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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITIES OF ETHANOLIC EXTRACT OF THE AERIAL PARTS OF *BRASSICA NIGRA*

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ABSTRACT

Plants contain a wide variety of phytochemical constituents, which are secondary metabolites used directly or indirectly in the pharmaceutical industry. Most of the fast research indicated that plants are a good source for treating many diseases affecting mankind. For centuries, man has effectively used various components of plants or their extracts to treat many diseases. Since *Brassica nigra* seeds have numerous pharmacological properties, the present study analysed its phytochemicals qualitatively and invitro anti-oxidants studies. Ethanolic extract of this plant was discovered in the phytochemical analysis, and results indicated that the extract has a high amount of phenol, flavonoids, alkaloids, steroids, saponins, terpenoids and mucilage and protein compounds. Ethanolic extract of the plant of *Brassica nigra* aerial parts was subjected to antioxidant activities such as DDPH assay, reducing power assay, hydroxyl free radical scavenging activity, ABTs+ radical scavenging activity and superoxide anion scavenging activities. The plant has shown effective antioxidant activity in all assay techniques. The results obtained in this study indicate that the plant *Brassica nigra* is a potential source of natural antioxidants. The phytochemical profile and antioxidant potential of *Brassica* plants make them desirable candidates for nutritional and pharmaceutical applications.

KEYWORDS: *Brassica nigra*, Flavonoids, Phytochemical analysis, Ethanol, Antioxidant.

INTRODUCTION

Mustard seeds are from the mustard plant, a cruciferous vegetable related to broccoli, Brussels sprouts and cabbage. While there are approximately forty different varieties of mustard plants, there are three principal types used to make mustard seeds: black mustard (*Brassica nigra*), white mustard (*Brassica alba*) and brown mustard (*Brassica juncea*). Black mustard seeds have the most pungent taste, while white mustard seeds, which are yellow in colour, are the mildest and are used to make American yellow mustard. Brown mustard, which is dark yellow in colour, has a pungent, acrid taste and is the type used to make Dijon mustard. Mustard seeds are sold either whole or as a ground powder. Mustard seed is a rich source of oil and protein. The seed has oil as high as 46-48%, and the whole seed meal has 43.6% protein. Mustard is used as a food flavouring, for forage, as an emetic, and the medicinal parts were the seeds from which oil is extracted (Joseph et al 2004). It is commonly used as a spice. It is native to tropical regions of North Africa, temperate regions of Europe, and Asia. Among the diversity of medicinal plants, Khardal (*Brassica nigra*) is one of the medicinal plants used extensively in various non-communicable/ chronic degenerative diseases and has potential pharmacological effects on cancer.

Brassica nigra is an annual herbaceous plant. It grows up to 2 m (a little over 6 ft), with many branches. The lower leaves are dentate (toothed), pinnatifid (deeply lobed) or lyrate (deeply lobed, but with an enlarged terminal lobe and smaller lateral lobes) and are often hairy, at least on the underside. Upper leaves on flowering stems are narrow and oblong. In contrast to many *Brassica* species, the leaves are glaucous (waxy). The yellow, four-parted, and cross- shaped flowers occur in many racemes (spike-like clusters) and produce 4-sided siliques capsular fruit that dehisces (splits open) when mature that may be up to 2.5 cm (1 in) long. Each silique contains 2 to 12 or more reddish-brown to black round seeds. A single plant may produce thousands of seeds, which must be harvested by hand or mechanically before they fully ripen because the siliques spontaneously split and disperse the seeds when they are mature (Larsson et al 2006).

Mustard seed (*Brasicca nigra*) contains the following chemical constituents are allyl isothicyanate; myrosinase; sulphoraphane; flavonols- flavones; flavan-3-ols; anthocyanidins; flavanones; isoflavones; non-flavonoids - phenolic acids, hydroxyl cinnamates, stilbenes etc;

flavonoids and hydroxycinnamic acids; anthocyanins and glucosinolates (Cartea et al 2011; Kalpana et al 2013).

