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Research Article

QUANTIFICATION OF TOTAL PHENOLICS, FLAVONOID AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *ARISTOLOCHIA BRACTEATA* RETZ.

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ABSTRACT

Objective: To assess the total phenolics and flavonoids and *in vitro* antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of whole plant of *Aristolochia bracteata* Retz. using various antioxidant model system viz, DPPH, hydroxyl, superoxide, ABTS and reducing power.

Methods: Total phenolic content was estimated by folin-ciocalteau method. Flavonoids were determined by Aluminium chloride method. *In vitro* antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts was evaluated by studying 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, ABTS radical cation scavenging activity and reducing power using standard procedure.

Result: The total phenolics and flavonoids in methanol extract were found to be 1.32 g 100 g^{-1} and 3.48 g 100 g^{-1} respectively. Among the solvent tested, ethanol extracts of whole plant of *A. bracteata* showed potent *in vitro* antioxidant activities.

Conclusion: This study indicates significant free radical scavenging potential of whole plant of *A. bracteata* which can be exploited for the treatment of various free radical mediated ailments.

Keywords: *Aristolochia bracteata,* Antioxidant activity, Methanol, DPPH, ABTS.

INTRODUCTION

Antioxidants are any substance that when present at low concentration compared with those of an oxidizable substrate significantly delays/ prevents oxidation of the substrate. Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are widely used in the food industry to prevent food deterioration and to extent shelf life of foods [1]. However, the usage of synthetic antioxidants was found to increase risk of cancer occurrence and liver damage in human [2,3]. It has been suggested that it could be due to the rigorous toxicity that they possess compared to natural antioxidants [4]. Therefore, the search for alternative source of natural antioxidant is becoming increasingly important.

Aristolochia bracteata Retz. is a shrub distributed throughout India, belonging to the family Aristolochiaceae. *A. bracteata* is used in traditional medicine as gastric stimulant and in the treatment of cancer, lung inflammation, dysentery, and snake bites [5]. Root powder is combined with honey and given internally in the case of gonorrhea, boils, ulcers and other skin diseases [6]. In Indigenous system of medicine, it is reported that the leaves were used for skin diseases, rheumatism and analgesic [7]. The whole plant was used as purgative and anthelmintic, antipyretic and anti-inflammatory agents [8]. Along with its use against health disorder, it was felt worthwhile to screen the antioxidant activity of *A. bracteata* so that its further role in natural antioxidants is explored. With this perspective this study has been undertaken. Hence the medicinal plants are recognized as sources of natural antioxidants *A. bracteata* has been selected for this study.

MATERIALS AND METHODS

The whole plant of *Aristolochia bracteata* Retz. was freshly collected from Kouvandampalayam, Coimbatore district, Tamil Nadu. The plant specimen was identified and authenticated in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology unit, Research department of Botany, V. O. Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of Plant extract

Freshly collected whole plant of *A. bracteata* were dried in shade, and then coarsely powdered separately in a Wiley mill. The coarse powder (100g) was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 ml in a Soxhlet apparatus for 24 hrs. All the extracts were filtered though Whatman No.41 filter paper. All the extracts were concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of Total Phenolics

Total phenolic content was estimated using Folin-Ciocalteau reagent based assay as previously described [9] with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteau reagent (diluted ten-fold) and 4mL (75g/L) of Na2CO3 were added. The mixture was allowed to stand at 20ºC for 30min and the absorbance of the developed colour was recorded at 765nm using UV-VIS spectrophotometer. 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).

Estimation of Flavonoids

The total flavonoid content was determined according to Eom *et al* [10]. An aliquot of 0.5 mL of sample (1mg/mL) was mixed with 0.1mL of 10% aluminium chloride and 0.1mL of potassium acetate (1M). In this mixture, 4.3mL of 80% methanol was added to make 5mL volume. The mixture was vortexted and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H [11].

