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In Vitro Free Radical Scavenging and Membrane Stabilizing Activity of *Paederia foetida* Leaves.

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ABSTRACT

Paederia foetida is a native Bangladeshi plant, used for the treatments of various diseases in folklore medicine. In this study, the antioxidant potential of *Paederia foetida* extract was evaluated by using a series of well established antioxidant assay systems. *Paederia foetida* extract scavenge DPPH radical in a dose dependent manner. The extract was also showed potent free radicals inhibitory activity generated from sodium nitroprusside as nitric oxide and free radicals from H_2O_2. Furthermore, *Paederia foetida* extract showed moderate reducing power and potent membrane stabilizing activity of RBC membrane in hypotonic medium. The extracts exhibited remarkable antioxidant activities in all system studied. Scavenging of reactive oxygen species (ROS) may be attributed to the high amount of phenolic compounds present in the extract. Further study is necessary for isolation and characterization of the antioxidants present in the extract, which may serve as a potential source of natural healer for oxidative stress and inflammatory diseases.

INTRODUCTION

Oxidative stress plays a crucial role in various degenerative diseases**.** Cellular antioxidant systems are compromised in oxidative stress and cells are not able to scavenge free radicals sufficiently which may cause further damage. Therefore, supplementation with antioxidants would be beneficial. Currently, the possible toxicity of synthetic antioxidants has been criticized. Plants are rich in wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids etc which are strong antioxidants and showed various pharmacological activities. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea, and herbs may contribute an adequate antioxidant status in various diseases [1,2]. The *Rubiaceae* is one of the five largest plant families which contain 611 genera and 13,143 species has so far been recognized [3]. A large number of members of this family are ethnomedicinally important and showed a wide variety of biological activities. *Paederia foetida* belongs to *Rubiaceae* family, is a climbing, herbaceous, hairy or quite smooth, slender vine distributed almost all over Bangladesh [4]. *Paederia foetida* has been used extensively in traditional medicine. It is claimed to promote sexual vigour, increases the quantity of semen and body strength and produces a youthful glow [5]. Leaves are considered as a good remedy for diarrhea and dysentery and used as household remedy during convalescence from acute illness [4]. Leaves are also used as a poultice to relieve distension of the abdomen due to flatulence and also used to treat herpes and earache [4]. Decoction of leaves is considered as diuretic. Seeds are useful for the treatment of piles and leucoderma [4]. The whole plant is also used both externally and internally for rheumatic diseases. Several investigations were also reported on *Paederia foetida* extracts showed interesting biological activities. Anti-inflammatory activity against carrageenan-induced rat paw edema was reported [6]. Recent reports suggest that *Paederia foetida* extracts are hepatoprotective[7] and possesses antioxidant activity [8].

As part of our ongoing investigations on natural antioxidants from local medicinal plants of Bangladesh [9,10,11,12], this study reports *in vitro* antioxidant activity of *Paederia foetida* extracts. The evaluation of antioxidant

activities were investigated by a series of *in vitro* assays such as DPPH scavenging assay, reducing power assay, NO scavenging assay, H_2O_2 scavenging assay, total antioxidant capacity assay and erythrocyte membrane stabilizing assays.

MATERIALS AND METHODS

Chemicals

DPPH (1, 1-diphenyl, 2-picryl hydrazyl), NBT (Nitro blue tetrazolium), TCA (Trichloro acetic acid) and Ferric chloride were obtained from Sigma Chemical Co., USA. Ascorbic acid was obtained from SD Fine Chem. Ltd., Biosar, India. Hydrogen peroxide (H_2O_2) was obtained from Qualigens Fine Chemicals, Mumbai, India. Naphthyl ethylene diamine dihydrochloride was obtained from Roch-light Ltd., Suffolk, England. Sodium nitro prusside was obtained from Ranbaxy Lab., Mohali, India. Potassium ferricyanide was obtained from May and Backer, Dagenham, UK.

