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Antioxidant activity of Algerian *Nigella sativa*total oil and its unsaponifiable fraction

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ABSTRACT

Background/Aim: *Nigella sativa*(NS) oil is usually considered as oxidatively stable, the aim of this study is to evaluate its antioxidant capacity in vitro and that of its unsaponifiable fraction by several mechanisms. **Methods:** NS seed total oil (TO) was extracted with n-hexane and this oil was further the matrix to extract the unsaponifiable fraction (UF). TO and its UF were investigated for the antioxidant activity using the following spectrophotometric assays:ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid), DPPH (2, 2'-diphenyl-1-picrylhydrazyl)and H_2O_2 (Hydrogen peroxide) radicals scavenging tests and lipid peroxidation inhibition capacity assay. All tests were realized by spectrophotometric methods. **Results:** Quantitative determination of phenols and flavonoids in TO and UF showed that UF has the highest phenolic and flavonoid concentrations with 24,73±0,51 µg GAE/g extract and 8,31±0,40 µg QAE/g extract respectively. All antioxidant assays showed that UF has the strongest antioxidant capacity with; IC_{50} 0,455 ±0,01 mg/ml in ABTS scavenging assay, IC_{50} 113,24 ±0,425 mg/ml in DPPH scavenging assay and IC_{50} of 0,324±0,003 mg/ml in H_2O_2 scavenging assay, equally, UF has the strongest Lipid peroxidation inhibition capacity With a percentage inhibition of 57.5%. **Conclusion:** The results obtained in this investigation showed that the UF has an important antioxidant capacity, which make NS an attractive source of new therapeutic components.

Keywords: Antioxidant capacity, Flavonoid content, Lipid peroxidation inhibition capacity assay, *Nigella sativa*, Phenolic content.

INTRODUCTION

It is generally accepted that free radicals (also called pro-oxidants) and reactive oxygen species (ROS) play an important role in the development of tissue damage and pathological changes. An antioxidant is defined as a compound capable of preventing the pro-oxidation process, or biological oxidative damage. The organism must confront and control the presence of both pro-oxidants and antioxidants continuously [1]. Changing the balance toward an increase in the pro-oxidants over the capacity of antioxidants is defined as oxidative stress which is the origin of most pathologies [2]. There is increasing evidence that oxidative stress leads to many biochemical changes and is an important contributing factor in several human chronic diseases, such as atherosclerosis and cardiovascular diseases, cancer, several neurodegenerative disorders, diabetes and likely the aging process.

Medicinal plants constitute an inexhaustible source of interesting bioactive compounds, that's why there in an increased interest in their use as therapeutic agents [3, 4]; in this respect, *Nigella sativa*is one of the most studied plants, it has a large therapeutic spectrum and has long been used as a natural remedy for a number of human illnesses and disorders [5, 6].

The oil of *Nigella sativa*is usually considered as oxidatively stable, it prevents the deterioration through the reducing of radical reactions responsible for lipid oxidation, also, the presence of tocopherol, carotenoid, phospholipids and phenolics improves its stability and its antioxidant properties [7, 8].

The unsaponifiable fraction contains carbohydrates, phospholipids, liposoluble vitamins, pigments, tocopherols, phytosterols and polyphénols [9] which are the most important natural antioxidants in the oil. The consumption of this oil in our food may protect us against oxidative damages. These natural antioxidants may function (a) as reducing agents, (b) as free radical scavengers, (c) as complexes of pro-oxidants metals, and (d) as inhibitors of the formation of singlet oxygen [10].

MATERIALS AND METHODS

Plant material

*Nigella sativa*seeds come from the same batch of seeds procured from an herbalist. They are local, grown in the highlands of Northeastern Algeria (Batna and Biskra). Their identification was confirmed in the laboratory.

Seeds were washed, dried and powdered with an electric micronizer.

Total oil extraction

Seed powder was extracted in a Soxhlet-extractor with methanol solvent to get the methanol extract, to which we added n-hexane for obtaining the total oil. During the extraction, the extracted lipids required the addition of 0, 75 % aqueous sodium chloride solution. The solvent was removed on a rotary evaporator at 40°C. Total oil was collected in a flask and stored at 4°C for further analysis [11].

