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Phytochemical Analysis of Indian and Ethiopian Black Cumin Seeds (*Nigella Sativa*)

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Abstract

In the present investigation, phenolic content and antioxidant activities of methanolic extracts of *Nigella sativa* seeds relative to Ethiopian and Indian black cumin were examined. Ferric thiocyanate test was carried out to find the reducing power of both types of *Nigella sativa* seeds. This test was utilized in explaining antioxidant activity of methanolic extracts of seeds. The phenolic composition of the methanolic extracts was estimated by Folin-Ciocalteu spectrophotometric method. Significantly higher amount of total phenolic content was exhibited by Ethiopian black cumin seeds compared to Indian type with a mean value of 437.00µg Gallic acid equivalents per g of powdered seed and the lowest mean value was 89.53µg Gallic acid equivalents per g of powdered seed. Similarly, high DPPH radical scavenging activity, with IC_{50} value of 7.22± 0.27 and 8.30± 1.34mg of Gallic acid equivalents per mL respective to Ethiopian and Indian Nigella seeds were observed. In addition, ABTS radical scavenging assay showed IC_{50} value of 9.29± 0.31 and 6.82 ± 1.9mg of Trolox equivalents per mL were recorded in Ethiopian and Indian Nigella seeds respectively.

Keywords: Antioxidants; Black cumin; *Nigella sativa*; Indian black cumin; Ethiopian black cumin

Introduction

Black cumin (*Nigella sativa*) belongs to the *Ranunnclaceae*-family native to Mediterranean region and claimed as a medicinal spice worldwide. There is a good potential for spices and herbs to be used as sources of natural antioxidants as they contain phenolic deter pines, flavonoids, tannins and phenolic acids etc[1]. The Nigella seeds have been studied most extensively, both phytochemically and pharmacologically. The aqueous and oil extracts of the N. sativaseeds have been shown to possess antioxidant, anti-inflammatory, anticancer, analgesic and antimicrobial activities[2]. In addition, phenolic compounds are found to be capable of regenerating α tocopherol into its active form of the antioxidant[3]. It is known that seeds are digestive stimulants as well as carminative, aromatic, diuretic, diaphonetic, stomachic, anthelmintic, asthmatic sources[4]. Increasing interest in using natural antioxidants, tocopherols, flavonoids, for the preservation of food is very useful in modern society. These phytochemicals naturally present as secondary metabolites in all plant materials prominently in herbs, vegetables, fruits and seeds. In this case presence of food phenolics in *Nigella sativa* is analyzed further. There are many beneficial implications of antioxidants due to their free radical scavenging and metal chelating activities(antioxidant capacity) in human health, such as in the treatment and prevention of cancer, cardiovascular disease, and other pathologies, recent interest in food phenolics has increased greatly[5].These natural antioxidants support in avoiding toxicity problems [1].The published findings provide clear evidence that both the oil and its active ingredients, in particular Thymoquinone (TQ), possess reproducible anti-oxidant effects through enhancing the oxidant scavenger system, which as a consequence lead to antitoxic effects induced by several causes. Thymoquinone, Thymol and other functional compounds present in black cumin seeds contribute to have its medicinal properties [6]. Since Nigella seeds consist of many phytochemicals having antioxidant property it is reasonable to investigate the ability of the seed extract to act as a radical scavenger.

Materials and Methods

Materials

Indian and Ethiopian origin black cumin seeds were purchased from a Sri Lankan company and seeds were tested for their quality parameters and were stored in the laboratory conditions. Powdered samples of each type were prepared by grinding and ground samples were kept in glass containers separately for subsequence used in the study. Extraction process was carried out by adding 5g of powder into 50ml of methanol

and was kept on shaker overnight. Then extracts were centrifuged 1100× for 10 minutes, followed by filtration by using a Whataman grade 1 qualitative filter paper and solutions were stored in brown bottles at 4 °C. To determine the total phenolic content, samples of prepared extracts in triplicates were utilized. In the case of determining antioxidant potential by using DPPH radical scavenging assay and ABTS assay, to increase the effectiveness of the results, the samples were prepared in triplicates and duplicate measurements were taken from each replicate. The extracted solutions were kept at 40°C for subsequent analysis. Furthermore, Total phenolic content and antioxidant capacity under DPPH and ABTS assays were determined in seed coat, cotyledon and whole seed with respect to two types.

