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Evaluation of antioxidant and free radical scavenging activities of different fractions of *Pterospermum suberifolium* leaf extract

Dibyajyoti Saha[1] and Swati Paul[1]

[1]*Department of Pharmacy, BGC Trust University Bangladesh, Chittagong*

Abstract

The study was aimed an evaluating the antioxidant activities of the various leaf extracts (ethyl acetate, n-butanol and chloroform) of *Pterospermum suberifolium* by using various in vitro antioxidant assays. The total polyphenols were also determined spectrophotometrically with the Folin-Ciocalteu reagent. For evaluation of antioxidant activity various methods such as 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging, nitric oxide radical (NO.), superoxide anion radical scavenging by riboflavin/methionine/illuminate system, hydroxyl radical scavenging assays by Thiobarbituric acid (TBA), reducing power assay by trichloroacetic acid (TCA) and total reducing ability determination by Fe^{3+}- Fe^{2+} transformation method were carried out. The results thus obtained were compared with standard antioxidant compounds such as ascorbic acid, butylated hydroxyl toluene (BHT), trichloroacetic acid (TCA), rutin, curcumin and catechin. In DPPH radical scavenging assay the IC_{50} value of ethyl acetate fraction (EF) 26.2 µg/ml, n-butanol fraction (NF) 66.2 µg/ml and chloroform fraction (CF) (>100 µg/ml) were found. Effectiveness in nitric oxide scavenging assay the IC_{50} value of EF, NF and CF were found 32.4 µg/ml, 78.5 µg/ml and >100 µg/ml respectively. Whereas in superoxide anion scavenging assay the IC_{50} value of EF (51.8 µg/ml) was low other than NF (91.4 µg/ml) and CF (>100 µg/ml). It was also found that the IC_{50} of NF (86.6 µg/ml) and CF (>100 µg/ml) were greater than the EF (28.7 µg/ml) in hydroxyl radical scavenging assay. IC_{50} for Fe^{2+} chelating activity assay of EF (55.6 µg/ml), NF (86.2 µg/ml) and CF (>100 µg/ml) respectively. The total phenolic compound was calculated as quite high in EF (169.2 ± 12.5 µg mg^{-1} pyrocatechol equivalent) other than NF (32.7 ± 8.92 µg mg^{-1}) and CF (21.2 ± 6.13 µg mg^{-1}). The results of all six *in vitro* antioxidant assays exhibited that *P. suberifolium* possess relatively moderate antioxidant property than standards. The data obtained in the *in vitro* models clearly establish the antioxidant potency of all leaf extracts.

Key Words: DPPH, Phenolics content, *Pterospermum suberifolium*, Reducing power, Scavenging assay

Introduction

The genus *Pterospermum suberifolium* , (Sterculiaceae) represents of about 40 species in the world, of which 12 species were reported from India [1] and 8 species has been reported from Tamil Nadu state [2]. Ethnomedicinally, the leaves are used for headache [3], treatment of fractured bones [4], small pox [5] and antimicrobial properties [6]. The plant has been reported to contain β-amyrin, betulin,

Correspondence to: Dibyajyoti Saha, Department of Pharmacy, BGC Trust University Bangladesh, Chittagong.
Tel:+88 01755588624, E-mail: saha.dibyajyoti@gmail.com