Unsaponifiable fraction extraction

UF was isolated following conventional procedures, as described IUPAC 2401.

In a 250 ml flask fitted with a condenser, we introduced 5 g of the total oil of *Nigella sativa* seeds, which we added 50 ml of alcoholic potassium hydroxide solution 2 M. The mixture is heated in a water bath for 1 hour and circular stirring. After cooling to a temperature below 25 ° C the content was transferred into a separatory funnel and 100 ml of water was added.

The mixture was washed (3 x 100 ml) with petroleum ether 40-60 ° C. Ethereal liquids are then combined in another separatory funnel containing 40 ml of water. After separating the two phases, the ethereal phase is recovered and washed initially with 40 ml of a potassium hydroxide solution at 30 g / l then with 40 ml of water. This washing is repeated several times until the aqueous phase is not alkaline anymore. The ethereal phase is transferred to another flask. After removal of the ether on a rotary evaporator at 30°C, the residue is dried at 100-105 °C to constant mass [12]. The yield in all samples was 2,5%.

Total phenolics and flavonoid content

Total phenolics content was determined using the Folin-Ciocalteu product, which based on spectrophotometric method [13].The reaction mixture was prepared by mixing 0,5 ml of extract solution, 2,5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2,5 ml of $NaHCO_3$ (7,5%) solution then, samples were incubated at 45°C for 45min.

The absorbance was determined using spectrophotometer at 765 nm. Samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. Phenolic content was measured using garlic acid equivalent per gram of dried extract (µg GAE/g extract) by means of the garlic acid calibration curve.

Total flavonoid content has been measured by a spectrophotometric method using aluminum chloride as chromophore reagent [14].The samples contained 1 ml of extract solution and 1 ml of 2% of $AlCl_3$ solution dissolved in methanol and then, they were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at 415 nm. All samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. Flavonoid content was measured using quercetin equivalent per gram of dried extract (µg QAE/g extract).

ABTS radical scavenging assay

The ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity is based on the estimation of ABTS radical cation formation, the color of this ABTS radical compound is suppressed in the presence of antioxidant molecules, which have the ability of scavenging this radical.

$ABTS^{*}+$ is generated by mixing 7mM ofABTS solution with 2,45 mM of potassium sulfate then the mixture is stored at room temperature for 16h. The solution is diluted to get an absorbance of 0,7±0,05 at 734 nm. To assess the antiradical activity of TO and UF, we added 2 ml of ABTS solution to 0,3 ml of test samples with different concentrations, measurements were taking after 30 min at 734 nm [15].

The antioxidant activity was estimated by calculating the percentage of the decrease in absorbance of different samples concentrations, using the following equation:

$$\text{Inhibition (\%)} = [(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}})/\text{Abs}_{\text{Control}}] \text{ X } 100$$

Where; A Control is the absorbance of the control reaction and A Sample is the absorbance of the sample. IC_{50} value indicated the concentration of sample required to scavenge 50% of ABTS radicals, low IC_{50} is equivalent of high scavenging capacity, and it's calculated by plotting percentage inhibition against different concentrations of oil [16].

DPPH radical scavenging activity

The second antioxidant activity is the DPPH (2, 2'-diphenyl-1-picrylhydrazyl) radical scavenging activity, that is based on the reduction of a methanol solution of $DPPH^{*}$ by an antioxidant molecule which play a role of a hydrogen donating and lead to the formation of the non-radical form DPPH [17]. The sample solutions were prepared by dissolving TO and UF in DMSO (Dimethylsulfoxide) solvent with different concentrations. The reaction mixture prepared by blending samples with DPPH solution, then we kept the mixture in the dark for 30 min before measuring the absorbance at 715 nm. The antioxidant capacity was estimated by changing in the reaction color from purple to yellow and it's calculated using the following formula:

$$\text{Inhibition (\%)} = [(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}})/\text{Abs}_{\text{Control}}] \text{ X } 100$$

Where; A Control is the absorbance of the control reaction and A Sample is the absorbance of the sample at 517 nm. IC_{50} value indicated the concentration of sample required to scavenge 50% of DPPH radicals.

