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SECONDARY METABOLITES, ANTIOXIDANT ACTIVITY, PHYTONUTRIENT ANALYSIS OF *Nigella sativa* and *Brassica hirta* seeds

M. Krishnaveni*& S. Saranya[1]

***Assistant Professor &[1] M.Phil Student Department of Biochemistry, School of Bio-Sciences, Periyar University, Salem-636 011**

***Corresponding Author Email: krishnavenim2011@gmail.com**

ABSTRACT

Nigella sativa, Brassica hirta seeds were selected for the present study. Since, these seeds offer many pharmacological properties; it was decided to study the phytochemicals, secondary metabolites, antioxidant activities. From our results, it was known, that qualitative analysis of phytochemical showed positive results for all the tests performed. Among the secondary metabolites tested, the total phenolic content was higher in both the seeds studied but comparatively higher phenolic content (6.26 ±3.00mg/g) was observed in Brassica hirta seeds. Nitric oxide Scavenging activity was higher (10.33±2.33mg/g) with Nigella sativa seeds. Whereas, reducing power activity was higher (6.40±1.03mg/g) with Brassica hirta seeds. Protein content was higher with Nigella sativa (11.06±0.28mg/g) and Brassica hirta seeds (7.70±2.94mg/g) studied. Hence, it is concluded that Nigella sativa and Brassica hirta seeds could be used as a therapeutic agent in drug formulations for various diseases.

KEY WORDS

Antioxidant, Brassica hirta, Nigella sativa, Phytochemicals, Seeds.

INTRODUCTION

The medicinal values of plants have been discovered through its extracts from various parts of plants which contain not only minerals, primary metabolites but also secondary metabolites with antioxidant potential. The antioxidant capacity exhibited by phenolic compounds was mainly due to its redox properties able to function as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators. Apart from its antioxidant properties it was also antiallergic, antiinflammatory, antimicrobial, antithrombotic, cardioprotective, vasodilatory effects. The antioxidant activity induced by phenolics depend on its structures, especially the electron delocalization over an aromatic nucleus. Those compounds on reaction with free radicals causes delocalization of the gained electron over the phenolic antioxidant and also its stabilization by the resonance effect of the aromatic nucleus so as to prevent the continuation of the free radical chain reaction.[1] Antioxidants possess free radical chain reaction attacking properties thus eliminate as well as diminish the action of free radicals causing oxidative stress via its antioxidative defense mechanisms. Among the naturally occurring antioxidants, ascorbic acid, carotenoids, phenolic compounds are more effective [2] in inhibiting lipid peroxidation in order to scavenge free radicals, active oxygen species by proliferating a reaction cycle and in chelating heavy metal ions.[3] Any imbalance between the oxidants - antioxidants causing damage leads to aging, diseases of various types in humans. The free radical scavenging ability was measured using hydrogen atom transfer reactions, single electron transfer reactions.[4,5] The antioxidant is able to quench free radicals by hydrogen donation: $X + AH \rightarrow XH + A$ via hydrogen atom transfer reactions. The antioxidant transfers one electron to reduce metals, carbonyl groups, radicals etc via single electron transfer method based on deprotonation and in ionization potential of the reactive functional group.[6-8]

Antioxidant based drug formulations prevent and treat atherosclerosis, stroke, diabetes, Alzheimer's disease,cancer.[9] Hence, the present study was undertaken to study the phytochemical analysis qualitatively as well as secondary metabolites, antioxidant, phytonutrients quantitatively in the aqueous extract of *Nigella sativa, Brassica hirta* seeds.

MATERIALS AND METHODS

Sample collection

Nigella sativa, Brassica hirta seeds were purchased from the local shop at Salem. The seed purchased was identified for its botanical name through online sources.

Extract preparation

Aqueous extract was prepared by dissolving 15g of powdered sample – *Nigella sativa* and *Brassica hirta* seed powder in 200ml of distilled water. The mixture was heated on a hot plate with continuous stirring at 30-40°C for 20minutes. Then the water extract was filtered through filter paper and used for qualitative analysis Harborne (1984), Kokate et.al (1995).[10,11]

Test for carbohydrate

Molisch's test: To the extract added few drops of alcoholic alpha napthol solution, few drops of concentrated Sulphuric acid along the sides of test tube. Positive result gives purple or violet colored ring at the junction.

