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Original Article

Antioxidant capacity of *piper longum* and *piper nigrum* fruits grown in Bangladesh

Proity Nayeeb Akbar[1,2], Ismet Ara Jahan[1*], M. Hemayet Hossain[1], Rajib Banik[1], Husna Purvin Nur[1] and Md. Tofazzal Hossain[1]

[1]Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Quadrat-E-Khuda Road, Dhanmondi, Dhaka-1205
[2]Randolph College, 2500 Rivermont Avenue, Virginia, USA

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ABSTRACT

The study is performed to determine and compare the antioxidant capacity of water and ethanol extracts of *Piper longum* and *Piper nigrum* fruits grown in Bangladesh. DPPH, ABTS and nitric oxide free radical scavenging activity, FRAP, superoxide dismutase, Fe^{2+} ion chelating ability, total antioxidant capacity, reducing power assay, total flavonoid, and total phenolic content determination assays were used for evaluation of antioxidant capacity. The ethanol extracts of *P. longum* (long pepper from Dhaka, long pepper from Rajshahi) and *P. nigrum* (white pepper, black pepper) fruits showed significant activities compared to the water extracts in most of the antioxidant assays in a dose dependent manner. The total phenolic and flavonoid contents ranged between 32.83 to 174.92 mg of GAE/g of dry extract and 33.44 to 172.98 mg of QE/g of dry extract, respectively. In total antioxidant capacity and FRAP assay, the activity of the extracts ranged from 9.05 to 199.17 mg AAE/g extract and 5.32 to 18.33 mg $FeSO_4$E/g extract. In DPPH scavenging and SOD assay, the percent inhibitions were found to be similar. However, ABTS, Fe^{2+} scavenging, and reducing power assay showed better results for ethanol extracts, while nitric oxide scavenging activity showed better activity for water extracts.

Keywords: *Piper Longum*, *Piper Nigrum*, Antioxidant Activity, Total Phenolic, Total Flavonoid.

INTRODUCTION

Many plant-derived molecules have shown a promising effect in therapeutics [1]. Spices and herbs are recognized as sources of natural antioxidants and thus play an important role in the chemoprevention of diseases and aging. Among the plants investigated to date, one showing enormous potential is the pepper family otherwise known as *Piperaceae* [2]. *Piper longum* and *Piper nigrum* are the two flowering vine in the family *Piperaceae*. *P. longum*, also known, as Long Pepper is one of the most widely used Ayurvedic herbs original to northeastern and southern parts of Bangladesh, India and Srilanka. It is used for the treatment of respiratory tract diseases like cough, bronchitis, asthma, cold, as counter-irritant and analgesic [3]. It is applied locally for muscular pain and inflammation and used internally as a carminative in conditions such as loss of appetite and sleeplessness [4]. *P. nigrum* is another well-known climbing vine native to Bangladesh, southern India and Srilanka and is cultivated almost everywhere in the tropical regions especially for its fruits, which are used as spices. *P. nigrum*, also known as black Pepper or in many cases white pepper, is produced from the still-green drupes of the pepper plant. Once dried, the spice is called black pepper. White pepper, however, consists of the seed of the pepper plant alone, with the darker-colored skin of the pepper fruit removed. It has a slightly different flavor than black pepper, due to the lack of certain compounds present in the outer fruit layer of the drupe, but not found in the seed. Externally, both black and white pepper are used for its rubefacient and as a local application for relaxed sore, throat and some skin disorder. It has anti-microbial [5] and anti-mutagenic properties [6]. Inhalation of black pepper oil also increases the reflexive swallowing movement [7]. In addition, an animal study showed that piperine, a constituent of black and white pepper beneficially improved the plasma levels of insulin [8].

***Corresponding Author Address:** Ismet Ara Jahan, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Quadrat-E-Khuda Road, Dhanmondi, Dhaka-1205, Bangladesh; E-mail: ismet0103@yahoo.com*

The major chemical constituents of the plants are volatile oil, resin and alkaloids viz. piperine, piperlongumine, piperlonguminine, pipperin, pippalartin, piplartine etc [9]. It also contains polyphenols, which includes flavanols, flavonoids, flavandiols and phenolic acids and amides including N-isobutyleicosa-2,4-dienamide, N-Isobutyleicosa-2,4,14-trienamide, N-Isobutylocatadeca-2,4,14-trienamide, piperine, guineensine etc [10]. Excessive generation of reactive oxygen species (ROS) and other radicals can damage proteins, carbohydrates, polyunsaturated fatty acids, and DNA, and may thus lead to oxidative stress and to a variety of degenerative processes and diseases such as aging, immunodeficiencies, neurologic disorders, inflammation, arthritis, ischemia, arteriosclerosis, coronary heart disease, stroke, diabetes mellitus, Parkinson's disease, Alzheimer's disease and certain cancers [11, 12, 13] ROS are continuously produced during normal physiologic events and removed by antioxidant defense mechanisms [14]. Therefore, the great interest has been recently focused on the natural foods, medicinal plants and phytoconstituents due to their well known abilities to scavenge free radicals (i.e. antioxidant power). As a part of our ongoing research on investigation of the antioxidant capacity of medicinal plants and spices grown in Bangladesh, we have performed a study on the antioxidant capacity of ethanol and water extracts of *P. longum* from two cultures region Dhaka and Rajshahi, and *P. nigrum* (white pepper and black pepper) from Dhaka, Bangladesh,

MATERIALS AND METHODS

Chemicals: DPPH, ABTS, ascorbic acid, gallic acid, quercetin, BHA, folin-ciocalteu's phenol reagent, griess reagent, NBT, NADH, PMS, TPTZ, Trolox, and ferrozine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium molybdate, sodium acetate, glacial acetic acid, $FeCl_3.6H_2O$, $FeCl_2.4H_2O$, potassium ferricyanide, trichloro acetic acid, phosphate buffer pH 6.6, sodium nitroprusside, phosphate buffer saline pH 7.4, aluminium chloride, potassium persulfate, Tris-HCL buffer, and the solvents were of analytical grade and obtained from SD Fine Chem. Ltd. (Biosar, India).

