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IN-VITRO ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL STUDIES OF BOERHAAVIA DIFFUSA LINN. ROOTS.

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ABSTRACT

The present study was carried out to evaluate the antioxidant activity and phytochemical constituents of *Boerhaavia diffusa* Linn. (Nyctaginaceae) dried roots. The root extracts were prepared with chloroform, ethanol and water and screened for *in vitro* antioxidant activities by using assay of DPPH radical scavenging, antiproteolytic activity, polyphenol oxidase inhibition, hydroperoxide inhibition and ferric reducing power. The activity was compared with standard antioxidant like quercetin, salicylic acid, L-cysteine and ascorbic acid. Among these three extracts, ethanol extract has shows better antioxidant activity as compared to remaining two. Phytochemical screening of *Boerhaavia diffusa* L. root powder revealed the presence of carbohydrates, saponins, proteins, flavonoids, steroids, fats and alkaloids.

INTRODUCTION: Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radicals is responsible for many adverse reactions causing extensive tissue damage. Lipids, proteins and DNA are susceptible to attack by free radicals. Antioxidant shows resistance against oxidative stress by scavenging free radicals and inhibiting lipid peroxidation [1].

Free radicals are atoms or molecules with single unpaired electron that makes them highly reactive. Metabolic reactions are responsible for generation of oxidative free radicals which create a chain reaction leads to lipid peroxidation, DNA damage, etc. This has been implicated in atherosclerosis, cancer, neurodegenerative diseases and inflammatory bowel diseases [2]. Antioxidants play a vital role in delaying, intercepting or preventing oxidative reactions, catalyse by free radical [3].

Phytoconstituents like aegeline, agelinine, rutine, sterol, tannins, flavonoids, quercetin, volatile oils, ß-sitosterols, alkaloids etc. are already reported in pants [4]. *Boerhaavia diffusa* Linn. is a medicinal plant widely used in the Ayurvedic medicine. The plant was named in honor of Hermann Boerhaave, a famous Dutch physician of the 18th century [5]. *Boerhaavia diffusa* is called as Spreading Hogweed in English *viz.* belonging to the family of the Nyctaginaceae [6]. 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 tumors, and cancers, then as a diuretic, digestive aid, laxative and a menstrual promoter [7].

The first pharmacological studies have demonstrated that the root of *Punarnava* exhibits a wide range of properties: anti-inflammatory [8], diuretic [9], laxative [10], antiurethritis [11], anticonvulsant [12], antinematodal [13], antifibrinolytic [14], antibacterial and antihepatotoxic [15] activities. *Boerhaavia diffusa* is a widely used plant in Ayurvedic medicine for the treatment of several illnesses, so the present study was undertaken to investigate phytochemical nature and potential antioxidant effect of the root extracts of *Boerhaavia diffusa.*

MATERIALS AND METHODS:

Solvents used: The solvents used were chloroform, ethanol, methanol, and cychlohexane of AR grade.

Chemicals: 2, 2-diphenyl-1-picryalhydrazine (DPPH) was obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Quercetin, L-Cysteine and L-DOPA (3, 4-dihydroxyphenyl L- alanine) were obtained from S.D. Fine Chemicals Ltd., Mumbai. All other chemicals used were of AR grade and were obtained from commercial sources.

Plant Material and Extraction: *Boerhaavia diffusa* roots were collected from fields near by Swami Ramanand Teerth Marathwada University campus, Nanded (Maharashtra) in the month of September. The plant material was identified and voucher specimen (No. BSI/WC/Tech./2011/320) was collected in Botanical Survey of India, Pune. Roots were washed with running tap water, and then rinsed with distilled water to remove the soil and dust particles adhered on the surface of the sample. The sample was dried in shade. Dried root powder of *B. diffusa* was extracted by Soxhlet apparatus [16].

250 gm of dried roots were grind in mixture, finely grinded powder was serially extracted by using Soxhlet apparatus with chloroform for 6 hrs at 40˚C, then with ethanol for 6 hrs for 60˚C and finally with water for 6 hrs at 80˚C. The solvent was removed by distillation and semisolid mass was dried by using hot water bath at 40-50˚C and the yield was weighted. % yield of crude extracts calculated. The yield of chloroform extract was 1.33 gm (0.532% w/w), ethanol extract was 5.00 gm (2% w/w) and water extract was 4.85 gm (1.94%).