Fehlings test: To the extract added equal amount of Fehlings A and B solution, heat the tubes in a boiling water bath. Brick red precipitation of cuprous oxide is formed, if reducing sugar is **present.**

Benedicts test: To the extract added Benedict's reagent, the tubes were heated in a boiling water bath. Red precipitation indicates positive result.

Test for alkaloids

Wagners test: To the extract added few drops of iodine solution in potassium iodide. Reddish brown precipitate shows positive result.

Hagers test: To the extract added few drops of saturated solution of picric acid. Yellow colour precipitation signifies positive result.

Test for steroids and sterols

Libermann-Burchard test: To the extract added 2ml chloroform, 10 drops of acetic anhydride, 2 drops of concentrated sulphuric acid. Bluish red to cherry red color in chloroform layer shows positive result.

Salwoski test: To the extract added few drops of chloroform, concentrated sulphuric acid. Bluish red to cherry red color.

Test for Glycosides

Legal test: To the extract added pyridine, sodium nitroprusside. Positive result shows pink red color.

Baljet test: To the extract added picric acid. Appearance of orange color signifies positive result.

Test for saponins

Foaming test: Foams produces when the extract is mixed with water.

Test for tannin and phenolic compounds

Ferric chloride test: To the extract added ferric chloride. Formation of greenish black color showed positive result.

Potassium dichromate test: To the extract added potassium dichromate solution. Positive result showed brown precipitate formation.

Gelatin test: To the extract added 1% gelatin solution containing 10% sodium chloride. Appearance of white precipitation confirms the presence of gelatin.

Test for protein and amino acids

Biuret test: To the extract added 4% sodium hydroxide, few drops of 15% copper sulphate. Appearance of purple color confirms the presence of protein.

Ninhydrin test: Bluish violet color forms when a solution of ninhydrin and extract mixture was heated.

Heat test: Protein coagulation showed positive result when test solution was heated on a boiling water bath.

Test for fixed oil

Copper sulphate test: Blue colour formed when the extract was mixed with 1ml of 1% copper sulphate and 10% sodium hydroxide.

SECONDARY METABOLITES

The phenol and flavonoid content of aqueous leaf extract was analysed.

Determination of Total phenol content

Total phenolic content were determined by Folil-ciocalteau method Nabavi et.al.[12] The extract samples 0.1ml were mixed with folinciocalteau reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous $NaCo_3$ (4ml, 1M) were added. The mixture was allowed to stand for 15min and the phenols were determined by colorimetric method at 765 nm. The standard curve was prepared. Total phenol values are expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

Estimation of flavonoids

The aluminium chloride method Mervat et.al [13] was used for the determination of the total flavonoid content. To 0.1ml of the extract solution added 0.1ml of $AlCl_3$ (10%) sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was recorded after 30min of incubation. A standard calibration plot was generated by using known concentration of quercetin. The concentration of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent/g of sample.

ANTIOXIDANT ASSAYS

Nitric oxide scavenging assay, Reducing power, Total antioxidant assay, Metal chelating activities were performed.

Nitric oxide scavenging activity

This was estimated by the method of Ebrahimzadeh et.al.[14] The procedure is based on the principle that, sodium nitroprusside in aqueous solution, at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with extract and incubated at room temperature for 150min. After the incubation period 0.5ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546nm. Quercetin was used as positive control.