Plant Material: Seeds of *P. longum* and *P. nigrum* were collected from Dhaka and Rajshahi, Bangladesh during April 2013, and identified by the experts of BCSIR.

Sample Preparation: The fruits of *P. longum* and *P. nigrum* were cleaned, dried and coarsely powdered. The dried powdered samples were stored in an airtight container and kept in a cool place until used for the current research purpose.

Extraction of Plant Materials: 25 g of each of the powdered samples (two sets) were taken and soaked in 250 ml of 95% ethanol and distilled water (250 ml) separately. The samples were shaken for 24 hours using an orbital shaker (VRN-480, Gemmy, Taiwan). The solutions were then filtered through Whatman filter paper (Bibby RE200, Sterilin Ltd., UK) under reduced pressure. Finally, the filtrates were concentrated separately by a rotary vacuum evaporator (R-215, Buchi, Switzerland) at low temperature and then freeze dried. The dried ethanol and water extracts of *P. longum*, Dhaka (2.42 g & 1.94 g); *P. longum*, Rajshahi (2.58 g & 2.01 g); *P. nigrum* (black, 2.95 g & 2.15 g) and *P. nigrum* (white, 2.73 g & 2.08 g) were obtained respectively. The dried extracts were stored in a cool place for further research.

Preliminary Phytochemical Screening: Phytochemical tests were performed on the ethanol and water extracts of *P. longum* and *P. nigrum* for different chemical groups as alkaloids, flavonoids, gums, reducing sugars, saponins, steroids and tannins following the standard methods (Evans, 1989).

Determination of Total Phenolic Content: The total phenolics were determined by the modified folin-ciocalteu method [15]. 1 ml of the aqueous and ethanolic extracts was collected in two 10 ml volumetric flasks separately. To each flask, 5 ml of Folin-Ciocalteu reagent (1: 10 v/v distilled water) and 4ml (75 g/L) of sodium carbonate were added. The solutions were Vortex-ed for 15 seconds and allowed to stand for 30 min at 40°C for color development. The absorbance was measured against the blank in a double beam UV/Visible spectrophotometer (Analykjena, Model 205, Jena, Germany) at absorption maxima 765 nm. Three readings were taken per solution to get reproducible results. The total phenolic content was determined and expressed as mg Gallic acid equivalents per gram of dry extract using the equation obtained from a standard Gallic acid calibration curve, $y = 6.2548x - 0.0925$, $R^2 = 0.9962$.

Determination of Total Flavonoid Content: Aluminium chloride colorimetric method was used for the determination of total flavonoid concentration of the extracts [16]. 1 ml of the extracts was individually mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The solution was allowed to stand for 30 min at room temperature and the absorbance of the

reaction mixture was measured at 415 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). The total flavonoid content was determined as mg of Quercetin equivalent per gram using the equation obtained from a standard Quercetin calibration curve, y = 4.7385x + 0.0355; R^2 = 0.9993.

Methods for Evaluation of Antioxidant capacity

DPPH free radical scavenging activity: The method of Govindarajan [17] was used for performing the DPPH radical scavenging activity. Serial dilutions from the stock solutions were carried out to obtain concentrations of 100, 200, 300, 400 and 500µg/ml. An equal amount of the sample solution and 0.1 mM of solution of DPPH were mixed. The mixture was vortexed and allowed to stand in the dark at 25 °C for 30 min. After incubation, the absorbance of the mixture was read against a blank at 517 nm using a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). The radical scavenging activity was expressed as the inhibition percentage (I %) and calculated as per the equation:
I (%) = (A_{blank} – A_{sample} / A_{blank}) x 100, where A_{blank} is the absorbance of the control (containing all the reagents except the testing compound), and A_{sample} is the absorbance of the experimental sample with all reagents. The IC_{50} value (the concentration of a sample required to scavenge 50% DPPH radical) was calculated from the plot of inhibition (%) against the concentration of the extract. All determinations were carried out in triplicate and the average was noted. Ascorbic acid was used as the standard antioxidant.

ABTS radical scavenging activity: The antioxidant capacity of the extracts was determined by ABTS radical cation as described by Fan YJ and coworkers [18], with some modifications. The ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 16 h. The ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. 1 ml of sample at different concentrations (100 to 500µg/ml) was added to 1 ml of the ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min before the absorbance at 734 nm was recorded. The ABTS scavenging effect was calculated as per the equation:
ABTS scavenging effect = (A_o – A_s / A_o) x 100, where, A_o = absorbance of control and A_s = absorbance of sample.

Nitric Oxide radical Scavenging activity: Nitric oxide (NO) scavenging activity was measured following the method of Dinis and co-workers [19]. Sodium nitroprusside (5 mmol) in phosphate buffered saline was mixed with different concentrations of the different extracts (10–500µg/ml) dissolved in ethanol and incubated at 25°C for 30 min. A control without the test compound but with an equivalent amount of ethanol was taken. After 30 min, 1.5 ml of the incubation solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm. The nitric oxide radical scavenging activity was expressed as the inhibition percentage (I %) and calculated as per the equation:
I (%) = (A_{blank} – A_{sample} / A_{blank}) x 100, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound with all reagents. IC_{50} was calculated from the plot of inhibition (%) against extract concentration. All the tests were carried out in triplicate and average of the absorptions was noted. Ascorbic acid was used as positive control standard for this study.

