IJPSR (2011), Vol. 2, Issue 2 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 September, 2010; received in revised form 23 November, 2010; accepted 14, January 2011

ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF THE LEAF EXTRACT OF *BRASSICA NIGRA*

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Keywords:

Anti-oxidant,
Anti-Inflammatory,
Brassica nigra,
In vitro,
In vivo

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ABSTRACT

Crude ethanolic extract of *Brassica nigra* was studied to detect the chemical compounds as well as to evaluate the antioxidant and anti-inflammatory activities. The different antioxidants assays including total antioxidant activity, DPPH, nitric oxide (NO) scavenging, reducing power, total phenolic content and flavonoid content were studied. Phytochemical screening showed the presence of alkaloids, flavonoids, glycosides and carbohydrates in the extract. Total phenol content in the plant was 6.67 mg/g of galic acid. *Brassica nigra* was found to contain 2.04 mg/g of quercetin in flavonoid assay. Total antioxidant capacity of the extract was found to be 97.08 mg/g of ascorbic acid. *Brassica nigra* showed IC_{50} value of 63.09µg/ml whereas the standard antioxidant showed IC_{50} value 14.45 µg/ml in DPPH method. The standard antioxidants ascorbic acid, galic acid and quercetin showed the reducing power 485.75%, 736.30% and 763.01%, respectively whereas *Brassica nigra* showed the value 263.69%. IC_{50} value in NO scavenging activity of the extract was found to be 118.21 µg/ml whereas ascorbic acid showed the value 5.47 µg/ml and quercetin had the value 15.24 µg/ml. *In vivo* and *in vitro* anti-inflammatory activity of the crude extract was evaluated using carrageenan induced rat paw edema and protease enzyme inhibition assay, respectively. *In vivo* anti-inflammatory test of the ethanolic extract of *Brassica nigra* (500 mg/kg) gave 17.9% inhibition whereas standard Phenylbutazone (100mg/kg) gave 39.38%. *In vitro* anti-inflammatory test of *B. nigra* by protease inhibition method also gave 42.57% inhibition of trypsin at dose 250 µg/ml.

INTRODUCTION: Most of the potentially harmful effects of oxygen are believed to be due to the formation and activity of reactive oxygen species acting as oxidants frequently in the form of free radicals [1]. Cell damage caused by free radicals appears to be a major contributor to aging, cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction [2]. Overall, free radicals have been implicated in the pathogenesis of at least 50 diseases [3]. Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. Synthetic antioxidants are widely used because they are effective and cheaper than natural types.

However, the safety and toxicity of synthetic antioxidants have been important concerns [4]. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea, and herbs may contribute to shift the balance toward an adequate antioxidant status[5]. The interest in natural antioxidant, especially of plant origin, has greatly increased in recent years[6]. Also many other plant species have been investigated in the search for natural antioxidants but generally there is still a demand to find more information concerning the antioxidant potential of plant species. The study of plants that have been traditionally used for inflammation is still fruitful and logical research strategy in the source of new anti-inflammatory drugs[7].

Brassica nigra (Family- Brassicaceae) popularly known as mustard has both edible and medicinal value. The plant is used in the treatment of rheumatism, as a means of reducing congestion in internal organs. Traditionally the plant is also applied in neuralgia and spasms, alopecia, epilepsy, snakebite, and toothache. It is also used to treat carcinoma, throat tumors, and imposthumes. A liquid prepared from the seed, when gargled, is said to help tumours of the sinax. The seed is eaten as a tonic and appetite stimulant. Hot water poured onto bruised mustard seeds makes a stimulating foot bath and can also be used as an inhaler where it acts to throw off a cold or dispel a headache. Mustard Oil is said to stimulate hair growth. Mustard is also recommended as an aperients ingredient of tea, useful in hiccup. Mustard flour is considered antiseptic[8]. Our aim in this study is to evaluate the antioxidant and anti-inflammatory properties of ethanolic extract of *Brassica nigra*.

MATERIALS AND METHODS:

Plant Materials: The plant *Brassica nigra* was collected from the village Uthali under Jibannagar thana of Chuadanga district, Bangladesh during the month of August 2009 and was identified by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka (Ref no 33168).

