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Secondary Metabolites and Antioxidant Activity of the Crude Leaf Extract of *Bacopa monniera* (L.) Pennel. and *Coccinia grandis* (L.) J. Voigt

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ABSTRACT
Leaf extracts of *Bacopa monniera* (L.) Pennel. and *Coccinia grandis* (L.) J. Voigt. were assessed for their secondary metabolites content and antioxidant activities, which were measured by spectrophotometer using 2, 2- diphenyl-1- picrylhydrazyl (DPPH) radical. Antioxidant activity was dose dependent and showed inverse relation with decoloration of DPPH from purple to yellow due to its reduction by antioxidants. The IC_{50} values of the antioxidants were determined by linear regression analysis. Results of the antioxidant activities of crude leaf extracts were discussed in relation to the presence of secondary metabolites and compared with standard antioxidant ascorbic acid

Keywords: Leaf Extract, Secondary Metabolites, Antioxidants, DPPH, Free Radical, IC_{50} Value.

1. Introduction
Medicinal plants produce a vast array of secondary metabolites [1] and such metabolites have been found to have a broad range of therapeutic properties [2], including antioxidant activities [3, 4]. Through oxidation reactions, living cell generates a number of reactive oxygen species (ROS) like superoxide, hydroxyl, peroxyl, alkoxy, nitric oxide etc., which induce oxidative stress and initiate chain reactions leading to cell damage and various diseases [5] while antioxidants prevent the oxidation of other molecules, cancel out the cell-damaging effects of free radicals [6] and lower the risk of different diseases [7]. Several enzymes as well as non enzymatic secondary metabolic compounds of plant origin are able to scavenge ROS [8] and thus can protect the organism from oxidative damage. In the present work, ethanolic extracts of the leaf of *Bacopa monniera* (L.) Pennel. and *Coccinia grandis* (L.) J. Voigt. were examined for their secondary metabolites content and antioxidant activities.

2. Materials and Methods
a. Preparation of leaf extract
Leaves of *Bacopa monniera* (L.) Pennel. and *Coccinia grandis* (L.) J. Voigt., collected from their natural habitat of Chittagong University campus at flowering stage, were used for extraction. Collected samples were cleansed, chopped, air- dried at room temperature and finally grinded into powder. From each plant sample, 100g powder was macerated with ethanol (1:5) in a sealed container for 5 days at room temperature with occasional shaking. Extract of each sample was filtered separately through Whatman No.1 filter paper and was evaporated to dryness under vacuum below 50 ^{o}C to get about 3g blackish-green colored extract. The leaf extract thus prepared was kept at 4 ^{0}C for future use.

b. Determination of secondary metabolites
Dragendroff, Hager, Mayer, Wagner and Tannic acid reagents, prepared following Cromwell [9], were used for alkaloid assessment following the method developed by Webb [10] and others [11, 12]. Flavonoids were assessed following Wall *et al.* [13] and Farnsworth [14] and tannins according to Wall *et al.* [13]. Terpenes and sterols were determined following Bhattachrjee and Das [15] and Libermann- Burchard's test, respectively. To a small amount of alcoholic extract dissolved in 1 ml dist. H_2O a few drops of aqueous solution of sodium hydroxide were added and development of yellow color indicated the presence of glycosides. Water extract producing a persistent frothing on vigorous shaking (permanent foam which remained stable on heating) indicated the presence of saponins.

c. Antioxidative capacity

The antioxidative activity of the ethanolic extract was examined by comparing it with that of a known antioxidant compound, ascorbic acid, by the assays of scavenging stable free radical, 2, 2- diphenyl-1- picrylhydrazyl (DPPH) following Cuendet *et al.*[16] with slight modifications.

