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PHYTOCHEMICAL, ANTIOXIDANT AND CYTOTOXICITY STUDY OF THE METHANOLIC EXTRACTS OF LEAVES OF *TRAPA BISPINOSA* ROXB.

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ABSTRACT: About 80% of the population living in rural areas in developing countries depends on traditional medicine for their health care needs. Many of the powerful drugs used in modern medicines originated in plants. Today's plant-based drugs treat a range of diseases, from headaches to cancer. The objective of the present study was to evaluate phytochemical, antioxidant potential, and cytotoxicity of methanolic extract of leaves of *Trapa bispinosa* Roxb. The phytochemical screening demonstrated the presence of carbohydrates, glycosides, alkaloid, saponins, and tannins. Total phenolic and flavonoid content of the *Trapa bispinosa* Roxb. extracts was determined by using the Folin- Ciocalteu reagent and aluminum chloride (AlCl₃) method, respectively. Antioxidant activity was evaluated by using 1, 1-diphenyl-2-picryl-hydrazil (DPPH) free radical assay. In DPPH assay, free radical scavenging activity of the extracts was evaluated comparing with ascorbic acid at the wavelength of 517 nm and the IC₅₀ value of *Trapa bispinosa* Roxb. extracts were found to be 1.58 µg/ml, whereas ascorbic acid showed the value of 1.129 µg/ml. Moreover, it showed moderate reducing power. In the case of brine shrimp cytotoxicity assay, LC₅₀ value was obtained to be 2.87 µg/ml. The present study might be extended for the formulation and evaluation of different antioxidant herbal dosage forms.

INTRODUCTION: Many diseases are linked with "oxidative stress" that results from an imbalance between the formation and neutralization of pro-oxidants. Oxidative stress is initiated by free radicals, which cause protein and DNA damage along with lipid peroxidation and these changes contribute to various diseases such as cancer, atherosclerosis, cardiovascular diseases, aging and inflammatory diseases ¹.

To counter pro-oxidants, the body is endowed with another category of compounds called antioxidants which are produced either endogenously or received from exogenous sources ². Synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have recently been reported to be dangerous for human health. Current research is directed towards finding naturally-occurring antioxidants of plant origin ³.

Trapa bispinosa Roxb. (family Trapaceae) is a small herb well known for its medicinal properties ⁴. In Bangladesh, there exists a variety of water chestnut locally known as Paniphal or Singhara. The fruit (nut) of water chestnut is eaten by humans in raw or cooked form ⁵. Water chestnut (*Trapa bispinosa*) is the major source of starch which

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contains approximately 72% starch. Starch is extracted from *Trapa bispinosa*, and it is used in yoghurt as stabilizing agent⁶. It has been used in traditional system of medicine like Unani and Ayurveda since centuries for many medical conditions like strangury, dysuria, polyuria, sexual debility, general debility, sore throat, lumbago, bilious affections and dysentery, etc.⁷ The fruits *Trapa bispinosa* Roxb. are reported to be used in the treatment of leprosy, burning sensation, fatigue, inflammation, biliousness, strangury and fractures^{8, 9}. Earlier studies reported that *Trapa bispinosa* possesses antimicrobial and cytotoxic¹⁰, antiulcer¹¹, antidiabetic¹² nootropic¹³, and neuroprotective activity¹⁴.

The aim of the present study was to evaluate the phytochemical, *in-vitro* antioxidant activities and cytotoxicity of *Trapa bispinosa* Roxb.

MATERIALS AND METHODS:

Plant Material: Fresh leaves of *Trapa bispinosa* Roxb. were collected from Savar lakes, Savar, Dhaka area and was identified by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka (Accession No. 38204).

Extraction: The leaves of the plant were collected in fresh condition. It was sun-dried first and then, dried in an oven at reduced temperature (< 70 °C) to make it suitable for grinding. The powdered plant materials were submerged in sufficient volume of methanol in an air-tight flat bottomed container for seven days, with occasional shaking and stirring. The extracts were then filtered and dried on an electrical water bath.

Drugs and Chemicals Used: 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid, quercetin, gallic acid were obtained from Sigma Chemical Co (MO, USA). Folin-Ciocalteu reagent (FCR), was purchased from E Merc. All other chemicals and reagents were of analytical grade.

Phytochemical Tests: Various phytochemical tests that include Molisch's test for carbohydrates, test for glycosides, Borntrager's test for anthraquinone glycosides, Mayer's reagent; Hager's reagent and Dragendorff's reagent for alkaloids, Frothing test for saponins, Hydrochloric acid test for flavonoids, Salkowski's test for steroids and Ferric chloride test for tannins.

Determination of Total Phenol: Total phenols were determined by Folin Ciocalteu reagent (Folin and Ciocalteu, 1927)¹⁵. A dilute extract of plant extract (0.5 mL of 1:10 g/mL diluted with distilled water) or Gallic acid (Standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) and aqueous Na₂CO₃ (4 mL, 1 M). The mixtures were allowed to stand for 15 min, and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/l solutions of Gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound. The result was calculated from the regression equation of the calibration curve ($y=0.013x+0.127$, $r^2=0.988$).