Reactive oxygen species (ROS) such as O_2, H_2O_2 and OH• are highly toxic to cells. Cellular antioxidant enzymes and the free radical scavengers usually protect a cell from the toxic effects of ROS. When the generation of the ROS overtakes the antioxidant defense of the cells, oxidative damage of the cellular macromolecules (lipids, proteins and nucleic acid) occurs, leading finally to various pathological. Reactive nitrogen species (RNS) are the products of normal cellular metabolism. NO• is a small molecule with one unpaired electron on the antibonding ($2\pi^*y$) orbital and is, therefore, a radical. NO• is generated in biological tissues by specific nitric oxide synthases (NOSs). The overproduction of reactive nitrogen species is called nitrosative stresses. This may occur when the generation of reactive nitrogen species in a system exceeds its ability to neutralise and eliminate them. Nitrosative stresses may lead to nitrosylation reactions that can alter the structure of proteins and inhibit their normal function (Gulcin et al 2003).

Free radicals play an essential role in the origin of life and biological evolution, implicating their beneficial effects on the organism. The cytotoxic effect of free radicals is deleterious to mammalian cells. It mediates the pathogenesis of many chronic diseases, but it is responsible for killing pathogens by activated macrophages in the immune system. Antioxidants fight against free radicals by protecting us from various diseases and scavenge of reactive oxygen radicals or protecting the antioxidant defence mechanism. ROS can damage biological macromolecules such as DNA, carbohydrates and proteins. ROS is a collective term which includes oxygen radicals (O_2 and OH.) and some non-radical derivatives of oxygen like H_2O_2, HOCl, and ozone (O_3). Suppose the human disease is believed to be due to the imbalance between oxidative stress and antioxidant defence. In that case, it is possible to limit oxidative tissue damage and prevent disease progression by antioxidant defence supplements. In addition, antioxidant activity may be considered a fundamental property important for life (Amarowicz et al 2004). In the present investigation, we have reported a phytochemical analysis of ethanol extract of the plant; from those chemical constituents, an in-vitro antioxidant analysis was carried out by different assay methods.

MATERIAL AND METHODS

Preparation of powder material

After authentication, the fresh, healthy aerial parts of *Brassica nigra* dried properly in the shade for three weeks, segregated, pulverized by a mechanical grinder and passed through a 40-mesh sieve. The powdered plant materials were stored in an airtight container and used for further studies.

Preparation of extracts (Harborne et al 1998)

About 1 kg of air-dried aerial parts of plant *Brassica nigra* was extracted in Soxhlet assembly successively with ethanol. The powdered material was dried at room temperature each time before extracting with the next solvent. Each extract was concentrated by using a rotary vacuum evaporator. The extract obtained with each solvent was weighed and the percentage yield was calculated in terms of the dried weight of the plant material. The colour and consistency of the extract were also noted. All the solvents used for this work were of analytical reagent grade (Merck, Mumbai).

ALKALOIDS (Peach and Tracey, 1995; Raaman, 2006)

Meyer's reagent (Potassium iodide)

1.3 g of mercuric chloride was dissolved in 60 ml of distilled water and 5.0 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water. To 1.0 ml of leaf extract, a few drops of reagent were added. The formation of white or pale-yellow precipitate showed the presence of alkaloids.

PHENOL

Ferric chloride test

To 1.0 ml of leaf extract, 2.0 ml of distilled water, followed by a few drops of 10 % aqueous $FeCl_3$ solution was added. The formation of blue or green colour indicates the presence of phenols.

FLAVANOIDS

In the test tubes containing 0.5 ml of leaf extract, 5-10 drops of dilute HCl and a small piece of zinc or magnesium were added, and the solution was boiled for a few minutes. In the presence of flavonoids, reddish-pink or dirty brown colour was produced.

TANNINS

Ferric chloride test

To 1- 2 ml of leaf extract, a few drops of 5 % aqueous $FeCl_3$ solutions were added. A bluish-black colour, which disappears on the addition of a few ml of dilute H_2SO_4, was followed by the formation of a yellowish-brown precipitate.

SAPONINS

A drop of sodium bicarbonate solution was added in a test tube containing about 5.0 ml of leaf extract. The mixture was shaken vigorously and kept for 3 minutes. A honey comb-like froth was formed and it showed the presence of saponins.

TERPENOIDS

Salkowski reaction

5.0 ml of leaf extract was mixed with 2.0 ml of chloroform, and concentrated H_2SO_4 (3.0 ml) was carefully added to form a layer. A reddish-brown colouration in the interphase was created to show positive results for the presence of terpenoids.