Preliminary study to determine reducing power

The ability of the plant extracts to reduce Fe3+ was analyzed by the method of Oyaizu 1986[7] with some modification, 1ml of *Nigella sativa* extract was mixed with 1ml of phosphate buffer (0.2M, pH 7.2) and 2ml of 1% $K_3Fe(CN)_6$. Then mixed solutions were incubated at 37°C for 30 minutes. 1ml of 10 % trichloroacetic acid was added and the mixture was centrifuged at 650rpm for 10min. Then upper layer was mixed with 4ml of distilled water and 1ml of 0.1% aqueous $FeCl_3$. The absorbance was measured at 700nm using spectrophotometer (UVMINI-1240). The average absorbance values were plotted against concentration and compared with the strength of reducing power. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of total phenolic content

Total phenolic contents of Black cumin extracts of Indian and Ethiopian origins were determined using the Folin-Ciocalteu (F-C) spectrophotometric method, described by Cheng et al. [8]. An aliquot of 0.125ml of diluted extract was added to 0.5ml of deionized water and 0.125ml of the (F-C) reagent. Then the mixture was shaken well, followed by incubation for 3minutes at room temperature. Next, 1.52ml of 7% Na_2CO_3 solution was added and the volume obtained was adjusted to 3ml using distilled water, mixed vigorously, and hold for 90 min at ambient temperature. The absorbance of the solution was then measured at 760nm against a blank using spectrophotometer (UVMINI-1240). The total phenolic content was expressed as mg of Gallic acid equivalents (GAE) per gram of dry weight through the calibration curve of Gallic acid.

Determination of the antioxidant content; DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

Antioxidant capacity of both Indian and Ethiopian types were measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method described in Mansouri et al. [9] with some modifications and results were given as percentage of inhibition. The stock solution was prepared by dissolving 3mg of DPPH in 100ml methanol to obtain an absorbance of 0.900±0.02 units at 517nm using UV-Vis Spectrophotometer and 3ml of methanol was used as the blank sample. Control sample was prepared by mixing 2ml DPPH solution in 2ml methanol. For each type of Black cumin sample, a series of dilution was prepared and 1.5ml of each diluted sample was mixed with 1.5ml DPPH solution. After 30 min of incubation in darkness at ambient temperature, the resultant absorbance was recorded at 517nm. The percentage inhibition of absorbance was calculated according to the following equation.

Inhibition % = [Absorbance of control−Absorbance of sample/ Absorbance of control] ×100%

Where, $A_{control}$= Absorbance value of the DPPH solution of the control sample

A_{sample}= Absorbance value of the DPPH solution of extracted black cumin

DPPH is a stable radical, widely used to evaluate the free radical scavenging activity in many plant extracts[10]. The stable DPPH shows a maximum absorbance at 515nm and it can be undergone reduction by an antioxidant as follows.

The disappearance of the DPPH radical due to the action of antioxidant and absorbance at 515nm is taken as a measurement of antioxidant potential. In this study ability of test material which is the methanolic extract of *Nigella sativa* to scavenge DPPH radical was assessed based on their IC_{50} values, defined as the concentration of the test material to decrease the concentration of DPPH. solution to half of its initial value. These IC_{50} values were obtained by reading a calibration curve prepared by inhibition percentage values using above equation as a function of concentration of test material (methanol extract of *Nigella sativa*).

$$DPPH.+ AH \rightarrow DPPH\text{-}H + A$$

Inhibition percentage of each Black cumin type was calculated using above equation and plotted as a function of concentration of extracted Black cumin sample. According to the graph, concentration which gives the 50% inhibition activity was estimated as the IC_{50} value from regression analysis using the software MINITAB R17. As Gallic acid was used as the standard antioxidant, different concentrations of Gallic acid; 1, 2, 3, 4, 5, 6mg/L were prepared and absorbance was measured at 517nm after following the same scavenging procedure. Percentage inhibition was calculated for each dilution of Gallic acid solution and was plotted as a function of concentration of standard antioxidant. The concentration which gives the 50% inhibition activity was estimated as the IC_{50} value from regression analysis using the software MINITAB 17 for standard reference.