Plant material

Paederia foetida herbs were collected from the Stamford University Bangladesh, Siddeswari campus, Dhaka in May 2011 and authenticated through Professor Dr. Abdul Ghani and a voucher specimen was deposited in the Pharmacognosy Laboratory.

Extraction

The shade-dried leaves were coarsely powdered and extracted with mixture of ethanol: water (7:3 ratios) by a Soxhlet apparatus. The solvent was completely removed by rotary evaporator and obtained gummy exudates. This crude extract was used for further investigation for potential antioxidant properties.

Phytochemical screening

Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorffs reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce suds. Gum was tested using Molish reagents and concentrated sulphuric acid.

Antioxidant Activity Test

DPPH radical scavenging activity

The free radical scavenging capacity of the extracts was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% ethanol [10]. Ethanol extract of *Paederia foetida was* mixed with 95% ethanol to prepare the stock solution (5 mg/mL). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and *Paederia foetida* extracts was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 mL and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV–visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolve in distilled water to make the stock solution with the same concentration (5 mg/mL). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% ethanol was served as blank. % scavenging of the DPPH free radical was measured by using the following equation:

% Scavenging Activity = (Absorbance of Control - Absorbance of Sample)/ Absorbance of Control x 100

The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition.

Nitric oxide radical inhibition assay

Nitric oxide radical inhibition was determined by the use of Griess Illosvoy reaction [10]. In this investigation, Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *Paederia foetida* extract (10 µg to 320 µg) or standard solution (ascorbic acid, 0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink coloured chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

Scavenging of hydrogen peroxide

A modified method based on that of Ruch *et al.*[13] was used to determine the ability of the extract to scavenge hydrogen peroxide [14]. Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Standards (ascorbic acid) and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of standard or extract solutions (3.4 mL) were added to 0.6 mL of hydrogen peroxide solution. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. For each concentration, a separate blank sample was used for background subtraction. The percentage of scavenging was calculated as follows-

$$\% H_2O_2 \text{ Scavenging} = (\text{Absorbance of Control} - \text{Absorbance of Sample}) / \text{Absorbance of Control} \times 100$$

Reducing power

The reducing power of *Paederia foetida* was determined according to the method described previously [15,16]. Different concentrations of *Paederia foetida* extract (100 µg – 1000 µg) 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 portion (2.5 ml) of trichloroacetic acid (10%) 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 was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard.

Determination of total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of the extract was evaluated by the phosphomolybdenum method described previously [17]. The assay is developed based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95^0C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer) against blank after cooling to room temperature. Ethanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Membrane stabilizing activity

Preparation of erythrocyte suspension

Whole blood was obtained with heparinized syringes from a rat through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 minutes at 3000 g.

Hypotonic solution-induced rat erythrocyte haemolysis

Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte haemolysis [10]. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (0.25-2.0 mg/ml) or indomethacin (0.1 mg/ml). The control sample consisted of 0.5 ml of RBC mixed with hypotonic - buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated as follows-

$$\% \text{ Inhibition of haemolysis} = 100 \times [OD1\text{-}OD2/OD1]$$

Where:
OD1 = Optical density of hypotonic-buffered saline solution alone
OD2 = Optical density of test sample in hypotonic solution

RESULTS

Phytochemical screening of the extracts indicated the presence of flavonoids, saponin, gum and tannins (Table 1).

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Table 1: Phytochemical screening of *Paederia foetida* extract.

Extract	Alkaloids	Gum	Flavonoids	Tannins	Saponins
Extract of *Paederia foetida*	-	+	++	+++	++

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *Paederia foetida* are given in Figure 1. The IC_{50} values of the extracts were found to be 98.13 µg/mL whereas IC_{50} for ascorbic acid was 13.40 µg/mL.

