPH radical was estimated according to the method of Hasani et al [18] with some modifications. Briefly, 50 µL of plasma were added to 950 µl of the DPPH methanolic solution (2.4 mg /100 ml of methanol). After 30 min of incubation in the darkness followed by a centrifugation, the absorbance of supernatant is measured at 517. The plasmatic antioxidant power was then calculated (see the part in vitro).

C. Statistical Analysis

Data obtained in vitro and in vivo are expressed as mean ± SD and mean ± SEM respectively. The sigmoid hemolysis curves were fitted by computer analysis Software (Graph Pad. Prism.V5.00). Differences between the control and the treatments in these experiments were tested for significance using analysis of variance followed by Dunnet's/tukey's test. A probability of $P < 0.05$ was considered significant.

III. RESULTS AND DISCUSSION

A. Preparation of Nigella Sativa Seed Extracts

Due to the complicated constituents and pharmacological diversities of plants, in vitro bioassay-guided fractionation has been effectively applied to screen the biological activities that contribute important indications for investigating the characteristics of active components [19]-[20]. As shown in "Fig. 1", the methanolic extract (ME) of Nigella sativa was fractionated through solvent–solvent partitioning to obtain four fractions of hexane (HE), chloroform (CF), ethyl acetate (EAE), and hexane (HE). The recoveries of ME, HE, CE,

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EAE, and WE were about 21,5 %,3,05 %, 0.41%, 0.62 %and 14,08% respectively, indicating that the constituents of *Nigella sativa* seed belong mainly to the two opposing extremes of polarity.

B. Total Phenolics and Flavonoids Content

A number of studies have focused on the biological activities of phenolic compounds and flavonoides , which are potential antioxidants and free radical-scavengers [21].the obtained results "Table I" show that CE in *Nigella sativa* samples contained the highest amount of total phenolic compounds and flavonoids (81.31 µg GAE/mg of extract, 5.20 QE/mg of extract) followed by EAE (72.43 µg GAE/mg of extract, 4.19 µg QE/mg of extract), ME (33.64µg GAE/mg, 3.80 µg QE/mg of extract) and WE (27.07µg GAE/mg of extract, 2.45 µg QE/mg of extract) while the HE contains only (15,95 µg GAE /mg of extract, 0.43µg QE/mg of extract).

TABLE I
PHENOLICS AND FLAVONOIDS CONTENT OF *NIGELLA SATIVA* SEED EXTRACTS

Extract	Total Phenolics (µ g GAE/mg of extract)	Flavonoids (µg QE/mg of extract)
ME	33.64 ± 0.34	3.80 ± 0.07
HE	15.95 ± 0.58	0.43 ± 0.06
CE	81.31 ± 1.97	5.20 ± 0.02
EAE	72.43 ± 2.94	4.19 ± 0.09
WE	27.07± 0.58	2.45 ± 0,06

The results are given as means ± SD of three measurements

C. DPPH Radical Scavenging Activity

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a stable radical with a maximum absorbance at 517 nm that can readily undergo reduction by an antioxidant. Because of the ease and convenience of this reaction, it has now widespread use in the free radical-scavenging activity assessment [22]. IC_{50} values for DPPH scavenging activities of *Nigella sativa* extracts and standards antioxidant are given in "Table II".

The CE and EAE show the best radical scavenging activity with IC_{50} values 106.56 and 121.62 µg /ml respectively, followed by ME (IC_{50}=237.79 µg/ml) and HF (IC_{50}=394.53 µg/ml). The WE show the lowest radical scavenging activity (IC_{50}=447.76 µg/ml). Compared with quercetin and BHT the CE and EAE were less active.The scavenging effect of CE and EAE is most likely due to their high phenolic and flavonoid content.The activity of HE which represents the poorest extract in phenolics and flavonoids seems to be ascribed to essential oils, whose radical scavenging activity has been widely studied. Carvacrol, t-anthenol, 4-terpineol and thymoquinone, are powerful scavengers of DPPH,hydroxyl radical [9].