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method [11]. Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was

added to 3 ml of the solution of all extracts in methanol at different concentration (50,100,200,400 & 800µg/mL).The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al* [12]. Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1mL EDTA , 0.01mL of $FeCl_3$,0.1mL H_2O_2, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (50,100,200,400 &800µg/mL)dissolved in distilled water,0.33mL of phosphate buffer (50mM , pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then incubated at 37^{0}C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan *et al* [13]. The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, P^H 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 ml NADH (0.936mM) solution, 1.0 mL extract of different concentration (50,100,200,400 & 800µg/mL), and 0.5 mL Tris – HCl buffer (16mM, P^H 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25^{o}C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula

Antioxidant activity by radical cation (ABTS. +)

ABTS assay was based on the slightly modified method of Huang *et al* [14]. ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power

The reducing power of the extract was determined by the method of Kumar and Hemalatha [15]. 1.0 mL of solution containing 50,100,200,400 &800µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50^{o}C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5^{o}C) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

RESULT

Total phenolic and flavonoid content:

The total phenolic and flavonoid content of methanol extract of *A. bracteata* were found to be 1.32 g 100 g^{-1} and 3.48 g 100 g^{-1} respectively.

DPPH radical scavenging activity

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. bracteata* was shown in fig.1. All the extracts of *A. bracteata* showed potent DPPH radical scavenging activity in concentration dependent manner. Among the solvent tested, methanol and ethanol extracts showed more DPPH radical scavenging activity than standard ascorbic acid. At 800µg/mL concentration methanol and ethanol extracts of *A. bracteata* possessed 37.68% and 25.33% increased scavenging activity on DPPH radical respectively when compared with standard ascorbic acid.

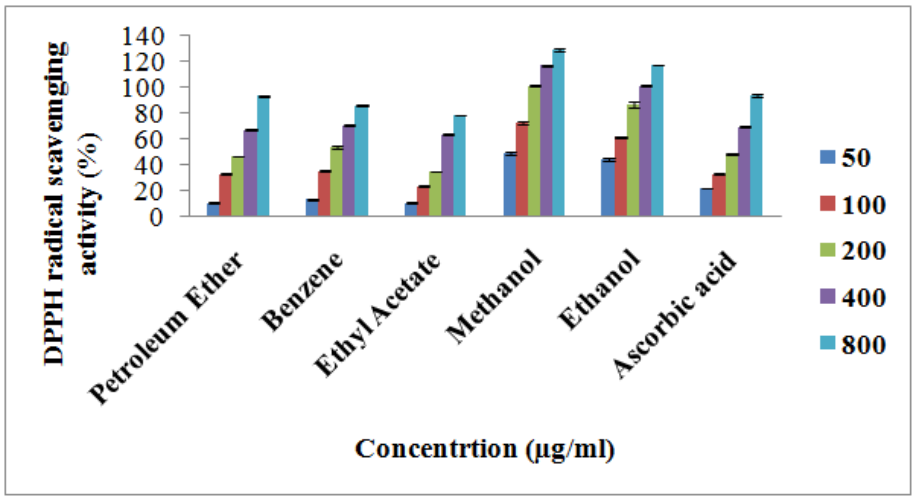

Fig. 1: DPPH radical scavenging activity of different extracts of *Aristolochia bracteata*

Hydroxyl radical scavenging activity

Fig. 2 showed hydroxyl radical scavenging activity of different extracts of *A. bracteata.* Hydroxyl scavenging effect of *A. bracteata* increases with the concentration of extracts. At 800µg/mL concentration ethanol and methanol extracts of *A. bracteata* showed 22% and 9.56% more scavenging activity on hydroxyl radical when compared with ascorbic acid.

Superoxide radical scavenging activity

Fig. 3 illustrated the superoxide radical scavenging activity of different extracts of *A. bracteata* at various concentrations. All the extracts of *A. bracteata* showed scavenging activity against superoxide in concentration dependent manner. At 800µg/mL concentration ethanol and methanol extracts of *A. bracteata* exhibited increased superoxide radical scavenging activity of 32.58% and 20.68% respectively than standard ascorbic acid. Ethyl acetate extract showed lesser activity at all the concentration when compared to standard ascorbic acid. Petroleum ether and benzene extract of *A. bracteata* possessed little higher activity than ascorbic acid.

ABTS radical cation scavenging activity

Different extracts of *A. bracteata were* subjected to ABTS radical cation scavenging activity and the results were shown in fig. 4. It depicts concentration dependent increased scavenging effect of different extracts of *A. bracteata* and standard trolox. At 800µg/mL methanol extract showed 8.39% increased scavenging activity on ABTS when compared with trolox. At 800µg/mL except methanol all the other solvents tested, showed lesser scavenging activity than standard trolox.

Reducing power

The reducing power of different extracts of *A. bracteata* and standard ascorbic acid was depicted in fig. 5. Reducing power of *A. bracteata* increased with the increase in the concentration of extracts. At 800µg/mL methanol, ethanol and ethyl acetate showed potent reducing power than standard ascorbic acid. Reducing power of petroleum ether extract is comparable to standard ascorbic acid.