Determination of the antioxidant content; ABTS [2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] radical scavenging activity

For the ABTS assay, the procedure followed according to the method of Arnao et al. [11] with some modifications. The stock solutions included 7mM ABTS solution and 2.45mM potassium persulfate solution. ABTS stock solution was prepared by dissolving 36mg ABTS in 10ml distilled water and the Potassium

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persulfate stock solution was prepared by dissolving 6.6mg of Potassium persulfate in 10ml distilled water. The working solution which contains the ABTS•+ radical was prepared by mixing the two stock solutions in equal amounts and allowed to stand in dark under room temperature for 12-16 hours. The solution "Phosphate Buffer Saline" (PBS) was prepared by mixing 0.77g monosodium dihydrogen orthophosphate, 5.18g disodium monohydrogen orthophosphate, 1.48g sodium chloride and 500ml distilled water. The solution was mixed well in a magnetic stirrer and the final pH was maintained at pH 7.4. The working solution was then diluted by mixing 5ml ABTS•+ solution with 150ml PBS to obtain an absorbance of 0.70±0.02 units at 734nm using UV-mini 1240 Spectrophotometer. A blank sample was prepared by mixing 400µl distilled water and 4ml PBS. A control sample was prepared by mixing 400µl distilled water and 4ml diluted ABTS•+ working solution. For each extracted sample a serial dilution was prepared and 400µl of each diluted sample was mixed with 4ml diluted ABTS•+ working solution and incubated at room temperature for 5 minutes in dark. The absorbance was read at 734nm. The percentage inhibition of absorbance was calculated according to the following equation.

$$\%inhibition = [A_{control} - A_{sample} / A_{control}] \times 100$$

The calculated percentage inhibition of absorbance at 734nm was plotted as a function of concentration of methanol extracted black cumin samples and the sample concentration which gives the 50% inhibition activity was estimated as the IC_{50} value from regression analysis using the software MINITAB R 17 using Trolox as the standard antioxidant.

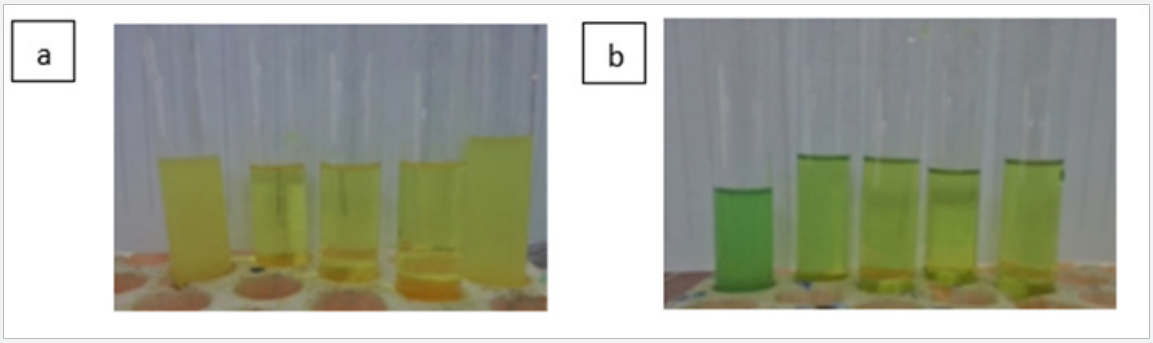

Figure 1: Colour of methanol extracts of black cumin (a) before reduction b) after reduction respectively.