STEROIDS

Libermann-Burchard's test

To 1.0 ml of leaf extract, 1.0 ml of conc. H_2SO_4 was added, followed by adding 2.0 ml of acetic anhydride solution. A greenish colour developed and turned blue indicates the presence of steroids.

CARBOHYDRATES (Raama et al 2006)

Benedict's test

173 g of sodium citrate and 100 mg of sodium carbonate were dissolved in 500 ml of water. To this solution, 17.3 g of copper sulphate dissolved in 100 ml of water was added. To 0.5 ml of the leaf extract, 5.0 ml of Benedict's reagent was added and boiled for 5 minutes. The formation of a bluish-green colour showed the presence of carbohydrates.

GLYCOSIDES

A small amount of leaf extract was dissolved in 1.0 ml of water, and then an aqueous sodium hydroxide solution was added. The formation of yellow colour indicates the presence of glycosides.

AMINOACIDS AND PROTEINS

Biuret's test

To 1.0 ml of leaf extract, 5-8 drops of 5 % sodium hydroxide solution were added, followed by one or two drops of 1 % copper sulphate. The formation of pink or purple colour confirmed the presence of proteins and amino acids.

IN VITRO ANTIOXIDANT ACTIVITY

The following methods, namely determined the invitro antioxidant activities of the various extracts of plant studies DPPH assay, reducing power assay, hydroxyl free radical scavenging activity, ABTs+ radical scavenging activity and superoxide anion scavenging activity.

DPPH RADICAL SCAVENGING ACTIVITY (Shimada et al., 1992)

Principle

DPPH radical is scavenging by antioxidants by donating a proton forming the reduced DPPH. The colour change from purple to yellow after reduction can be quantified by its decrease in absorbance at wavelength 517 nm.

Reagents

0.2 mM DPPH

80% Methanol

Butylated Hydroxyl Anisole.

Procedure

Various concentrations of the sample (4.0 ml) were mixed with 1.0 ml of a solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2mM. The mixture was shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517nm. Ascorbic acid was used as DPPH radical scavenging activity was calculated as follows a control. The percentage of inhibition in;

$\% \text{ Inhibition} = (((A_0\text{-}A_1))/A_0) \times 100$

Where, A_0 is absorbance of the control, A_1 is absorbance in the presence of the sample. It is represented in Table 2 and figure 1.

REDUCING POWER ASSAY (Oyaizu, 1986)

Reducing power was measured by direct electron donation in the reduction of Fe^{3+} $(CN\text{-})_6$ to $Fe^{2+}(CN\text{-})_6$. The product was visualised by the addition of free Fe^{3+} ions after the reduction

reaction by forming the intense prussian blue colour complex, and quantified by absorbance at 700 nm.

Procedure

The reaction mixture contained 2.5 ml of various concentrations of the sample, 2.5 ml of 1% potassium ferricyanide and 2.5 ml of 0.2 M sodium phosphate buffer. The control contained all the reagents except the sample. The mixture was incubated at 50° C for 20 min. and the addition of 2.5 ml of 10% (w/v) of trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 min. 5.0 ml of the supernatant upper layer was mixed with 5.0 ml of deionized water and1.0 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against blanks that contained distilled water and phosphate buffer. Increased absorbance indicates increased reducing power of the sample. The percentage effect was calculated as,

% Inhibition = $(((A_0-A_1))/A_0) \times 100$

and it is represented in Table 3 and figure 2.

HYDROXYL RADICAL SCAVENGING ASSAY (Rajeshwar et al., 2005)

Hydroxyl radicals were generated from $FeSO_4$ and hydrogen peroxide and detected by their ability to hydroxylate salicylate and the hydroxylated salicylate complex is measured at 562 nm.

Procedure

A reaction mixture of 3.0 ml volume contained, 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and 1.0 ml of different concentrations (5-100 µg / ml) of sample. After incubation for an hour at 37^o C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. Vitamin E was used as positive control. The percentage scavenging effect was calculated as,

% Inhibition = $((A_0-A_1))/A_0) \times 100$

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample; It is represented in Table 4 and figure 3.