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kaempferol, lupeol, quercetin, scopoletin and β - sitosterol [7] and α-sitosterol, 3, 7, 11, 15-tetramethyl-2-hexa decane-1-ol, ricinoleic acid, vitamin-E, phytol, α-tocopherol, diethyl phthalate, squalene, benzhydrazide-3-mthoxy-N2-(4-phenylcyclo hexylideno, benzoic acid, 4- heptyl-4-cyanophenyl ester and n-hexa decanoic acid [8]. It is increasingly being realized that a majority of the disease of today are due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals or due to the excessive oxidative stress of the current life or due to the poor scavenging/ quenching in the body due to the depletion of the dietary antioxidants [9]. Free radicals have been implicated in causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes etc. Reactive oxygen species (ROS) have been known to cause tissue injury through covalent binding and lipid peroxidation. Lipid per-oxidative process has been shown to augment collagen synthesis and fibrosis [10]. The most common reactive oxygen species are superoxide anion, hydrogen peroxide, peroxyl radical and reactive hydroxyl radical. The nitrogen derived free radicals are nitric oxide and peroxynitrite anion. Oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems. Antioxidant are the substances that when present in low concentration significantly delays or reduces the oxidation of the substrate [11]. Antioxidants protect the body damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body and hindering the process of oxidation. So diseases linked with free radicals can be prevented by antioxidant therapy. It was studied that, currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been suspected to cause or prompt negative health effects and therefore they should be substituted with naturally occurring antioxidants [12]. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability [13]. Flavonoids and phenolic compounds widely distributed in plants to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc [14]. They were also suggested to be a potential iron chelator [15]. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties. The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs. The need to identify antioxidants, which can scavenge several free radicals and prevent multiple diseases, can be achieved by simple in vitro test systems. Further the in vitro results can be confirmed with ex vivo and in vivo systems.

Materials and Methods

Plant material. The plant was collected from hilly areas of India in April 2008. The plant leaves were collected and identified by taxonomic experts in India. A voucher specimen has been deposited for future reference. The plant leaves were thoroughly washed with water and dried in hot air oven at 50 ºC for 3 days and at 40 ºC for the next 4 days.

Extraction and solvent-solvent partitioning. The dried leaves were coarsely powdered from which 500 g was extracted with a mixture of methanol: water (7:3, v/v) in a Soxhlet apparatus. The solvent was completely removed and obtained 18 g (yield 3.6 %) dried crude extract by using rotary evaporator Bibby RE200, Sterlin Ltd, England . The crude extract was dissolved in 10 % aqueous methanol to make the mother solution which was partitioned off successively by three solvents namely ethyl acetate fraction (EF) 3 × 100 mL, n-butanol fraction (NF) 3 × 100 mL, chloroform fraction (CF) 3 × 100 mL. All the three fractions and the residual hydromethanol fraction were subjected to dryness under reduced pressure. The dried extracts thus obtained were used for investigation [16].

Chemicals. 1, 1-diphenyl-2-picryl hydrazyl (DPPH), ferrous sulphate ($FeSO_4$), potassium iodide, $Na_2S_2O_3$, disodium hydrogen phosphate, potassium dihydrogen phosphate, Nitroblue terazolium (NBT), NADH, Phenazine methosulphate nicotinamide (PMS), ammonium molybdate, sodium phosphate, hydrogen peroxide and dimethyl sulfoxide (DMSO) were used.

Determination of total phenolics. The concentrations of phenolic content in all the fractions were determined with Folin Ciocalteu's phenol reagent (FCR). The 1 ml of the solution (contains 1 mg) of the fraction in methanol was added to 46 ml of distilled water and 1 ml of FCR, and mixed thoroughly. After 3 min, 3 ml of sodium carbonate (2 %) were added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was measured at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from standard pyrocatechol graph:

$$\text{Absorbance} = 0.001\times \text{ pyrocatechol } (\mu g) + 0.0033$$

DPPH radical scavenging activity. The DPPH assay measured hydrogen atom (or one electron) donating activity and hence provided an evaluation of antioxidant activity due to free radical scavenging. DPPH, a purple-coloured stable free radical, was reduced into the yellow-coloured diphenylpicryl hydrazine which is measured spectrophotometrically at 517 nm [17]. Briefly, 0.1 mM

solution of DPPH solution in methanol was prepared and 1ml of this solution was mixed with 3 ml of sample solutions in water at different concentrations. Finally, after 30 min, the absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. DPPH radical-scavenging activity was calculated according to the following equation:

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

Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract.