H_2O_2 radical scavenging assay

The ability of our samples to trap hydrogen peroxide was determined according to the method developed by [18]. This method is based on the absorption of H_2O_2 in UV.

The principle of the reaction is to neutralize hydrogen peroxide (H_2O_2) by an antioxidant which will facilitate its decomposition into molecules of water.

An H_20_2 solution (40 mM) was prepared in phosphate buffer (0.02M) (pH = 7.4), 1.2 ml of this solution are added to 2 ml of standard or extract already prepared in methanol, After incubation for 10 min at room temperature, the absorbance was measured at 230 nm.

The percentage of the scavenger activity of the H 2 O 2 radical is calculated according to the following formula:

$$\text{Inhibition (\%)} = [(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}})/\text{Abs}_{\text{Control}}] \text{ X } 100$$

Where; A Control is the absorbance of the control reaction and A Sample is the absorbance of the sample.

Lipid peroxidation inhibition capacity assay

This activity is determined according to the method of [19].

In a tube, 1 ml of pure ethanol is added to 10 mg of the extract, and 25.1 mg of linoleic acid are also added. Afterwards, 2 ml of phosphate buffer (0.05 M, pH = 7) are added to the above mixture and 1 ml of distilled water is then added, the solution thus prepared is incubated at 40 ° C. in the dark.

After 24 hours:

A volume of 25 µl of this solution is introduced into another test tube and 2.4 ml of 75% ethanol are added. 25 µl of ammonium thiocyanate are added thereafter. The tubes are set in the dark for 3 minutes.

25 µl of $FeCl_2$ / HCl is added and the absorbance is read at the spectrophotometer at 500 nm. The percentage inhibition of lipid peroxidation is calculated according to the following equation:

$$\text{Inhibition (\%)} = [(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}})/\text{Abs}_{\text{Control}}] \text{ X } 100$$

Where; A Control is the absorbance of the control reaction and A Sample is the absorbance of the sample.

Statistical analysis

The results were expressed as mean ±SD. The data were subjected to one-way analysis of variance (ANOVA), where the difference between groups and standards were determined by Tukey's test, using Graph Pad program, p value ≤ 0,05 was regarded as significant.

RESULTS

Determination of phenolics and flavonoids contents

Polyphenols are the most important compounds that are responsible for the antioxidant activity. To assess their levels in *Nigella sativa* TO and UF we used garlic acid as a standard compound. Result expressed as µg of GAE/g of extract, using the standard curve equation

$$y = 0{,}011x + 0{,}002,\ R^2 = 0{,}993.$$

For quantification flavonoids, we used quercetin as a standard and the result was expressed as µg of Quercetin Equivalent per g of extract following the standard curve equation

$$y = 0{,}018x + 0{,}009,\ R^2 = 0{,}991.$$

Our results indicated that *Nigella sativa*UF showed the highest amount of polyphenols with 24,73±0,51 µg GAE/g extract, the TO contains 16,67±0,35 µg GAE/g extract. Moreover, total flavonoids content was found to be highest in UF with 8,31±0,40 µg QAE/g extract than that of TO with3,83±0,02 µg QAE/g extract (table 1).

Table 1: Total phenolics and total flavonoids contents in *Nigella sativa* TO and UF

Fractions	Total phenolics (µg GAE/g extract)	Total flavonoids (µg QAE/g extract)
TO	16,67±0,35	3,83±0,02
UF	24,73±0,51	8,31±0,40

Each value is represented as mean±SD (n = 3)

ABTS, DPPH and H_20_2 radicals scavenging assays

The model of scavenging stable free radicals by hydrogen-donating antioxidants is widely used to evaluate the antioxidant properties in a relatively short time. To estimate this capacity, we used three assays: ABTS free radical scavenging assay, DPPH free radical scavenging assay and H_20_2 free radical scavenging assay.

