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Antioxidant and membrane stabilizing properties of *Ichnocarpus frutescens*

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Abstract

The antioxidant potential of the 70% methanolic extract of *Ichnocarpus frutescens* was assessed by the 1, 1-diphenyl-2-picrylhydrazyl (DPPH), the hydrogen peroxide, the nitric oxide, the reducing power and the lipid peroxidation inhibition (thiobarbituric acid-reactive substances production) and the red blood cell (RBC) membrane stabilization assays. The extract showed significant antioxidant activities in all assays in a dose dependent manner. The extracts displayed notable activities in reactive oxygen species (ROS) scavenging which could be attributed to the high phenolic content of this extract. Moreover, *I. frutescens* extract showed strong reducing power and an ability to suppress lipid peroxidation. Suppression of lipid peroxidation and nitric oxide scavenging would be the probable mechanism of the stabilization of the RBC membrane. In the DPPH radical scavenging assay the IC_{50} value of the extract was found to be 23.75 μg/mL. The extract inhibited the nitric oxide radicals generated from sodium nitroprusside and the IC_{50} value was found to be 69.69 μg/mL, comparable to that of 86.83 μg/mL for the positive control ascorbic acid.

Key words: *Ichnocarpus frutescens*, Apocynaceae, antioxidant, lipid peroxidation, reactive oxygen species.

1. Introduction

Ichnocarpus frutescens (L.) R. Br., known as 'Dudhilata' in Bengali (family: Apocynaceae) is a medicinally important evergreen climbing shrub that grows abundantly in all parts of Bangladesh as well as in India, SriLanka, Thailand, Malaysia, Indonesia, Philippines and Australia [1, 2]. The ethnomedicinal uses of this plant include its use as a laxative, demulcent, depurative, diaphoretic, diuretic and in the treatment of asthma, bronchitis, cholera, cough, dog-bites, diabetes, dysentery, fever, jaundice, measles, night blindness, small pox, snake-bites, sore, syphilis,

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tumour and wound [3]. The crude extract of the leaves of this plant have been shown to reduce fever, headache, inflammation and plasma glucose in diabetes [4, 5]. Leaves of *I. frutescens* have been reported to contain flavonoids and phenolic acids and stems to contain triterpene glycosides [6, 7]. As part of our on-going phytochemical and bioactivity studies on Bangladeshi medicinal plants [9-14], we now report on the antioxidant and the membrane stabilizing activity of the 70% methanolic extract of the leaves of *I. frutescens*.

2. Materials and methods

2.1 Plant material

The leaves of *Ichnocarpus frutescens* (L.) R. Br. were collected from Jahangirnagar University campus, Savar, Bangladesh in December 2006, and was identified at the Bangladesh National Herbarium, Mirpur, Dhaka, where the Voucher specimen (no:31.304) was deposited.

2.2 Extraction

Dried ground leaves (100 g) were Soxhlet-extracted with 70% aqueous MeOH (500 mL). The extract was concentrated by evaporation under reduced pressure at 40°C to yield a gummy concentrate of greenish color extract.

2.3 Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), ammonium molybdate, hydrogen peroxide (H_2O_2), sodium nitroprusside were purchased from Merck, Germany and ethylene diamine tetra acetic acid (EDTA), sodium phosphate, sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride from BDH, England. Ferric chloride was obtained from Thomas Baker and Potassium ferricyanide was from Guandong Chemical Reagent, China. All other reagents were of analytical grade.

2.4 The DPPH assay

The method used by Takao *et al*. [15] was adopted with suitable modifications [16, 17]. DPPH (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 μg/mL.

Qualitative analysis: Test samples were applied on a TLC plate and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour changes (purple to white) were noted.

Quantitative analysis: Serial dilutions were performed with the stock solution (10 mg/mL) of the plant extract to obtain concentrations of $5x10^{-1}$, $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$, $5x10^{-6}$, $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$, $5x10^{-10}$ mg/mL. Diluted solutions (2 mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in duplicate and the average absorption was noted for each concentration. The RC_{50} value, which is the concentration of the test material that reduces 50% of the free radical concentration, was calculated as mg/mL.