ABTS+ RADICAL SCAVENGING ACTIVITY (Giao et al., 2007)

ABTS+ decolourisation assay involves the generation of the ABTS+ chromophore by the oxidation of ABTS+ with potassium persulphate. It is applicable for both hydrophilic and lipophilic compounds. The scavenging activity of the leaf extracts on ABTS+ radical cation was measured at 734 nm.

Procedure

The reaction was initiated by the addition of 1.0 ml of diluted ABTS+ to 10 µl of different concentrations (5-100 µg / ml) of sample and also to 10 µl of ethanol as a control. Ascorbic acid was used as positive control. The absorbance was read at 734 nm after 6 minutes and the percentage inhibitions were calculated. The inhibition was calculated according to the equation,

% Inhibition = $(((A_0-A_1))/A_0) \times 100$

Where, A_0 is absorbance of control reaction, A_1 is absorbance of test compound. It is expressed in Table 5 and figure 4.

SUPEROXIDE RADICAL SCAVENGING ACTIVITY (Liu etal., 1997)

This method is based on the inhibition of production of nitro blue tetrazolium of the superoxide ion by the plant extract and is measured spectrophotometrically at 560 nm.

Procedure

The assay tubes containing various concentration of sample 0.2 ml of EDTA, 0.1 ml of nitro blue tetrazolium, 0.05 ml of riboflavin and 2.25 ml of phosphate buffer and control tubes were set up without the sample. Similarly, the activity of the standard antioxidants was also carried out. The initial optical uniform with a fluorescent lamp for 30 min. A 560 nm was measured again and difference in optical density was taken as the quantum of superoxide production. The percentage inhibition was calculated by comparing with the optical density of the control tubes.

% Inhibition = $(((A_0-A_1))/A_0) \times 100$

A_0- Absorbanace of control A_1- Absorbanace of sample. It is expressed in Table 6 and figure 5.

RESULTS AND DISCUSSION

Phytochemical Screening

In the Preliminary phytochemical analysis of the *Brassica nigra*, was revealed that ethanolic extract of plant was found to indicates the presence of various constituent such as alkaloids, carbohydrates, glycosides, flavonoids, steroids, amino acids, saponins, proteins, phenols and tannins were shown in the table.1. The ethanolic extract of *Brassica nigra* was found to have higher content of Alkaloid, steroids, proteins, phenolic and flavonoid components. Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups.

Flavonoids are potent antioxidants present in foods of plants. Most beneficial effects of flavonoids are attributed to their antioxidant and chelating abilities.

Antioxidant activity

Antioxidants that block the reactive oxygen species may be involved in preventing oxidative diseases like cardiovascular diseases, neurovascular diseases and autoimmune diseases. In this way, the antioxidant compounds minimize the oxidative stresses and prevent the oxidative damage to food materials and living organisms.

The present study plant extract of black mustard was also great value to prevent these diseases as it has good antioxidant property due to the presence of antioxidant phytochemicals mainly the phenols, flavonoids and ascorbic acid. Most of these phytochemical compounds act as antioxidants due to their hydrogen donating and reducing abilities. Phenols are the phytochemicals which act as metal ion chelators and interfere with oxidation reactions including lipid peroxidation by donating the proton to free radicals. Phenoxy radicals are relatively stable to stop the oxidation chain reaction. Therefore, they stop the initiation of new oxidation chain reaction and terminate the propagation routs by capturing free radicals (Mandal et al 2010). The DPPH radical scavenging activity of the extract increases with increasing concentration are shown in table 5. It shows the IC_{50} of the ethanolic extract of *Brassica nigra* and standard vitamin C was found to be 440.36 µg/ml and 295.51µg/ml. The antioxidant activity of the same extract in increasing concentration compared with the Vitamin C (standard) by reducing power assay are displayed in table 6 and the IC_{50} value of sample extract and the standard was 412.05 µg/ml and 286.56 µg/ml. The IC_{50} of the ethanolic extract of the plant and vitamin C by hydroxyl scavenging assay were found to be 393.56µg/m and 306.30µg/ml are shown in table.4. The IC_{50} of the ethanolic extract of Plant and Ascorbate in the method using ABTs+ radical scavenging activity was found to be 376.22µg/ml and 284.57µg/ml are presented in table.5. The IC_{50} of the ethanolic extract of Plant and Ascorbate in the method using superoxide radical scavenging activity was were found to be 439.73 µg/ml and 284.48µg/ml are shown in table.6. The invitro antioxidant activity of the ethanolic extracts of *Brassica nigra* was determined by the above methods showing that the plant was found to be more effective compared with the standard Ascorbic acid (Vitamin C). The extract of plant was capable of reducing DNA damage at all concentrations in various assay methods.