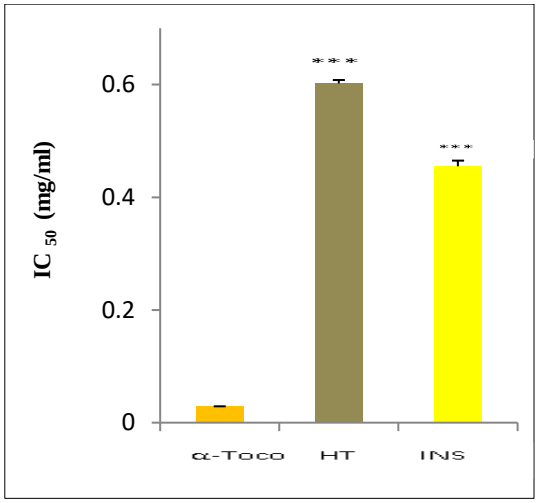
The results showed a similar tendency in the ability of the two fractions to reduce these radicals. UF showed the most important capacity followed by TO, as shown in figures 1, 2 and 3.

In the ABTS radical assay, the concentration for 50% inhibition were found to be 0,455 ±0,01 mg/ml and 0,602 ±0,006 mg/ml for UF and TO respectively. In the DPPH radical assay, IC_{50} were found to be 113,24 ±0,425µg/ml and 173,66 ±0,174 µg/ml for UF and TO respectively. In the H_20_2 radical assay, the IC_{50} were 0,324±0,003 mg/ml and 0,39±0,002 mg/ml for UF and TO respectively (table 2).

Table 2: ABTS, DPPH and H_20_2 radicals scavenging activities of TO, UF and standard

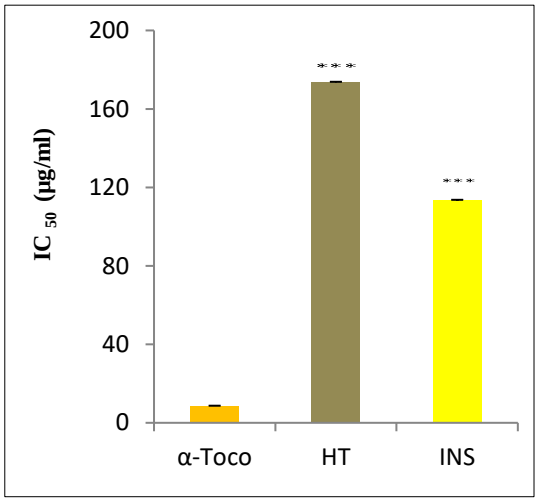
Fractions	IC_{50} for ABTS radical (mg/ml)	IC_{50} for DPPH radical (µg/ml)	IC_{50} for H_20_2 radical (mg/ml)
TO	0,602 ±0,006	173,66 ±0,174	0,39±0,002
UF	0,455 ±0,01	113,24 ±0,425	0,324±0,003
α-tocopherol	0,029 ±0	8,61 ±0,1	0,09±0,005

Each value is represented as mean±SD (n = 3), it has been reported that the lower IC_{50} indicated the higher activity, in this assay, the difference between radical scavenging capacities were statistically significant according to Tukey's test at $p < 0{,}05$.



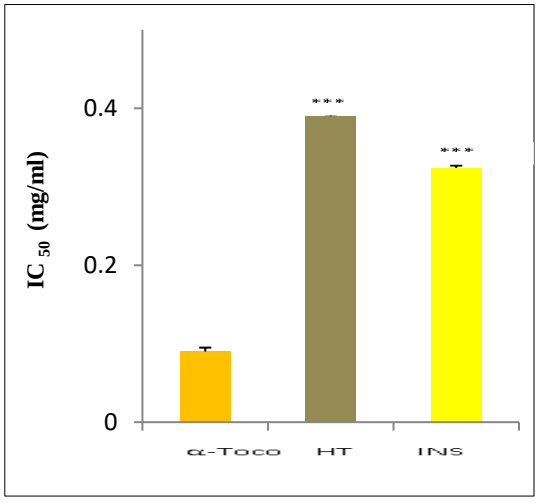
Values were expressed as the mean±SD of triplicate.
Comparisons are made with α-tocopherol ***: p ≤ 0,001

Figure 1: ABTS radical scavenging effect of TO, UF and standard



Values were expressed as the mean±SD of triplicate.
Comparisons are made with α-tocopherol ***: p ≤ 0,001

