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In vitro antioxidant activity of *Piper nigrum* Linn.

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ABSTRACT

The fractions R1, R2 and R3 obtained from pet ether extract of *Piper nigrum* Linn. (PEPN) were investigated for *in vitro* antioxidant activity.1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical, superoxide anion radical, nitric oxide radical, and hydroxyl radical scavenging assays were performed. The free radical scavenging activity of the different fractions PEPN increased in a concentration dependent manner. R3 and R2 fraction of PEPN in 500µg/ml inhibited the peroxidation of a linoleic acid emulsion by 60.48±3.33% and 58.89±2.51%, respectively. In DPPH free radical scavenging assay, the activity of R3 and R2 was found almost similar. R3 (100µg/ml) fraction of PEPN inhibited 55.68±4.48% nitric oxide radicals generated from sodium nitroprusside whereas curcumin in the same amount inhibited 84.27±4.12%. Moreover, PEPN scavenged the superoxide radical generated by the Xanthine/Xanthine oxidase system. The fraction R2 and R3 in the dose of 1000µg/ml also inhibited 61.04±5.11% and 63.56±4.17% respectively, the hydroxyl radical generated by Fenton's reaction. The amounts of total phenolic compounds were also determined and 56.98µg pyrocatechol phenol equivalents were detected in one mg of R3.

KEYWORD: Lipid peroxidation, phenolic compounds, *Piper nigrum*.

INTRODUCTION

The importance of reactive oxygen species (ROS) has attracted increasing attention over the last decade. ROS, include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-) and non free radicals such as H_2O_2 and singlet oxygen (O_2) and various forms of activated oxygen. They are involved in various physicochemical processes and diseases such as aging (1), cancer (2) & atherosclerosis (3). Several studies have reported that plants have potent antioxidants and represents as an important source of natural antioxidants (4-6). Butyl hydroxyl anisol (BHA), and butyl hydroxyl toluene (BHT) are most commonly used antioxidants but both are suspected to cause liver damage (7-8).

Black pepper (*Piper nigrum* Linn.), the king of spice, is one of the oldest and most popular spice in the world. It belongs to family piperacae and used in many Asian countries as a stimulant, for the treatment of colic, rheumatism, headache, diarrhea, dysentery, cholera, menstrual pain, removing excessive gas from gastrointestinal tract and increasing flow of urine. It is used in folk medicine for stomach disorders, digestive problems, neuralgia and scabies. Its active constituent, piperine has investigated to reduce liver damage in rats (9). The methanolic extract of *P. nigrum* fruits have hepatoprotective and antioxidant effects in rats (10). The purpose of the present study was to evaluate the *in vitro* antioxidant activity of different fractions obtained from pet ether extract of *Piper nigrum* (PEPN).

MATERIALS AND METHODS

Plant materials and extraction

Piper nigrum Linn. fruits were collected in the month of March 2003 from the Kottayam, Kerala, India. The plant materials were identified and authenticated in the Herbarium, Botany Department, Guru Nanak Dev University, Amritsar, Punjab, India. A voucher specimen (B-05) of the collected plant materials was also deposited in the Department of Pharmacy, Government Institute of Pharmaceutical Sciences & Engineering, Amritsar for future reference. The collected materials were washed thoroughly in water, air dried for a week at 35-40°C and pulverized in electric grinder. The powder obtained was extracted in pet ether. The extract was then again made to powder by using rotary evaporator under reduced pressure. This powder was further subjected to silica gel (# 60-120, BDH) column chromatogram and fraction R1, R2 and R3 was eluted from the mixture of pet ether and ethyl acetate in the ratio of 6:4, 5:5 and 4:6 respectively.

Chemicals

Ammonium thiocyanate was purchased from Merck, Darmstadt, Germany. Ferrous chloride, ferric chloride ($FeCl_2$), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), EDTA, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), α-tocopherol, ascorbic acid, catechin, pyrocatechol, curcumin, nitroblue tetrazolium (NBT), thiobarbituric acid, 2-deoxy-2-ribose, and trichloroacetic acid, were purchased from Sigma, St. Louis, USA. All other chemicals and reagents were of analytical grade.