Nitric oxide scavenging activity. Nitric oxide was generated from sodium nitroprusside, which at physiological pH liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions (NO^{2-}) which diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink color which can be measured at 546 nm [18]. Sodium nitroprusside (10 mM, 2 ml) in phosphate buffer saline was incubated with the test compounds in different concentrations at room temperature for 30 min. After 30 min, 0.5 ml of the incubated solution was added with 1 ml of Griess reagent and the absorbance was measured at 546 nm. The nitric oxide radicals scavenging activity was calculated according to the following equation:

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

Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract.

Superoxide anion scavenging activity assay. The scavenging activity of the different fractions towards superoxide anion radicals [19] with slight modification. Phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the reduction of nitroblue tetrazolium (NBT). About 1 ml of nitro blue tetrazolium (156 µM), 1 ml NADH (468 µM) in 100 mM phosphate buffer of pH 7.8 and 0.1 ml of sample solution of different concentrations were mixed. The reaction started by adding 100 µl PMS (60 µM). The reaction mixture was incubated at 25 ºC for 5 min and absorbance of the mixture was measured at 560 nm against blank samples. The percentage inhibition was determined by comparing the results of control and test samples.

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

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Hydroxyl radical scavenging activity. The formation of hydroxyl radicals ($OH^{\cdot}$) from Fenton reagents was quantified using 2-deoxyribose oxidative degradation [20]. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malonaldehyde, by its condensation with thiobarbturic acid (TBA). The reaction mixture contained deoxyribose (2.8 mM); $FeCl_3$ (100 mM); KH_2PO_4–KOH buffer (20 mM, pH 7.4); EDTA (100 mM); H_2O_2 (1.0 mM); ascorbic acid (100 mM), and various concentrations of the test compounds in a final volume of 1 ml. Ferric chloride and EDTA (when added) were premixed just before addition to the reaction mixture. The reaction mixture was incubated at 37 ºC for 60 min. After incubation at 37 ºC for 1 h, 1.0 ml of 2.8 % trichloroacetic acid and 1.0 ml of 1 % aqueous solution of TBA were added to the sample; test tubes were heated at 100 ºC for 20 min to develop the color. After a cooling, TBARS formation was measured spectrophotometrically at 532 nm against an appropriate blank. The hydroxyl radical-scavenging activity was determined by comparing absorbance of the control with that of test compounds.

Reducing power assay. The Fe^{3+} reducing power of different fractions were determined. The extract (2.5 ml) at various concentrations was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50 ºC for 20 min. The reaction was stopped by adding 2.5 ml of trichloroacetic acid (TCA) solution (10 %) and then centrifuged at 800 g for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride solution (0.1 %, w/v) and the absorbance was measured at 700 nm. Butylated hydroxyl toluene was used as reference standard. Higher absorbance of the reaction mixture indicated greater reducing power.

Fe^{2+} chelating activity assay. The chelating activity of the extracts for ferrous ions Fe^{2+} was measured [21]. To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as:

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

Statistical analysis. All data on all antioxidant activity tests are the average of triplicate analyses. The data were recorded as mean ± SD. The statistical significance of differences between groups was determined by analysis of

variance (ANOVA), followed by Dunnett's test for multiple comparisons among groups. Differences of $p < 0.05$ were considered statistically significant.

Results and Discussion

There are different models available for evaluation of antioxidant activities. The chemical complexity of different fractions and mixture of compounds present could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extracts would be more informative and even necessary. In this study, different free radical scavenging activities were measured and results were compared with standard antioxidant.

Total polyphenolics contents of the extracts. The total phenolic compound amount was calculated as quite high in ethyl acetate fraction of MEPA (169.2 ± 12.5 µg mg^{-1} pyrocatechol equivalent) other than n-butanol (32.7 ± 8.92 µg mg^{-1}) and chloroform fraction (21.2 ± 6.13 µg mg^{-1}). The high concentration of polyphenolics in the ethyl acetate fraction of methanol extract may be responsible for its high free radical scavenging activity. The FCR reducing capacity of different fractions are due presence of hydroxyl groups present in the polyphenolics and flavonoids. The key role of phenolic compounds as scavengers of free radicals is emphasized [22]. They were reported to eliminate radicals due to their hydroxyl groups, and they contribute directly to antioxidant effect of system and it also has an important role in stabilizing lipid oxidation.