Determination of Flavonoid Content: Aluminum chloride colorimetric method was used for flavonoids determination (Chang *et al.*, 2002)¹⁶. Each plant extracts (0.5 mL of 1:10 g/mL) in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% Aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. It remained at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 µg/mL in methanol.

Determination of Total Antioxidant Capacity: The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH (Prieto *et al.*, 1999)¹⁷. The antioxidant capacity is expressed as ascorbic acid equivalent (AAE). The plant extract (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against the blank. Total antioxidant capacity of the extract was measured from the regression equation prepared from the concentration versus optical density of ascorbic acid.

DPPH Scavenging Activity: DPPH scavenging activity of the *Trapa bispinosa* Roxb. was measured by the method developed by Manzorro *et al.*, (1998)¹⁸. The sample extract (0.2 mL) was diluted with methanol, and 2 mL of DPPH solution (0.5 mM) was added. After 30 min, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging was calculated.

Total Reducing Power Determination: The reducing power of the extract was determined according to the method of Oyaizu (1986)¹⁹. 10 mg of extract in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(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 FeCl₃ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Gallic acid, Quercetin, and Ascorbic acid were used as reference compounds. All the analyses were performed in triplicate, and the results were averaged. Increased absorbance of the reaction mixture indicated increasing reducing power.

Cytotoxic Study: Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of plant extracts (Meyer *et al.*)²⁰. It was carried out to investigate the cytotoxicity of the extract. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conically shaped vessel (1L), filled with sterile artificial seawater (prepared by using sea salt 38 g/L and adjusted pH 8.5) under constant aeration for 48 h. After hatching, active nauplii free from eggshells were collected from a brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a micropipette and placed in each test tube containing 4.5 mL of brine solution. In each experiment, 0.5 mL of the extract was added to 4.5 mL of brine solution and maintained at the ambient room temperature for 24 h, and surviving nauplii were counted. For the investigations test solution of the extract was prepared by dissolving 20 mg of the extract in 1 mL of pure dimethyl sulfoxide (DMSO). 500 µl of the solution was taken in test tubes, each containing 500 µl of simulated seawater.

A stock solution having the concentration 1 mg/mL was obtained by adding 9 mL of simulated seawater in the test tube. A series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. Tests were conducted along with negative control (DMSO treated), and different concentrations (1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL and 500 µg/mL) of the leaves extract of *T. bispinosa* in a set of two tubes per dose.

RESULT AND DISCUSSION:

Phytochemical Tests: The results of the various qualitative chemical tests for the detection of chemical constituents of the extract are placed in **Table 1**.

TABLE 1: QUALITATIVE RESULTS OF PHYTOCHEMICAL SCREENING

Test for Phytochemicals	<i>Trapa bispinosa</i> Roxb.
Carbohydrates	+
Glycosides	+
Alkaloids	+
Saponins	+
Flavonoids	-
Steroids	-
Tannins	+

‘+’ Indicates presence, ‘-’ Indicates absence

As evident from **Table 1**, the extract gave a positive reaction for carbohydrates, alkaloids glycosides, saponins, and tannins while gave a negative reaction for flavonoids, steroids. In a previous study, phytochemical screening showed the presence of flavonoids, alkaloids, and gum in the plant extract²¹. In another study, high quantity of saponins and alkaloid was found in *T. bispinosa*²².

Total Phenolic Compound Assay: The plant phenolics constitute one of the major groups of the compounds acting as primary antioxidants or free radical scavengers. The antioxidant activity of the phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Osawa, 1994)²³. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when ~1.0 g was daily ingested from a diet rich in vegetables and fruits (Tanaka, 1988)²⁴.

The content of total phenolics in the ethanolic plant extracts was determined using the Folin-Ciocalteu assay. The content of phenolics in the extract of *Trapa bispinosa* Roxb. was 0.45mg/g GAE.

Flavonoid Content Assay: Flavonoids protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxy radicals, hydroxyl radicals, and peroxynitrite. Epidemiological studies have shown that flavonoid intake is inversely related to mortality from coronary heart disease and the incidence of heart attacks.

Flavonoid content was calculated from the regression equation of the calibration curve ($y = 0.0098x - 0.0364$) and is expressed as Quercetin equivalents (QE). The flavonoid content was 0.74mg/g quercetin equivalent in *Trapa bispinosa* Roxb.

Total Antioxidant Assay: The total antioxidant capacities of the methanolic extracts of *T. bispinosa* were determined from the calibration curve ($y=0.0043x+0.1503$, $R^2 = 0.887$) established by ascorbic acid. The ascorbic acid equivalent of *T. bispinosa* was 0.84mg/g.