Determination of total antioxidant activity

The antioxidant activity of different fractions (R1, R2 and R3) obtained from PEPN was determined by the thiocyanate method (11). Various concentrations (50, 100, 250, and 500 µg/ml) of different fractions of PEPN were prepared in methanol and added to a linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0) and phosphate buffer (2 ml, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 ml 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 ml with 40 mM phosphate buffer, pH 7.0. The mixed samples were then incubated at 37ºC in a glass flask for 60 hr to accelerate the oxidation process (12). One milliliter of the incubated sample was removed at 12 hr interval and 0.1 ml 20 mM $FeCl_2$ and 0.1 ml 30% ammonium thiocyanate were added. The absorbance of this was measured at 500 nm using spectrophotometer (DU640i, Beckman). Alpha Tocopherol was used as a reference compound. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. All data reported are the average of triplicate analyses. Percent inhibition of lipid peroxide generation was calculated using following formula (Equation 1).

$$\% \ Inhibtion = \frac{Control \ \ absorbance - Test \ absorbance}{Control \ absorbance}$$

(1)

DPPH free radical scavenging activity of PEPN

The free radical scavenging activity of different fractions of PEPN was measured using DPPH by the method of Blois (13). One ml of each fractions of PEPN and reference compound in various concentrations (50, 100, 150, 200 & 250 µg/ml) was added into one ml of 0.1mM solution of DPPH in methanol. After 30 minutes, absorbance was measured at 517 nm by using spectrophotometer (DU640i, Beckman). A 0.01mM solution of DPPH in methanol was used as control where as BHA was used as a reference material. All tests were performed in triplicate. Percent inhibition was calculated using equation 1 mentioned above.

Nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (14). Three ml of 10 mM sodium nitroprusside in phosphate buffer was added into two ml of each fraction of PEPN and the reference compound in different concentrations (20, 40, 60, 80, and 100 µg/ml). The resulting solutions were then incubated at 25ºC for 60 minutes. The similar procedure was repeated with methanol as a blank which served as control. To 5ml of the incubated sample, 5 ml Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was measured using spectrophotometer (DU640i, Beckman) at 546 nm. All tests were performed in triplicate. Percent inhibition of the nitric oxide generated was measured by comparing the absorbance values of control and test preparations (equation 1). Curcumin was used as a reference material.

Superoxide anion radical scavenging activity

The method described by Chang et al (15) was used to investigate the superoxide anion scavenging activity of different fractions obtained from PEPN. The reaction mixture was prepared by dissolving Na_2CO_3 (0.53gm), EDTA (0.004gm) and xanthine in 100ml of distilled water. Ten ml of NBT solution (0.025mM) was added into the reaction mixture. 995µl of this solution with xanthine was further added to 5µl of each fractions of PEPN and reference compound in different concentrations in distilled water. After 15 minutes, the absorbance was measured using spectrophotometer (DU640i, Beckman) at 560 nm. The reaction mixture with xanthine oxidase was used as a control whereas BHT was used as reference compound. All tests were performed in triplicate. Percent inhibition was calculated by comparing the results of control and test samples (equation 1).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and PEPN for hydroxyl radical generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) by the method of Kunchandy and Rao (16). The reaction mixture containing (1.0ml), 100 µl 2-deoxy-2-ribose (28

mM in 20 mM phosphate buffer, pH 7.4), 500 µl of the each fraction of PEPN and reference compound in phosphate buffer (20 mM, pH 7.4), 200 µl 1.04 mM EDTA and 200 µM $FeCl_3$ (1:1 v/v), 100 µl 1.0 mM H_2O_2, and 100 µl 1.0 mM ascorbic acid, was incubated at 37ºC for 1 hr. One milliliter 1% thiobarbituric acid and 1.0 ml 2.8% trichloroacetic acid were added and incubated at 100ºC for 20 min. After cooling, absorbance was measured using spectrophotometer (DU640i, Beckman) at 532 nm against a control preparation containing deoxyribose and buffer. Catechin was used as a positive control. Reactions were carried out in triplicate. Percent inhibition was determined by comparing the results of the test and control samples (equation 1).

