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Antioxidant Properties of Methanolic Extracts of *Boerhavia diffusa*

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ABSTRACT

Natural antioxidants present are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Free radical scavengers are compounds like polyphenols, flavonoids and phenolic compounds. The present study was carried out to evaluate the antioxidant activity and phytochemical constituents of *Boerhaavia diffusa* L., a herbaceous member of the family Nyctaginaceae from methanolic extracts of different plant parts. Methanolic extracts of leaves, stems and roots were prepared and screened for *in vitro* antioxidant activities by using peroxidase assay, Ferric Reducing Ability of Plasma (FRAP), lipid peroxidation assay (LPO), ABTS and the quantitative estimation of the total phenolics as Gallic Acid Equivalent (GAE) per gram dry weight and total flavonoid as Quericitin Equivalent (QE) per gram dry weight. Maximum total phenolic content was recorded in leaves (24.5±1.703 mg GAE g^{-1} DW) While maximum total flavonoid content was found in leaves (79.86±3.751 mg QE g^{-1} DW). Leaves methanolic extract showed maximum radical scavenging acitvity for FRAP (96.664 Mm L^{-1} g^{-1}), while ethyl acetate and hexane extract of stems showed minimum activity for FRAP. Leaves showed maximum radical scavenging activity for LPO (26.66 MDA g^{-1} DW) and peroxidase (0.383 Mm min^{-1} g^{-1} DW), a good correlation with the total phenolic and flavonoidal content of plant is seen. It signifies that the plant-derived phenolics and flavonoids represents good sources of natural antioxidants. It is seen that this plant exhibits significant antioxidant activity.

Key words: ABTS, LPO, FRAP, peroxidase assay

INTRODUCTION

Plants not only provide food for man but also give a number of active compounds with potent and varied therapeutic value. *Boerhavia diffusa* (Family Nyctaginaceae) is a herbal plant which is common in the tropics in both dry and rainy seasons and subtropics in a wild perennial herb which may be encountered in different terrestrial habitats (Banjare *et al.*, 2012). Genus *Boerhaavia*, consisting of 40 species is distributed in tropical and subtropical regions and warm climate. It is found in Ceylon, Australia, Sudan and Malay Peninsula, extending to China, Africa, America and Islands of the Pacific. Among 40 species of Boerhaavia, 6 species are found in India, namely *B. diffusa*, *B. erecta*, *B. rependa*, *B. chinensis*, *B. hirsute* and *B. rubicunda*. *Boerhaavia diffusa* in India is found in warmer parts of the country (Scientific Name: *Boerhaavia diffusa* Linn. Syn. *B. repens*; *B. repens*, Family: Nyctaginaceae, Family Name: Hog weed, Horse Purslane, Common Indian Names Gujarati: Dholia-saturdo, Moto-satoda, Hindi:

Snathikari Kannada: Kommegida Marathi: Tambadivasu Sanskrit:Punarnava, Raktakanda, Shothaghni, Varshabhu Bengali: Punurnava Tamil: Mukaratee-Kirei Telugu: Punernava) and grows as common weed its, useful parts are root, leaves and seeds (Oudhia, 2003).

The root, leaves, aerial parts or the whole plant of *Boerhaavia diffusa* have been employed for the treatment of various disorders in the Ayurvedic herbal medicine daily used by millions of people in India, Nepal, Sri Lanka and indirectly through it being the major influence on Unani, Chinese and Tibetan medicines. The root is mainly used to treat gonorrhea, internal inflammation of all kinds, dyspepsia, oedema, jaundice, menstrual disorders, anaemia, liver, gallbladder and kidney disorders, enlargement of spleen, abdominal pain, abdominal tumours and cancers. It cures corneal ulcers and night blindness and helps restore virility in men. People in tribal areas use it to hasten childbirth. The juice of *Boerhaavia diffusa* leaves serves as a lotion in ophthalmia. It is also administered orally as a blood purifier and to relieve muscular pain, also is administered in diabetes (Rajpoot and Mishra, 2011; Chude *et al*., 2001).