Preparation of the extract: The leaves of the plant were collected in fresh condition. It was sun-dried and then, dried in an oven at reduced temperature (not more than 50^0C) to make suitable for grinding. The powdered plant materials were submerged into ethanol in an air-tight flat bottomed container for seven days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant materials were dissolved in the solvent.

Drugs and Chemicals: Carrageenan, phenylbutazone 1, 1- diphenyl- 2- picryl- hydrazyl (DPPH), trypsin, ascorbic acid, quercetin, galic acid were obtained from Sigma Chemical Co. (MO, USA). Folin- Ciocalteu reagent (FCR), tris-HCl buffer (pH 7.4), casein, Grise reagent were purchased from E Merc. All other chemicals and reagents were of analytical grade.

Animals: The experiments were carried out on Albino rats (Swiss strain). They were obtained from ICDDRB. Rats of 2-3 months old, weighing 130-170 gm were used. The rats were kept in groups of 5 in each polyvinyl cages (BIK industries, India) having

dimensions of 408mm X 280mm X 150 mm. The animals were given standard rat feed and water kept in the laboratory environment for seven days. They were fasted overnight and weighed before the experiment. The standard rat feed was developed by the Bangladesh Council for Scientific and Industrial research, Dhaka.

Phytochemical screening: Various phytochemical tests which were performed under the heading of phytochemical screening are Molisch's test for carbohydrates, general test for Glucosides, test for Glycosides, Borntragers' test for Anthraquinone glycosides, tests for alkaloids by Mayer's reagent; Hager's reagent; Wagner's reagent and Dragendroff's reagent, test for Saponins, test for Flavonoids, test for Steroids and test for Tannins.

Determination of Total Phenol: Total phenols were determined by Folin Ciocalteu reagent [9]. A dilute extract of plant extract (0.5ml of 1:10g/ml) or Gallic acid (Std. phenolic compound) was mixed with Folin Ciocalteu reagent (5ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4ml, 1M). The mixtures were allowed to stand for 15 minutes and the total phenols were determined by colorimetry at 765nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250mg/l solutions of Gallic acid in methanol: Water (50:50, v/v). Total phenol values are expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

Determination of Flavonoid content: Aluminum chloride colorimetric method was used for flavonoids determination[10]. Plant extracts (0.5ml of 1:10g/ml) in methanol were separately mixed with 1.5ml of methanol, 0.1ml of 10% Aluminum chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It remained at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415nm. The calibration curve was prepared by using quercetin solutions at concentrations 12.5 to 100 µg/ml in methanol.

Determination of total antioxidant capacity: The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH [11]. The antioxidant capacity is expressed as ascorbic acid equivalent (AAE). Plant extract (0.3ml) was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95^oC for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank. Total antioxidant capacity of plant extract was measured from the regression equation prepared from the concentration versus optical density of ascorbic acid.

DPPH Scavenging Activity: DPPH scavenging activity of the *Brassica nigra* was measured by the method developed by Manzocco *et.al* [12]. 0.2 ml of the sample extract was diluted with methanol and 2 ml of DPPH solution (0.5mM) was added. After 30 min, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging was calculated by the following equation:

$$\text{Scavenging activity (\%)} = (1 - A1/A0) \times 100,$$

Where: $A0$- absorbance of the blank (methanol replacing the extract), $A1$- absorbance in the presence of the sample extract

Total Reducing Power Determination: The reducing power of the plant was determined according to the method of Oyaizu [13]. 10 mg of extract in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50^0C for 20 minutes. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged

at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Gallic acid, Quercetin and Ascorbic acid were used as reference compounds. All the analyses were performed in triplicate. Increased absorbance of the reaction mixture indicated increasing reducing power.