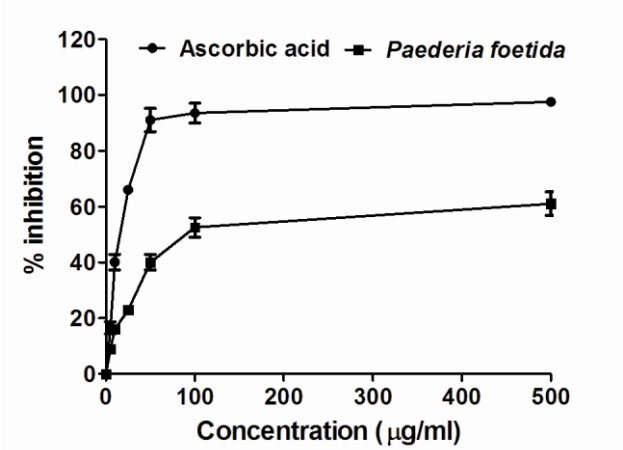

Figure 1: Scavenging of DPPH radical by ascorbic acid and extract of *Paederia foetida.*
Values are given as duplicate and expressed as Mean ± Standard deviation

Suppression of $NO\cdot$ release may partially be attributed to direct $NO\cdot$ scavenging activity. *Paederia foetida* extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro* (Figure 2). The scavenging of nitric oxide by the plant extract was increased in a dose-dependent manner. The IC_{50} value of the extract was 40.56 µg/mL whereas the IC_{50} value of vitamin C was 13.04 (Table 2).

Table 2: Scavenging of free radical by crude hydroethanolic extract of *Paederia foetida* and ascorbic acid in DPPH method, NO^- scavenging method and H_2O_2 scavenging method.

Sample	IC_{50} (µg/mL)		
	DPPH scavenging method	NO^- scavenging method	H_2O_2 scavenging method
Crude Hydroethanolic extract of *Paederia foetida* Linn.	98.13 µg/mL	40.56 µg/mL	133.00 µg/mL
Ascorbic acid	13.40 µg/mL	13.03 µg/mL	99.66 µg/mL,

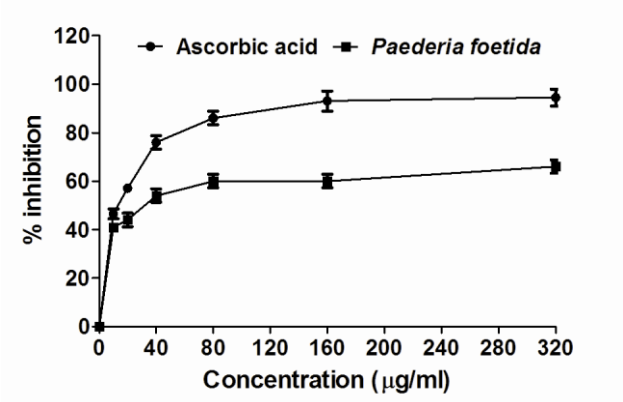

Figure 2: Scavenging of NO^- radical by ascorbic acid and extract of *Paederia foetida.*
Values are given as duplicate and expressed as Mean ± Standard deviation.

Our data on the reducing power of the tested extracts suggests moderate reducing properties. Like other antioxidant assays, the reducing power of *Paederia foetida* extracts was increased with increasing the amount of samples. Figure 3 shows the reducing ability of *Paederia foetida* extracts in comparison with ascorbic acid.

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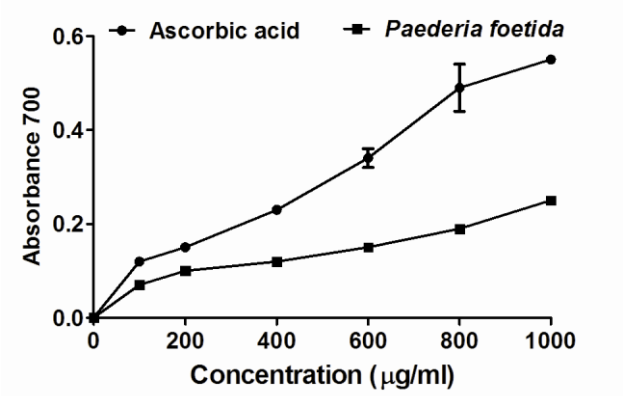

Figure 3: Reducing power of ascorbic acid and extract of *Paederia foetida.* Values are given as duplicate and expressed as Mean ± Standard deviation.

