

IN VITRO ANTIOXIDANT POTENTIAL OF WHOLE PLANT OF *ANDROGRAPHIS ECHIOIDES* (L.) NEES (ACANTHACEAE)

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Received: 03 Jan 2015 Revised and Accepted: 20 Apr 2016

ABSTRACT

Objective: Antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of the whole plant of *Andrographis echioides* have been tested using various antioxidant model systems viz; DPPH (1,1-Diphenyl-2-picryl-hydrazyl), hydroxyl, superoxide, ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) 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 DPPH (1,1-Diphenyl-2-picryl-hydrazyl) radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, ABTS radical cation scavenging activity and reducing power using the standard procedure.

Results: The total phenolic and flavonoids in methanol extract were found to be 1.26g/100 g and 1.18 g/100 g respectively. The methanol extract of *Andrographis echioides* is found to possess DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activity. The IC₅₀ value of DPPH, hydroxyl, superoxide, and ABTS radical cation scavenging activity were found to be 49.11, 36.18, 37.13 and 38.15 µg/ml respectively. Like the antioxidant activity, reducing the power of the extract increases with increase in concentration.

Conclusion: The finding indicated promising antioxidant activity of crude extracts of the above plant and needs further exploration for their effective are in the both modern and traditional system of medicines.

Keywords: Antioxidant activity, DPPH, ABTS, Phenol, Flavonoid.

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INTRODUCTION

Numerous persistent and degenerative diseases such as cardiovascular disease, cancer, diabetes, neurodegenerative and ageing involve oxidative damage triggered by free radicals [1, 2-4]. The oxidative damage caused by these diseases are influenced largely by Reactive oxygen species (ROS), which including superoxide free radical (O₂⁻), hydroxyl free radical (OH⁻) and hydrogen peroxide (H₂O₂); this could lead to DNA mutations, protein inactivation, lipid peroxidation, cell apoptosis or abnormal proliferation, causing the occurrence of diseases from the cellular and molecular levels [5, 6]. Several fruits and vegetables possess abundant quantities of natural antioxidants such as vitamins and polyphenol, and it has been established that the consumption of many fruits and vegetables is associated with the decline of cardiovascular diseases and cancer [7].

Plants and their products have abundant phytochemicals and have been proved to be good sources of potential antioxidants [8]. Today consumers prefer natural antioxidants for use as nutraceuticals, biopharmaceuticals and as food additives [9]. As these natural obtained antioxidants have fewer on no side effects, they are preferred over synthetically derived antioxidants which have genotoxic effects [10]. Therefore, investigations of biological activity and chemical composition of medicinal plants as a potential source of natural antioxidants are numerous.

Andrographis echioides (Acanthaceae) which is commonly known as false water willow is a herb commonly found throughout India. The genus *Andrographis* is a native of India contains 28 species of small annual shrubs essentially distributed in tropical Asia. Some of them are medicinally important. The plants from genus *Andrographis* are used in goiter, liver diseases [11], fever, fertility problems, bacterial [12], malarial, helminthic, fungal, diarrhea and larvicidal disorders [13, 14]. Leaf juice boiled with coconut oil used to control falling and graying of hair [15]. So our present study focuses on the whole plant

of *Andrographis echioides* to determine their free radical scavenging properties. The literature survey showed very little information on this plant and thus prompted us to analyze this plant.

MATERIALS AND METHODS

The whole plant of *Andrographis echioides* (Voucher No. VOCB6978) were collected from Surandai, Tirunelveli District, Tamil Nadu. The collected samples were cut into small fragments and shade dried until the fracture is uniform and smooth. The dried plant material was granulated or powdered by using a blender and sieved to get uniform particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant material.

Preparation of plant extract

The coarse powder (100g) of the whole plant of *Andrographis echioides* was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 ml in a Soxhlet apparatus for 24 h. All the extracts were filtered through Whatman No.41 filters 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.

Preparation of extracts for phytochemicals screening

Freshly collected the whole plant of *A. echioides* was dried in the shade, and then coarsely powdered separately in a Willy 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 h. All the extracts were filtered through Whatman No.41 filters paper. All the extracts (petroleum ether, benzene, ethyl acetate, methanol, and ethanol) were subjected to qualitative tests for the identification of various phytochemical constituents as per standard procedures [16-18].

FT-IR analysis

A little powder of plant specimen was mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Infrared spectra were recorded as KBr pellets on a Thermoscientific Nicot iS5 iD1 transmission, between 4000 – 400 cm⁻¹ [19].

Estimation of total phenolic content

Total phenolic contents were estimated using Folin-Ciocalteu reagent based assay as previously described [20] with little modification. To 1 ml of each extract (100µg/ml) in methanol, 5 ml of Folin-Ciocalteu reagent (diluted ten-fold) and 4 ml (75g/l) of Na₂CO₃ were added. The mixture was allowed to stand at 20 °C for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. 1 ml aliquots of 20, 40, 60, 80, 100 µg/ml methanolic gallic acid solutions were used as a standard for the calibration curve. The absorbance of the solution was compared with a 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 flavonoids content was determined according to Eom *et al.* [21] An aliquot of 0.5 ml of sample (1 mg/ml) was mixed with 0.1 ml of 10% aluminium chloride and 0.1 ml of potassium acetate (1M). In this mixture, 4.3 ml of 80% methanol was added to make 5 ml volume. This mixture was vortexed, and the absorbance was measured spectrophotometrically at 415 nm. 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 an 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 [22].