Figure 2: DPPH radical scavenging effect of TO, UF and standard

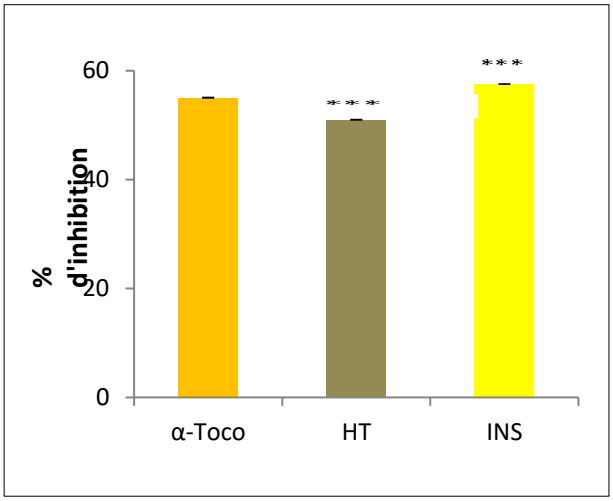


Values were expressed as the mean±SD of triplicate.
Comparisons are made with α-tocopherol ***: p ≤ 0,001

Figure 3: H_2O_2 radical scavenging effect of TO, UF and standard

Lipid peroxidation inhibition capacity assay

The inhibition of the peroxidation of linoleic acid by the extracts of the seeds of *Nigella sativa* was carried out according to the ferric thiocyanate method; the results showed that UF has the most important capacity to inhibit the process of lipid peroxidation with apercentage of 57.5% (figure 4).



Values were expressed as the mean±SD of triplicate.
Comparisons are made with α-tocopherol ***: p ≤ 0,001

Figure 4: Percentage of inhibition of lipid peroxidation by TO, UF and standard.

DISCUSSION

In the present study, the antioxidant activity of total oil and the unsaponifiable fraction of *Nigella sativa* was investigated, this is the first work that dealt with the unsaponifiable fraction extracted from the seeds of *Nigella sativa*.

The content of our oil in unsaponifiables has been estimated at 2.5%, a much lower rate than that of the *Nigella sativa* oil of Denizli in Turkey (5.38%) [20] India (4%), Kutahya (Turkey) (3.47%) and Konya (Turkey) (4.0%) but high compared to that of Egypt's *Nigella sativa* seeds (0.66%)[20].This fluctuation in the unsaponifiable contents is related to the extraction method and the origin of the seed [21].

Total oil of *Nigella sativa* contains a small amount of phenolics and traces of flavonoids, many studies were reported on the different extracts of this plant, and the results pointed out that the chloroform and ethyl acetate extracts showed the highest levels of phenolics and flavonoids following by aqueous and hexane extracts [22]. The unsaponifiable fraction has the highest phenolic and flavonoid concentrations with 24,73±0,51 µg GAE/mg extract and 8,31±0,40 µg QAE/mg extract respectively.

Unsaponifiable vegetable oils are considered natural antioxidants. They are able to minimize the oxidation of oils during storage. These unsaponifiable compounds are as well recommended in food as in medicine, for their anti-cancer and / or anti-inflammatory activities.

The antioxidant activity estimated by ABTS, DPPH and H_2O_2 radicals scavenging assays indicated that the unsaponifiable fraction is very efficient. We can relate this capacity to (i) the important amounts of

polyphenols and flavonoids, (ii) the diversity of its constituents and their synergism, such as sterols, tocopherols, higher aliphatic alcohols, pigments and natural hydrocarbons, and (iii) the different kinetic behaviours of potential antioxidants.

The effect of TO and UF against the peroxidation of linoleic acid showed that UF has the highest activity. A study reported that the apolar antioxidant has an important antioxidant activity because they have the capacity to dispose and concentrate on the center of lipid-water, which permit to prevent the creation of free radicals, for neutral lipids, they dispersed in an aqueous phase, which decrease its capacity of lipid protection [23].

CONCLUSION

This study showed clearly the antioxidant potential of *Nigella sativa* unsaponifiable fraction and this capacity is explained by the diversity of its antioxidant components. This finding illustrates the utility of this fraction as a natural antioxidant for use in therapy and lipid-containing foods. These bioactive substances could have extra nutritional properties and a central role in diet disease relationships. Further studies are required to disclose possible new bioactive constituents of the unsaponifiable fraction.

Conflicts of Interest: none

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