The scavenging of H_2O_2 by vitamin C and the extract of *Paederia foetida* after incubation for 10 min was increased with increased concentration of the sample. The extract exhibited higher H_2O_2 scavenging activity than vitamin C at similar concentrations. The IC_{50} values of the extracts and ascorbic acid were 133.00 µg/mL and 99.66 µg/mL, respectively (Table 2).

Total antioxidant capacity of the *Paederia foetida* extract, expressed as the number of equivalents of ascorbic acid, is shown in Figure 4. Total antioxidant capacity was also increased in a dose-dependent manner.

The extract of *Paederia foetida* at a concentration range of 0.50-2.0 mg/mL significantly protected the rat erythrocyte membrane against lysis induced by hypotonic solution (Table 3). In contrast, indomethacin (0.10 mg/mL) offered a significant protection of the rat red blood cells (RBC) against the damaging effect of a hypotonic solution.

Table 3: Membrane stabilizing activity of the extract of *Paederia foetida.*

Concentration	Absorbance	% Protection
Hypotonic medium 50 mM	0.35± 0.01	-
P. foetida 0.25 mg/mL	0.247±0.0057	16.51±1.15
0.5 mg/mL	0.219±0.0014	25.23±0.69
1.0 mg/mL	0.186±0.0057	42.17±1.46
1.5 mg/mL	0.084±0.0049	72.94±0.90
2.0 mg/mL	0.043±0.0035	86.55±1.29
Indomethacin (0.10 mg/mL)	0.051±0.003	85.26±0.41

Values are average of duplicate experiments and represented as mean ± standard deviation.

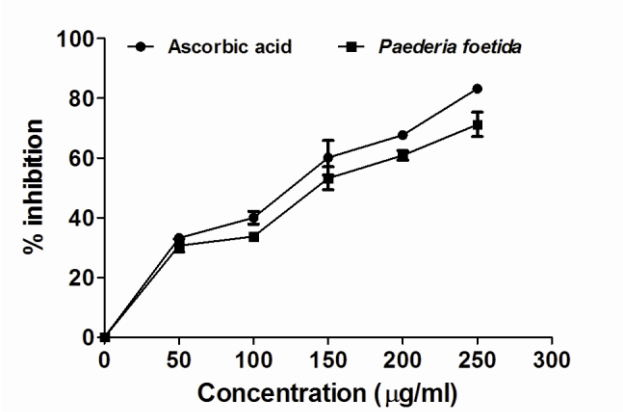

Figure 4: Scavenging of H_2O_2 radical by ascorbic acid and extract of *Paederia foetida.* Values are given as duplicate and expressed as Mean ± Standard deviation.

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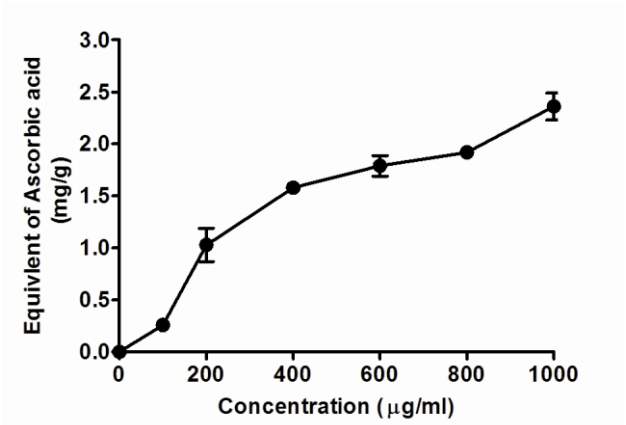

