

## EVALUATION OF *IN VITRO* ANTIOXIDANT POTENTIAL OF AQUEOUS EXTRACT OF *TRAPA NATANS* L. FRUITS

NEELESH MALVIYA, SANJAY JAIN, AVNISH JAIN, SOURABH JAIN and RAJENDA GURJAR

Department of Pharmacognosy, Smriti College of Pharmaceutical Education,  
Dewas Naka, Indore-452010 Madhya Pradesh, India

**Abstract:** In the present study, the *in vitro* antioxidant potential of aqueous extract of *Trapa natans* L. fruits rind was investigated. The extract was found to contain a large amount of polyphenols and also exhibited an immense reducing ability. The total content of phenolic, flavonoid and tannin compounds was estimated as 63.81 mg of gallic acid equivalents/g of dry material, 21.34 mg of rutin equivalents/g of dry material and 17.11 mg of total tannin equivalent /g of dry material, respectively. IC<sub>50</sub> values for different antioxidant model were calculated as 128.86 µg/mL for DPPH radicals, 97.65 µg/mL for O<sub>2</sub><sup>•-</sup>, 148.32 µg/mL for H<sub>2</sub>O<sub>2</sub> and 123.01 µg/mL for NO, respectively. Reducing power and inhibition of •OH radical-induced BSA oxidation were also determined. The data obtained in the present study suggest that the aqueous extract of *Trapa natans* L. fruit rind have significant antioxidant activity against free radicals.

**Key words:** *Trapa natans* L., aqueous extract, antioxidant activity

Oxidative stress is a crucial etiological factor implicated in several chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in the ageing processes (1, 2). However, human organism has multiple mechanisms, especially enzymatic and nonenzymatic antioxidant systems, to protect the cellular molecules against reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide anion, hydrogen peroxide, hydroxyl radicals, nitric oxide and peroxynitrite. The capacity of the defensive system is affected by age, diet, health status of an individual. Therefore, to balance the equilibrium between reactive species and defensive system, exogenous antioxidants are constantly required. The antioxidants may mediate their effect by directly reacting with reactive species and quenching them. Several synthetic antioxidants, e.g., butylated hydroxyanisole and butylated hydroxytoluene are commercially available, but are quite unsafe and their toxicity is a problem of concern. Natural antioxidants, especially phenolics and flavonoids are safe and bioactive (3). Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for

human consumption. *Trapa natans* L. belongs to the family Trapaceae; shingoda is its local Indian name, it has a folkloric reputation as a cure of various diseases. It has been used since ancient days in Ayurvedic medical practices for the treatment of dyspepsia, haemorrhages, diarrhea and dysentery. It improves taste and acts as an astringent, aphrodisiac, cooling, diuretic and rejuvenating agent (4, 5). To the best of our knowledge, there is no scientific report available in support of the antioxidant activity of *Trapa natans* L. fruit rind. The aim of the present study was to evaluate the *in vitro* antioxidant activity of aqueous extract of *Trapa natans* L., and investigation of the presence of bioactive components, such as flavonoid, tannin and phenolic content.

### MATERIALS AND METHODS

#### Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), nitro blue tetrazolium (NBT), ascorbic acid and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemicals, St. Louis, MO, USA.

\* Corresponding author: Mob.: 09424430324, e-mail: neelesh\_brcop@rediffmail.com

### Plant resources and preparation of crude drug extract

The fruits of *Trapa natans* were collected from local market of Indore, district of Madhya Pradesh (MP) state, India and identified at the Government Agriculture College, Indore (M.P.). The herbarium specimen has been submitted to Pharmacognosy Department of the college (Voucher specimen no. – 001/T). Fruits were air dried and macerated with water. The extract was concentrated to dryness under reduced pressure in a rotary evaporator to yield dried aqueous extract, which was 9.96% of the starting material.

### Total phenolics

The total phenolics in the alcoholic extract of *Trapa natans* fruits was determined using Folin-Ciocalteu reagent (6). Hundred mg of the dry extract was leached with 250 mL of methanol/water (60:40, v/v, 0.3% HCl) and filtered through a 0.45 µm Millipore filter. To 100 µL of the filtrate, 100 µL of 50% Folin-Ciocalteu reagent, and 2 mL of 2.5% sodium carbonate were added and mixed. After 2 h, the absorbance of the solution was measured at 750 nm with a spectrophotometer (Shimadzu UV-1700). Quantitation was based on the standard curve of gallic acid (0–0.5 mg/mL), which was dissolved in methanol/water (60:40, v/v, 0.3% HCl).