Reducing power assay

Reducing power assay was performed according to the method of Yen et.al.[15] Aqueous extract was mixed with phosphate buffer (2.5ml, 0.2M, P^H 6.6) and potassium ferriccyanide (2.5ml %). The mixture was incubated at 50^0c for 20min. 1.0 ml of trichloro acetic acid (10%) was added to stop the reaction, which was then centrifuged at 3000rpm for 10min. The upper layer of solution (1.5ml) was mixed with distilled water (1.5ml) and $FeCl_3$ (0.1ml, 0.1%) after mixing, the contents were incubated for 10 min and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Total antioxidant capacity

Total antioxidant capacity by phospho-molybdenum method assay is based on the reduction of Mo (V1) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as number of equivalents of ascorbic acid. Assay was carried out according to the method of Prieto P et.al.[16]

Metal chelating activity

The chelating ability of ferrous ion was estimated by the method of Ebrahimzadeh MA.[17] Add extract to a solution of 2mM $FeCl_2$ (0.05ml). The reaction was initiated by the addition of 5mM Ferrozine (160µl), the mixture was shaken vigorously and left standing at room temperature for 10min. Absorbance of the solution was then measured spectrophotometrically at 562nm. Standard curve was plotted using ascorbic acid. Distilled water (1.6ml) instead of sample solution was used as a control. Distilled water (160µl) instead of ferrozine was used as a blank, which is used for error correction because of unequal color of sample solution.

Analysis of phytonutrients

Total carbohydrates, proteins, aminoacids were performed according to the standard prescribed methods.

Estimation of carbohydrate

The total carbohydrate was estimated by Anthrone method Hedge.[18] To 0.1 ml of extract added 4ml of anthrone reagent and contents were heated in a boiling water bath for 8 minutes. The tubes were cooled and read at 630nm using spectrophotometer Schimadzu Model - UV 1800. The standards were developed with glucose. Standard graph plotted was used to find out concentration of glucose present in the unknown/ sample.

Estimation of protein

The total protein was estimated by Lowry's method Lowry et.al.[19] To 0.1ml of extract added 2ml of alkaline copper reagent, mixed well and incubated for 10minutes. After the incubation period 0.2ml of Folin ciocalteau reagent (diluted in the ratio of 1: 2) was added and allowed for 30minutes incubation, then read at 660nm using spectrophotometer Schimadzu - Model UV 1800. The standards were developed with Bovine serum albumin. Standard graph plotted was used to find out concentration of protein present in unknown/ sample.

Estimation of aminoacids

The amino acid was estimated by Ninhydrin method Yemm et.al.[20] To 0.1 ml of sample added 1 ml of ninhydrin solution dissolved in Butanol: Acetone. Cover the test tube with a piece of paraffin film to avoid the loss of solvent due to evaporation. With gentle stirring, the reaction mixture was heated at 80-100ºC for 4-7 minutes. Cool the test tubes and the color developed was read at 570nm. Tyrosine was used for developing standards.

For all estimations readings were taken using UV spectrophotometer Schimadzu Model 1800. Standard graph was plotted for all experiments using their respective standards and the samples were plotted against the standard by taking concentration in X axis and OD in Y axis.

STASTICAL TOOL

The Mean and Standard deviation (S) was calculated by using the following formula:

Mean = Sum of x values / n (Number of values)

$$s = \frac{\sqrt{\sum(X-M)^2}}{n-1}$$

RESULTS AND DISCUSSION

Table.1 Phytochemicals in Aqueous extract of *Nigella sativa, Brassica hirta* seeds

S.No	Name of the test	Results
1.	**Test for carbohydrate**	
	a)Molisch's test	+++
	b)Fehlings test	+++
2.	**Test for alkaloids**	
	a)Wagners test	---
	b)Hagers test	+++
3.	**Test for steroids and sterols**	
	a)Libermann - Burchard test	++
	b)Salwoski test	++
4.	**Test for Glycosides**	
	a)Legal test	++
	b)Baljet test	+
5.	**Test for saponins**	
	Saponin test	+
6.	**Test for tannin and phenolic compounds**	
	a)Ferric chloride test	+++
	b)Potassium dichromate test	++
	c)Gelatin test	+

7.	**Test for protein and amino acids**	
	a)Biuret test	+
	b)Ninhydrin test	+++
	c)Heat test	+
8.	**Test for fixed oil**	
	a)Copper sulphate test	+++

++ Strong, ++ Moderate, + low

The results of Qualitative analysis are shown in Table.1. The results of qualitative analysis showed positive answers for all the compounds tested.