Phytochemical Analysis: The phytochemical analysis for the presence of carbohydrates, saponins, proteins, tannins, coumarins, glycosides, flavonoids, steroids, fats and alkaloids was carried out [17].

Interaction with DPPH Radical [18]: The ability of *Boerhaavia diffusa* root extracts towards DPPH radical scavenging was carried out by mixing 0.5 ml of 0.1 mM solution of DPPH and 0.5 ml individual extract prepared in absolute ethanol. After 10 min. of reaction time, the samples were observed spectrophoto-metrically at 517 nm and Quercetin (1mM) was used as a standard compound.

Assay of Antiproteolytic Activity: Individual extract (1mg/ml, 0.1ml) was incubated with trypsin (0.0-75 mg/ml) for 20 min viz. followed by addition of bovine serum albumin (BSA, 6 g/100ml, in 0.1 M phosphate buffer, pH 7.6). After 20 min. of incubation at 37˚C, the reaction was terminated by using trichloroacetic acid (3 ml, 5%). The resultant precipitates were separated by centrifugation at 5000 rpm. The acid soluble protein fractions were estimated by using Lowery method [19]. Salicylic acid (1mM) was used as a reference compound.

Inhibition of Hydroperoxide Formation: The RBC membrane solution was prepared [20] with slight modification [21]. To the membrane solution (1.0 ml), 5 ml of chloroform: methanol (2:1) was mixed followed by centrifugation at 1000 g for 15 min. for separation of the two phases. The chloroform layer was taken in a test tube and dried at 45˚C in water bath. The lipid residue leftover was dissolved in 1.5 ml cychlohexane. The amount of hydroperoxides generated was measured at 233 nm against a cychlohexane blank. Aspirin (acetyl salicylic acid. 1mM) was used as a reference drug.

Inhibition of Polyphenol Oxidase [22]: Peeled pieces of potato pieces (100gm) were blended and extracted in chilled 20 ml citrate buffet (pH 4.8, 0.1 M), which was filtered through 8 layers of cheesecloth and centrifuged at 5000-8000 rpm for 10 min (3-4˚C) and collected the supernatant and stored this crude enzyme extract at 4˚C. The enzyme assay was carried out with reaction mixture containing L-DOPA (2Mm, 1ml), enzyme solution (0.5ml), 1 ml individual extract and citrate buffer (pH4.8, 0.1 M) to a final volume of 3 ml. It was incubated for 5 min and O.D. was taken at 470 nm. L-Cysteine (1mM) was used as a reference drug for comparative study. The observations of the above studied parameters were calculated as % activity by using following formula;

$$\% \text{ activity} = [(A0-A1)/A0] \times 100$$

Where, A0 was the absorbance of the control, and A1 was the absorbance of standards.

Ferric Reducing Power Assay: Reducing power was investigated using he method developed by Oyaizu [23]. A 0.75 mL fraction of each extract individually mixed

with 0.75 mL of phosphate buffer (200mM, pH 6.6) and 0.75 mL of 1% potassium ferricyanide. The mixture was placed in a water bath for 20min at 50°C. The resulting solution was cooled rapidly, mixed with 0.75 mL of 10% trichloroacetic acid and centrifuged at 3,000rpm for 10min. A 5.0mL fraction from the supernatant was mixed with 5mL of distilled water and 1mL of 1% ferric chloride. Absorbance of the resultant mixture was measured at 700nm after 10min. The higher the absorbance value has the stronger reducing power.

RESULTS AND DISCUSSION:

Phytochemical screening: Phytochemical screening of *Borehaavia diffusa* L. root powder revealed the presence of carbohydrates, saponins, proteins, flavonoids, steroids, fats and alkaloids; while tannins, coumarins and glycosides were absent.

Interaction with DPPH Radical: Reactive oxygen species produced *in vivo* include superoxide radical, hydrogen peroxide and hypochlorous acid. Superoxide and hydrogen peroxide can interact in the presence of certain transition metal ions to yield a highly reactive oxidizing species i.e. hydroxyl radical. The antioxidants react with the stable free radical DPPH (deep violet color) and convert it to 1, 1- diphenyl-2-picryl hydrazine with discoloration [24].