Ferrous Ion Chelating Ability: The ferrous ion chelating activity was investigated according to the method of Dehpour and coworkers [20]. Different concentrations of the extracts (100-500µg/ml) were added to 0.1 ml solution of 2 mM ferrous chloride ($FeCl_2$). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured at 562 nm by spectrophotometer. The percentage of inhibition of ferrozine-Fe^{+2} complex formations was calculated according to the following equation:
Ferrous ion chelating ability (%) = [(A_o – A_s) /A_o] × 100, where A_o is the absorbance of the control solution (containing all reagents except for the extract); A_s is the absorbance in the presence of the sample (plant extracts). All the tests were carried out in triplicate and the mean value was taken. EDTA was used as the standard.

Superoxide radical scavenging activity: The superoxide anion scavenging activity was measured as described by Gow-Chin and coworkers [21]. The superoxide anion radicals were generated in 3.0 ml of Tris-HCL buffer (16 mM, pH 8.0), containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936

mM) solution, 1.0 ml extract of different concentration (100 to 500 µg/ml), and 0.5 ml Tris-HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12 mM) to the mixture. The mixture was then incubated at 25°C for 5 min before the absorbance was measured at 560 nm against a blank sample. The percent inhibition was calculated by using the following equation:

Superoxide radical scavenging activity= $\{(A_o - A_1)/A_o)*100\}$, where, A_o is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. The process was done in triplicates and the results were averaged.

Determination of total antioxidant capacity: The total antioxidant capacity of the extracts were evaluated by the phosphomolybdenum assay method [22] which is based on the reduction of Mo (VI) to Mo (V) and the subsequent formation of a green phosphate-Mo (V) complex in acidic condition. The extracts were allowed to mix with 3.0 ml of the reagent solution (0.6 M H_2SO_4, 28 mM Na_3PO_4, 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min. After letting the solution cool back to room temperature, the absorbance was measured at 695 nm using a UV-Visible spectrophotometer against a blank solution. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

Ferric Reducing Antioxidant Power (FRAP assay): A modified method [23] was adopted for the FRAP assay. The fresh FRAP solution was prepared by mixing 25 ml acetate buffer (300 mM, pH 3.6, 3.1 g $C_2H_3NaO_2{\cdot}3H_2O$ and 16 ml $C_2H_4O_2$), 2.5 ml TPTZ (10 mM in 40 mM HCl) and 2.5 ml $FeCl_3{\cdot}6H_2O$ (20 mM). The temperature of the solution was raised to 37°C before use. Plant extracts were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark. Readings of the product (ferrous tripyridyltriiazine complex) were taken at 593 nm. The standard curve was linear between 100 and 500 µM $FeSO_4$. Results were expressed in µM Fe (II)/g dry mass and compared with that of Quercetin.

Reducing Power assay: The reducing power of the piper extracts was determined according to the method followed by Dehpour and co-workers [20]. Different concentrations of the plant extracts (100-500µg/ml) in 1 ml of distilled water were 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°C for 20 min. A 10% solution of trichloroacetic acid (2.5 ml) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 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 of the mixture was measured at 700 nm with spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. All the tests were carried out in triplicate and average of the absorptions was recorded. Ascorbic acid was used as the standard reference compounds.

Statistical analysis

Three replicates of each sample were used for statistical analysis and the values were reported as mean ± SD.

RESULTS

Chemical group test: Qualitative analysis carried out for ethanol and aqueous extracts of *P. longum* and *P. nigrum* showed the presence of some medicinally active constituents (Table I). Phytochemical screening revealed the presence of phenolic, flavonoid, reducing sugar, steroid, and tannins at different concentration in both ethanol and water extracts of *P. longum* and *P. nigrum* fruits.

Total Phenolic content: The total phenolic content of the ethanol extracts of PNB, PNW, PLD and PLR were 140.50 ± 0.38, 174.92 ± 0.33, 121.47 ± 0.57 and 81.24 ± 0.32 mg of GAE/ gram, while that of the water extracts were found to be 34.59 ± 0.11, 32.83 ± 0.15, 34.64 ± 0.22 and 105.51 ± 0.82 mg of Gallic acid equivalent/ gram, respectively (Table II). The total phenolic content in the different extracts was found to be in the following order: PNW-E> PNB-E> PLD-E> PLR-W> PLR-E> PLD-W> PNB-W> PNW-W.

Total Flavonoid content: The total flavonoid content of the ethanol extracts of PNB, PNW, PLD and PLR were 110.5 ± 0.73, 62.73 ± 0.19, 41.65 ± 0.15 and 172.98 ± 0.62 mg of QE equivalent/ gram of dry extract, while that of the water extracts were 33.44 ± 0.13, 38.09 ± 0.14, 83.13 ± 0.17 and 46.85 ± 0.18 mg of QE equivalent respectively (Table II). The flavonoid content in different extracts of piper was found to be in the following order: PLR-E> PNB-E> PLD-W> PNW-E> PLR-W> PLD-E> PNW-W> PNB-W.

Antioxidant Activities: As phenolic and flavonoid content in the different extracts of PNB, PNW, PLD, PLR were found to be significant, all the extracts of *P. longum* and *P. nigrum* fruits were screened for their possible antioxidant capacity. For this analysis, eight complementary test systems, like DPPH free radical scavenging, ABTS free

radical scavenging, nitric oxide scavenging, ferrous ion chelating ability, superoxide dismutase radical scavenging (SOD), total antioxidant capacity, ferric reducing antioxidant power (FRAP), and reducing power assay were followed.