### Total flavonoids

Dried powdered fruit (1 g) was extracted by 100 mL of methanol using hot decoction method for 1 h and followed by filtration. One milliliter samples of the prepared extract were placed in a six separate 10 mL volumetric flask. Three mL of methanol and 0.3 mL of aqueous NaNO<sub>2</sub> solution (1:20, w/v) were added in each flask and 3 mL of aqueous AlCl<sub>3</sub> solution (1:10, w/v) was added 5 min later. After 6 min, 2 mL of 1 M NaOH was added and the volume was made up to 10 mL with methanol. Absorbance was measured against a blank at 510 nm with a UV-VIS spectrophotometer. Rutin was used as the standard for a calibration curve. Analysis was done in triplicate (7). The total flavonoid content was calculated using the following linear equation:

$$A = 0.01069C - 0.001163, \quad r^2 = 0.9998$$

where A is the absorbance, C is the flavonoid content in µg /g of dry material.

### Total tannins content

Dried powdered fruit was refluxed with 100 mL of acetone for 2 h, filtered and the filtrate was concentrated up to 25–30 mL. The concentrated solution was extracted with diethyl ether (25 mL, 3

portions) followed by *n*-butyl alcohol previously saturated with water (25 mL, 3 portions). Combined *n*-butyl layers were dried and then *n*-butyl alcohol was removed under vacuum at room temperature until constant weight was attained. Analysis was done in triplicate.

Total tannins content was calculated by following formula (8):

$$\% \text{ w/w total tannins content} = (\text{weight of } n\text{-butanol fraction in g} / \text{weight of sample taken in g}) \times 100.$$

### Free radical scavenging assays

#### Total reducing power of the extract

Reducing power of the aqueous extract was determined according to the procedure described by Yen (9), with slight modification. Equivalent volume of extract containing 50–300 mg of dry matter was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%); the mixture was incubated at 50°C for 20 min. Then, 5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 1000 rpm for 10 min at 5°C in a refrigerated centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and color was developed by adding ferric chloride solution (1.0 mL, 0.1%). Absorbance of resultant solution was measured at 700 nm. The measurement was run in triplicate and the results were averaged. Ascorbic acid was used as the control. Increased absorbance of the reaction mixture indicated increased reducing power of the sample.

#### DPPH assay

One milliliter solution of the extract (50–300 mg) was added to 0.5 mL of 0.15 mM DPPH solution (in methanol). The content was mixed vigorously and allowed to stand at 20°C for 30 min. The absorbance was read at 517 nm. The measurement was run in triplicate and the results were averaged. Ascorbic acid was used as the control (10).

#### Scavenging of O<sub>2</sub><sup>•-</sup>

The method was adapted from Yen (9). The reaction mixture, comprising 1 mL *Trapa natans* fruits extract solution (50–300 mg) in distilled water, 1 mL of phenazine methosulfate (60 µM) in phosphate buffer (0.1 M, pH 7.4), 1 mL of NADH (450 µM) in phosphate buffer, was incubated at 25°C for 5 min. The absorbance was read at 560 nm against blank sample. Triplicate determinations were made at each dilution. Ascorbic acid was used as the control.

### Scavenging of H<sub>2</sub>O<sub>2</sub>

Scavenging of H<sub>2</sub>O<sub>2</sub> by the extract was determined by the method of Ruch (11). One milliliter of *Trapa natans* fruits extract solution (50–300 mg) (prepared in phosphate buffered saline (PBS)) was incubated with 0.6 mL of 4 mM H<sub>2</sub>O<sub>2</sub> solution (prepared in PBS) for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution containing the extract without H<sub>2</sub>O<sub>2</sub>. The concentration of H<sub>2</sub>O<sub>2</sub> was spectrophotometrically determined from absorption at 230 nm using the molar absorbance of 81 M<sup>-1</sup> cm<sup>-1</sup>. The measurement was run in triplicate and percentage inhibitions were calculated. Ascorbic acid was used as the control.

### NO scavenging

Scavenging of NO was determined by incubating sodium nitroprusside (5 mM, in PBS) with different concentrations of *Trapa natans* fruits extract (50–300 mg) at 25°C. After 120 min, 0.5 mL of incubated solution was withdrawn and mixed with 0.5 mL of Griess reagent. The absorbance was measured at 550 nm. The amount of nitrite was calculated from standard curve constructed by sodium nitrite (12). The measurement was run in triplicate and percentage inhibitions were calculated. Ascorbic acid was used as the control.