The results obtained during this experimentation given in **table 1** indicated that the crude extracts of *B. diffusa* were found to interact with DPPH radical and stabilize their hyperactivity. Among the tested extracts of *B. diffusa* roots, ethanol extract (56.13%) was found to be more effective as DPPH radical scavenger, while the minimum effect was observed in case of water extract (15.03%).

The chloroform extract was showing 35.03 % as compared to quercetin (93.00%), a standard free radical stabilizing agent. DPPH stable free radical method is an easy, rapid and sensitive phytometric assay method commonly employed for evaluating the antioxidant activity of a specific compound or plant extracts based on their capabilities to donate hydrogen ion [25]. The fall in extinction and potential of antioxidant to scavenge free radicals are correlated [26]. DPPH radical gives strong absorption at 517 nm with purple color. The ethanolic solution gives purple coloration which when reduced by an antioxidant molecule give rise to a yellow solution. The presence of sufficient amount of vitamin C, alkaloids and flavonoids can be cause for reduction of DPPH into hydrazine.

Assay of Antiproteolytic Activity: Water extract of *B. diffusa* roots was good inhibitor of the trypsin induced hydrolysis of bovine serum albumin as compared to salicylic acid i.e. 42.3 %. While ethanol and chloroform extracts have shown moderate inhibition (**table 1**). In initiation and progression of inflammatory process proteases e.g., trypsin are highly responsible and trypsin inhibition is target for anti-inflammatory drug [27]. Plant polyphenols like flavonoids inhibits proteolytic enzymes responsible for the process of inflammation [28]. So, all the three extracts showing moderate inhibition of trypsin contained bioactive flavonoids as they are known to possess anti-inflammatory activity.

Inhibition of Hydroperoxide Formation: The results of the inhibition of formation of hydroperoxides shows that, all extracts of *B. diffusa* have a potential to inhibit lipid peroxidation especially the effect was more prominent in case of ethanol extract (96.93%), whereas the activity of chloroform extract was (86.19%)as shown in **table 1**. All extracts were found to be excellent as good inhibitors of lipid peroxidation. The mechanism for the inhibition of lipid peroxidation by *B. diffusa* root extract is may be due to the donation of electron to conjugated dienes.

Conjugated diene or hydroperoxide formation is one of the intermediate steps during lipid peroxidation takes place due to hydrogen capture from the unsaturated fatty acids. The mechanism for the inhibition of lipid peroxidation by *B. diffusa* root extract *is* may be due to the donation of electron to conjugated dienes. In pathological processes, lipid peroxidation is results due to formation of hydroperoxides. The oxidation of unsaturated fatty acid present in biological membranes results into lipid radical formation and spread.

The oxygen uptake by these radicals is responsible for membrane destruction due to the rearrangement of double bonds in unsaturated lipids. These reactions can also results in formation of malondialdehyde responsible for mutagenic and carcinogenic activity [29]. Oxidative stress is responsible for the generation of free radicals and considered as one of the agent for the lipid peroxidation.

Flavonoids and non-flavonoids which are commonly found in herbs, react as an antioxidant by inhibiting the oxygen radical formation through the enhanced oxidation of Fe^{2+} ion as the pro-oxidant and strongly inhibited the formation of thiobarbituric acid-reactive substances responsible for lipid peroxidation [29] .

Inhibition of Polyphenol Oxidase: Among the tested extracts of polyphenol oxidase inhibition; chloroform extract (45.40%) showed moderate inhibition of polyphenol oxidase as compared to L-cysteine (61.01%). The order of reactivity is chloroform extract (45.40%)> ethanol extract (10.00%)> water extract (9.23%) as shown in **table 1**.

Polyphenol oxidase enzyme is copper containing enzyme widely present in plant kingdom which is easily detectable and catalyzes oxidation of endogenous monophenols to ortho-dihydroxyaryl compounds to ortho-quinones.

Subsequent nucleophilic addition reactions of phenols, amino acids, and proteins with the electrophilic ortho-quinones form brown, black, or red-colored secondary products associated with the undesired discoloration of fruit and vegetables [30].

Inhibition of polyphenol oxidase is given in **table 1**. PPO inhibition may be due to the interaction of *B. diffusa* root extract with PPO generated quinones converting it into the stable colorless compound [22]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. 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 [32].