DPPH free radical scavenging activity: Table 3 shows the dose-response activity for DPPH radical scavenging activity of the ethanol and water extracts of PNB, PNW, PLD and PLR compared with the standard, ascorbic acid. At a concentration of 500 µg/ml, the scavenging activities of the ethanol extracts of PNB-E, PNW-E, PLD-E and PLR-E were found to be 77.88 ± 0.04, 26.67 ± 0.07, 67.18 ± 0.08 and 76.86 ± 0.04% inhibition, while that of the water extracts of PNB-W, PNW-W, PLD-W and PLR-W were 73.02 ± 0.03, 56.61 ± 0.04, 68.07 ± 0.07 and 56.47 ± 0.05%, respectively. However, the IC_{50} values of ethanol extracts PNB-E, PNW-E, PLD-E and PLR-E were found to be 243.15 ± 1.6, 448.20 ± 3.9, 89.18 ± 0.24 and 266.35 ± 1.7 µg/ml and that of the water extracts of PNB-W, PNW-W, PLD-W and PLR-W were 155.55 ± 2.8, 358.60 ± 2.3, 118.29 ± 2.5 and 424.26 ± 3.5 µg/ml, respectively, against the standard, ascorbic acid with an IC_{50} value of 6.09 ± 0.04 µg/ml (Table III). The % inhibition was found to be in the order: PNB-E> PLR-E> PNB-W> PLD-W> PLD-E> PNW-W> PLR-W> PNW-E.

ABTS free radical scavenging activity: The ABTS radical scavenging activity of the ethanol and water extracts of piper extracts exhibited a dose-dependent relation in comparison to the standard, ascorbic acid (Table IV). The overall activity of the ethanol extracts was observed to be higher than that of the water extracts. The scavenging capacity of the ethanol extracts of PNB-E, PNW-E, PLD-E and PLR-E were 55.88 ± 0.03, 74.87 ± 0.09, 77.96 ± 0.04 and 81.80 ± 0.03%, while that of the water extracts of PNB-W, PNW-W, PLD-W and PLR-W were 79.83 ± 0.09, 44.91 ± 0.05, 65.28 ± 0.05 and 43.56 ± 0.03%, respectively, at the same concentration, where AA showed 96.02 ± 0.03% inhibition. IC_{50} values of the ethanolic extracts of PNB-E, PNW-E, PLD-E and PLR-E were 224.3 ± 1.61, 184.2 ± 1.92, 238.4 ± 1.59 and 224.3 ± 2.01 µg/ml and that of the water extracts of PNB-W, and PLD-W were 154.02 ± 1.59, and 364.2 ± 2.66 µg/ml, respectively, while the standard, ascorbic acid giving a value of 4.02 µg/ml. The % inhibition was found to be in the following order: PLR-E> PNB-W> PLD-E> PNW-E> PLD-W> PNB-E> PNW-W> PLR-E.

Nitric oxide radical scavenging activity: The nitric oxide radical scavenging activity of the ethanol and water extracts of PNB, PNW, PLD and PLR exhibited a dose-dependent relation in comparison to the standard, ascorbic acid (Table V). The NO scavenging activity of the water extracts was found to be higher than those of the ethanol extracts. At 500 µg/ml concentration, the scavenging activities of the ethanolic extracts of PNB, PNW, PLD and PLR were 18.16 ± 0.06, 13.28 ± 0.06, 11.40 ± 0.07 and 8.81 ± 0.09%, while that of the water extracts of PNB, PNW, PLD and PLR were 36.41 ± 0.08, 37.32 ± 0.10, 27.69 ± 0.08 and 20.67 ± 0.08%, respectively. The scavenging activity of AA, at the same concentration, was 77.92 ± 0.03%. The percent inhibition was found to be in the following order: PNW-W> PNB-W> PLD-W> PLR-W> PNB-E> PNW-E> PLD-E> PLR-E.

Ferrous-ion Chelating Ability: Table VI shows the ferrous-ion chelating ability of the different extracts of piper species in a dose-dependent manner. The ethanol extracts showed a higher ferrous-ion chelating ability than the water extracts. At 500 µg/ml, the scavenging activities of the ethanolic extracts of PNB, PNW, PLD and PLR were 65.90 ± 0.07, 69.36 ± 0.07, 72.83 ± 0.08 and 63.06 ± 0.04% respectively, while that of the water extracts of PNB, PNW, PLD and PLR were 27.99 ± 0.03, 20.42 ± 0.05, 39.27 ± 0.05 and 19.95 ± 0.06%. The IC_{50} values of the ethanolic extracts of PNB, PNW, PLD and PLR were 82.36 ± 0.44, 340.32 ± 4.25, 180.51 ± 2.35 and 150.81 ± 2.21 µg/ml, while the standard, ascorbic acid, gave a value of 5.58 ± 0.05 µg/ml. The percent inhibition was found to be in the following order: PLD-E> PNW-E> PNB-E> PLR-E> PLD-W> PNB-W> PNW-W> PLR-W.

Superoxide Dismutase radical scavenging activity: The superoxide dismutase radical scavenging activity of the ethanol and water extracts of piper species is exhibited in Table VII. The ethanol and water extracts showed a similar superoxide radical scavenging activity. At 500 µg/ml, the scavenging activities of the ethanolic extracts of PNB, PNW, PLD and PLR were 55.02 ± 0.09, 52.12 ± 0.04, 52.69 ± 0.05 and 60.33 ± 0.05%, while that of the water extracts of PNB, PNW, PLD and PLR were 60.16 ± 0.06, 58.32 ± 0.06, 55.16 ± 0.06 and 32.48 ± 0.04% with ascorbic acid being 93.85 ± 0.09%. The IC_{50} values of the ethanol extracts of PNB, PNW, PLD and PLR were 320.2 ± 3.25, 482.60 ± 5.21, 482.3 ± 4.17 and 372.81 ± 3.5 µg/ml and that of the water extracts of PNB, PLD and PNW were 447.7 ± 4.15, 381.5 ± 3.90 and 441.2 ± 4.11 µg/ml, respectively. The activity of the standard, ascorbic acid, was observed to be 82.01 ± 0.41 µg/ml. The % inhibition was found to be in the following order: PLR-E> PNB-W> PNW-W> PLD-W> PNB-E> PLD-E> PNW-E> PLR-W.