Amount of total phenolic compounds

Total soluble phenolic compounds present in the different fractions of PEPN were determined with the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (17). To 0.1ml of each fraction of PEPN (1mg/ml in distilled water) in Erlenmeyer flask, one ml Folin-Ciocalteu reagent was added. After three minutes 3 ml 2% Na_2CO_3 was added. Subsequently, the mixture was shaken for 2 hr at room temperature and absorbance measured using spectrophotometer (DU640i, Beckman) at 760 nm. All tests were performed in triplicate. The standard graph was prepared with 0.1 ml of pyrocatechol solution containing 0-400 mg of pyrocatechol in place of PEPN. To determine the amount of total phenolic compounds in the extracts, the absorbance of a sample, which contains 1000 µg of dried extracts, was measured at 760 nm using a spectrophotometer. The concentration of total phenolic compounds in samples was determined as µg pyrocatechol equivalents using the following equation:

Absorbance = 0.001 x pyrocatechol (µg) + 0.0033

Statistical analysis

Data are reported as the mean ± SD of three measurements. Statistical analysis was performed by the Student *t*-test and ANOVA. $P < 0.05$ was regarded as significant.

RESULTS

Total antioxidant activity

In the present study, the antioxidant activity of the different fractions (R1, R2 & R3) of PEPN was determined by the ammonium thiocyanate method. All the fractions showed antioxidant property (Table 1) in a concentration dependent manner. Percent inhibition was more in case of R3 than R2. The fraction R1 showed negligible percent inhibition. All the fractions showed less percent inhibition than reference (α-tocopherol). The results indicate that the fractions R2 and R3 of PEPN significantly ($P < 0.05$) inhibited linoleic acid peroxidation.

DPPH free radical scavenging activity

The DPPH radical is considered to be a model of lipophilic radical. A chain reaction in lipophilic radicals was initiated by lipid autoxidation. The positive DPPH test suggests that the samples are free radical scavengers. The scavenging effects of different fractions of PEPN and BHA on the DPPH radical are illustrated in table 2. R2 and R3 had significant scavenging effects on the DPPH radical which increases with increasing concentration from 50-250 µg/ml. The scavenging effect of all the fractions was lower than that of BHA. The fraction R1 showed negligible percent inhibition. The fraction R2 and R3 have statistically significant DPPH free radical scavenging activity ($P < 0.05$).

Nitric oxide radical scavenging activity

In the present study, the fractions (R1, R2 & R3) of PEPN were investigated for their inhibitory effects on nitric oxide production. The percent inhibition of nitric oxide generation by different fractions of PEPN was shown in Table 3. The antioxidant activity of all the three fractions was less than curcumin which was used as a reference compound. It was also observed that R3 more actively inhibit the production of nitric oxide radicals than R2 and R1.

Superoxide anion radical scavenging activity

All the fractions PEPN was found to possess scavenging effects on superoxide anions at concentrations dependent manner (Table 4). R3 in the dose of 100µg/ml inhibited the production of superoxide anion radicals by 70.22±3.55%, showing strong superoxide radical scavenging activity. However activity remains below the BHT.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test (R1, R3 and R3) compounds for hydroxyl generated by $Ferric^{3+}$ascorbate-EDTA-H_2O_2 system (Fenton reaction). The R2 and R3 fraction of PEPN was capable of reducing DNA damage at all concentration whereas R1 showed negligible or no effect (Table 5). Catechin used as a standard was more effective than fractions of PEPN in inhibiting the oxidative DNA damage.

Amount of total phenolic compounds

The fraction R1, R2 and R3 were investigated to have 25.23, 38.56 and 56.98 µg pyrocatechol equivalents of

Table 1. Inhibition (%) of lipid peroxidation in a linoleic acid emulsion by different fractions (R1, R2 &R3) of pet ether extract of Piper nigrum (PEPN) and by a-tocopherol (α -Tph)

Quantity of the fraction used	% of Inhibition			
in µg	R1	R2	R3	α-Tph
50	4.33±5.25*	47.12±5.24*	49.94±5.78*	60.36±4.35*
100	7.58±2.80	50.56±1.32*	52.59±4.85*	65.32±5.48*
250	8.18±6.50	54.97±2.86	56.19±5.22*	68.44±2.57*
500	10.21±3.95	58.89±2.51*	60.48±3.33*	76.47±5.12*

Data are reported as means ± SD of three measurements. ; *P < 0.05 compared to control

Table 2. 1,1-diphenyl-2-picryl-hydrazol (DPPH) free radical scavenging activity of different fractions (R1, R2 &R3) of pet ether extract of *Piper nigrum* (PEPN) and butylated hydroxy anisole (BHA)