NO Scavenging Activity: The scavenging effect of the *Brassica nigra* on nitric oxide was measured according to the method of Alisi and Onyeze [14]. 4 ml of extract solution at different concentrations were added in the test tubes to 1ml of sodium nitroprusside solution (5mM) and the tubes incubated at 29°C for 2 h. An aliquot (2 ml) of the incubation solution was removed and diluted with 1.2 ml Griess reagent (1% Sulfanilamide in 5% H_3PO_4 and 0.1% Naphthylethylenediamine dihydrochloride). The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550nm.

***In vivo* anti-inflammatory activity:** The method was essentially that of Winter *et al.,* [15] with minor modifications [16]. The animals were weighed and randomly divided into three groups consisted of 5 rats in each group. Group-I received the test compound ethanolic extract at the doses of 500mg/kg of body weight. Group II received phenylbutazone (PBZ) as standard drug at a dose of 100mg/kg of body weight while Group- V was kept as control giving 1% Tween-80 in distilled water. After half an hour of drug administration, 0.1 ml of 1% (w/w) Carrageenan solution was injected through 26 gauge needle into the subplanter surface of the right hind paw of each rat of each group. The maximum linear cross section of the joint was measured before Carrageenan administration and similar measurements were made 3 hours after the administration of Carrageenan injection administration was calculated to observe the time dependent activity of drugs. The average percent increase in paw diameter with time was calculated and compared against the control group. Percent inhibition was calculated using the formula:

Inhibition of inflammation= (Vc-Vt)/Vc x 100,

Where Vc and Vt represents the average paw diameter of control and treated animals respectively.

***In vitro* anti-inflammatory activity:** The reaction mixtures (2.0 ml) contained 0.06 mg trypsin, 1.0 ml of 25 mM tris-HCl buffer (pH 7.4) and 1.0 ml of aqueous solution of ethanolic extract of *Brassica nigra* (50-250 µg/ml). The mixtures were incubated at 37°C for 5 minutes. 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 minutes. 2.0 ml of 70 % perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged. Absorbance of the supernatant was read at 280 nm against buffer as blank [7, 17]. The percentage of inhibition was calculated as above. Each experiment was done in triplicate and taken the average.

Statistical analysis: All the in vitro experimental results were mean±SEM of three parallel measurements. Results of *in vivo* study were given as mean±SEM and data were evaluated by using student's t test. *P* values<0.001 were regarded as significant.

RESULTS AND DISCUSSION:

Phytochemical screening: The results of various qualitative chemical tests for the detection of chemical constituents of *Brassica nigra* is shown in the **Table 1**.

TABLE 1: RESULTS OF PHYTOCHEMICAL SCREENING

Test for	*Brassica nigra*
Carbohydrates	+
Glycosides	+
Glucosides	+
Anthraquinone glycosides	-
Alkaloids	+
Saponins	-
Flavonoids	+
Steroids	-
Tannins	-

'+' Indicates Positive Reaction '-' Indicates No Reaction

Since the chemical constituents present in a plant are directly responsible for its therapeutic and other pharmacological properties, the constituents of the plant which are detected during this investigation should have some direct relationship with local medicinal uses. Brock *et al.,* [18] reported the presence of nortropane alkaloids in *Brassica nigra*. The presence of sinigrin which is one of the gycosides in *Brassica nigra* has been well established [19]. Khan isolated a flavanoid called isorhamnetin from *Brassica nigra* [20]. This investigation and other earlier researches indicate the presence of flavonoids in the plant. Thus, it was expected that the selected plants analyzed under this research work should have some direct therapeutic effects against oxidative stresses as well as inflammation.

Determination of Total Phenol: The content of total phenolics in the ethanolic plant extracts was determined using the Folin- Ciocalteu assay, calculated from the regression equation of the calibration curve ($y=0.013x+0.127$, $r^2=0.988$) and is expressed as galic acid equivalents (GAE). It was observed that the content of phenolics in the extracts under this investigation correlates with the antioxidant activity. The phenolic content in *Brassica nigra* was 6.67 mg/g GAE. Phenolic compounds are commonly found in both edible and inedible plants that are reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of the phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [21].