d. DPPH assay

The reaction mixture contained 3ml 0.004% DPPH in 100% ethanol and 5ml (20 to 800µg/ml) crude leaf extract or ascorbic acid solution in case of experiment or standard control, respectively. After 30 min incubation period at room temperature (19 ^{0}C) in the dark, the absorbance, optical density (OD), was measured against a blank at 517 nm in UV-Visible Spectrophotometer (Shimadzu, Japan). The degree of decolonization of DPPH from purple to yellow following reduction (Fig. 1) indicated the scavenging efficiency of the extract or ascorbic acid solution. Lower absorbance followed by the degree of decoloration of the reaction mixture indicated the free radical scavenging efficiency of the substances. The percentage of DPPH decoloration (scavenging) activity was calculated with the help of the following formula:

$$\% \text{ of scavenging activity} = \left(\frac{A-B}{A}\right) \times 100$$

where, *A* was the absorbance of control (DPPH solution without the sample), *B* was the absorbance of DPPH solution in the presence of the sample (leaf extract / ascorbic acid solution).Values are presented as mean with ± SE of the mean of three replicates. The % scavenging activity was plotted against log concentration and the IC_{50} (inhibition concentration 50, µg/ml) value of leaf extract was calculated by using linear regression analysis.

3. Results and Discussion

Secondary metabolites such as alkaloids, flavonoids, sterols, resins, tannins, glycosides and saponins contents were assessed qualitatively in the leaf extract of *B. monniera* and *C. grandis* (Table 1). Qualitative test of alkaloid in the leaf extracts of two plant species indicated its moderate (2+ and 3+) presence, a bit higher concentration was being noted in *C. grandis* than *B. monniera* because *C. grandis* gave more 3+ responses with different reagents. Dragendorff (D) and Wagner (W) reagents appeared more effective in the assay. Alamgir *et al.* [17, 18] noted the presence of alkaloid in 7 plant species while Krishnaiah *et al.* [19] and Koche *et al.* [20] found alkaloids distribution in all 14 medicinal plants they examined. All other secondary metabolites considered in the present work were present in both the species except sterols in *B. monniera* and saponins in *C. grandis*. Din *et al.*[21], in a phytochemical screening of alkaloids, steroids, triterpenes and saponins carried out on 103 leaf samples from 102 plant species representing 78 genera and 41 families of Malaysia, reported that a total of 4, 19 and 53 leaf samples gave positive results for alkaloids, steroids/triterpenes and saponin, respectively. Edeoga *et al.* [22] detected the presence of alkaloids, cardiac glycosides, flavonoids, phlobatannin, saponins, steroids, tannins and terpenoids in all 10 medicinal plant species they examined. Sporadic and uneven distributions of all these secondary metabolites in different medicinal plant species, however, are reported by others [19, 20, 23].

In the present work, the scavenging activities of the ethanolic extract of leaf of *B. monniera* and *C. grandis* were determined and compared with that of the standard antioxidant ascorbic acid. DPPH is a radical and a trap (scavenger) for other radicals. DPPH free radical in solution is purple in color with its odd electron and gives a strong absorption maximum at 517 nm and the color of DPPH turns from purple to yellow (Figures 1, 2) when the odd electron becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H when the molar absorptivity of the DPPH radical undergoes reduction. The % of scavenging activity (reverse to decoloration) of both ascorbic acid and leaf extracts were increased almost linearly with the rise of their concentrations similar to that of the first order reaction of enzyme kinetics (Figure 3). The resulting dose dependent decolorization is stoichiometric with respect to number of electrons captured and, therefore, the rate of reduction of a chemical reaction upon addition of DPPH is used as an indicator for antioxidant activity and thus explains the mechanism DPPH free radical scavenging activity.

The scavenging kinetics of both ascorbic acid and leaf extracts after the zone of the first order reaction later became nonlinear and, however, showed dose dependent activity (Table 2). The spectrometric absorptive activities at 517nm for different concentrations were inversely related to respective free radical scavenging activities. Among the eight different concentrations (20 to 800µg/ml) of ascorbic acid and leaf extracts of two plant sources, the highest scavenging activities were 96.86%, 75.85% and 86.02%, respectively for ascorbic acid, *B. monniera* and *C. grandis* at 800µg/ml concentration. Ghosh *et al.* [24] with similar extract of the aerial parts of *B. monniera* also noted concentration dependent scavenging activities.