Figure 5: Total antioxidant capacity of extract of *Paederia foetida.* Values are given for two consecutive experiment and expressed as Mean ± Standard deviation

DISCUSSION

Phenolic compounds and flavonoids showed a wide range of biological activities *in vivo* and serve as strong antioxidants which scavenge singlet oxygen molecules and free radicals [18-20]. Medicinal plants are a good source of phenolic compounds and flavonoids. Previous report suggests that *Paederia foetida* extract prevented oxidation of β-carotene and linoleic acid and showed antioxidant activities in ABTS free radical scavenging assay system [8]. In this report, we showed that *Paederia foetida* leaves extract possess strong antioxidant activity in other *in vitro* antioxidant assay systems such as DPPH radical scavenging assay, reducing power, H_2O_2 radical scavenging assay, total antioxidant capacity assay and membrane stabilization assay.

DPPH antioxidant assay principal is mainly developed based on the idea that in presence of antioxidants, a stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) will decolorize [21]. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also develops a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. *Paederia foetida* showed scavenging activity of DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. This activity was increased with the increasing concentration of sample extract.

Nitric oxide is important for inflammation, cancer and other pathological conditions [22]. The toxicity and damage caused by $NO^{\bullet}$ and $^{\bullet-}O_2$ is multiplied as they react to produce reactive peroxynitrite ($^{\bullet-}OONO$) which leads to serious toxic reactions with biomolecules [22]. Plant extracts may have the property to counteract the effect of NO formation which in turn preventing the deleterious effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO to more reactive peroxynitrate ($^{\bullet-}OONO$). Suppression of $NO^{\bullet}$ released may be partially attributed to direct $NO^{\bullet}$ scavenging by *Paederia foetida* extracts which decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. The scavenging of nitric oxide by the *Paederia foetida* extract was also increased in a dose-dependent manner.

Reducing power assay was developed based on the conversion of Fe^{3+} to Fe^{2+} transformation in the presence of extract samples and serves as a significant indicator of potential antioxidant activity [15]. A direct co-relation between antioxidant activity and reducing power of certain plant extracts were reported earlier [23,24]. The reducing properties are generally associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [24,25]. In our study, reducing power of *Paederia foetida* extract was also increased in a dose dependent manner.

Hydrogen peroxide itself is not very reactive, but sometimes is toxic to cell because it may give rise to singlet oxygen ($^1O_2$) and a hydroxyl radical, which then become very powerful oxidizing agents in the cells [26,27]. Therefore, removing of H_2O_2 is very important for antioxidant defense in cell or food systems. Dietary polyphenols protects mammalian cells from cytotoxicity induced by hydrogen peroxide, especially compounds with the orthodihydroxy phenolic structure such as quercetin, catechin, gallic acid ester, caffeic acid ester[28,29] and terpenoids [30,31]. Therefore, the antioxidant compounds present in *Paederia foetida* extract may probably be involved in removing the H_2O_2. In our study, ascorbic acid and *Paederia foetida* extract scavenge H_2O_2 by a concentration dependent manner.

The phosphomolybdenum method working on the principle that reduction of Mo(VI) to Mo(V) by the antioxidant compounds which may form a green phosphate/Mo(V) complex [17]. This green phosphate/Mo(V) complex chromophore thus can be measured at maximum absorption at 695 nm with spectrophotometer. Total

antioxidant capacity was increased by increasing the concentration of *Paederia foetida* extract in assay medium. Total antioxidant capacity of the *Paederia foetida* extract was expressed as the number of equivalents of ascorbic acid.