An in-vitro antioxidant activity of the *Brassica nigra* extract revealed that good results by increasing the concentration of the compounds at (100, 200, 300, 400 and 500) using different methods. Normally ascorbic acid (vitamin) is used as standard; it showed highest antioxidant activity at all concentrations. Chemical constituents such as phenols and flavonoids predominantly associated with this antioxidant property of the plant *Brassica nigra*.

Table 1: Qualitative analysis of phytoconstituents present in ethanolic extract of *Brassica nigra.*

Phytochemicals	Ethanol
Alkaloids	+++
Phenols	+++
Flavonoids	+++
Tannins	+
Saponins	++
Terpenoids	++
Steroids	+++
Carbohydrates	+
Glycosides	+
Amino acids	+
Proteins	++

Antioxidant activities of ethanolic extract of plant *Brassica nigra* (Black mustard)

Table 2: DPPH scavenging assay.

S. No.		% inhibition				
		100(µg/ml)	200(µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)
1	Sample	11.45± 0.20	24.22 ± 0.50	36.20 ± 0.30	45.25 ± 0.90	58.20 ± 0.20
2	Standard (Vitamin C)	17.55 ± 0.20	36.83 ± 0.10	50.78±0.30	71.49 ± 0.90	83.20±0.10

The experiment was conducted in triplicates (n = 3)

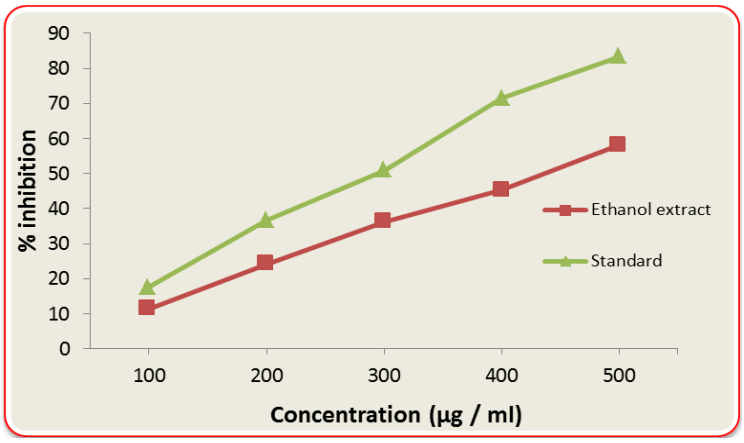

Figure 1: DPPH scavenging assay.

IC_{50} value of Sample : 440.36 µg/ml

IC_{50} value of Vitamin C (standard) : 295.51µg/ml

Table 3: Reducing power assay.

S. No.		% inhibition				
		100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)
1	Sample	14.83± 0.45	25.28 ± 0.65	37.38 ± 0.50	48.83 ± 0.20	57.38 ± 0.43
2	Standard (vitamin C)	17.73 ± 0.70	36.82 ± 0.95	51.84± 0.20	64.88 ± 0.90	77.37± 0.20

The experiment was conducted in triplicates (n=3).