### Protein oxidation

The protein (BSA) was oxidized by a Fenton-type reaction. BSA (1 mg/mL) was incubated at 25°C in a solution with 2.5 mM H<sub>2</sub>O<sub>2</sub>, 1.0 mM FeCl<sub>3</sub>, 1.0 mM ascorbate and 3.0 mM EDTA in the presence or absence of *Trapa natans* fruits extract (50–300 mg). After incubation for 45 min, protein was precipitated with 10% trichloroacetic acid, centrifuged (5000 rpm, 4°C, 10 min) and the supernatant was decanted. Protein pellets were dissolved in 1 mL of 50 mM potassium phosphate buffer, pH 7.5. Total sulfhydryl (-SH) group determination was performed according to the method of Sedlak (13) using Ellman's reagent. Ascorbic acid served as control, measurements were run in triplicate and the results were averaged.

## RESULTS AND DISCUSSION

The extract was found to contain 63.81 ± 4.1 mg/g of total phenolics expressed as gallic acid equivalent (GAE, mg/g of GAE), 21.34 ± 1.32 mg/g of total flavonoid content and 17.11 ± 0.22 mg/g of total tannins, respectively. The interest in the phenolics has increased outstandingly due to their prominent free radical scavenging activity. Phenolic com-

pounds could be classified as simple phenols (a single aromatic ring bearing at least one hydroxyl group), and polyphenols (with at least two phenol subunits like flavonoids or three and more phenol subunits called tannins). Since polyphenols are responsible for the antioxidant activity, the obtained amount of total polyphenols in the extract indicated that the extract possesses a high antioxidant activity. Measurement of reducing potential would reflect antioxidant activity in the extract. In this method ferric ions are reduced to ferrous ions with color change from yellow to bluish green. The intensity of change in color depends on the reducing potential of the compounds present in the medium. The reducing potential of the extract measured for the concentration up to 300.0 µg/mL, showed a general increase in activity when concentration increased. As showed in Fig. 1, the reducing power of *Trapa natans* fruits extract was 0.502 at the concentration of 300 µg mL<sup>-1</sup>. However, ascorbic acid showed slightly higher activity with a reducing power of 0.631 at the same concentration. The capacity of *Trapa natans* fruits extract to scavenge DPPH, O<sub>2</sub><sup>•-</sup>, •OH and NO was measured and the results are shown in Figure 2 A–D. The antioxidants reacted with DPPH and convert it into a colorless α,α-diphenyl-β-picryl hydrazine. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. The extract significantly and dose dependently reduced DPPH radicals. Ascorbic acid showed an excellent scavenging activity (IC<sub>50</sub> = 76.56 µg mL<sup>-1</sup>). It was observed that *Trapa natans* fruits extract also had strong scavenging activity, with an IC<sub>50</sub> value of 128.86 µg mL<sup>-1</sup> (Fig. 2A). The DPPH scavenging ability of the extract may be attributed to

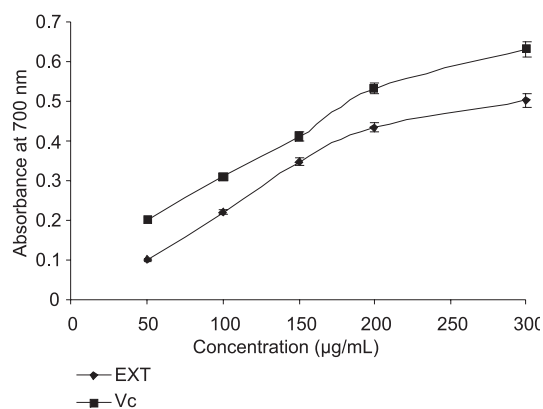


Figure 1. Reducing power of *Trapa natans* fruits extract (EXT). The absorbance values were directly plotted as the mean of replicate absorbance values ± SD. (n = 3) against extract concentration in µg per mL of reaction volume. Vc = ascorbic acid

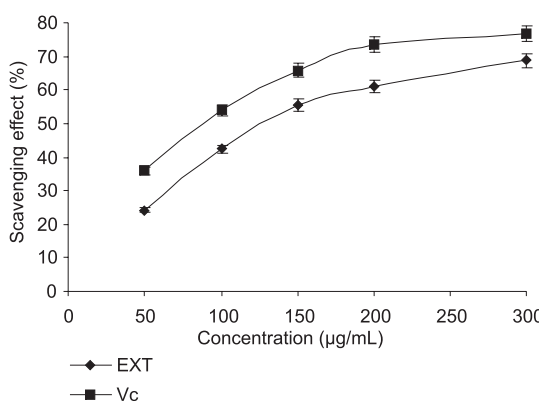


Figure 2A. DPPH radical-scavenging activities of *Trapa natans* fruit extract (EXT) and ascorbic acid (Vc). The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values  $\pm$  S.D. (n = 3) against extract concentration in  $\mu\text{g}$  extract per mL reaction volume