DPPH radical scavenging activity. The results of the free radical scavenging potentials of different fractions tested by DPPH method are depicted (Figure 1). Antioxidant reacts with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm and convert it to 1, 1-diphenyl-2-picryl hydrazine, due to its hydrogen donating ability at a very rapid rate [23]. The degree of discoloration indicates the scavenging potentials of the antioxidant. The IC_{50} value of EF was found as 26.2 µg/ml, whereas the IC_{50} value of NF and CF was found as 66.2 µg/ml and >100 µg/ml respectively. The low IC_{50} value of ethyl acetate fraction is due presence of high polyphenolics and flavonoids in it.

Nitric oxide scavenging assay. Different fractions of methanol extract of P. suberifolium also moderately inhibited nitric oxide in dose dependent manner (Figure 2). IC_{50} value of EF, NF and CF was found as 32.4 µg/ml, 78.5 µg/ml and >100 µg/ml respectively. Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities. Studies in animal models have suggested a role for NO in the pathogenesis of inflammation and pain and NOS inhibitors have been shown to have beneficial effects on some aspects of the inflammation and tissue changes seen in models of inflammatory bowel disease [24].

Anion scavenging assay. In the PMS/NADH-NBT system, superoxide anion is generated using a non-enzymatic reaction of phenazine methosulphate in the presence of NADH and molecular oxygen [25]. Superoxide anion reduces NBT into formazan at pH 7.8 at room temperature and formazan generation can be determined spectrophotometry at 560 nm. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion by the active fractions of the extracts. In this assay, ethyl acetate fraction shows maximum superoxide anion scavenging activity and the results are presented (Figure 3). The IC_{50} value of EF (51.8 µg/ml) was low other than NF (IC_{50} = 91.4 µg/ml) and CF (IC_{50} = >100 µg/ml).