2.5 Nitric oxide radical inhibition assay

Nitric oxide radical inhibition could be estimated by the use of Griess Illosvoy reaction [18]. 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 *I. frutescens* extract (10 μg to 160 μg) or standard solution (BHT, 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, naphthyl ethylene diamine dihydrochloride (1 mL) was

added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Ascorbic acid was used as a positive control.

2.6 Hydrogen peroxide scavenging assay

A modified method based on that of Ruch *et al.* [19] was used to determine the ability of the extracts to scavenge hydrogen peroxide. Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Positive control (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. The percentage of scavenging was calculated as follows:

% H_2O_2 Scavenging = 100 x (Absorbance of Control- Absorbance of Sample) / Absorbance of Control

2.7 Reducing power

The reducing power of *I. frutescens* was determined by the method described by Oyaizu [20]. Different concentrations of the extract (100 μg – 1000 μg) in 1 mL of distilled water was mixed with phosphate buffer (2.5 mLl, 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. Butylated hydroxy toluene (BHT) was used as a positive control.

2.8 Determination of total antioxidant capacity

The total antioxidant capacity of the *I. frutescens* extract was assessed by the phosphomolybdenum method [21]. The assay is 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 3 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C 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. Methanol (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.

2.9 Membrane stabilizing activity

Preparation of erythrocyte suspension: Whole blood was obtained with heparinized syringes from rats 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 min at 3000 g.

Hypotonic solution-induced rat erythrocyte haemolysis: Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte haemolysis [22]. 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 according to modified method described by Shinde *et al.* [22].

% Inhibition of haemolysis = 100 x {OD1-OD2/ OD1}

Where: OD1 = Optical density of hypotonic-buffered saline solution alone

OD2 = Optical density of test sample in hypotonic solution

3. Results and discussion

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *I. frutescens* is presented in Table 1. The DPPH antioxidant assay is 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 the absorbance at 515-517 nm and also for 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. In the TLC-based qualitative antioxidant assay using the DPPH spray, the extract of *I. frutescens* showed prominent free radical scavenging properties indicated by the presence of a yellowish-white spot on a reddish purple background on the TLC plate. From the quantitative assay, the IC_{50} value of the extract was determined 23.75 µg/mL, as opposed to that of ascorbic acid (IC_{50} 56.96 µg/mL), which is a well known antioxidant.

Table 1. Antioxidant potential of *I. frutescens* in various *in vitro* assays

Test material	IC_{50} value in µg/mL		
	DPPH[a]	NOS[b]	HPS[d]
I. frutescens extract	23.75	69.69	190.89
Ascorbic acid	56.96	86.83	197.00

[a]1, 1-Diphenyl-2-picryl-hydrazyl assay; [b]Nitric oxide scavenging assay; [c]Hydrogen peroxide scavenging assay

Table 2. Effect of the methanolic extract of *Ichnocarpus frutescense* on rat erythrocyte haemolysis

Sample	Extract Concentration	Optical Density	% Inhibition of haemolysis
Hypotonic medium	50mM	0.462 ± 0.05	-
	0.25 mg/mL	0.368 ± 0.04	20.36
	0.50 mg/mL	0.364 ± 0.06	21.214
Ichnocarpus Frutescens	1.00 mg/mL	0.362 ± 0.05	21.645
	1.50 mg/mL	0.158 ± 0.08	65.80
	2.00 mg/mL	0.150 ± 0.04	67.53
Indomethacin	0.10 mg/mL	0.230 ± 0.02	50.216