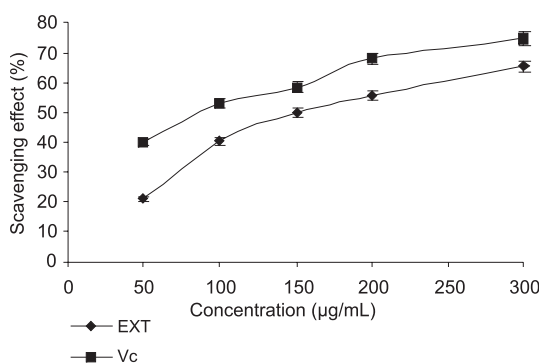


Figure 2C. H<sub>2</sub>O<sub>2</sub> scavenging activity of *Trapa natans* fruit extract (EXT) and ascorbic acid (Vc). The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values  $\pm$  S.D. (n = 3) against extract concentration in  $\mu\text{g}$  per mL of reaction volume

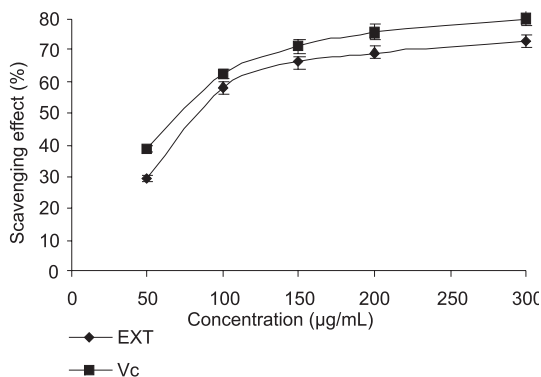


Figure 2B. O<sub>2</sub><sup>-</sup> scavenging activity of *Trapa natans* fruit extract (EXT) and ascorbic acid (Vc). The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values  $\pm$  SD (n = 3) against extract concentration in  $\mu\text{g}$  per mL of reaction volume

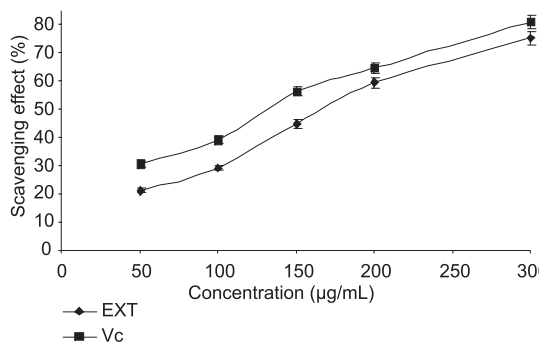


Figure 2D. NO scavenging activity of *Trapa natans* fruit extract (EXT) and ascorbic acid (Vc). The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values  $\pm$  S.D. (n = 3) against extract concentration in  $\mu\text{g}$  per mL of reaction volume

its hydrogen donating ability. The primary free radical in most biological systems is O<sub>2</sub><sup>-</sup>. Although O<sub>2</sub><sup>-</sup> itself is quite unreactive compared to other radicals, the biological systems convert it into more reactive species, e.g. •OH radicals (14). *Trapa natans* fruits scavenged O<sub>2</sub><sup>-</sup> significantly and dose dependently. The O<sub>2</sub><sup>-</sup> scavenging activity was determined by phenazine methosulfate/NADH-NBT system, wherein O<sub>2</sub><sup>-</sup> derived from dissolved oxygen by phenazine methosulfate/NADH coupling reaction reduces NBT. With this assay, the IC<sub>50</sub> value of *Trapa natans* fruits extract was estimated as 97.65  $\mu\text{g mL}^{-1}$ , while the value of ascorbic acid was 63.42  $\mu\text{g mL}^{-1}$  (Fig. 2B). The spontaneous or catalytic dismutation of O<sub>2</sub><sup>-</sup> leads to formation of H<sub>2</sub>O<sub>2</sub>, which in

the presence of a transition metal ion like Fe<sup>3+</sup>, decomposes into •OH radicals, highly damaging species in free radical pathology (15). The extract also scavenged H<sub>2</sub>O<sub>2</sub>, however, as compared to O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> was weakly scavenged by the extract. Compared with *Trapa natans* fruits extract, ascorbic acid was more effective for scavenging H<sub>2</sub>O<sub>2</sub>. *Trapa natans* fruits extract scavenged 50% of H<sub>2</sub>O<sub>2</sub> at the concentration of 148.32  $\mu\text{g mL}^{-1}$ , whereas ascorbic acid exhibited a scavenging rate of 50% at the concentration of 73.32  $\mu\text{g mL}^{-1}$  (Fig. 2C).