Results

Ferric thiocyanate test (FTC)

The radical scavenging activity using DPPH or reducing power are two established *in vitro* methods employed for the evaluation of antioxidant activity. Prior to quantification of antioxidants using DPPH and ABTS like radical scavenging methods, determining the reducing power of extracts of Black cumin using Ferric thiocyanate test is very advantageous. Therefore, two dilution series relative to Indian and Ethiopian types were subjected to react with ferric thiocyanate including other reagents. Since this method is a qualitative method, colour difference observed as given in the Figure 1a & 1b.

The presence of reducing agents in methanol extract of black cumin is directly causing the conversion of Fe^{3+}/ferricyanide complex formed in the test to the Fe^{2+} form. After reaction has taken place, colour change could observe as yellow colour to bright green colour. This colour change qualitatively shows the reducing power of the methanol extract of *Nigella sativa* which confirms the presence of reducing agents in black cumin. Some authors have observed a direct correlation between antioxidant activities and the reducing power. Therefore, presence of these reducing agents indicates the availability of antioxidant compounds in *Nigella sativa*.

There is a prominent colour difference prior and after the reduction has occurred as shown in Figure 1. Even the colour intensity decreases as concentration of methanol extract of the *Nigella sativa* decreases which emphasis that as concentration of the extract decreases, reducing agents present in the sample also decreases. Both types, Indian and Ethiopian were tested for the presence of reducing power and higher absorbance was observed in Ethiopian type than the Indian. Intensity of the colour can be compared by absorbance relevant to the concentration of the extract of black cumin using the graph given in Figure 2.

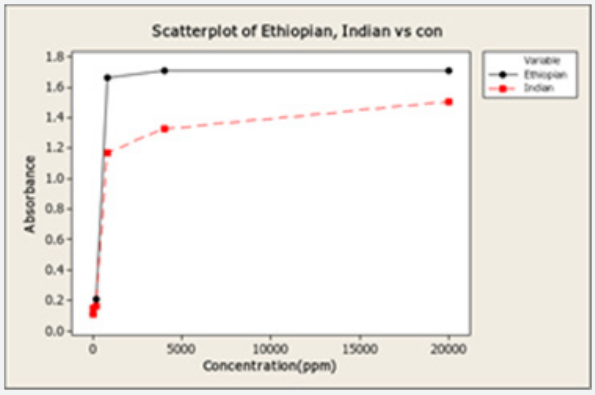

Figure 2: Comparison of colour intensities relevant to absorbance versus concentration of Indian and Ethiopian black cumin.

According to Figure 2, both Indian and Ethiopian origins showed the similar colour change, but intensity of the colour was different. It can be identified by measuring the formation of

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Perl's Prussian blue due to the reaction at 700nm. It is possible to monitor the concentration of Fe^{2+} observing the absorbance; higher the absorbance at 700nm, higher the concentration of Fe^{2+} which indicates that reducing power is also high. Therefore, it can be concluded that Ethiopian origin shows a higher reducing power than that of the Indian.

Determination of total phenolic content (TPC)

Total phenolic content of Indian and Ethiopian types of *Nigella sativa* with respect to three parts of the seeds are given in Table 1 based on the absorbance values of methanol extract of black cumin seed reacted with the Folin–Ciocalteu reagent and compared with the standard solutions of Gallic acid equivalents.

Table 1: TPC contents of Nigella sativa (Black cumin).

Part of the Seed	Ethiopian Type	Indian Type
Whole seed	542.56^{a}± 1.50	437.00^{a}± 1.27
Seed coat	203.12^{b}± 0.52	186.17^{b}± 4.38
Cotyledon	105.67^{c}± 6.95	89.53^{c}± 1.23

*Data presented as mean values for triplicates with duplicate measurements in each replicate ± S.D (n=6). a,b,c, letters in same column are significantly different at (p<0.05) level.