Concentrations of the antioxidants (leaf extracts/ascorbic acid standard) required for the inhibition of DPPH radical scavenging effect up to 50%, IC_{50}, were calculated by linear regression analysis from a graph constructed by plotting the % of scavenging values against the respective log concentration of antioxidatants (Figure 4). The calculated IC_{50} values for ascorbic acid, leaf extracts of *B. monniera* and *C. grandis* were 2.19, 224.08 and 106.25µg/ml, respectively (Table 2, Figure 4). The IC_{50} value denotes 50% inhibition of absorbance; a lower value would reflect greater antioxidant activity of the sample and vice-versa. Ghosh *et al.* [24] with *B. monniera* noted IC_{50} value 238.22µg/ml, a higher value than that of the present work, while Mishra *et al.* [25] with the methanolic extract of *C. grandis* noted IC_{50} value 54.6 µg/ml, a lower value, than that of the present work. While examining the antioxidant activities of the methanolic and ethanolic extracts of a large number of plant species different investigators noted a wide range of variation in IC_{50} values [26]. Both the plants of the present work showed a high level free radical (DPPH) scavenging or antioxidant activity, *C. grandis* had higher activity than that of *B. monniera*. The free radical scavenging (antioxidant) activities of these plants species are due to the different secondary metabolites and they probably contribute to their effectiveness in therapeutic use.

Table 1: Secondary metabolites (alkaloids, flavonoids, sterols, resins, tannins, glycosides and saponins) contents in the leaf of *Bacopa monniera* (L.) Pennel. and *Coccinia grandis* (L.) J. Voigt.

Scientific name and family	Plant part used	Secondary metabolites of the leaf extracts										
		Alkaloids					Metabolites other than alkaloids					
		Reagents used					Flavonoids	Sterols	Resins	Tannins	Glycosides	Saponins
		D	H	M	T	W						
Bacopa monniera (L.) Pennel. (Scrophulariaceae)	Leaf	3+	2+	2+	2+	3+	+	-	+	+	+	+
Coccinia grandis (L.) J. Voigt. (Cucurbitaceae)	Leaf	3+	3+	3+	2+	3+	+	+	-	+	+	+

Table 2: DPPH free radical scavenging activity of ascorbic acid, *Bacopa monniera* (L.) Pennel. and *Coccinea grandis* (L.) J. Voigt (leaf) ethanol extract.

Bacopa monniera Conc. (µg/ml)	Log Conc.	Absorbance at 550 nm			Scavenging activity, %			IC_{50}, µg/ml of different antioxidant agents		
		Ascorbic acid	*B. monniera*	*C. grandis*	Ascorbic acid	*B. monniera*	*C. grandis*	Ascorbic acid	*B. monniera*	*C. grandis*
Control	-	0.568	0.421	0.472	-	-	-	2.19	224.08	106.25
20	1.30	0.170	0.374	0.345	68.02	20.76	26.91			
40	1.60	0.147	0.360	0.332	72.08	23.73	29.66			
60	1.78	0.118	0.351	0.310	76.30	25.64	34.32			
80	1.90	0.090	0.334	0.275	80.60	29.24	41.74			
100	2.00	0.065	0.323	0.226	85.65	31.57	52.12			
200	2.30	0.053	0.255	0.156	89.37	45.97	66.95			
400	2.60	0.031	0.190	0.116	93.85	59.75	75.42			
800	2.90	0.015	0.114	0.066	96.86	75.85	86.02			