Flavonoid content Assay: Flavonoid content was calculated from the regression equation of the calibration curve ($y = 0.009 - 0.036$) and is expressed as Quercetin equivalents (QE). *Brassica nigra* extract contains 2.04 mg/g quercetin equivalent. Nair S reported the similar flavonoid content in mustard seed [22]. Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health such as antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities. Flavonoids also protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite.

Total Antioxidant Assay: The total antioxidant capacities of the ethanolic extracts of the selected plants were determined from the calibration curve established by ascorbic acid at 695 nm. The regression line was $y=.005x-0.028$. Ascorbic acid equivalent of *Brassica nigra* was found to be 82.08 mg/g. The study reveals that the antioxidant activity of the extracts is in the increasing trend with the increasing concentration of the plant extract. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed method and hence, it was decided to extend its application to plant extracts.

DPPH Scavenging Activity: Figure 1 showed the decrease in absorbance of DPPH free radical due to the scavenging ability of the soluble solids in different concentrations of plant extract and standard ascorbic acid. IC_{50} Value (µg/ml) of *Brassica nigra* was found to be 63.09±1.25 while ascorbic acid showed the value 14.45±0.03 (Table 2). Seong Soo Han reported that *Brassica juncea* has IC_{50} value 42 µg/ml which does not deviate so far from these study[23].

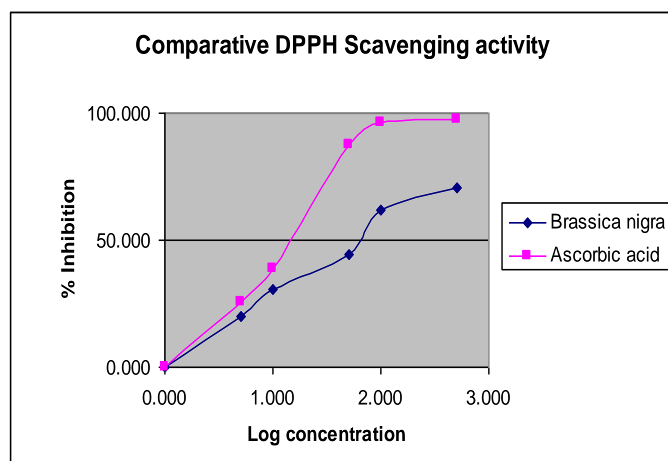

FIG. 1: DPPH SCAVENGING ACTIVITY OF BRASSICA NIGRA WITH STANDARD

Nitric Oxide Scavenging Activity: IC_{50} value of *Brassica nigra* was found to be 118.21±1.29µg/ml whereas ascorbic acid and quercetin showed the value 5.47±0.29µg/ml and 15.24±0.96µg/ml respectively (**Table 2**) during nitric oxide scavenging activity determination. The plant extracts showed NO scavenging activity in a concentration dependent manner. So it can be claimed that the constituents of the plant have the property to counteract the effect of NO formation and in turn may be of considerable benefit in nitrosative stress. Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes.

TABLE 2: COMPARATIVE NO AND DPPH SCAVENGING ACTIVITY OF THE EXTRACTS WITH STANDARDS

Sample	NO (IC_{50} µg/ml) Mean±SE.	DPPH (IC_{50} µg/ml) Mean±SE.
Brassica nigra	118.21±1.29*	63.09±1.25*
Ascorbic Acid	5.47±0.29	14.45±0.03
Quercetin	15.24±0.96	

[a]NO is the inhibition of NO production (IC_{50}: µg/ml), [b]DPPH is the free radical scavenging activity (IC_{50}: µg/ml), *$p < 0.001$ by student's test for values between the sample and the control

Reducing Power Assessment: At 200 µg/ml, the reducing power of the crude extract was 263.69±1.12 where as ascorbic acid, quercetin and galic acid showed 485.653±0.19, 763.01±0.36 and 736.301±0.54 respectively (**Table 3**). The reducing power increased as the extract concentration increased, indicating some compounds in the extracts is both electron donors and could react with free radicals to convert them in to more stable products and to terminate radical chain reactions. The antioxidant activity has been reported to be concomitant with the development of reducing power. The reducing power of the ethanolic extracts of the plant might be due to its hydrogen donating ability, as described by Shimada *et al.,* [24].