Total Antioxidant Capacity: The total antioxidant capacity of the ethanol extracts of PNB, PNW, PLD and PLR were 132.34 ± 0.92, 123.32 ± 0.65, 199.17 ± 0.78 and 175.10 ± 0.99 mg of ascorbic acid equivalent/ gram, while that of the water extracts were 9.05 ± 0.08, 26.29 ± 0.21, 40.47 ± 0.15 and 10.10 ± 0.05 mg of ascorbic acid equivalent (Table II). The total antioxidant capacity was found to be in the following order: PLD-E> PLR-E> PNB-E> PNW-E> PLD-W> PNW-W> PLR-W> PNB-W.

Ferric Reducing Antioxidant Power (FRAP assay): The ferric reducing antioxidant power of the ethanol extracts of PNB, PNW, PLD and PLR were 6.62 ± 0.02, 5.82 ± 0.03, 18.33 ± 0.06 and 14.50 ± 0.09 mg of ferrous sulfate equivalent respectively, while that of the water extracts were 6.51 ± 0.01, 5.32 ± 0.08, 7.20 ± 0.09 and 6.75 ± 0.06 mg of ferrous sulfate equivalent (Table II). The reducing power was found to be in the following order: PLD-E> PLR-E> PLD-W> PLR-W> PNB-E> PNB-W> PNW-E> PNW-W.

Reducing Power assay: Table VIII shows the absorbance of the ethanol and water extracts of PNB, PNW, PLD and PLR compared with the standard, ascorbic acid. At a concentration of 500 µg/ml, the average absorbance of PNB, PNW, PLD and PLR in ethanol was 0.25 ± 0.008, 0.42 ± 0.007, 0.19 ± 0.005 and 0.21 ± 0.007, while that of the water extracts of PNB, PNW, PLD and PLR was 0.11 ± 0.006, 0.08 ± 0.006, 0.13 ± 0.007 and 0.14 ± 0.007, respectively. The standard, ascorbic acid, gave an absorbance of 1.99 ± 0.009. Absorbance is directly related to the reducing power. So, the reducing power was observed to be in the following order: PNW-E> PNB-E> PLR-E> PLD-E> PLR-W> PLD-W> PNB-W> PNW-W.

DISCUSSION

Preliminary phytochemical screening of the plants, *P. longum* and *P. nigrum* showed the presence of phenols, flavonoids and tannins (Table I). In the past few years, there has been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. Phenolic compounds and flavonoids have been reported to show antioxidant activity in biological systems and acting as scavengers of singlet oxygen and free radicals [24, 25]. Many plants contain substantial amounts of antioxidants to scavenge the excess free radicals from the human body [26, 27].

The phenolic content in the investigated plant material analyzed is presented in Table II. According to the results of this study, it can be revealed that the high inhibition value in the ethanol extract might be due to the high concentration of phenolic compounds present in the extract. It has been reported that phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups [28]. Polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans [29].

Flavonoids are a type of phenolic compound. They can contribute to a decrease in oxidative stress via inhibition or activation of key regulating enzymes such as xanthine oxidase, phospholipase and nitric oxide synthase [30]. The inhibition of the enzymes by some flavonoids could be due to a reaction of the flavonoid with free radicals generated at the active site of the enzymes. The antioxidant activity of flavonoids is due for both the presence of aromatic OH groups and their number per molecule who are considered to play a pivotal role [31].

P. longum and *P. nigrum* extracts were tested for their antioxidant activity by various *in vitro* assays. The DPPH radical scavenging activity of the extracts is given in Table III. This activity was increased by increasing the concentration of the sample extract. DPPH antioxidant assay is developed based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for a visible deep purple color of DPPH in alcoholic solution and can be measured at absorbance 515 nm. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Moreover, the DPPH radical scavenging activity has been shown to be directly related with the total phenolic content present in the extracts as suggested by many previous reports [32, 33]. This justifies for the low and moderate inhibition of the extracts for DPPH scavenging activity.

ABTS radical scavenging property reflects the ability of an antioxidant species to donate electron and hydrogen atom to inactive these radical species [34, 35]**.** The IC_{50} (µg/ml concentration of antioxidant required to quench 50% of the stable free radical) concentration of the ethanol extracts exhibited better activities than the water extracts did. A standard antioxidant ascorbic acid was used to compare the antioxidant potential and it was found that none of the IC_{50} values of the two extracts were anywhere near to ascorbic acid. The highest inhibition was shown by PNBW, while the rest of the extracts showed relatively lower activity.

Nitric oxide is very unstable under aerobic condition. It reacts with O_2 to produce the stable product nitrates and nitrite through the

intermediates NO_2, N_2O_4 and N_3O_4. It is estimated by using the Griess reagent. In the presence of test compound, also known as the scavenger, the amount of nitrous acid decreases. The extent of decrease reflects the extent of scavenging. Based on the percentage inhibition of aqueous and ethanol extracts, it was found that none of the extracts show a 50% or more inhibition in comparison to the standard ascorbic acid (136 ± 0.08), respectively.

The ferrous ion chelating ability of the extracts was estimated by the method of Dinis [19]. Ferrozine can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals [36]. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. Based on our results, PNB-E showed significant inhibition activity, while the other extracts showed moderate and low inhibitory effect. The most active extract (ethanol) interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine.

Superoxide anion is another harmful reactive oxygen species as it damages cellular components in biological systems. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complexes such as cytochrome [37]. The present study showed potent superoxide radical scavenging activity of *P. longum* and *P. nigrum* fruit extracts. The ethanol extract showed potent antioxidant activity in superoxide radical scavenging method.