TABLE II
DPPH-SCAVENGING ACTIVITIES OF *NIGELLA SATIVA* SEED EXTRACTS, QUERCETIN AND BHT

	IC_{50} (µg /ml)
BHT	43.48±0.58
Quercetin	1.48 ± 0.03
ME	237.79 ± 3.15
HE	394.53 ± 4.78
CE	106.56 ± 1.28
EAE	121.62 ± 2.60
WE	447.76 ± 1.48

The results are given as means ± SD of three measurements

The obtained results indicate the existence of significant linear correlation between IC_{50} values of radical-scavenging activity and total phenolic ($r^2 = 0.98$, $p < 0.01$) and flavonoids ($r^2 = 0.70$, $p < 0.01$) contents of all extracts. Indeed, phenolic compounds, especially flavonoids are recognized as potentially antioxidant substances with the ability to scavenge free radical species and reactive forms of oxygen, the scavenging effect of flavonoids (FlOH) is attributed to their low potential redox making them thermodynamically able of reducing free radicals ($R^•$) by a transfer of hydrogen from hydroxyl groups. This reaction gives rise to aroxyle radical ($FLO^•$) and the stabilized radical (RH), the ($FLO^•$) subsequently undergo a structural rearrangement allowing the redistribution of the unpaired electron on the aromatic ring and the stabilization of aroxyl radicals [23].

D. Superoxide Anion Scavenging Activity.

In the PMSNADH- NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.

The obtained results "Table III" show that HE and CE are the most active with IC50 values 361.86 µg/ml and 371.80µg/ml respectively. The scavenging effect of these two extracts (HE and CE) was statistically similar (p <0.001) to that of standard antioxidant BHT (IC_{50} = 344.59 µg/ml) which is 10 times less active than quercetin (IC_{50} = 33.69). Unlike CE and HE, the scavenging effect of EAE, ME, WE is very low with IC_{50} values 5.58 mg/ml, 6.88 mg/ml and 9.24 mg/ml respectively.

TABLE III
SUPEROXYDE ANION SCAVENGING ACTIVITIES OF *NIGELLA SATIVA* SEED EXTRACTS, QUERCETIN AND BHT

	IC_{50}
BHT	344.59 ± 35.3 µg/ml
Quercetin	33.69 ± 0.35 µg/ml
ME	6.88 ± 0.068 mg/ml
HE	371.80 ± 7.10 µg/ml
CE	361.86 ± 22.42 µg/ml
EAE	5.58 ± 0.28 mg/ml
WE	9.24 ± 0.067 mg/ml

The results are given as means ± SD of three measurements.

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The effect of CE appears to be related to its richness in polyphenols and flavonoids. However, this is not the case for the HE, the poorest in polyphenols and flavonoids. Its activity is probably attributed to the presence of other substances more active than those found in other extracts. These substances are probably essential oils which represent between 0.18 to 0.50% in seed weight and between 1.4-1.9% by weight of the fixed oil [24]. The scavenging effect of reactive oxygen species by pure compounds contained in the essential oil of *Nigella sativa* seeds has been widely studied. Thymoquinone, one of the major compounds of this oil, inhibits the generation of $O2 \bullet^-$ radical by xanthine / xanthine oxidase ($IC_{50} = 3.35$ μM) without having any effect on the activity of the enzyme [25].

Except the HE, all extracts tested in this study show a significant linear correlation between their superoxide anion scavenging effect and their phenolics ($r^2 = 0.76$, p< 0.01) and flavonoids ($r^2 = 0.91$, p< 0.01) contents.

E. Chelating Activity

It has been well recognized that transition metal ions such as those of iron and copper are important catalysts for the generation of the first few free radicals to initiate the radical chain reaction or the radical mediates lipid peroxidation [26]. Chelating agents may inhibit radical generations by stabilizing transition metals, consequently reducing free radical damage. To better estimate the potential antioxidant activities of the *Nigella sativa* seed extracts, chelating activity of each extract was evaluated against Fe^{2+}.

The results are shown in "Table IV". The IC_{50} values of the different extracts showed a wide variation ranging from 0.169 mg / ml to 13.137 mg/ml. The WE and EAE appear to have the most important chelating activity with IC_{50} values of 0.169 mg / ml and 0.173 mg/ml respectively (40.578 and 39.704 and mg EDTA-E/g extract), followed by the ME (IC_{50}= 0.316 mg / ml).compared with quercetin, these three extracts are from 1.75 to 3.27 times more active. This is probably explained by a synergistic effect between the components of these extracts. The chelating effect of CE and HE is very low (IC 50 = 4.10 and 13.137 mg / ml), 24 to 77 times less active than the WE and 7 to 24 times less active than quercetin.