TABLE 3: COMPARATIVE REDUCING POWER OF THE EXTRACTS WITH STANDARDS (200 µG/ML)

Sample	% Reducing Power Mean±SE
Brassica nigra	263.69±1.12
Ascorbic Acid	485.653±0.19
Galic Acid	736.301±0.36
Quercetin	763.01±0.54

In vitro Anti-inflammatory activity: The protease inhibition of the extracts is shown in the following (**Table 4**) that indicates the significant anti-inflammatory activity of *Brassica nigra.*

TABLE 4: PERCENT ((%)) PROTEASE INHIBITION OF BRASSICA NIGRA

Dose	%Protease Inhibition
50 μg/ml	15.26
100 μg/ml	22.14
150 μg/ml	25.36
200 μg/ml	32.89
250 μg/ml	42.57

Enzymes and proteins play an essential role in inflammation and other functions of the immune system. Proteolytic enzymes such as bromelain, papain, trypsin and chymotrypsin are essential regulators and modulators of the inflammatory response [25].

Trypsin has been shown to induce *in vivo* epidermal proliferation, vasodilatation and inflammatory infiltration in the upper epidermis by the activation of PAR2 family. The expression of PAR2 on endothelial cells and inflammatory cells including neutrophils and macrophages, determines the involvement of PAR2 in both proinflammatory and anti-inflammatory responses of different experimental models of inflammation [26]. An earlier report indicates flavonoid to be a competitive inhibitor of trypsin[27]. Since *Brassica nigra* contains flavonoids that are responsible for inhibition of trypsin enzyme and thus act as anti-inflammatory agent.

***In vivo* anti-inflammatory activity:** Anti-inflammatory activity of the ethanolic extracts of *Brassica nigra* was comparable to that phenylbutazone. Although the extracts exhibited significant inhibition of paw edema but the percent inhibition of paw edema 17.90%, was much less than standard drug phenylbutazone 39.38% of paw edema (**Table 5**).

TABLE 5: COMPARATIVE EFFECTS OF THE EXTRACT & PBZ ON CARRAGEENAN INDUCED RAT PAW EDEMA

Sample	Dose	Increment of paw diameter in mm at 3rd hour(mean)	% inhibition of inflammation
Phenylbutazone	100 mg/kg	0.982	39.382
Plant extract	500mg/kg	1.333	17.901*

Values are mean ± SE, (n =5); * $p < 0.001$, Student's t test as compared to control

It has been established that the initial phase of the edema can be attributed to the release of histamine & serotonin (5-HT), the maintenance of edema during the plateau phase can be attributed to kinin-like substances and the second accelerating phase of swelling are due to prostaglandin like substance. Since the test material exhibited significant inhibition of edema at 3rd hour after administration of carrageenan in comparison to control, the possible mechanism of anti-inflammatory activity of the test material might be its ability to inhibit the biosynthesis of prostaglandins from arachidonic acid by inhibiting the enzyme cyclooxygenase.

However, the inhibitory effect of the extract on the release of histamine or serotonin or kinin like substances could not be ruled out, probably all the extracts might be showed significant inhibition of rat paw edema during 1st hour & 2nd hour of carrageenan administration. From phytochemical study, it was observed that flavonoids were present in the plant extracts. Universally it is established that flavonoids have anti-inflammatory effects. The anti-inflammatory effects of flavonoids are due to actions on blood vessels, inflammatory cells & inflammatory mediators. However, the exact mechanism of action as an anti-inflammatory agent still remains obscured. Better information can be obtained by further studies including fractionation of the extracts, isolation& purification of the active constituents. Synthesis of active constituent may be done if proved possible.

CONCLUSION: Based on the results of this study, it is clear that *Brassica nigra* has powerful *in vitro* antioxidant capacity against various antioxidant systems. At the same time it also shows *in vitro* and *in vivo* anti-inflammatory activity. However, further studies are necessary to examine underlying mechanisms of antioxidant and anti-inflammatory effects and to isolate the active compound(s) responsible for these pharmacological activities.

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