The total antioxidant capacity (TAC) of the plant extract is shown in Table II. TAC mainly concentrates on the thermodynamic conversion and measures the number of electrons or radicals donated or quenched by a given antioxidant molecule and measure the capacity of biological samples under defined conditions. The phosphor-molybdenum method was based on the reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH with a maximal absorption at 695 nm [38]. In this assay ethanol extracts were found to have a higher activity, as compared to the water extracts. This study reveals that the antioxidant activity of the extract exhibited increasing trend with the increasing concentration of the plant extract. Thus, the extract demonstrated electron donating capacity, may act as radical chain terminators, transforming reactive free radical species into stable non reactive products [39, 22]. Hence, the ethanol extracts exhibited significant TAC.

The ability of plant extract to reduce ferric ions was determined in FRAP assay. The change in absorbance at 593 nm owing to the formation of blue colored Fe^{+2} tripyridyl triiazine (TPTZ) compound from the colorless oxidized Fe^{+3} form by the action of electron donating antioxidants [23]. The FRAP values of the ethanol extracts of *P. longum* were found to be significantly higher than the other extracts (Table VIII). Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present, thus it can be said that the piper extracts may act as free radical scavenger, capable of transforming reactive free radical species into stable non radical products. The antioxidant potentials of the extracts were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II) at 593 nm and its antioxidant activity increased proportionally with the polyphenolic content.

The reducing power might be due to hydrogen donating ability. Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferricyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. This transformation of Fe^{3+} into Fe^{2+} determines the reducing power ability. The reducing ability of a compound generally depends on the presence of antioxidants, which exert the antioxidant response by donating a hydrogen atom and breaking the free radical chain [40]. The antioxidant principles present in the ethanol and aqueous extracts cause the reduction of Fe^{3+}/ ferricyanide complex to its ferrous form. However, none of the extracts showed significant reducing power in this assay.

The phenolic content was found in higher amount in the ethanol extract of PNW than PNB. The flavonoid content was found significant in both the varieties of *P. longum*. The total antioxidant capacity was also observed to be significant for all the extracts, but *P. longum* ethanol extract was found to be the highest. In FRAP assay, *P. longum* ethanol extract showed better activity. In DPPH assay, *P. longum* ethanol extract from Dhaka

showed better activity in comparison to the other extracts. In ABTS activity, all the ethanol extracts exhibited a better activity than the water extracts. In ferrous ion chelating ability test, all the ethanol extracts were found to show good activity and the *P. nigrum* (black) ethanol extract demonstrated the best activity among all.

CONCLUSION

The antioxidant capacity of any plant extract largely depends on both the composition of the extract and the test system. It can be influenced by a large number of factors, and cannot be fully evaluated by one single method. It is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action [41]. The present study suggested that between the two types of extracts, (ethanol and water) the ethanol extracts of the plants, *P. longum* and *P. nigrum* fruits possess good antioxidant activity that might be helpful in preventing or slowing the progress of various oxidative stress related diseases. These plants can also be seen as a potential source of useful drugs. Further studies are going on these plants in order to isolate, identify, and characterize the bioactive compounds. This study is the most comprehensive comparison between two species of piper plants in different solvents on the basis of their antioxidant properties, phenolic and flavonoid contents. Between the two samples of *P. nigrum*, the activity of *P. nigrum* black was found to be better than that of the white. Of the two *P. longum* samples, the sample from Rajshahi was found to show better activity. The overall activity analysis of the four samples of piper reveals that *P. nigrum* black was the most potent among all the samples. Further studies on the effective antioxidants contained in these fruit fractions and the mechanism by which they protect against disease development are highly recommended.

Abbreviations

PNB-E: *Piper nigrum* (black) ethanol extract; PNB-W: *Piper nigrum* (black) water extract; PNW-E: *Piper nigrum* (white) ethanol extract; PNW-E: *Piper nigrum* (white) water extract; PLR-E: *Piper longum* (Rajshahi) ethanol extract; PLR-W: *Piper longum* (Rajshahi) water extract; PLD-E: *Piper longum* (Dhaka) ethanol extract; PLD-W: *Piper longum* (Dhaka) water extract; AA: Ascorbic acid; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: Azinobis (3-ethyl-benzothiazoline-6-sulfonic acid); BHA: Butylated Hydroxy Anisol; NBT: Nitroblue Tetrazolium; NADH: Nicotinamide Adenine Dinucleotide Hydrazine; PMS: Phenazine methosulfate; TPTZ: 2,4,6-tripyridyl-s-triazine; GAE: Gallic Acid; QC: Quercetin.

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Table I: Phytochemical screening carried out on *P. longum* and *P. nigrum* ethanol and water extracts

Compounds	PNB-E	PLD-E	PNW-E	PLR-E	PNB-W	PLD-W	PNW-W	PLR-W
Anthraquinone	-	-	-	-	-	-	-	-
Alkaloid	+++	+++	+++	+++	+++	+	+++	++
Flavonoid	+++	++	++	+++	++	++	++	++
Phenols	+++	+++	+++	++	+	+	++	+++
Anthocyanoside	-	-	-	-	-	-	-	-
Reducing sugar	++	++	++	++	++	++	++	++
Aminoacid	-	-	-	-	-	-	-	-
Saponin	-	-	-	-	-	-	-	-
Steroid	++	++	++	++	++	++	++	++
Tannin	++	++	++	++	++	++	++	++
Triterpenoid	-	-	-	-	-	-	-	-

Table II: Total phenolic, total flavonoid, total antioxidant capacity and FRAP content of *P. longum* and *P. nigrum* extracts