The chelating activity of methanolic extract of the stems and roots of *Nigella sativa* was investigated by Bourgou *et al*[27], this study reveals a very low activity , the obtained IC_{50} values were7.5 mg/ml and 3.8 mg/ml for roots and stems respectively. Phenolic compounds have been reported to be chelators of free metal ions [28] [29]. But in this study a low and no significant correlation ($r^2 = 0.15$, p $\square$ 0.05) was observed between the chelating extracts of *Nigella sativa* seeds and their contents in phenolic compounds. Similar results have been reported in other works, for example in a study on extracts of fourteen varieties of barley, Zhao *et al* [26] have found a very weak correlation between the chelating activity of these extracts and their contents in phenolic compounds. This might indicate that phenolic compounds might not be the main chelators of iron. In a complex mixture, organic acids, amino acids and sugars can be sequesters of transition metal ions. [30]. In addition, the ability for phenolic compounds to chelate metal ions depends on the availability of properly oriented functional groups [31]. Therefore, a sample high in polyphenols might not chelate metal if the polyphenols present did not have suitable groups that could chelate the cation. Also, when a phenolic group is conjugated with a carbohydrate group, as in naturally-occurring phenolic glycosides, it can no longer bind metals [30].

F. β -Carotene Bleaching Assay

In this assay, oxidation of linoleic acid produces hydroperoxide-derived free radicals which attack the chromophore of b-carotene, resulting in a bleaching of the reaction emulsion. An extract capable of retarding/inhibiting the oxidation of α-carotene may be described as a free radical scavenger and primary antioxidant [32]

As can be seen in "Fig. 2" all the extracts were capable of inhibiting the bleaching of b-carotene by scavenging linoleate-derived free radicals.

TABLE IV
CHELATING ACTIVITIES OF EDTA, QUERCETIN AND *NIGELLA SATIVA* SEED EXTRACTS

	IC_{50} (mg/ml)	ChelatingActivity (mgEDTA-E/g extract)
EDTA	6.87 ± 0.131^{a}	–
Quercetin	0.55 ± 0.024	12.402 ± 0.535
ME	0.316 ± 0.003	22.021 ± 0.227
HE	13.137 ± 0.376	0.523 ± 0.007
CE	4.103 ± 0.104	1.675 ± 0.042
EAE	0.173 ± 0.011	39.704 ± 2.637
WE	0.169 ± 0.000	40.578 ± 0.114

[a]μg/ml
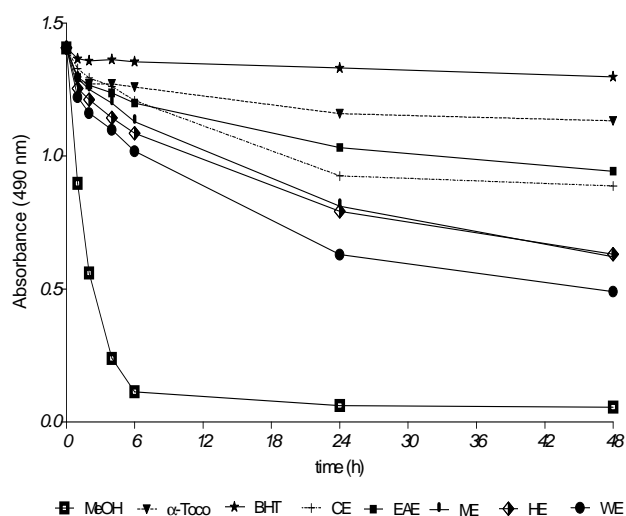The results are given as means ± SD of three measurements

Fig. 2 Kinetics of β-carotene bleaching at 490 nm in the absence and presence of *Nigella sativa* extracts, BHT, α-tocopherol (Each value is the average of three tests)
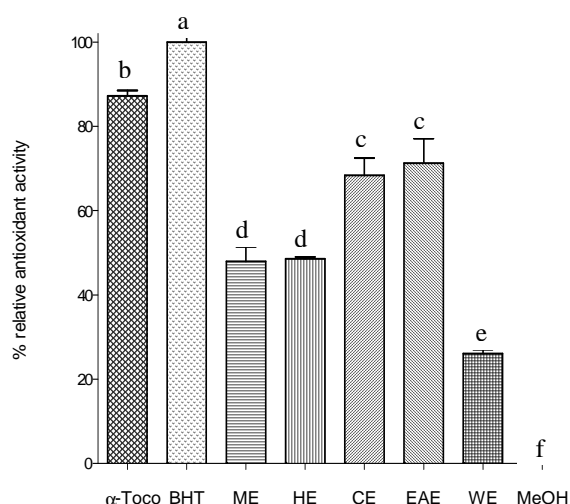
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Fig. 3 Relative antioxidant activities of *Nigella sativa* seeds extracts, BHT and α-tocopherol in β-carotène/ linoleic acid system (values are the average of three measurements ± SD), bars with different letters indicate activities significantly different ($p \leq 0.01$).