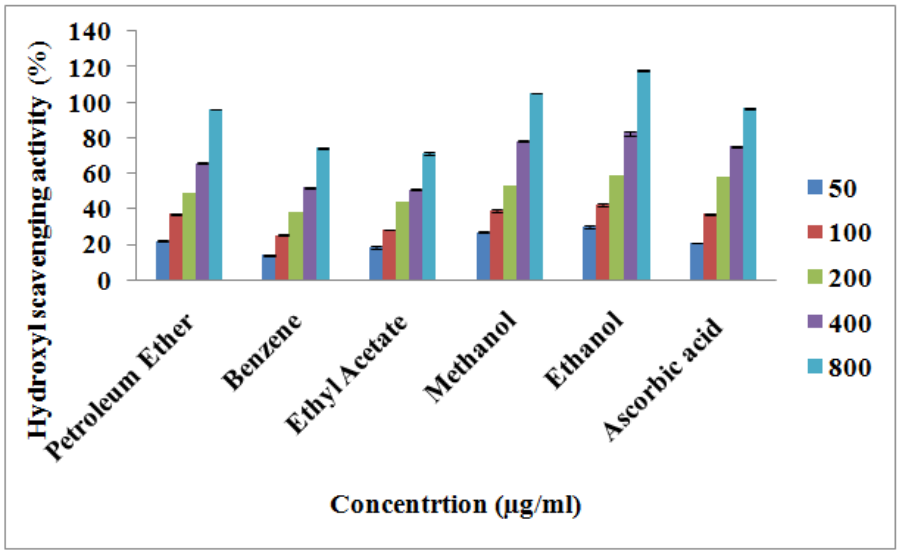

Fig. 2: Hydroxyl radical scavenging activity of different extracts of *Aristolochia bracteata*

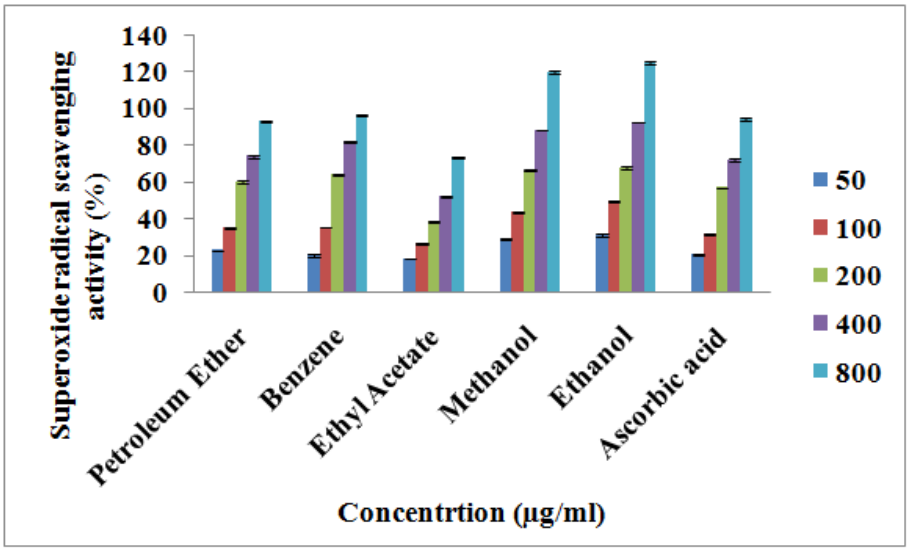

Fig. 3: Superoxide radical scavenging activity of different extracts of *Aristolochia bracteata*