Plant extract	Total phenolic mg GAE/g extract	Total flavonoids mg QCE/g extract	Total antioxidant capacity mg AA/g extract	FRAP mg $FeSO_4$/g extract
PNB-E	140.50 ± 0.38	110.5 ± 0.73	132.34 ± 0.92	6.62 ± 0.02
PLD-E	121.47 ± 0.57	41.65 ± 0.15	199.17 ± 0.78	18.33 ± 0.06
PNW-E	174.92 ± 0.33	62.73 ± 0.19	123.32 ± 0.65	5.82 ± 0.03
PLR-E	81.24 ± 0.32	172.98 ± 0.62	175.10 ± 0.99	14.50 ± 0.09

PNB-W	34.59 ± 0.11	33.44 ± 0.13	9.05 ± 0.08	6.51 ± 0.01
PLD-W	34.64 ± 0.22	83.13 ± 0.17	40.47 ± 0.15	7.20 ± 0.09
PNW-W	32.83 ± 0.15	38.09 ± 0.14	26.29 ± 0.21	5.32 ± 0.08
PLR-W	105.51 ± 0.82	46.85 ± 0.18	10.10 ± 0.05	6.75 ± 0.06

Table III: DPPH radical scavenging activity of *P. longum* and *P. nigrum* extracts

Concentration (µg/ml)	DPPH radical scavenging activity (% inhibition)								
	AA	PNB-E	PLD-E	PNW-E	PLR-E	PNB-W	PLD-W	PNW-W	PLR-W
100	97.06 ± 0.02	39.39 ± 0.02	52.89 ± 0.02	34.40 ± 0.05	25.62 ± 0.02	41.76 ± 0.08	48.54 ± 0.01	36.47 ± 0.02	26.64 ± 0.02
200	97.24 ± 0.03	43.79 ± 0.03	54.10 ± 0.03	23.24 ± 0.03	42.74 ± 0.09	57.76 ± 0.05	59.25 ± 0.07	39.39 ± 0.03	34.03 ± 0.03
300	96.87 ± 0.04	59.25 ± 0.02	61.83 ± 0.05	30.90 ± 0.08	54.45 ± 0.03	61.76 ± 0.09	61.76 ± 0.04	46.03 ± 0.03	39.39 ± 0.03
400	95.12 ± 0.05	71.69 ± 0.03	64.20 ± 0.07	37.86 ± 0.02	67.35 ±0.07	68.61 ± 0.04	64.81 ± 0.06	53.62 ± 0.04	48.47 ± 0.04
500	95.70 ± 0.06	77.88 ± 0.04	67.18 ± 0.08	26.67 ± 0.07	76.86 ± 0.04	73.02 ± 0.03	68.07 ± 0.07	56.61 ± 0.04	56.47 ± 0.05
IC_{50}	6.09 ± 0.04	243.15 ± 1.6	89.18 ± 0.24	448.20 ± 3.9	266.35 ± 1.7	155.55 ± 2.8	118.29 ± 2.5	358.60 ± 2.3	424.26 ± 3.5

Table IV: ABTS radical scavenging activity of *P. longum* and *P. nigrum* extracts

Concentration (µg/ml)	ABTS radical scavenging activity (% inhibition)								
	AA	PNB-E	PLD-E	PNW-E	PLR-E	PNB-W	PLD-W	PNW-W	PLR-W
100	94.87 ± 0.02	22.88 ± 0.02	39.87 ± 0.02	37.87 ± 0.06	21.63 ± 0.08	35.50 ± 0.02	27.40 ± 0.04	22.21 ± 0.09	22.95 ± 0.02
200	94.93 ± 0.03	49.33 ± 0.05	44.92 ± 0.07	52.82 ± 0.05	47.64 ± 0.05	62.91 ± 0.08	38.27 ± 0.06	31.59 ± 0.05	29.19 ± 0.07
300	95.34 ± 0.02	51.54 ± 0.08	58.95 ± 0.05	69.14 ± 0.08	58.28 ± 0.09	72.03 ± 0.07	46.35 ± 0.07	32.18 ± 0.08	34.90 ± 0.08
400	95.68 ± 0.02	52.10 ± 0.07	77.08 ± 0.06	71.16 ± 0.07	71.95± 0.04	75.04 ± 0.04	52.39 ± 0.08	37.92 ± 0.07	39.29 ± 0.05
500	96.02 ± 0.03	55.88 ± 0.03	77.96 ± 0.04	74.87 ± 0.09	81.80 ± 0.03	79.83 ± 0.09	65.28 ± 0.05	44.91 ± 0.05	43.56 ± 0.03
IC_{50}	4.01 ± 0.06	224.3 ± 1.61	238.4 ± 1.59	184.2 ± 1.92	224.3 ± 2.01	154.02 ± 1.59	364.2 ± 2.66	---	---

Table V: Nitric oxide radical scavenging activity of *P. longum* and *P. nigrum* extracts

Concentration (µg/ml)	NO radical scavenging activity (% inhibition)								
	AA	PNB-E	PLD-E	PNW-E	PLR-E	PNB-W	PLD-W	PNW-W	PLR-W
100	45.33 ± 0.05	3.64 ± 0.02	3.16 ± 0.04	5.59 ± 0.01	5.50 ± 0.02	16.49 ± 0.04	4.59 ± 0.01	20.59 ± 0.05	3.49 ± 0.01
200	57.94 ± 0.09	4.08 ± 0.04	3.65 ± 0.08	6.88 ± 0.02	5.99 ± 0.05	20.40 ± 0.07	11.85 ± 0.03	22.64 ± 0.06	6.19 ± 0.03
300	68.11 ± 0.08	4.69 ± 0.05	4.28 ± 0.05	7.62 ± 0.05	6.75 ± 0.03	24.15 ± 0.05	20.63 ± 0.07	23.88 ± 0.08	8.31 ± 0.04
400	72.31 ± 0.04	5.65 ± 0.09	5.01 ± 0.09	10.88 ± 0.08	7.96 ± 0.07	29.84 ± 0.06	22.53 ± 0.09	31.56 ± 0.08	15.39 ± 0.09
500	77.92 ± 0.03	18.16 ± 0.06	11.40 ± 0.07	13.28 ± 0.06	8.81 ± 0.09	36.41 ± 0.08	27.69 ± 0.08	37.32 ± 0.10	20.67 ± 0.08
IC_{50}	136 ± 0.08	---	---	---	---	---	---	---	---