The relative antioxidant activities are shown in "Fig. 3" The order of decreasing efficacy was EAE (71.29%) and CE (68.34%) (Statistically indistinguishable) > HF (48.58%) and ME (47.91%) (Statistically indistinguishable) > WE (26.02%). Compared to standards antioxidants, the CE and EAE are 1.2 times less active than α-tocopherol and 1.4 times less active than BHT.

The high activity of CE and EAE is most likely attributed to their phenolic and flavonoid content, the low activity of WE is probably explained by its high polarity. Indeed, Frankel and Meyer [33] have suggested that the polarity of an extract is important in water:oil emulsions, viz., apolar extract exhibit most important antioxidant properties as they are concentrated within lipid-water interface, thus helping to prevent radical formation and lipid oxidation of β-carotene. While polar extract are diluted in the aqueous phase and are thus less effective in protecting lipids.

G.Blood Total Antioxidant Capacity

It is largely accepted that diet enriched with fruits, vegetables and vegetal oils increase the antioxidant potential of the body resulting in many beneficial effects on health [34]. In this context, the effect of treatment with the ME and the FO of *Nigella sativa* seeds, on the total antioxidant status of mice was evaluated using the KRL assay which is based on the whole blood resistance to free radical aggression, taking into account all the body reserves since blood contains every kind of the molecular and enzymatic antioxidant equipment [35]. These antioxidants are mobilized during an ROS attack induced *ex vivo* by t-BHP, to protect erythrocytes integrity and then delay their hemolysis.

From the obtained sigmoid hemolysis curves "Fig. 4", it appears that treatment with the ME and the FO causes a delay of hemolysis which is evident in a shift of the curves to the right. The HT_{50} values "Fig.5" reveal an extension of half hemolysis time in all treated groups compared with controls (CTL). For the group ME1 treated with ME (300 mg / kg), the increase in HT_{50} (HT_{50}=117.3±3.23 min) is statistically no significant compared with the control group (HT_{50}=106.77±2.36min). However, administering a higher doses of ME for the group ME2 (500 mg / kg), ME3 (800mg / kg) caused a significant delay of hemolysis (HT_{50} = 125.17 ± 7.15 min, $p \leq 0.05$), (HT_{50}= 136.01 ± 3.11, min, $p \leq 0.001$). These results indicate that the ME increases the total antioxidant capacity in dose dependent manner.