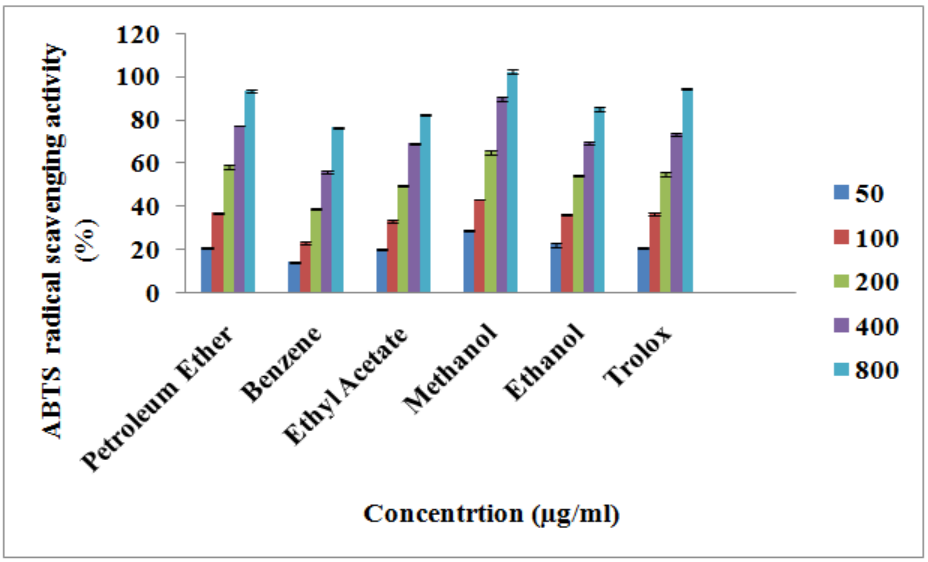

Fig. 4: ABTS radical cation scavenging activity of different extracts of *Aristolochia bracteata*

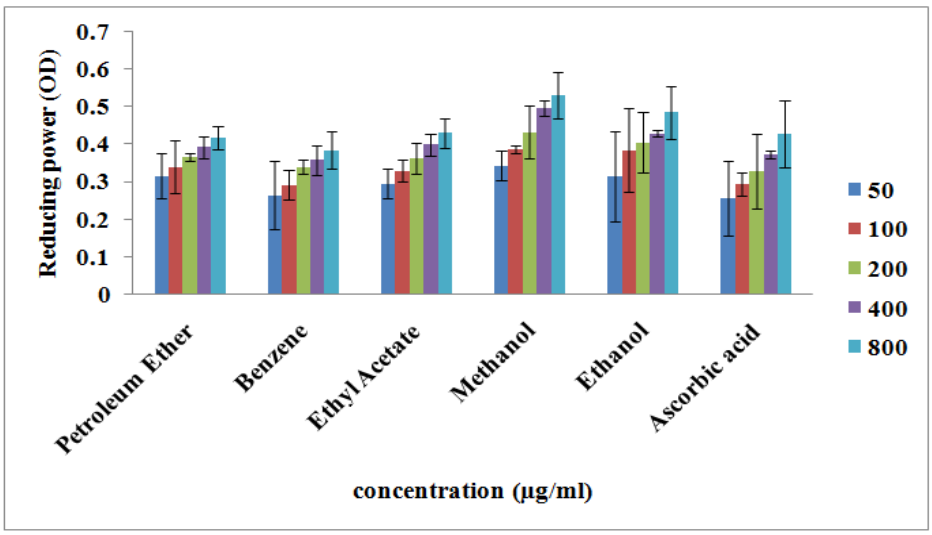

Fig. 5: Reducing power ability of different extracts of *Aristolochia bracteata*

IC_{50} values

IC_{50} values of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. bracteata* and standard ascorbic acid for DPPH radical scavenging were found to be 20.114µg/mL; 18.74µg/mL; 17.38µg/mL; 22.98µg/mL; 22.13µg/mL and 20.83µg/mL respectively.

IC_{50} values of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. bracteata* and standard ascorbic acid exhibited IC_{50} value of 20.13µg/mL; 18.54µg/mL; 18.04µg/mL; 21.96µg/mL; 22.48µg/mL and 20.96µg/mL for hydroxyl radical scavenging activity respectively.

IC_{50} values of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. bracteata* and standard ascorbic acid for superoxide radical scavenging were found to be 22.54µg/mL; 21.98µg/mL; 22.86µg/mL; 18.34µg/mL; 23.73µg/mL and 24.28µg/mL respectively.

IC_{50} values of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. bracteata* and standard trolox possessed IC_{50} value of 21.06µg/mL; 17.47µg/mL; 18.84µg/mL; 23.95µg/mL; 19.42µg/mL and 21.65µg/mL for ABTS radical scavenging activity respectively (Table 1).