Total Phenolic contents of the two types of black cumin seeds were evaluated as phenols may act as antioxidants and protect lipids from peroxidation. TPC varied significantly between the two types as well as between seed coat and the cotyledon as given in the Table 1. According to 95% confidence level TPC content varies among the seed coat, cotyledon and whole seed($p<0.05$). Highest TPC content was observed in whole seed and lowest in the cotyledon whereas Ethiopian origin was higher as a whole. An ascending order of TPC was recorded in cotyledon, seed coat and whole seed. The highest TPC content in Ethiopian type was recorded as a mean value of 542.56µg Gallic acid equivalents per g of powdered seed and the lowest was recorded as a mean value of 105.67µg Gallic acid equivalents per g of powdered seed. The highest TPC content was recorded in Indian origin as a mean of 437.00µg Gallic acid equivalents per g of powdered seed and the lowest was recorded as a mean of 89.53µg Gallic acid equivalents per g of powdered seed. These results are comparable with the results (36.05mg Gallic acid/kg oil) recorded in Kiralan et al. [12].

Most studies have been conducted to find out TPC of *Nigella sativa* as whole and very little evidence is available for seed coat and cotyledon. Furthermore, Nigella seed samples which have been identified as having high number of functional compounds contain high phenolic content. These facts suggest that both Indian and Ethiopian types of black cumin contain a significant level of phenolic compounds which may partially contribute to the stability of the oil extracted from these seeds and those phenolic compounds may have a positive impact in preventing coronary heart disease and cancer [13]. Since most of the phenolic compounds possess antioxidant abilities higher TPC may attributes higher antioxidant capacity.

DPPH radical scavenging assay

Methanol extracts of black cumin with respect to Indian and Ethiopian origins were subjected to evaluate radical DPPH with a view to investigate antioxidant capacities after confirming their reducing power qualitatively. Results of IC_{50} value respect to two types considering three parts of the seeds are illustrated in the Table 2.

Table 2: DPPH radical scavenging assay based on IC_{50} value (mg/mL) in different Black cumin types under different parts of the seed.

Part of the Seed	Ethiopian Type (IC_{50} value)	Indian Type (IC_{50} value)	Standard Antioxidant (Gallic Acid)
Whole seed	7.22^{a}± 0.27	8.00^{a}± 1.34	0.0036 ± 0.00
Seed coat	11.32^{b}±0.48	12.46^{b}±0.76	
Cotyledon	15.50^{c}±0.13	16.23^{c}±0.52	

*Data presented as mean values for triplicates with duplicate measurements in each replicate ± S.D (n=6). a,b,c, letters in same column are significantly different at ($p < 0.05$) level.

As shown in Table 2, assessment of antioxidant activity shows that both N. sativa Indian and Ethiopian types were able to scavenge this radical. According to the DPPH radical scavenging assay, IC_{50} value is the antioxidant concentration in seed extract that shows 50% inhibition activity of the DPPH free radical and it is indicated as mg of Gallic acid equivalents per ml of methanol extract. If the IC_{50} value is low that will indicate high antioxidant activity whereas high IC_{50} value indicates low antioxidant capacity.

Concerning the type, Ethiopian Nigella seeds displayed a higher activity than Indian type as shown in table 2(IC_{50} =7.22mg of Gallic acid per ml and 8.30mg of Gallic acid per ml respectively). Antioxidant capacity with respect to the type is significantly different at 95% confidence level ($p<0.05$). Similarly, according to the section wise of the Nigella seed such as seed coat, cotyledon and whole seed, antioxidant capacity varies as shown in Table 2. The ascending order of IC_{50} value indicates the descending order of antioxidant capacity. The ascending order of IC_{50} value under each section of the seed is whole seed, seed coat and cotyledon. Therefore, the highest antioxidant activity was recorded in whole seed in both types where the IC_{50} is the lowest and lowest antioxidant activity was recorded in cotyledon in both cases, where the IC_{50} value is the highest. The lowest IC_{50} value in Indian type was recorded as a mean value of 8.00mg of Gallic acid per ml in the whole seed and highest IC_{50} value was recorded as a mean of 16.23mg of Gallic acid per ml in the cotyledon. The lowest IC_{50} value in Ethiopian type was recorded as 7.22mg of Gallic acid per ml in whole seed and highest` IC_{50} value was recorded as 15.50mg of Gallic acid per ml in cotyledon. The antioxidant activity of seed coat was observed higher than the cotyledon due to the influence on the variability of phenolic profiles and levels. The IC_{50} value of the Gallic acid standard was recorded as 0.0036mg/ml.