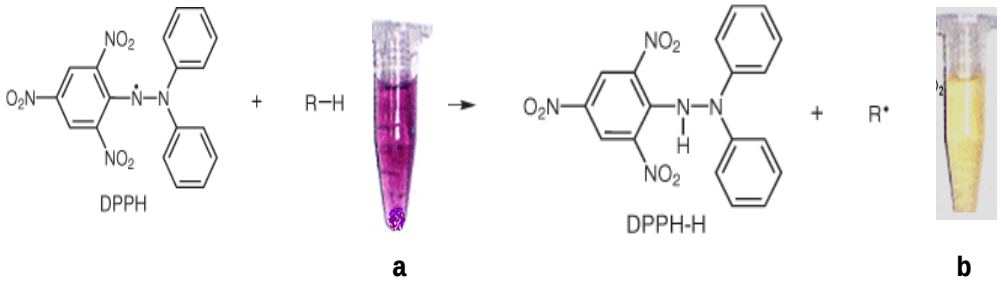
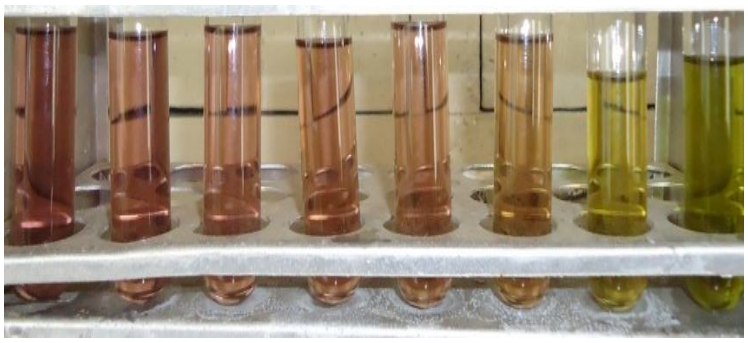


Fig 1: Principle of DPPH free radical scavenging mechanism and color transformation from purple(a) to yellow(b) on abstraction of hydrogen in the antioxidant reaction.



L → R

Fig 2: Dose dependent decolorization of DPPH from purple to yellow (L→R) dependent on the degree of reduction (DPPH→DPPH-H) by antioxidant (ascorbic acid or leaf extract).

Each test tube contained 3ml DPPH (0.004%) and 5ml of ascorbic acid or leaf extract at different concentrations (20 to 800µg/ml). Test tubes showing gradual color change from purple to yellow signify increased reduction of free radical (DPPH), increased antioxidant and decreased absorptive activity.

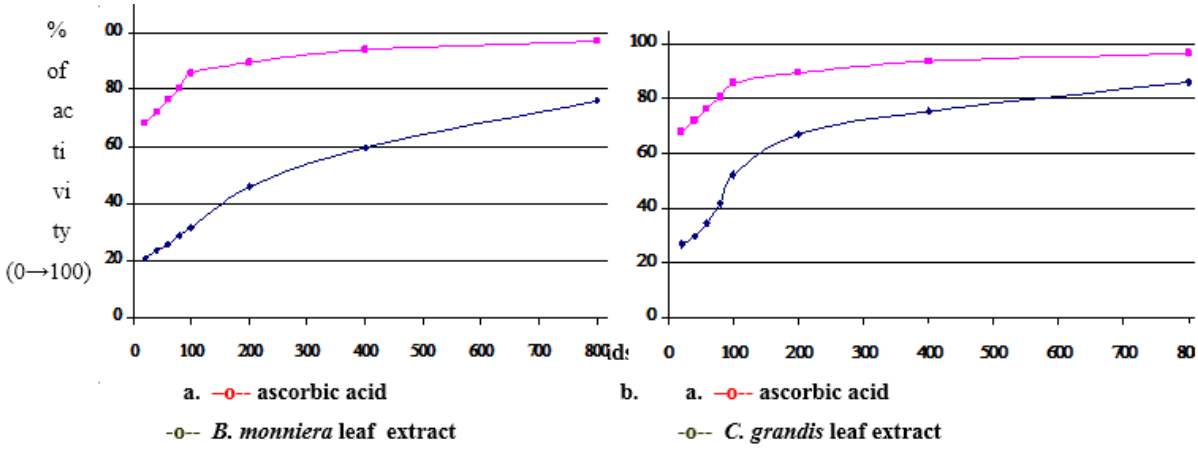


Fig 3: The % scavenging activity of standard antioxidant ascorbic, *B. monniera* and *C. grandis* leaf extract.