Table 1: IC_{50} values of different extracts of *Aristolochia bracteata*

Solvent	IC_{50} (µg/mL)			
	DPPH	Hydroxyl	Superoxide	ABTS
Petroleum ether	20.14	20.13	21.98	21.06
Benzene	18.74	18.54	22.86	17.47
Ethyl acetate	17.38	18.04	18.34	18.84
Methanol	22.98	21.96	23.73	23.95
Ethanol	22.13	22.48	24.28	19.42
Ascorbic acid	20.83	20.96	22.54	-
Trolox	-	-	-	21.18

DISCUSSION

In recent days many scientific research substantiate that free radicals play a major role in the development of cancer, heart disease, aging, cataracts and impairment of the immune system. Antioxidants are the substance that may inhibit the rate of oxidation and protect the cells from the damage by scavenging these unstable free radicals [16]. Allopathic medicines may cure a wide range of diseases; however, its high prices and side effects are causing many people to return to herbal medicines which have fewer side effects [17]. Therefore, the importance of natural antioxidants has greatly increased in recent years and we have to scout wide range of herbs containing natural antioxidants which are used to cure various diseases locally.

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate and phenyl propanoid pathways in plants. Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. They have also metal chelation properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action [18]. Some evidences suggest that the biological action of these compounds are related to their antioxidant activity [19,20].

The present study revealed that the methanol extract of *A. bracteata* contain high amount of phenols and flavonoids. Since it possess natural antioxidants like phenols and flavonoids. Hence, the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. bracteata* is tested for their antioxidant property.

DPPH is relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. DPPH method allows estimation of hydrogen radical donating ability of the extract [21]. This model represents the situation in metabolic system where an antioxidant will stabilize a free radical by reacting with hydrogen radical. The result of the present study revealed that the different extracts of *A. bracteata* expressed concentration dependent scavenging effect against DPPH radical. This showed that the *A. bracteata* contained high amount of radical scavenging compounds with proton-donating ability.

The hydroxyl radical is extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging biomolecules of the living cells. These radical combines with nucleotides in DNA and cause strand breakage leading to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity [22]. Ferric- EDTA incubated with H_2O_2 and ascorbic acid during which hydroxyl radicals were formed in the free solution and were detected by their ability to degrade 2-deoxyl-2-ribose into fragments that on heating with thiobarbituric acid form a pink chromogen [23]. The present study clearly divulges that the different extracts of *A. bracteata* scavenged hydroxyl radicals were proportional to the concentration of the extract.

Superoxide anions are the major ROS generated by spermatozoa. Superoxide anions can be generated either from a membrane associated NADPH oxidase or as a result of electron leakage from mitochondrial electron transport [24]. It is hypothesized that the low-level generation of superoxide anions from an NADPH oxidase is important to cell signaling events in spermatozoa. However, the synthesis of superoxide anions exhibited a significant increase in diabetes mellitus, hypertension and obesity. The superfluous superoxide anions can generate H_2O_2 which is the major ROS associated with cytopathic diseases in equine spermatozoa [25]. Therefore the control of superoxide anion is helpful to prevent the generation and development of some diseases. In the present study different extracts of *A. bracteata* showed potent superoxide scavenging activity when compared to the standard ascorbic acid.

ABTS radical scavenging assay involves a method that generates blue/green $ABTS^{+}$chromophore via the reaction of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidant is measured spectrophotometrically at 745nm [26]. The present study publicized that all the extracts of *A. bracteata* showed higher ABTS radical scavenging activity in concentration dependent manner than the standard trolox.

The reducing capacity of the extract is another significant indicator of antioxidant activity. Reducing power of biological fluids and tissues are highly correlated with the overall scavenging capacity of the samples. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} complex can then be monitored by measuring the formation of Perl's blue at 700nm. Increasing absorbance indicates an increase in reductive ability [27]. The result showed that there was an increase in reducing power of the different extracts of *A. bracteata* as the extract concentration increases.

In conclusion, the observed different scavenging activities of the different extracts against various systems may be referred to the different mechanism of the radical antioxidant reactions in the different assays. Phenolic compounds present in the plant kingdom are mainly responsible for the antioxidant potential of plants. Hagerman *et al.* [28] have reported that the high molecular weight phenolics have more abilities to quench free radicals and their effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups. Free radical scavenging activity of different extracts of *A. bracteata* might be due to the presence of high molecular weight phenolics. The different extracts exhibited remarkable radical scavenging capacity rendering their utilization in different ailments associated with oxidative stress [29,30]. Thus it can be concluded that the whole plant of *A. bracteata* can be used as an accessible source of natural antioxidants with consequent health benefits.

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