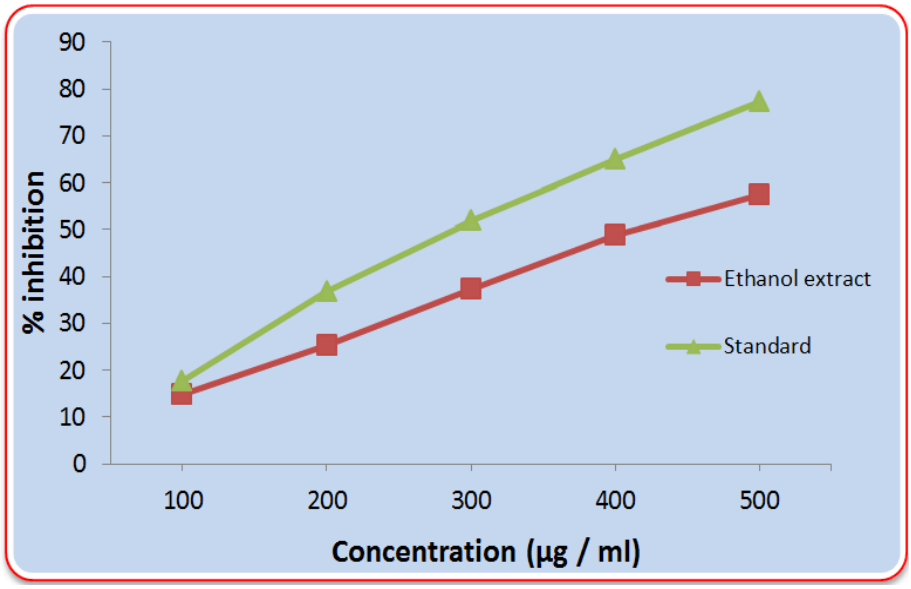

Figure 2: Reducing power assay.

IC_{50} value of Sample : 412.05 µg/ml

IC_{50} value of Vitamin C (standard): 286.56 µg/ml

Table 4: Hydroxyl free radicle scavenging assay.

S. No.		% Inhibition				
		100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)
1	Sample	13.72± 0.89	26.42 ± 0.85	38.42 ± 0.40	50.82 ± 0.62	63.82 ± 0.30
2	Standard (Vitamin E)	17.53 ± 0.30	36.90 ± 0.20	49.20± 0.80	62.85 ± 0.90	73.38± 0.50

The experiment was conducted in triplicates (n=3)

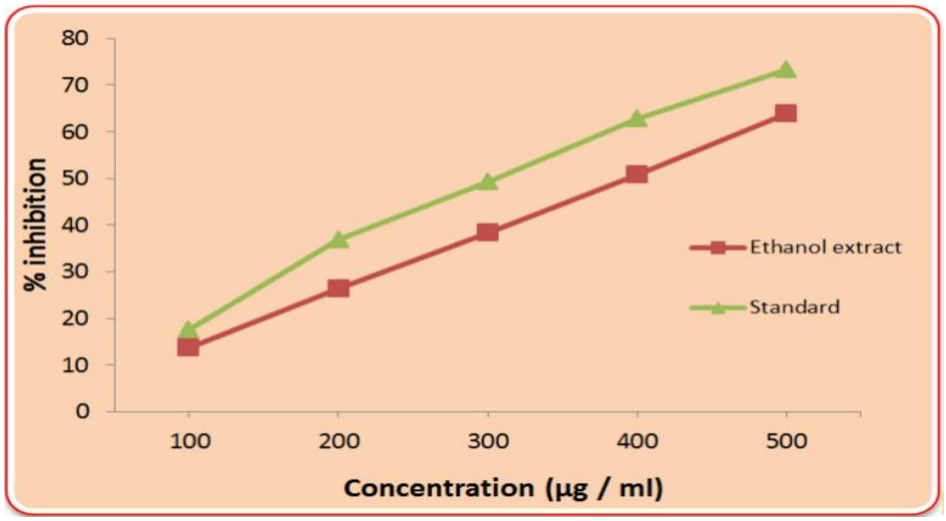

Figure 3: Hydroxyl free radicle scavenging assay.

IC_{50} value of Sample : 393.56 µg/ml

IC_{50} value of Vitamin E (standard): 306.30 µg/ml

Table 5: ABTS+ radical scavenging assay.

S. No.		% Inhibition				
		100 (µg/ml)	**200 (µg/ml)**	**300 (µg/ml)**	**400 (µg/ml)**	**500 (µg/ml)**
1	Sample	18.12± 0.30	28.59 ± 0.30	41.48 ± 0.20	52.59 ± 0.10	64.38 ± 0.50
2	Standard (Vitamin C)	20.11 ± 0.50	37.25 ± 0.59	52.48± 0.20	69.28 ± 0.50	80.26± 0.10

The experiment was conducted in triplicates (n=3)