*Trapa natans* fruits extract also quenched NO released by an NO donor, SNP. Incubation of SNP solution in PBS at 25°C for 120 min resulted in the release of NO. The extract effectively and dose

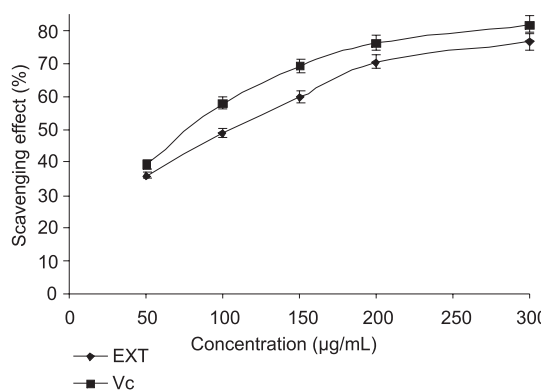


Figure 3. The scavenging of  $\bullet\text{OH}$  radical-induced BSA oxidation of *Trapa natans* fruit extract. The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values  $\pm$  SD, (n = 3) against extract concentration in  $\mu\text{g}$  per mL of reaction volume

dependently decreased the release of NO. Ascorbic acid showed an inhibitory activity ( $\text{IC}_{50} = 123.01 \mu\text{g mL}^{-1}$ ), while the  $\text{IC}_{50}$  value of *Trapa natans* fruits extract was  $152.62 \mu\text{g mL}^{-1}$  (Fig. 2D). Control experiments showed that even at high concentrations, the extract did not interfere with the reaction between nitrite and Griess reagent. ROS like  $\text{O}_2^{\cdot-}$  may react with NO and give rise to various other reactive nitrogen species (RNS) such as  $\text{NO}_2$ ,  $\text{N}_2\text{O}_4$ , and peroxynitrite. Both ROS and RNS together attack and damage various cellular molecules. Virtually all cellular components, including lipids, proteins, nucleic acids and carbohydrates, are susceptible to oxidative damage (16). *Trapa natans* fruits extract, owing to its radical scavenging ability may provide protection against oxidative damage induced to the biomolecules, thus in the next series of experiments, the ability of *Trapa natans* fruits extract was assessed to prevent oxidative damage to the major biomolecules: proteins and lipids. *Trapa natans* fruits extract effectively inhibited BSA oxidation induced by  $\text{Fe}^{2+}/\text{H}_2\text{O}_2$  system that generates  $\bullet\text{OH}$  radicals (Fig. 3). BSA oxidation was determined by Ellman reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] in terms of  $-\text{SH}$  group loss. Ellman reagent rapidly forms a disulfide bond with the SH group and releases a thiolate ion, which is yellow colored and absorbs at 412 nm. The oxidized thiols are unable to bind with Ellman reagent. Thus, the oxidation of a protein is monitored by measurement of a reduction in the absorbance at 412 nm. The incubation of BSA with  $\text{Fe}$ -ascorbate- $\text{H}_2\text{O}_2$  system caused the oxidation of SH groups. *Trapa natans* fruits extract dose dependent-

ly inhibited this SH oxidation. Ascorbic acid showed an excellent scavenging activity ( $\text{IC}_{50} = 66.54 \mu\text{g mL}^{-1}$ ), while the  $\text{IC}_{50}$  value of *Trapa natans* fruits extract was  $97.34 \mu\text{g mL}^{-1}$  (Fig. 3). The SH groups of proteins are crucial for several important functions. These groups maintain the functional conformation of proteins and also participate in catalytic activity of several enzymes. The SH groups, due to their ability to be reversibly oxidized, are recognized as key components involved in the maintenance of redox balance. Most DNA binding proteins are also redox sensitive, performing their functions by virtue of the SH groups. These proteins are involved in the regulation of cellular processes such as replication, recombination, viral integration and transcription. Therefore, oxidation of SH groups by oxidants may thus lead to disruption of various cellular functions and even lead to cell death. Only a few plant extracts/constituents have so far been shown to inhibit protein oxidation (17). The inhibition of protein oxidation by *Trapa natans* fruits extract extends its utility as an antioxidant, making it an antioxidant of choice. The inhibition of protein oxidation is thus a crucial property of antioxidant compounds by virtue of which they can inhibit the induction/progression of a number of diseases implicating oxidative stress. Thus, intake of *Trapa natans* fruits may be helpful in protection from numerous free radical generated diseases.

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