The results revealed that methanol extracts of N. sativawere different in types as well as in different parts of the seed were

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free radical scavengers, can act possibly as primary antioxidants. According to Erkan et al. [1] the reducing capacity of a compound may serve as an indicator of its potential antioxidant activity. Interestingly, high reducing power which was shown by Ethiopian type in the ferric thiocyante test similarly showed high antioxidant capacity in DPPH assay. These findings show that N. sativaseeds exhibit strong antioxidant capacity and that antioxidant potential varies in the two different genetic types as well as in different parts of the seeds.

ABTS radical scavenging assay

According to Lutterodt et al. [6], it is convenient to use at least two complimentary methods to evaluate the antioxidant capacity in vitro. Therefore, further confirmation of antioxidant potential of *Nigella sativa*, both Indian and Ethiopian types were subjected to be analyzed using ABTS radical.Results of the ABTS radical scavenging assay based on IC_{50} values of *Nigella sativa* under different types and different parts of the seeds are shown in the Table 3.

Table 3: Results of ABTS radical scavenging assay based on IC50 value (mg/mL) in different Black cumin types

Part of the Seed	Ethiopian Type (IC_{50} value)	Indian Type (IC_{50} value)	Standard Antioxidant (Trolox)
Whole seed	6.82^{a}± 1.90	9.29^{a}± 0.31	0.041±0.000
Seed coat	10.56^{b}±0.30	12.27^{b}± 0.02	
Cotyledon	13.95^{c}±0.22	14.68^{c}± 0.47	

According to the ABTS radical scavenging assay, IC_{50} value is the antioxidant concentration in the black cumin extract that shows 50% inhibition activity of the ABTS free radical and it is indicated as mg of Trolox equivalents per ml of extract. Low IC_{50} value indicates higher antioxidant activity whereas high IC_{50} value indicates low antioxidant capacity. There is a significant difference among the IC_{50} values obtained for different Nigella types at 95% confidence level. Furthermore, it is significantly different according to the part of the seed also. Although many studies have been done to investigate the antioxidant capacity of *Nigella sativa*, fewer findings are available on parts of the seeds separately. The lowest IC_{50} value in Indian type was recorded as a mean value of 9.29mg of Trolox per ml in the whole seed and highest IC_{50} value was recorded as a mean value of 14.68mg of Trolox per ml in the cotyledon. The lowest IC_{50} value in Ethiopian type was recorded as 6.82mg of Trolox per ml in whole seed and highest IC_{50} value was recorded as 14.68mg of Trolox per ml in cotyledon. The IC_{50} value of the Trolox standard was recorded as 0.041mg/ml. The highest antioxidant activity was recorded in whole seed in both types as mentioned in Table 3 where the IC_{50}value is lowest in both types. The lowest antioxidant activity was recorded in cotyledon in respect to both Indian and Ethiopian types and it shows highest IC_{50} value. There are several reports which have stated that antioxidant potential of phytochemicals presents in the *Nigella sativa* out of which most studied are thymol and thymoquinone. The strong antioxidant activity of N. sativaseeds assessed by the different systems could be attributed to their high total polyphenolic contents; in fact, it has been found that polyphenols are one of the most effective antioxidative constituents. The medicinal plants receive much attention with respect to phytochemical activities like antioxidative and medicinal properties now a-days and they are believed to be safe[14].

Conclusion

In relation to the material investigated antioxidant capability and phenolic content are higher in Ethiopian black cumin than the Indian origin whereas phytochemical content varies in each part of the seed. Seed coats of the *Nigella sativa* are rich in phytochemicals rather than cotyledon as many chemical compounds are concentrated into seed coat. Hence, Black cumin confirms to be a medicinal plant rich in phytochemicals. Further investigation will be necessary to confirm the results obtained on different genotypes cultivated in different environments.

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