Oxygen-free radicals, more generally known as Reactive Oxygen Species (ROS) along with Reactive Nitrogen Species (RNS) are well recognised for playing a dual role as both deleterious and beneficial species (Valko *et al*., 2006). The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants (Alam *et al*., 2013). Oxidative stress contributes to many pathological conditions and diseases including cancer, neurological disorders, atherosclerosis, hypertension, ischemia/perfusion, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease and asthma (Rahmatullah *et al*., 2010). Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical. This activity depends on the structure of the molecules and the number and position of the hydroxyl group in the molecules (Freeman and Crapo, 1982). Polyphenols are reducing agents and together with other dietary reducing agents, such as vitamin C, E and carotenoids, referred to as antioxidants, protect the body's tissues against oxidative stress and associated pathologies such as cancers, coronary heart disease and inflammation (Tapiero *et al*., 2002). During heavy metal stress, phenolic compounds can act as metal chelators and on the other hand, phenolics can directly scavenge molecular species of active oxygen (Apel and Hirt, 2004). Zn is an essential component of numerous proteins involved in the defense against oxidative stress (Florence, 1995).

MATERIALS AND METHODS

Plant material: Different plant parts (leaves, stem and roots) of *Boerhavia diffusa* were collected in month of October-December from University of Rajasthan campus. It was washed with tap water, dried at room temperature and ground to fine powder. The species specimen was submitted to herbarium, Department of Botany, University of Rajasthan. Jaipur, Rajasthan, India and got the voucher specimen No. RUBL211300.

Chemicals: All the chemicals used were of analytical graded and purchased from Hi Media from Hi-media Laboratory Pvt. Ltd. Mumbai.

Total phenolic and flavanoidal content

Plant extraction: Two gram the each of dry material (leaves, stems and roots) was extracted with 25 mL of methanol at room temperature for 48 h, filtered through Whatman paper No. 1 filter paper, stored and used for quantification.

Total phenolic content: Total phenolic compound contents were determined by the Folin-Ciocalteau method (McDonald *et al*., 2001; Ebrahimzadeh *et al*., 2008a, b; Nabavi *et al*., 2008). The extract samples (0.5 mL, 1:10 diluted) were mixed with Folin Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 mL, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200 and 250 mg mL^{-1} solutions of gallic acid in methanol. Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass) which is a common reference compound. Total phenolic content can be calculated from the equation:

$$T = \frac{C.V}{M}$$

Where:

T = Total phenolic concentration

C = Concentration of gallic acid from caliberation curve (mg mL^{-1})

V = Volume of extract (mL)

M = Wt. of methanol plant extract

Total flavanoidal content: Total flavonoid content was determined by using aluminium chloride colorimetric method ($AlCl_3$) according to the known method (Dewanto *et al*., 2002; Sakanaka *et al*., 2005) with slight modifications using Quercetin as standard. One mililiter of test material was added to 10 mL volumetric flask containing 4 mL of water. To above mixture, 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After 6 min, 2 mL of 1M NaOH was added and the total volume was made upto 10 mL with distilled water. Then the solutions were mixed well and absorbance was measured against blank at 510 nm. Total flavanoidal content of the extracts was expressed in milligram of quercetin equivalents g^{-1} DW. Total flavanoidal content can be calculated from the equation:

$$T = \frac{C.V}{M}$$

Where:

T = Total flavanoidal concentration

C = Concentration of gallic acid from calibration curve (mg mL^{-1})

V = Volume of extract (mL)

M = Wt. of methanol plant extract

Determination of antioxidant activity

Reducing ability (FRAP assay): The determination of the total antioxidant activity (FRAP assay) in the extract is a modified method of Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer (0.3 M acetic acid and 0.3 M sodium acetate), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM Ferric chloride ($Fecl_3.6H_2O$) solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $Fecl_3.6H_2O$. The temperature of the solution was raised to 37°C before use. Plant extracts (100 μL each of methanolic ethyl acetate and hexane) were allowed to react with 2900 μL of the FRAP solution for 30 min in the dark condition. Readings of the coloured product

(ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 100-1000 μM $FeSO_4$. Results are expressed in mM Fe (II) g^{-1} dry mass and compared with that of BHT, ascorbic acid, quercetin and catechin.