DPPH Scavenging Activity: DPPH easily accepts an electron or hydride radical to become a stable

diamagnetic molecule. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine.

The solution, therefore, loses color stoichiometrically depending on the number of electrons taken up. **Fig. 1** shows the amount of each extract needed for 50% inhibition (IC_{50}). The IC_{50} value of *Trapa bispinosa* Roxb. extracts were found to be 1.58 $\mu\text{g/ml}$, whereas ascorbic acid showed the value of 1.129 $\mu\text{g/ml}$.

In another study, the ethanolic extract of fruits of *Trapa bispinosa* Roxb. showed antioxidant activity (IC_{50} about $\sim 08 \mu\text{g/ml}$) which was comparable to standard drug ascorbic acid (IC_{50} about $\sim 10 \mu\text{g/ml}$)²⁰.

Reducing Power Assessment: Earlier, authors (Tanaka et al., 1988)²⁴ have observed a direct correlation between antioxidant activity and reducing the power of certain plant extracts. Figure 2 shows the reductive capabilities of the plant extract. The reducing power of extract of *T. bispinosa* was found moderate, and the reducing power of the extract was observed to rise as the concentration of the extract gradually increased.

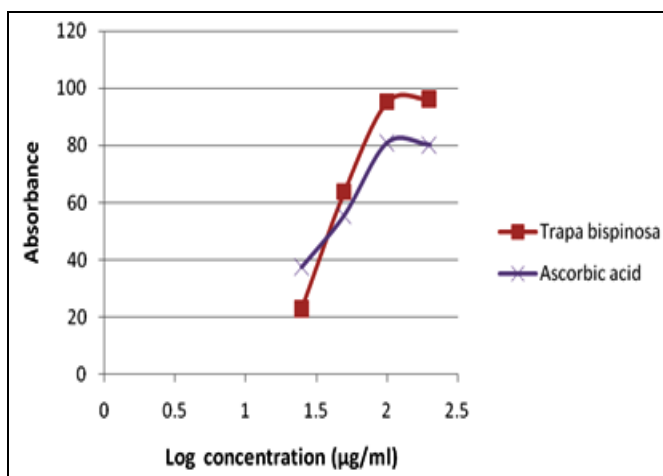


FIG. 1: COMPARATIVE DPPH SCAVENGING ACTIVITY

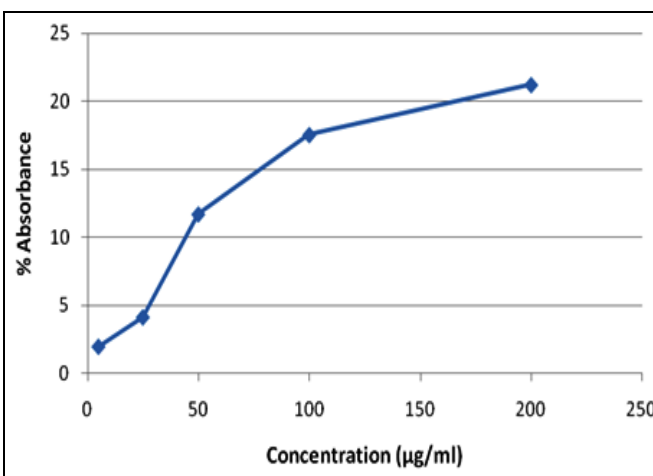


FIG. 2: REDUCING POWER OF *TRAPA BISPINOSA* ROXB.

Cytotoxic Study: The methanolic extract of *Trapa bispinosa* Roxb. was tested for Brine shrimp lethality bioassay using brine shrimp nauplii and DMSO as a solvent.

Control was used to see whether DMSO had any effect on brine shrimp lethality. The control group

of brine shrimp nauplii with and without DMSO exhibited no mortality.

For the extract, the number of nauplii died, and percent mortality was counted. The result is shown in the following **Table 2**.

TABLE 2: CYTOTOXICITY OF METHANOLIC EXTRACT OF *TRAPA BISPINOSA* ROXB.

Extract	Concentration of samples (µg/ml)	Log C	% mortality	LC ₅₀ (µg/ml)
Leaves part of methanolic extract of <i>Trapa bispinosa</i> Roxb.	500	2.698	80	2.87
	200	2.301	20	
	100	2	20	
	50	1.698	20	
	20	1.301	20	
	10	1	-10	
	5	0.698	0	
	1	0	10	

The LC₅₀ value of methanolic extract of *Trapa bispinosa* Roxb. leaves was 2.87 µg/ml

CONCLUSION: The methanolic extracts of *Trapa bispinosa* Roxb. leaves exhibited significant antioxidant activity. In the present study, the observed DPPH scavenging activity of the methanolic extracts of leaves might be useful for the development of newer and more potent natural antioxidants. The plant extract exhibited a strong cytotoxic effect. Further phytochemical and pharmacological studies are also required to use their medicinal and pharmaceutical potentialities.

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CONFLICT OF INTEREST: Nil

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