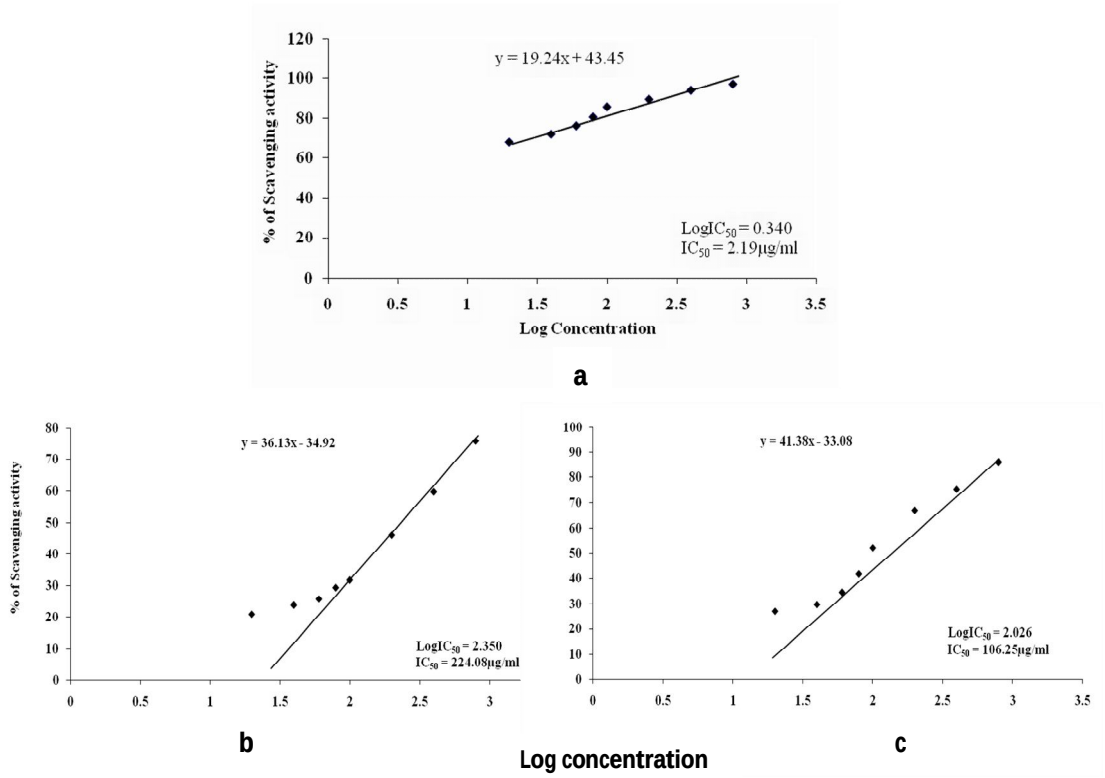


Fig 4: Regression line for IC_{50} value of ascorbic acid (a), *B. monniera* (b), and *C. grandis* (c) leaf extracts was constrated by putting % of scavenging activity against log concentration. For a. ascorbic acid, y= 19.24x + 43.45, log IC_{50}=0.340, IC_{50}=2.19µg/ml; b. *B. monniera*, y= 36.13x-34.92, log IC_{50}= 2.350, IC_{50}=224.08 µg/ml; and c. *C. grandis*, y= 41.31x-33.08, logIC_{50}=2.026, IC_{50}= 106.25 µg/ml.

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