Polyphenols are free radical scavengers and widely distributed in plants. So they are having therapeutic importance [33].

TABLE 1: PROFILE OF % INHIBITION ACTIVITY OF DPPH, TRYPSIN, HYDROPEROXIDES AND POLYPHENOL OXIDASE BY *BOERHAAVIA DIFFUSA* L. ROOT EXTRACTS

Boerhaavia diffusa L. root extracts (1mg ml^{-1})	DPPH radical scavenging (%)	Inhibition of trypsin (%)	Inhibition of lipid peroxidation (%)	Inhibition of PPO (%)
Chloroform	35.03(±0.86)	39.53(±0.1.24)	86.19(±0.15)	45.40(±0.36)
Ethanol	56.13(±1.276)	37.83(±0.96)	96.93(±0.14)	10.00(±0.21)
Water	15.03 (±0.61)	42.3 (±0.34)	44.48 (±0.28)	9.23 (±0.21)
Quercetin (1mM)	93.00(±1.72)	ND	ND	ND
Acetyl salicylic acid (1mM)	ND	56.34(±0.32)	55.10 (±0.96)	ND
L-Cysteine (1mM)	ND	ND	ND	61.01 (±0.25)

Percent activity shown here are the mean ± standard deviation of *n*=3; ND, Not determined

Ferric Reducing Power Assay: The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. 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.

Table 2 reveals the reductive capacity of water, ethanol and chloroform extracts of *Boerhaavia diffusa* and is compared with ascorbic acid as standard for the reduction of the Fe^{3+} - Fe^{2+} transformation [32]. Ethanol extract showed higher reducing power as compared to water and chloroform extracts **(fig. 1**).

TABLE 2: REDUCING POWER DETERMINATION OF CHLOROFORM, ETHANOL AND WATER EXTRACTS OF *BOERHAAVIA DIFFUSA* LINN ROOT EXTRACTS AND STANDARD ASCORBIC ACID

Boerhaavia diffusa Linn root extracts (1mg ml^{-1})	Absorbance
Chloroform	0.6604 ±0.005
Ethanol	0.7566 ±0.003
Water	0.6707 ±0.005
Ascorbic acid (1mM)	1.6534 ±0.004

Each value represents the mean ± standard deviation of *n*=3

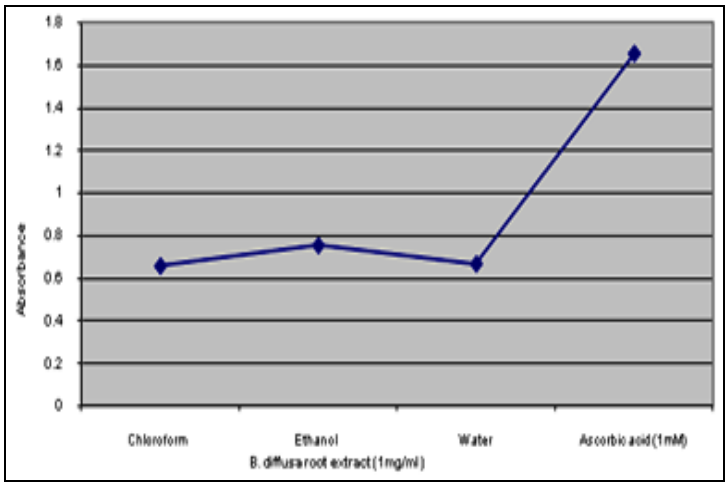

FIG. 1: REDUCING POWER DETERMINATION OF CHLOROFORM, ETHANOL AND WATER EXTRACTS OF *BOERHAAVIA DIFFUSA* LINN ROOT EXTRACTS AND STANDARD ASCORBIC ACID

CONCLUSION: It is well known that free radicals are one of the causes of several diseases. The findings of current study reveals that the root powder extracts of *Boerhaavia diffusa* had significant antioxidant activity. Ethanol extract of *B. diffusa* have the strongest radical scavengers among all extracts screened. Ethanol extract is promising extract for more detailed investigation of their antioxidant properties and application possibilities. From current study, *B. diffusa* roots could be exploited as a source of health-promoting antioxidants for use by both food and pharmaceutical industries.

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