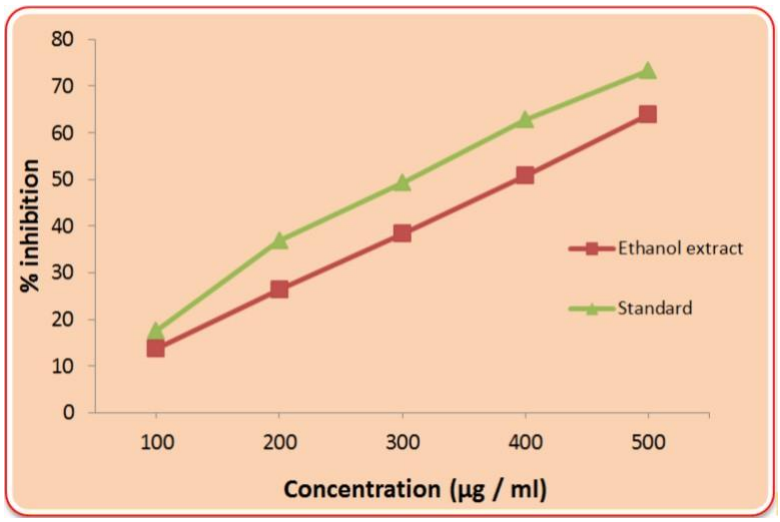

Figure 4: ABTS+ radical scavenging assay.

IC_{50} value of Sample : 376.22 µg/ml

IC_{50} value of Vitamin C (standard): 284.57 µg/ml

Table 6: Superoxide radical scavenging assay.

S. No.		% inhibition				
		100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)
1	Sample	11.49± 0.10	23.69 ± 0.50	35.37 ± 0.60	44.80 ± 0.20	59.36 ± 0.10
2	Standard (Vitamin C)	15.69 ± 0.10	34.70 ± 0.22	52.69± 0.30	68.27 ± 0.20	79.49± 0.50

The experiment was conducted in triplicates (n=3)

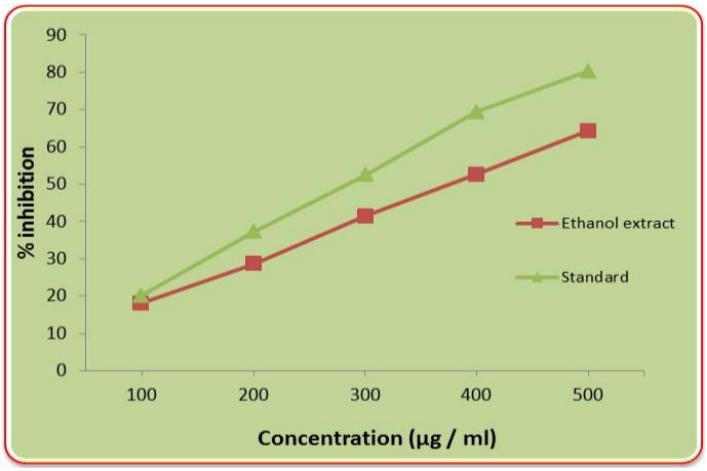

Figure 5. Superoxide radical scavenging assay.

IC_{50} value of Sample : 439.73 µg/ml

IC_{50} value of Vitamin C (standard) : 284.48µg/ml

CONCLUSION

The present work was focused on evaluating the resultant aerial parts ethanolic extract from *Brassica nigra* using the Soxhlet extraction method. This extract has high amounts of phenols, flavonoids, saponin, tannins steroid, terpenoid and also, exhibited the strong antioxidant potential were confirmed through the DDPH assay, reducing power assay, hydroxyl free radical scavenging activity, ABTs+ radical scavenging activity and superoxide anion scavenging activities. Extracts from the aerial parts of *Brassica nigra* are a valuable source of compounds that promote health in this context, fulfilling at the same time the promising antioxidant activity that can be practically used as food supplements, to delay lipid oxidation and heal from specific ailments via its free-radical scavenging ability. It would be interesting to conduct more researches to inspect the role of bioactive components which responsible for these activities. Hence, more studies are necessary to estimate the antioxidant, anticancer and antimicrobial efficiencies of their individual purified fractions which contain a novel compound helps in curing disease, promoting rapid healing and formation of new

tissues. The results showed in the present study indicate that the plant *Brassica nigra* (Black mustard) is a potential source of natural antioxidants.

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