Vitality of cells depends on the integrity of the cell membranes [32]. Red blood cell exposure to injurious substances such as hypotonic medium and phenyl hydrazine may cause rupture of the membrane accompanied by haemolysis and oxidation of haemoglobin [32]. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation [32]. Flavonoids are effective antioxidants protecting human red blood cells from free radical-induced oxidative haemolysis [33]. It is suggested that flavonoids can bind with the plasma membrane proteins and able to penetrate lipid bi-layers and alter the membrane fluidity [34,35]. Therefore, it is expected that compounds having membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances [33,34]. The extract of *Paederia foetida* at concentration range of 0.25-2.0 mg/mL significantly protected the rat erythrocyte membrane against lysis induced by hypotonic solution compared to indomethacin (0.10 mg/ml). Similar membrane stabilizing activity was also observed in a previously published report [6].

CONCLUSION

The plant *Paederia foetida* contains various phytoconstituents triterpenoid saponins such as ursolic acid, epifriedelinol and friedelin [36]. Triterpenoids are generally strong antioxidants effective against oxidative injuries. Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpenoid, exists widely in natural plants, also possess strong antioxidant activities[37] and protective against inflammation, hyperglycemia, hyperlipidemia and liver damage [31]. The present study demonstrated the effectiveness of *Paederia foetida* leaves extract as a potent antioxidant in various *in vitro* antioxidant assays. However, further studies on *Paederia foetida* leaves are solicited as progress in this area will extend the frontiers of exploiting the plant for neutraceutical and pharmaceutical purposes.

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REFERENCES

1. Ha HL, Shin HJ, Feitelson MA, Yu DY. Oxidative stress and antioxidants in hepatic pathogenesis. World J Gastroenterol. 2010;16:6035-43.
2. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol. 2009;7:65-74.
3. Davis AP, Govaerts R, Bridson DM, Ruhsam M, Moat J, Brummitt NA. A global assessment of distribution, diversity, endemism, and taxonomic effort in the *rubiaceae*. Ann Missouri Bot. 2009;96:68-78.
4. Ghani A. Medicinal Plants of Bangladesh. The Asiatic Society of Bangladesh, Dhaka, Bangladesh; 2003.
5. Raghunatban K, Mitra R. Pharmacognosy of Indigenous Drugs. Central Council for Research in Ayurveda and Siddha, New Delhi, India; 1982.
6. De S, Ravishankar B, Bhavsar G. Investigation of the anti-inflammatory effects of *Paederia foetida*. J Ethnopharmacol. 1994;43:31-8.
7. Uddin B, Nahar T, Basunia M, Hossain S. *Paederia foetida* protects liver against hepatotoxin-induced oxidative damage. Adv Biol Res. 2011;5:267-72.
8. Osman H, Rahim AA, Isa NM, Bakhir NM. Antioxidant activity and phenolic content of *Paederia foetida* and *Syzygium aqueum*. Molecules. 2009;14:970-8.
9. Akter R, Hasan SMR, Siddiqua SA, Majumder MM, Hossain MM, Alam MA, et al. Evaluation of analgesic and antioxidant potential of the leaves of *Curcuma alismatifolia* Gagnep. S J Pharm Sci. 2008; 1:3-9.
10. Alam MA, Ghani A, Subhan N, Rahman M, Haque M, Majumder M, et al. Antioxidant and membrane stabilizing properties of the flowering tops of *Anthocephalus cadamba*. Nat Pro Commun. 2008;3:65-70.
11. Alam MA, Nyeem M, Awal M, Mostofa M, Alam MS, Subhan N, et al. Antioxidant and hepatoprotective action of the crude methanolic extract of the flowering top of *Rosa damascena*. Orient Pharm Exp Med. 2008;8:164-70.
12. Saha MR, Alam MA, Akter R, Jahangir R. In vitro free radical scavenging activity of *Ixora coccinea* L. Bangladesh J Pharmacol. 2008;3:90-6.
13. Ruch R, Chung S, Klaunig J. Spin trapping of superoxide and hydroxyl radicals. Meth Enzymol. 1984;105:198–209.
14. Shon MY, Kim TH, Sung NJ. Antioxidants and free radical scavenging activity of *Phellinus baumii* (Phellinus of Hymenochaetaceae) extracts. Food Chem. 2003;82:593-7.
15. Berker K, Demirata B, Apak R. Determination of total antioxidant capacity of lipophilic and hydrophilic antioxidants in the same solution by using ferric–ferricyanide assay. Food Anal Methods. 2012;5:1150-8.

16. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Jpn J Nutr. 1986;44:307−15.
17. Prieto P, Ineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal Biochem. 1999;269:337–41.
18. Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci. 2012;196:67-76.
19. Atmani D, Chaher N, Atmani D, Berboucha M, Debbache N, Boudaoud H. Flavonoids in human health: from structure to biological activity. Curr Nutr Food Sci. 2009;5:225-37.
20. Mustafa RA, Hamid AA, Mohamed S, Bakar FA. Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants. J Food Sci. 2010;75:C28-C35.
21. Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol. 2004;26:211-9.
22. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991;43:109-42.
23. Bursal E, Köksal E. Evaluation of reducing power and radical scavenging activities of water and ethanol extracts from sumac (*Rhus coriaria* L.). Food Res Int. 2011;44:2217-21.
24. Wang J, Zhang Q, Zhang Z, Song H, Li P. Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from *Laminaria japonica.* Int J Biol Macromol. 2010;46:6-12.
25. Sun ZW, Zhang LX, Zhang B, Niu TG. Structural characterisation and antioxidant properties of polysaccharides from the fruiting bodies of *Russula virescens*. Food Chem. 2010;118: 675-80.
26. Halliwell B. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch Biochem Biophys. 2008;476:107-12.
27. Halliwell B, Hoult JR, Blake DR. Oxidants, inflammation, and anti-inflammatory drugs. FASEB J. 1988;2:2867-73.
28. de Groot H, Rauen U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. Fundam Clin Pharmacol. 1998;12:249-55.
29. Seibert H, Maser E, Schweda K, Seibert S, Gülden M. Cytoprotective activity against peroxide-induced oxidative damage and cytotoxicity of flavonoids in C6 rat glioma cells. Food Chem Toxicol. 2011;49:2398-407.
30. Graßmann J. Terpenoids as Plant Antioxidants. In: Gerald L, Editor. Vitamins & Hormones. Academic Press, p. 505-35; 2005.
31. Liu J. Oleanolic acid and ursolic acid: Research perspectives. J Ethnopharmacol. 2005;100: 92-4.
32. Ferrali M, Signorini C, Ciccoli L, Comporti M. Iron release and membrane damage in erythrocytes exposed to oxidizing agents, phenylhydrazine, divicine and isouramil. Biochem J. 1992;285:295-301.
33. Dai F, Miao Q, Zhou B, Yang L, Liu ZL. Protective effects of flavonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells. Life Sci. 2006;78: 2488-93.
34. Blasa M, Candiracci M, Accorsi A, Piacentini M, Piatti E. Honey flavonoids as protection agents against oxidative damage to human red blood cells. Food Chem. 2007;104:1635–40.
35. Sánchez-Gallego JI, López-Revuelta A, Sardina JL, Hernández-Hernández Á, Sánchez-Yagüe J, Llanillo M. Membrane cholesterol contents modify the protective effects of quercetin and rutin on integrity and cellular viability in oxidized erythrocytes. Free Radical Bio Med. 2010;48:1444-54.
36. Shukla YN, Lloyd HA, Morton JF, Kapadia GJ. Iridoid glycosides and other constituents of *Paederia foetida*. Phytochem. 1976;15:1989-90.
37. Ramachandran S, Prasad NR. Effect of ursolic acid, a triterpenoid antioxidant, on ultraviolet-B radiation-induced cytotoxicity, lipid peroxidation and DNA damage in human lymphocytes. Chem Biol Interac. 2008;176:99-107.