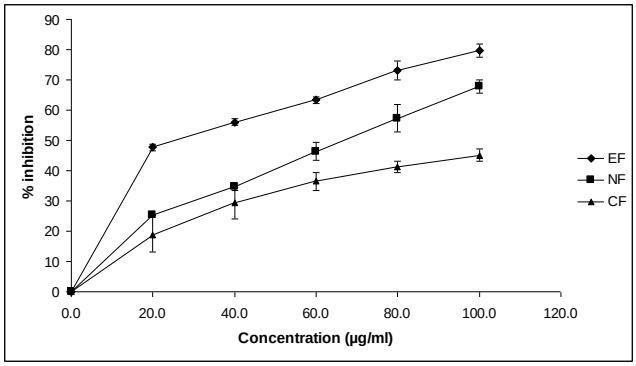

Figure 1 DPPH radical scavenging activity

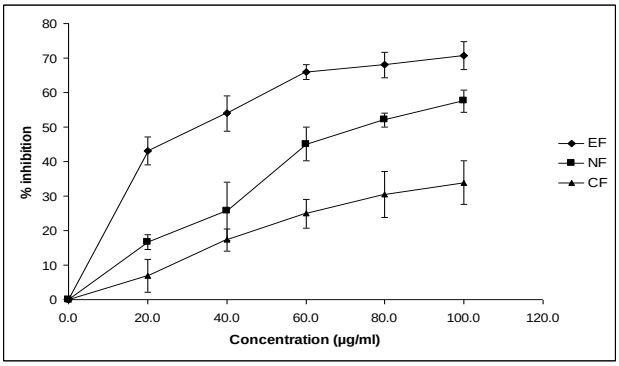

Figure 2 Nitric oxide scavenging assay

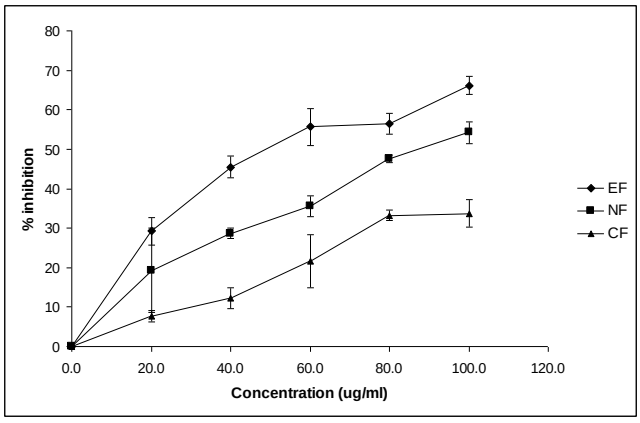

Figure 3 Superoxide anion scavenging assay

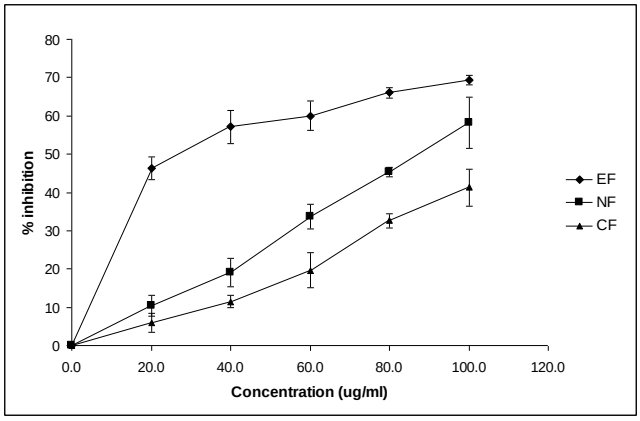

Figure 4 Hydroxyl radical scavenging

Hydroxyl radical scavenging activity. Free radicals and other species are constantly generated in vitro both by accidents of chemistry and for specific metabolic purposes. The most important reactions of free radicals in aerobic cells involved molecular oxygen and its radical derivatives, peroxides and transition metals. Reactive oxygen species are thought to play an important role in aging and in the pathogenesis of numerous degenerative or chronic disease, diabetics and atherosclerosis [26]. Different fractions of MEPA showed concentration-dependant reductive effect (Figure 4). In this assay, n-butanol fraction shows maximum hydroxyl radical scavenging activity (IC_{50} = 86.6 µg/ml). The IC_{50} value of EF (28.7 µg/ml) was low other than NF (IC_{50} = 86.6 µg/ml) and CF (IC_{50} >100 µg/ml).

Reducing power assay. The reducing power assay serve as a significant indicator of its potential antioxidant activity. Although, different mechanism was proposed for their antioxidants activity such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [27]. Different fractions of MEPA showed concentration-dependant reductive effect (Figure 5). The highest reducing activity was observed for the ethyl acetate fraction. The reducing properties are generally associated with the presence of different reductones [28]. The antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The reductive power of different fractions may be the reason for their antioxidant activity.

Fe^{2+} chelating activity assay. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and