Similarly, treatment of groups FO1 and FO2 by two doses (2ml/Kg and 4ml/kg) of FO has also caused a significant ($p \leq 0.01$) delay of hemolysis with HT_{50} values of 132.15 ± 2.96 min and 133.6 ± 4.46 min respectively. Similar results were obtained by Kökdil *et al.* [36]-[37]which showed that oral administration of the fixed oil of *Nigella unguicularis, Nigella orientalis* and *Nigella segetalis* seeds at a dose of 1ml/kg in rats during four weeks, resulting in improved blood parameters and a significant increase in total antioxidant status without affecting the level of serum MDA. In the same context, *in vitro* studies have shown that extracts of *Nigella sativa* seeds protect erythrocytes against lipid peroxidation, degradation, loss of deformability and increased osmotic fragility caused by the hydrogen peroxide H_2O_2[38].
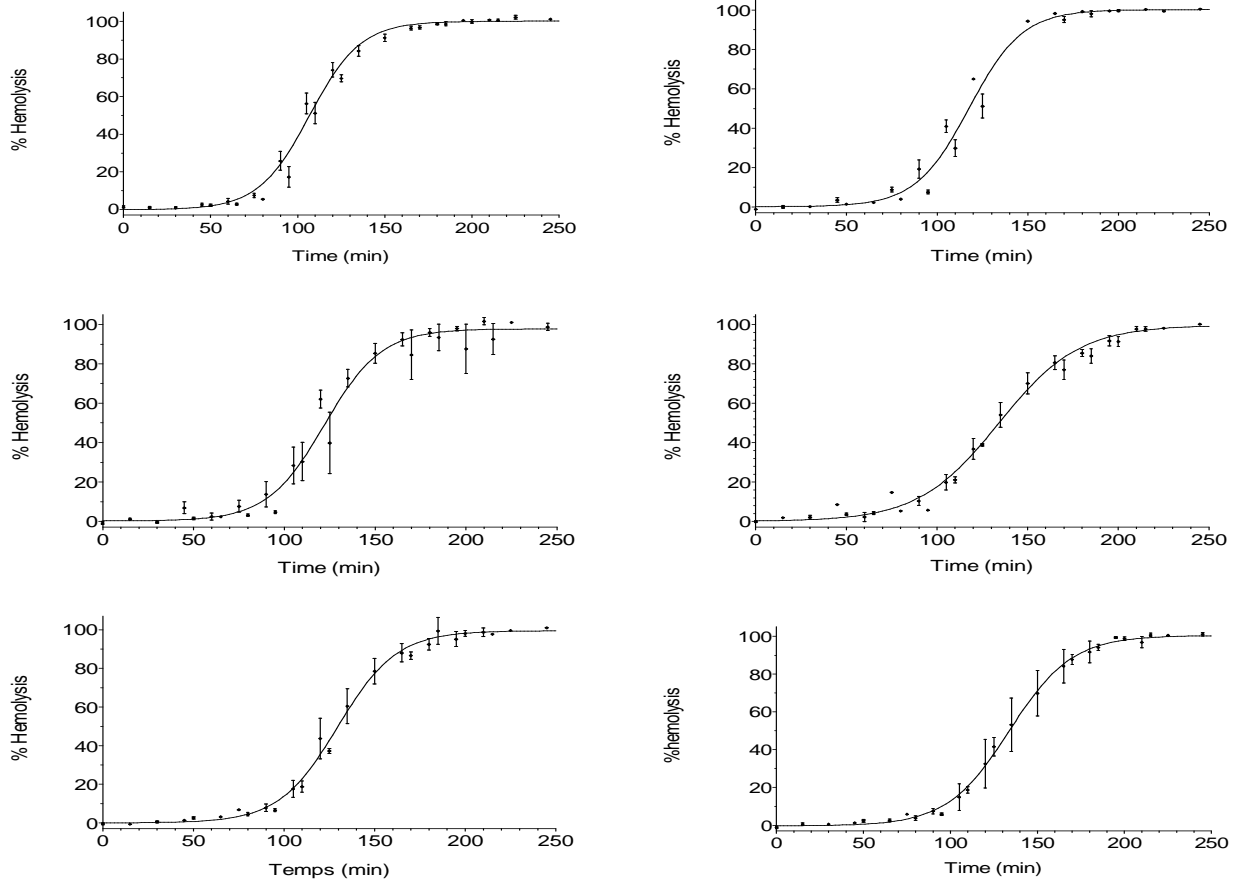
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Fig. 4 Kinetics of hemolysis in the presence of t-BHP for different studied groups. The values are means ± SEM (n = 6-7).
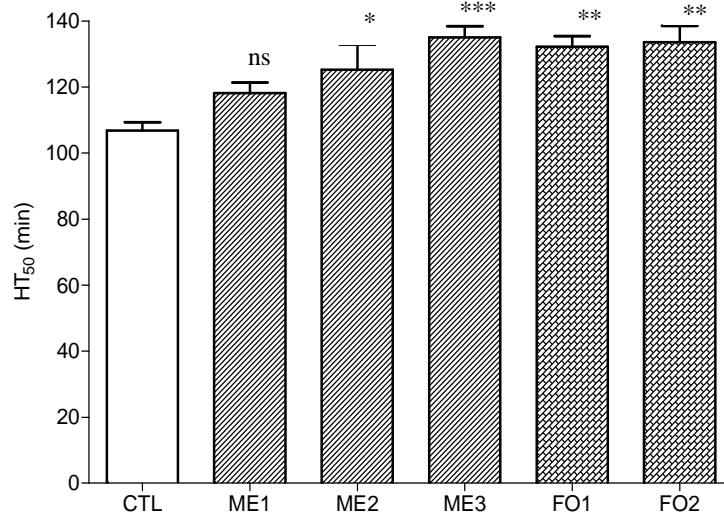

Fig. 5 Half-Hemolysis Time (HT50) for different studied groups.The values are means ± SEM (n = 6-7). Comparisons are made relative to
The control group, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ns: not significant

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H.Plasma Antioxidant Capacity