Quantity of the fraction used in	% of Inhibition			
µg	R1	R2	R3	BHA
50	3.56±1.32	12.58±2.8*	12.32±5.48*	38.59±4.85*
100	5.89±2.51	38.21±3.95*	38.47±5.12*	62.48±3.33*
150	8.97±2.86	43.18±6.55*	43.44±2.57*	70.19±5.22*
200	10.12±5.24	52.33±5.25*	52.36±4.35*	78.94±5.78*
250	12.41±6.62	61.24±7.58	61.11±4.98*	82.59±4.26*

*Data are reported as the means ± SD for three measurements ;*P < 0.05 compared to control*

Table 3. Inhibition of nitric oxide radicals by different fractions (R1, R2 &R3) of pet ether extract of Piper nigrum (PEPN) and curcumin

Quantity of the fraction used in	% of Inhibition			
µg	R1	R2	R3	Curcumin
20	5.23±3.28*	10.54±2.96*	28.54±3.98*	51.22±3.55
40	8.57±2.24*	21.23±4.58	42.29±5.88*	70.06±2.54*
60	12.45±3.39	35.68±5.18*	48.52±4.19	75.45±2.48*
80	15.56±4.17	38.95±4.28*	52.04±5.11*	80.87±5.02*
100	18.22±4.31*	40.23±7.47*	55.68±4.48*	84.27±4.12*

*Data are reported as the means ± SD for three measurements ; *P < 0.05 compared to control*

Table 4. Superoxide anion scavenging activity of different fractions (R1, R2 &R3) of pet ether extract of Piper nigrum (PEPN) and butylated hydroxy toluene (BHT)

Quantity of the fraction used	% of Inhibition			
in µg	R1	R2	R3	BHT
20	8.95±4.28	25.56±4.17*	48.87±5.02*	65.04±5.11*
40	11.23±7.47	46.22±4.31*	57.27±4.12*	45.68±4.48*
60	15.68±5.18*	57.45±3.39*	64.45±2.48*	78.52±4.19*
80	17.23±4.58*	60.57±2.24*	68.06±2.54*	80.29±5.88
100	18.54±2.96*	62.23±3.28*	70.22±3.55*	81.54±3.98*

*Data are reported as the means ± SD for three measurements ; *P < 0.05 compared to control*

Table 5. Hydroxyl radical scavenging activity of different fractions (R1, R2 & R3) of pet ether extract of Piper nigrum (PEPN) and catechin on deoxyribose damage

Quantity of the fraction used in	% of Inhibition			
µg	R1	R2	R3	Catechin
50	17.27±4.12	35.23±3.28*	44.68±4.48*	50.22±3.55*
100	19.54±3.98	45.23±7.47*	55.54±2.96*	65.22±4.31*
250	20.08±2.54*	56.29±5.88	59.57±2.24*	68.23±4.58*
500	21.45±2.48*	58.52±4.19*	62.45±3.39*	69.67±5.18*
1000	21.87±5.02*	61.04±5.11*	63.56±4.17*	70.95±4.28

*Data are reported as the means ± SD for three measurements ; *P < 0.05 compared to control*

phenols respectively. The phenolic compounds may contribute directly to the antioxidative action (18). Thus, the antioxidant properties of *Piper nigrum* may be possibly attributed to the phenolic compounds present.

DISCUSSION

All the fractions showed free radical scavenging activity in different models of this study. On comparison it was found that fraction R3 has highest antioxidant activity whereas R1 has least free radical scavenging activity. The antioxidant activity of these fractions might be due to inactivation of free radical or complex formation with metal ions, or combinations of both.

Most of the mammals have inherent mechanism to prevent and neutralize the free radical induced damage. In biochemical system superoxide radical and H_2O_2 reacts together to form the singlet oxygen and hydroxyl radical, these can attack and destroy almost all known biochemical (19). Hydroxyl radical produced may cause sugar fragmentation, base loss and leakage of DNA strand (20). Hydroxyl radicals are the major ROS causing lipid oxidation and enormous biological damage (21). PEPN scavenge off these free radicals and hence inhibit cellular damage. It is apparent from the present study that the PEPN not only scavenges off the free radicals but also inhibits the generation of the free radicals. It was already reported that naturally occurring phenolic compounds have free radical scavenging properties due to their hydroxyl groups (22). Further phenolic compounds are effective hydrogen donors which make them antioxidant (23).

It may be concluded that fractions obtained from pet ether extract of *P. nigrum* have significant antioxidant activity. The antioxidant potential potential may be attributed to the presence of polyphenolic compounds. These results are encouraging enough to pursue characterization of these fractions.

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