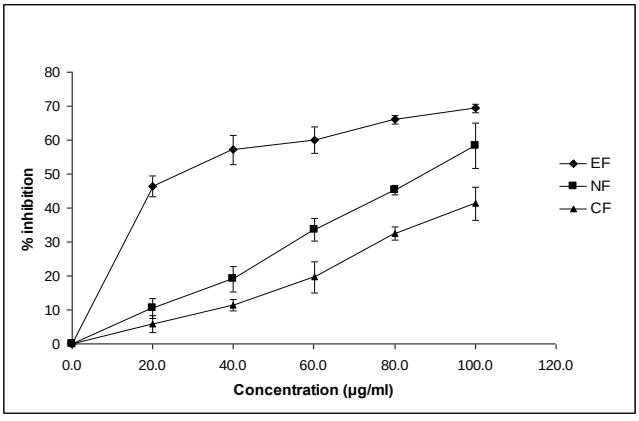

Figure 5 Reducing power assay

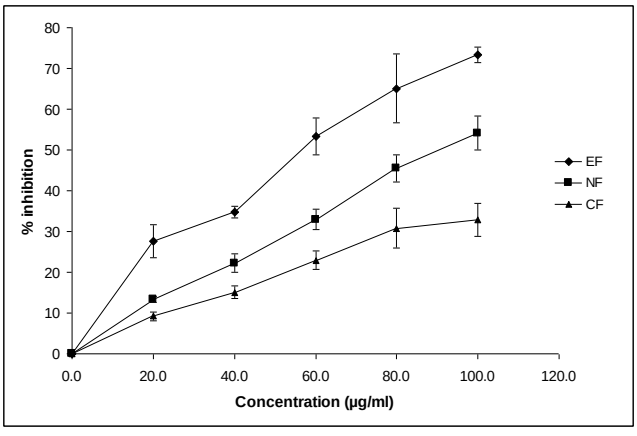

Figure 6 Fe^{2+} chelating activity assay

as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine– Fe^{2+} complex is interrupted in

Table 1 Comparative antioxidant activities of different fractions

Fractions	TPC	DPPH radical scavenging activity	NO scavenging activity	SO anion scavenging activity	Hydroxyl radical scavenging activity	Ferrous ion chelating activity
Ethyl acetate fraction	169.2 ± 12.5	79.7 ± 2.25	70.7 ± 4.04	66.1 ± 2.30	69.4 ± 1.21	73.4 ± 3.75
n- Butanol fraction	32.7 ± 8.90	73.8 ± 2.25	57.6 ± 3.20	54.2 ± 2.71	58.31 ± 6.70	54.1 ± 4.20
Chloroform fraction	21.2 ± 6.13	45.08 ± 1.97	33.9 ± 6.30	33.7 ± 3.51	41.3 ± 4.80	32.8 ± 4.10

*Activity (% inhibition) observed at 100 µg/ml
EF – Ethyl acetate fraction of leaf extract
NF - n- Butanol fraction of leaf extract
CF - Chloroform fraction of leaf extract

the presence of aqueous extract of *Pterospermum suberifolium*, indicating that have chelating activity with an IC_{50} of ethyl acetate fraction is 55.6 µg/ml followed by 86.2 µg/ml of n- butanol fraction and >100 µg/ml of chloroform fraction (Figure 6).

Correlation between different antioxidant activities and total phenolic contents. In general fraction with higher radical scavenging activity and antioxidant activity show a higher phenolic content and some good correlation were found among these parameters. It observed that highest correlation coefficient was exhibited between TPC and ferrous ion chelating activity and lowest correlation coefficient found between TPC and DPPH radical scavenging activity (Table 1 and Table 2).

Comparative study among different fractions. The behavior of different extract and fractions in relation to their activities were checked independently. Ethyl acetate fraction exhibit the highest DPPH radical scavenging activity, superoxide anion scavenging activity, hydroxyl free radical scavenging activity and ferrous ion chelating activity. The lowest activities exhibited by chloroform fraction (Figure 7 and Figure 8).

Fractions were taken at same concentration (100 µg/ml)

- DPPH free radical
- SO- superoxide anion
- NO- Nitric oxide
- HR- Hydroxyl radical
- FIC- Ferrous ion chelating activity

Comparative study with standard antioxidant. The results of different activities of the standard substances and fractions studied in this work rutin was found to have highest free radical scavenging activity (IC_{50} = 12.9 µg/ml) whereas curcumin have highest NO radical scavenging activity (IC_{50} = 20.8 µg/ml) and SO radical scavenging activity (IC_{50} = 8.9 µg/ml) and catechin showed highest hydroxyl radical scavenging activity (IC_{50} = 6.75µg/ml). None of the fraction exhibited a higher antioxidant activity than that of the standard substance (Table 3).