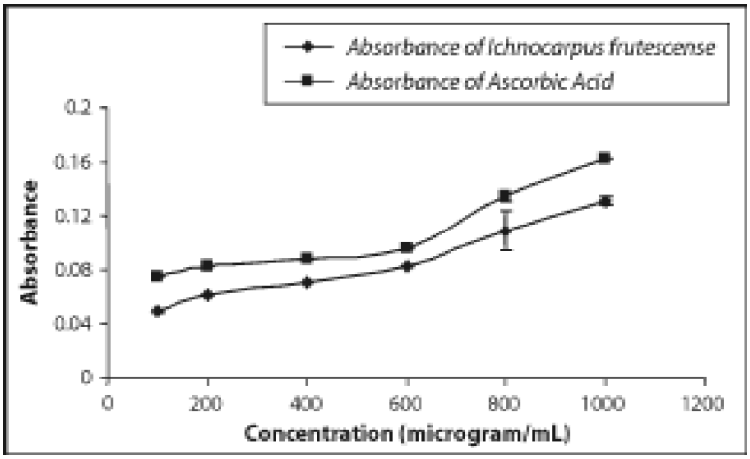

Fig. 1. Reducing power of the crude plant extract *I. frutescens*. Values are the average of duplicate experiments and represented as mean ± standard deviation.

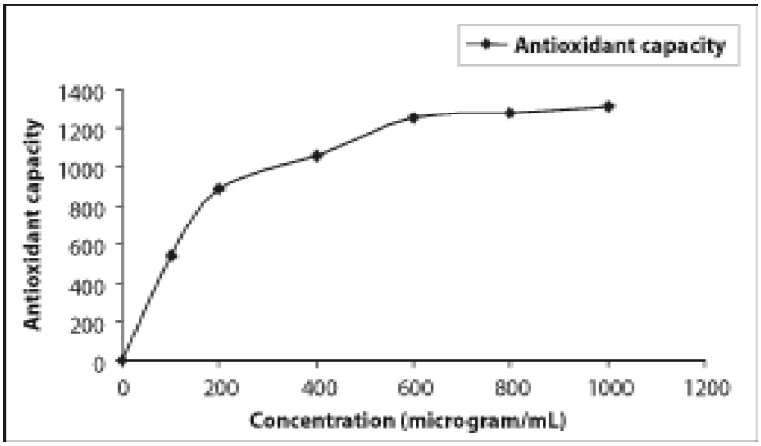

Fig. 2. Total antioxidant capacity of the extract of *I. frutescens*. Values are the average of duplicate experiments and represented as mean ± standard deviation.

The extract of *I. frutescens* decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro* (Table 1). Suppression of NO· released might be partially attributed to direct NO· scavenging. The scavenging of NO· by the plant extract increased in a dose-dependent manner. While the IC_{50} value of the extract was 69.69 μg/mL, that of ascorbic acid was 86.83 μg/mL. It is well known that a direct correlation exists between the antioxidant activity and the reducing power of plant extracts. 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 [23]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. From the observed reducing power with the test extract in this study, it could

be assumed that this reducing power might have contributed towards the observed antioxidant effect of this extract. Like the antioxidant activity, the reducing power of methanol extract increased with increasing amount of sample (Figure 1).

The scavenging of H_2O_2 by ascorbic acid and the extract of *I. frutescens* after incubation for 10 min increased with the increase of concentration. The extract exhibited higher H_2O_2 scavenging activity than ascorbic acid at similar concentrations. The IC_{50} values of the extract and ascorbic acid were 190.89 and 197.00 µg/mL, respectively (Table 1). Removal of H_2O_2 is important for antioxidant defense in cell or food systems. Dietary polyphenols are known to protect mammalian and bacterial cells from cytotoxicity induced by hydrogen peroxide, especially compounds with the orthodihydroxy phenolic structure e.g. quercetin, catechin, gallic acid ester, caffeic acid ester [24].

Therefore, the phenolic compounds of the test extract might be responsible for removing the H_2O_2. Total antioxidant capacity of the *I. frutescens* extract, expressed as the number of equivalents of ascorbic acid, is shown in Figure 2. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. The vitality of cells depends on the integrity of their membranes. Exposure of red blood cell to injurious substances such as hypotonic medium and phenyl hydrazine results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin [25]. The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation. Compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reactions, namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators [26]. The extract of *I. frutescens* at a concentration range of 0.50-2.0 mg/mL significantly protected the rat erythrocyte membrane against lysis induced by hypotonic solution (Table 2) and indomethacin (0.10 mg/mL) offered a significant protection of the rat red blood cells (RBC) against the damaging effect of a hypotonic solution. At a concentration of 2.0 mg/mL, the extract produced 67.53% inhibition of the RBC haemolysis, as compared to 50.216% produced by indomethacin.

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