Table VI: Fe^{2+} Chelating activity of *P. longum* and *P. nigrum* extracts

Concentration (µg/ml)	Fe^{2+} radical scavenging activity (% inhibition)								
	EDTA	PNB-E	PLD-E	PNW-E	PLR-E	PNB-W	PLD-W	PNW-W	PLR-W
100	99.15 ± 0.09	60.98 ± 0.02	41.27 ± 0.04	37.75 ± 0.03	44.17 ± 0.04	20.73 ± 0.02	2.16 ± 0.05	10.24 ± 0.03	13.50 ± 0.03
200	99.21 ± 0.08	64.71 ± 0.05	52.66 ± 0.05	39.62 ± 0.05	55.84 ± 0.05	21.36 ± 0.07	7.56 ± 0.09	17.73 ± 0.02	15.25 ± 0.04
300	99.37 ± 0.06	64.47 ± 0.08	68.14 ± 0.09	41.45 ± 0.09	59.59 ± 0.07	24.13 ± 0.08	27.03 ± 0.07	16.28 ± 0.07	19.56 ± 0.05
400	99.48 ± 0.07	64.35 ± 0.06	69.23 ± 0.07	63.79 ± 0.06	62.20 ± 0.03	24.86 ± 0.04	34.40 ± 0.04	19.17 ± 0.04	19.90 ± 0.08
500	99.62 ± 0.09	65.90 ± 0.07	72.83 ± 0.08	69.36 ± 0.07	63.06 ± 0.04	27.99 ± 0.03	39.27 ± 0.05	20.42 ± 0.05	19.95 ± 0.06
IC_{50}	5.58 ± 0.05	82.36 ± 0.44	180.51 ± 2.35	340.32 ± 4.25	150.81 ± 2.21	---	---	---	---

Table VII: Superoxide dismutase radical scavenging activity of *P. longum* and *P. nigrum* extracts

Concentration (µg/ml)	Superoxide Dismutase radical scavenging activity (% inhibition)								
	AA	PNB-E	PLD-E	PNW-E	PLR-E	PNB-W	PLD-W	PNW-W	PLR-W
100	60.67 ± 0.06	1.18 ± 0.01	7.06 ± 0.02	7.94 ± 0.07	6.83 ± 0.02	29.00 ± 0.03	10.72 ± 0.08	16.83 ± 0.01	30.84 ± 0.03
200	84.88 ± 0.08	23.67 ± 0.03	7.79 ± 0.08	10.04 ± 0.08	22.37 ± 0.05	31.56 ± 0.04	30.18 ± 0.05	23.08 ± 0.04	19.79 ± 0.06

300	89.42 ± 0.09	49.33 ± 0.05	19.01 ± 0.06	27.57 ± 0.04	39.86 ± 0.03	37.94 ± 0.06	41.22 ± 0.03	39.65 ± 0.08	10.78 ± 0.07
400	91.49 ± 0.07	53.84 ± 0.06	37.31 ± 0.03	39.63 ± 0.09	53.80 ± 0.07	41.03 ± 0.07	52.27 ± 0.04	44.25 ± 0.03	28.86 ± 0.09
500	93.85 ± 0.09	55.02 ± 0.09	52.69 ± 0.05	52.12 ± 0.04	60.33 ± 0.05	60.16 ± 0.06	55.16 ± 0.06	58.32 ± 0.06	32.48 ± 0.04
IC_{50}	82.01 ± 0.41	320.2 ± 3.25	482.3 ± 4.17	482.60 ± 5.21	372.81 ± 3.5	447.7 ± 4.15	381.5 ± 3.90	441.2 ± 4.11	---

Table VIII: Reducing power assay of *P. longum* and *P. nigrum* extracts

Concentration (µg/ml)	Average Absorbance at 700 nm								
	AA	PNB-E	PLD-E	PNW-E	PLR-E	PNB-W	PLD-W	PNW-W	PLR-W
100	0.78 ± 0.002	0.07 ± 0.003	0.06 ± 0.003	0.14 ± 0.002	0.07 ± 0.001	0.05 ± 0.002	0.09 ± 0.001	0.06 ± 0.002	0.10 ± 0.003
200	1.11 ± 0.003	0.10 ± 0.005	0.09 ± 0.008	0.21 ± 0.004	0.09 ± 0.005	0.06 ± 0.004	0.09 ± 0.004	0.06 ± 0.007	0.11 ± 0.008
300	1.52 ± 0.004	0.16 ± 0.004	0.13 ± 0.002	0.25 ± 0.005	0.11 ± 0.006	0.08 ± 0.005	0.10 ± 0.003	0.06 ± 0.004	0.12 ± 0.005
400	1.76 ± 0.003	0.22 ± 0.005	0.16 ± 0.003	0.36 ± 0.008	0.16 ± 0.008	0.09 ± 0.009	0.11 ± 0.005	0.07 ± 0.008	0.13 ± 0.002
500	1.99 ± 0.009	0.25 ± 0.008	0.19 ± 0.005	0.42 ± 0.007	0.21 ± 0.007	0.11 ± 0.006	0.13 ± 0.007	0.08 ± 0.006	0.14 ± 0.007

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