Secondary metabolites

Table.2 Secondary metabolites, Antioxidant activity *Nigella sativa, Brassica hirta* seeds

S.No	Parameters assessed	*Nigella sativa* (mg/g)	*Brassica hirta* (mg/g)
	Secondary metabolites		
1.	Total phenolics	3.66±0.57	6.26±3.00
	Total flavonoids	1.06±0.05	3.10±1.55
	Antioxidant activities		
	Phosphomolybdenum assay	03.16±1.44	4.00±2.25
2.	Nitric oxide Scavenging assay	10.33±2.33	2.80±1.73
	Reducing power assay	05.50±0.86	6.40±1.03
	Metal chelating activity	01.80±0.17	1.96±0.40

Values are Mean ± SD for three experiments

Table.2 shows the results of secondary metabolites and antioxidant activity of *Nigella sativa* and *Brassica hirta* seeds. The observed total phenolics was found to be 3.66 ±0.57mg/g in *Nigella sativa* and 6.26 ±3.00mg/g in *Brassica hirta.* Similarly, the total flavonoid calculated was 1.06 ± 0.05mg/g and 3.1 ± 1.55mg/g in *Nigella sativa* and *Brassica hirta* seeds. Antioxidant activities were tested using several different parameters such as phosphomolybdenum assay, Nitric oxide scavenging assay, reducing power assay, metal chelating assay. Among the antioxidant activity tested in *Nigella sativa* seeds, nitric oxide scavenging activity was found to be showing higher activity(10.33±2.33mg/g) followed by reducing power assay (5.5±0.86mg/g), phosphomolybdenum assay (3.16±1.44mg/g), metal chelating activity (1.8±0.17mg/g). In *Brassica hirta* seeds, the reducing power activity was found to be high (6.4±1.03mg/g), phosphomolybdenum activity (4.00 ±2.25mg/g), nitric oxide scavenging activity (2.8 ±1.73mg/g), metal chelating activity (1.96±0.40mg/g). The results of two seeds selected when compared for their antioxidant properties, it was evident that *Nigella sativa* seeds showed higher activity compared to *Brassica hirta* seeds. A positive correlation was observed between phenolic content and antioxidant activity. Phenolic compounds like flavonoids, phenolic acid, tannin are anti-inflammatory, anti-carcinogenic, anti-atherosclerotic in nature and show other antioxidant activities too. [21,22] Flavonoids, flavonols participate in stabilizing oxidation of lipid as well as associated with antioxidant activity[23] and the antioxidant properties of flavonoid depend on the structure especially hydroxyl position in the molecule and its ability to donate electron to a free radical.[24]

Table 3: Phytonutrients of *Nigella sativa, Brassica hirta* seeds

S.No	Parameters assessed	*Nigella sativa* (mg/g)	*Brassica hirta* (mg/g)
1	Protein	11.06±0.28	7.70±2.94
2	Aminoacid	1.00±0.00	1.30±0.00
3	Carbohydrate	2.26±0.23	1.30±0.17

Values are Mean ± SD for three experiments

The results of phytonutrients are depicted in the Table.3. The protein content was found to be 11.06±0.28mg/g, carbohydrate 02.26±0.23mg/g, aminoacid 01.00±0.00mg/g in *Nigella sativa* seeds. Whereas, the protein, aminoacid, carbohydrate content of *Brassica nigra* seeds were found to be 7.70±2.94mg/g, 1.30±0.00mg/g, 1.30±0.17mg/g. Phytonutrient assay also showed highest amount of nutrients in *Nigella sativa* seeds compared to *Brassica hirta* seeds. This shows that *Nigella sativa* seeds are more potent than *Brassica hirta* seeds. The increased protein content specifies increased food value, which might have been useful in the isolation of protein bioactive compound. The phytochemicals analysis of *Nigella sativa* seeds using analytical techniques was reported by Krishnaveni et.al.[25]

CONCLUSION

The results of the present study suggests that selected seeds could be used for pharmacological preparations as they possess anti-inflammatory, anti-asthmatic, antibacterial, antifungal, antidiabetic, hypocholestremic properties and also used in industries as flavoring agent since it have antioxidant property in significant amount.

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***Corresponding Author:**
M. Krishnaveni
Email: krishnavenim2011@gmail.com