Conclusion

The presence of wide variety of flavonoids in the leaves of *P. suberifolium* has been found earlier in general identification tests. Flavonoids comprise a large group of

Table 2 Correlation between different antioxidant activities and total phenolic contents

Co-efficient of Correlation (R)					
N	DPPH/TPC	N O/TPC	SO/TPC	HR/TPC	FIC/TPC
6	0.806	0.814	0.822	0.830	0.935

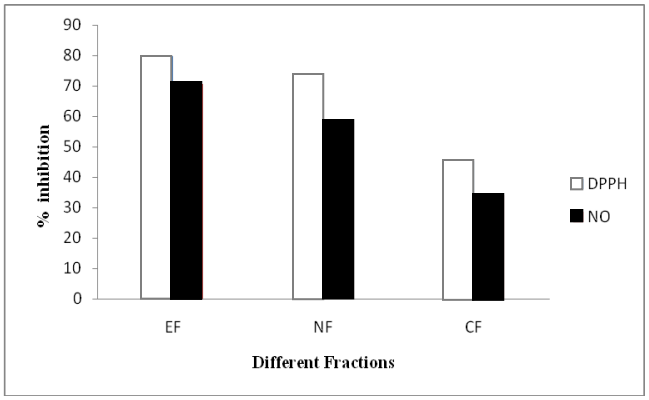

Figure 7 Comparative % scavenging activity of different fractions of plant extract by DPPH and NO scavenging assay

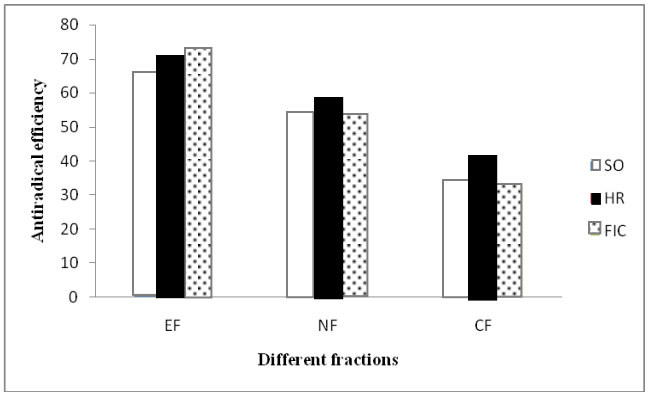

Figure 8 Comparative % scavenging activity of different fractions of plant extract by SO, HR and FIC scavenging assay

secondary metabolites have been shown to possess various biological properties related to antioxidant mechanism. The most widely acknowledged behavior of antioxidants is the interaction with oxidative free radicals. There is increasing interest in antioxidants such as flavonoids, particularly in those intended to prevent the presumed deleterious effect of free radicals in the human body and prevent the inhibition of lipid peroxidation. The antioxidant effect of flavonoids, can be reside both in their radical scavenging activity or in their metal chelating properties, of which the former may dominate. Some flavonoids included in this plant showed free radical scavenging activity. Dose dependent interaction of EF with 1, 1- diphenyl-2-picryl-hydrazyl radical and hydroxyl radicals establishes the capability of the constituents to scavenge the free radicals. Thus, it indicates that some of the therapeutic constituents of leaves of *P. suberifolium* may be due to its free radical scavenging property. Free radical scavenging property and total phenolic contents of EF can also be accepted an indication of antioxidant potential. It is difficult to decide in a screening for antioxidants from natural sources which of the plant species studied considered besting one, as each of them exhibits different antioxidant or scavenging activities. The results expressed in this study are the first information on the antioxidant activities of P. suberifolium. All the fraction of MEPA showed free radical scavenging activity when tested in different models. Among all the fractions the highest activity was observed in the ethyl acetate fraction followed by n-butanol fraction and chloroform fraction. Particularly, ethyl acetate fraction found to be rich source of polyphenolics compounds. The scavenging effect on DPPH radicals and superoxide radicals represent direct radical scavenging activity. However, in the hydroxyl radical scavenging assay, hydroxyl radicals are generated by the Fenton reaction and the inhibition of deoxyribose degradation could be attributed to the inhibition of radicals. It is well documented that free radicals are responsible for several diseases. The present result confirms the free radical scavenging activity of the plant which can be accounted for the traditional uses of the plant in treating several diseases.

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