Peroxidase assay: The method of assay measures the oxidation of pyrogallol to purpurogallin by peroxidase when catalyzed by peroxidise at 420 nm and at 20°C. Plant sample (200 mg) was homogenized with 10 mL of phosphate buffer and refrigerated centrifuged at 10000 rpm for 20 min. The clear supernatant was taken as the enzyme extract. The activity was assayed after the method of Chance and Maehley (1955) with following modifications. The 2.4 mL of phosphate buffer, 0.3 mL pyrogallol (50 μM) and 0.2 mL of H_2O_2 (30%) were added. The amount of purpurogallin formed was determined by taking the absorbence at 420 nm immediately after adding 0.1 mL enzyme extract. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used in calculating the enzyme activity that was expressed in terms of mM min^{-1} g^{-1} DW.

Lipid peroxidation assay (LPO): 0.5 g of dry material was homogenized with 10 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged for 5 min (15000 g, 4°C). Supernatant was collected and 1 mL of supernatant was mixed with 4 mL of 0.5% (w/v) TBA (Thiobarbituric acid) in 20% (w/v) TCA and then incubated in water bath at 95°C for 30 min. Reaction was quickly ended by incubating on an ice bath. In case the solution is not clear, centrifuge at 10000 g for 10 min and the absorbance was measured at 532 and 600 nm. OD_{600} values were subtracted from MDA-TBA complex values at 532 nm and MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient εM = 155 mM^{-1} cm^{-1}. Results were presented as μmols MDA g^{-1}.

ABTS radical scavenging assay: To determine ABTS radical scavenging assay, the method of Re *et al.* (1999) was adopted. The stock solutions included 0.002M ABTS solution and 0.07 M potassium persulphate solution. The working solution was then prepared by mixing the 25 mL of ABTS stock and 0.1 mL of potassium persulphate stock and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing ABTS solution with ethanol to obtain an absorbance of 0.706±0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 mL) at varying concentration were allowed to react with 3 mL of the ABTS solution and the absorbance was taken at 734 nm after 6 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as:

$$\text{Inhibition (\%)} = 1 - \frac{ABS_{sample}}{ABS_{control}} \times 100$$

where, $ABS_{control}$ is the absorbance of ABTS radical+methanol, ABS_{sample} is the absorbance of ABTS radica +sample extract/standard.

Statistical analysis: Experimental results are expressed as (M±SD). All measurements were replicated three times. IC_{50} values were also calculated by linear regression analysis. Experiments results were further analyzed for Pearson correlation coefficient (r) between total phenolic, flavanoid and free radical scavenging assay using the microsoft Excel 2007 software. The values were considered to be significantly different at $p<0.05$.

RESULTS AND DISCUSSION

In the present study, we have investigated the antioxidant activity of *Boerhavia diffusa* by different assays and found their scavenging activity. Table 1 shows the total flavonoid content maximum in leaves (79.86±3.757 mg GAE g^{-1} DW) while minimum in roots (7.08±0.36 mg GAE g^{-1} DW) and total phenolic content was seen maximum in leaves (24.5±1.703 mg QE g^{-1} DW) while minimum in roots (0.25±0.243). Table 2 shows FRAP activity of ethylacetate, hexane and methanol extracts. In ethyl acetate extract, maximum activity is seen in stem (53±2.645 Mm L^{-1} g^{-1}) while minimum in roots (27.551±1.392 Mm L^{-1} g^{-1}). In hexane extract maximum activity is seen in stem (89±3.605 Mm L^{-1} g^{-1}) while minimum in root (16.887±1.293 Mm L^{-1} g^{-1}). In methanol extract maximum activity is seen in leaves (137.67±2.516 Mm L^{-1} g^{-1}) while minimum in roots (74.33±2.081 Mm L^{-1} g^{-1}). Table 3 shows peroxidase activity maximum in leaves (0.124±0.11 Mm min^{-1} g^{-1} DW) while minimum in roots (0.0589±0.040 Mm min^{-1} g^{-1} DW). Table 4 shows LPO activity maximum in leaves (23.377±1.108 mg QE g^{-1} DW) while minimum in root (4.481±0.357). Table 5 shows the IC50 of methanolic extracts of which leaves shows highest activity for ABTS assay (58.721±2.460 Mm min^{-1} g^{-1}) while stem show minimum activity (732.344±9.844 Mm min^{-1} g^{-1}). Table 6 shows correlation values of Total Phenolic Content (TPC) and Total Flavonoidal Content (TFC) with the different free radical scavenging assays performed.