Plasma antioxidant capacity (PAC) is one of the most commonly used biomarkers to assess the effectiveness of dietary supplementation or antioxidant treatment. Due to the large number of antioxidants present in plasma, several methods have been developed: the ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), total radical absorption potential (TRAP) and the DPPH radical scavenging activity [39]-[40]. In our study, the obtained results "Fig.6" show that oral administration of ME (300 mg / ml) for the group ME1 leads to increased plasma antioxidant capacity ($41.63\pm4.36\%$ for EM1 group *versus* $26.34\pm6.14\%$ for the control group). this increase is statistically not significant compared with the control group, however the administration of two higher doses 500 mg/kg and 800 mg/kg for the groups ME2 and ME3 respectively has significantly increased ($p \leq 0.01$) the antioxidant capacity ($49.62\pm2.26\%$ and $48.18\pm3.73\%$). Similarly, the administration of FO (2 ml / kg and 4 ml / kg) for the groups FO1 and FO2 has significantly increased the plasma antioxidant capacity ($44.32 \pm 4.98\%$. $P \leq 0.05$) ($49.44 \pm2.45\%,p\leq0.01$).

In fact, the plasma contains a network of endogenous antioxidants such as (albumin, bilirubin, reduced glutathione and uric acid) as well as exogenous antioxidants derived from food. These antioxidants may act in a complementary and synergistic manner to provide a better protection against ROS. The increase in plasma antioxidant capacity is probably attributed to the elevated levels of exogenous antioxidants acquired following treatment with *Nigella sativa* seeds extracts (fixed oil, methanolic extract).
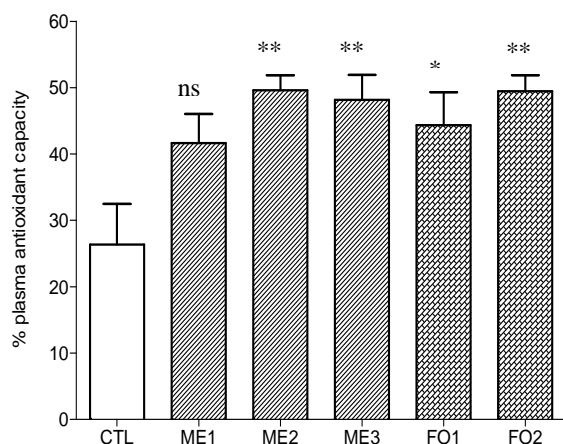
Fig. 6 Plasma antioxidant capacity toward DPPH radical for different groups. Values are means $\pm$ SEM (n = 6-7). Comparisons are made relative to the control group, *: $p \leq 0.05$, **: $p \leq 0.01$, ns: not significant

The study of the relation between total antioxidant capacity of blood (HT50) and plasma antioxidant capacity (%) shows a significant positive linear correlation ($r^2 = 0.48$, $p \square 0.01$) "Fig.7", this suggests that the blood total antioxidant capacity is partially attributed to antioxidant and free radical scavengers present in the plasma.
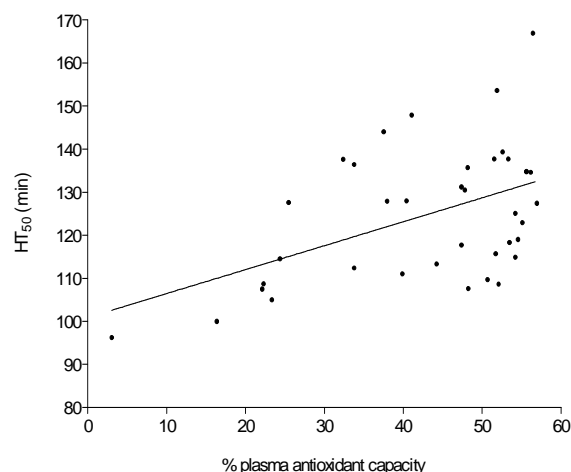
Fig. 7 Relationship between plasma antioxidant capacity and blood total antioxidant capacity (HT50) in mice of different groups (N = 37)

IV. CONCLUSION

From the obtained data in the present study we can say that *Nigella sativa* seeds have a considerable antioxidant activity *in vitro* and *in vivo*. This activity is mainly concentrated in the extracts CE, EAE and HE (fixed oil). The activity of CE and EAE is probably related to their high phenolic content. However, the activity of HE could be attributed to essential oils. Consequently, further work should be made to isolate the active constituents in each extract and to clarify theirs mode of action.

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