Table 1: Total phenolic and flavonoidal contents in different plant parts of *B. diffusa*

Plant part	Total flavonoidal content (mg GAE g^{-1} DW)	Total content phenolic (mg QE g^{-1} DW)
Leaves	79.860±3.757	24.50±1.703
Stem	39.375±1.653	7.77±0.780
Root	7.080±0.3600	0.25±0.243

Table 2: Total antioxidant (FRAP) activity of methanol, ethyl acetate and hexane extract of different plant parts of *B. diffusa*

Plant part	Methanol	Ethyl acetate	Hexane
Leaves	137.67±2.516	31.000±2.645	25.560±1.462
Stem	131.18±1.003	53.000±2.645	89.000±3.605
Root	74.33±2.081	27.551±1.392	16.887±1.293

Table 3: Peroxidase activity in different plant parts of *B. diffusa*

Plant part	Methanolic extract
Leaves	0.1240±0.011
Stem	0.1016±0.002
Root	0.0589±0.040

Table 4: LPO activity in different plant parts of *B. diffusa*

Plant part	Methanolic extract
Leaves	23.377±1.108
Stem	12.163±1.031
Root	4.481±0.357

Table 5: IC_{50} values of different plant parts of *B. diffusa* of ABTS radical scavenging assay

Plant part	IC_{50} values (µg mL^{-1})
Leaves	58.721±2.460
Stem	732.344±9.844
Root	228.672±9.781

Table 6: Correlation value of total phenolic contents and total flavonoidal contents

Antioxidant ctivity	Total phenol content	Total flavonoid content
ABTS		
Leaves	0.9633	-0.3575
Stem	0.9496	-0.1062
Root	0.6877	-0.2848
LPO		
Leaves	0.4684	0.8356
Stem	-0.8178	-0.7348
Root	-0.7855	-0.9286
Peroxidase		
Leaves	0.3768	0.8868
Stem	-0.6835	0.5694
Root	-0.5903	-0.9942

For ABTS, we find a positive correlation with the phenolic content of the plant in all plant parts (leaves, stem and roots) while a negative correlation with the flavonoidal content for all plant parts, this may be a reason for the highest activity of the stem methanolic extracts for the ABTS assay. For the LPO activity assay we see a positive correlation with both phenolic and flavonoidal content which may explains its highest activity in leaves but in contrast to LPO, for peroxidase assay highest activity is seen in leaves which shows positive correlation with the phenolic and flavonoidal content. Phenolic and flavonoidal content have shown a good correlation with antioxidant activity, this may be due to structural differences. According to the Singleton and Rossi (1965) various phenolic compounds have different responses in this assay. The molar response of this method is roughly proportional to the number of phenolic hydroxyl groups in a given substrate but the reducing capacity is enhanced when two phenolic hydroxyl groups are oriented ortho or para. Since these structural features of phenolic compounds are reportedly also responsible for antioxidant activity, measurements of phenols in infusions may be related to their antioxidant properties (Katalinic *et al.*, 2006). Phenolic compounds such as flavonoids, phenolic acid and tannins, possess anti-inflammatory, anti-carcinogenic, anti-atherosclerotic and other properties that may be related to their antioxidant activities (Chung *et al.*, 1998; Wong *et al.*, 2006). Flavonoids and flavonols are two polyphenolic compounds that play an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen *et al.*, 1993). Phenolic compounds may contribute directly to antioxidative action (Duh *et al.*, 1999). Polyphenolic compounds may have an inhibitory effect on mutagenesis and carcinogenesis in humans when as much as 1.0 g is ingested daily from a diet rich in fruits and vegetables (Tanaka *et al.*, 1998).

CONCLUSION

With the above study conducted, it may be concluded that the polyphenols present in plant *Boerhavia diffusa*, provide an understanding of its beneficial effects against the reactive oxygen species which in turn help in checking the pathological conditions like cancer, degeneration, autoimmune diseases, Alzheimers and aging caused due to them. With this study we conclude that this plant has a great potential and put to further research would help in putting it to commercial use as a marketed medicine.

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