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 [22]. Briefly, an 0.1 mM solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 3 ml of the solution of all extracts at different concentration (50,100,200,400 & 800µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. 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 to scavenging the DPPH radical was calculated by using the following formula.

$$\text{Radical scavenging activity (\%)} = \left\{ \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \right\}$$

Where, A₀ is the absorbance of the control reaction, and A₁ is the absorbance in the 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 Halli well [23]. Stock solutions of EDTA (1 mM), FeCl₃ (10 mM), Ascorbic Acid (1 mM), H₂O₂ (10 mM) and Deoxyribose (10 mM) were prepared in distilled deionized water.

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of FeCl₃, 0.1 ml H₂O₂, 0.36 ml of deoxyribose, 1.0 ml of the extract of different concentration (50,100,200,400 & 800 µg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.9), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37 °C for 1 hour. 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10%TCA and 1.0 ml of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured

at 532 nm. 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.* [24]. 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 (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract of different concentration (50,100,200,400 & 800 µg/ml), and 0.5 ml Tris-HCl buffer (16 mM, P^H 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25 °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.* [25]. ABTS radical cation (ABTS+) was produced by reacting 7 mM 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 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 min. 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 [26]. 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 °C for 20 min. Then 5 ml of 10% trichloroacetic acid was added and centrifuged at 980 g (10 min at 5 °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, radical superoxide 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, the standard error for aforesaid parameters was calculated.

RESULTS

Preliminary phytochemical analysis

The results of preliminary phytochemical screening of different extracts of the whole plant are presented in table 1. The methanol and ethanol extract of the whole plant of *A. echioides* revealed the presence of alkaloids, coumarins, flavonoids, phenols, quinones, glycosides, terpenoids, steroids, tannins, xanthoprotein, sugar and fixed oil (table 1).

Total phenolic and total flavonoid contents

The total phenolic and total flavonoid contents of the methanol extract of *A. echioides* whole plant were found to be 1.26g/100gand 1.18g/100grespectivity.

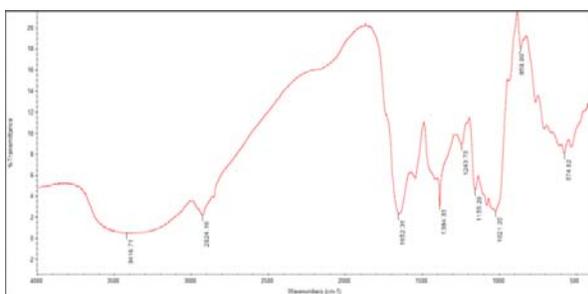
FT-IR

The FT-IR spectral studies of *A. echioides* the whole plant exhibited the following characteristics absorption peaks as shown in fig. 1 and table 2. From the spectral data, the presence of C-I, C-F, SO₂, SO₃, C=O, C=C, C-H and O-H were identified.

Table 1: Preliminary phytochemical screening of whole plant of *A. echioides*

Test	Petroleum ether	Benzene	Ethyl acetate	Methanol	Ethanol
Alkaloid	+	+	+	+	+
Antraquinone	-	-	-	-	-
Catechin	-	-	-	-	-
Coumarin	+	+	+	+	+
Flavonoid	+	+	+	+	+
Phenol	+	+	+	+	+
Quinone	+	+	+	+	+
Saponin	+	+	+	-	-
Steroid	+	+	+	+	+
Tannin	+	+	+	+	+
Terpenoids	+	+	+	+	+
Sugar	+	+	+	+	+
Glycoside	+	+	+	+	+
Xanthoprotien	+	+	+	+	+
Fixed oil	+	+	+	+	+

+Presence-Absence

Fig. 1: FT-IR Spectrum of the whole plant of *A. echioides*

DPPH radical scavenging activity

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. echioides* the whole plant are shown in fig. 1. DPPH radical scavenging activity of five extracts was observed to be increased with the increase of dose (50-

800µg/ml) were represented in the graph. Among the solvent treated, methanol extract exhibited highest DPPH radical scavenging activity. At 800µg/ml concentration, the methanol extract of *A. echioides* the whole plant possessed 134.15% scavenging activity on DPPH. The concentration of *A. echioides* whole plant methanol extract needed for 50 % inhibition (IC_{50}) was 49.11 µg/ml, while ascorbic acid needed 32.18 µg/ml. (table 3).

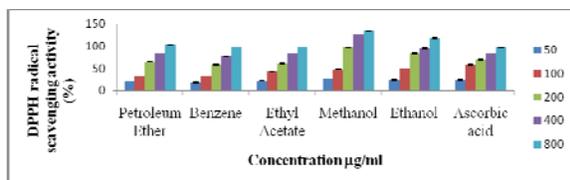
Table 2: FTIR spectroscopic data of the whole plant of *A. echioides*

S. No.	Group	Stretching frequency (cm ⁻¹)
1	O-H	3416.71
2	C-H	2924.16
3	C=C	1652.31
4	C=O	1384.33
5	SO ₃	1243.73
6	SO ₂	1155.29
7	C-F	1021.20
8	C-I	574.62

Table 1: IC_{50} values of different solvent extracts of the whole plant of *A. echioides**

Solvent	IC_{50} (µg/ml)			
	DPPH	Hydroxyl	Superoxide	ABTS
Petroleum ether	39.86	30.17	23.86	26.56
Benzene	37.27	26.28	23.65	25.15
Ethyl acetate	39.16	29.14	24.93	27.29
Methanol	49.11	36.18	37.13	38.15
Ethanol	42.65	34.56	36.83	32.93
Ascorbic acid	32.18	29.57	30.16	-
Trolox	-	-	-	28.41

* All values are mean of triplicate determinations

Fig. 2: DPPH radical scavenging activity of different extracts of *A. echioides*

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of petroleum ether, benzene, and ethyl acetate, methanol and ethanol extract of *A. echioides* the

whole plant are depicted in fig. 2. Methanol extract showed very potent activity. At 800µg/ml concentration, *A. echioides* the whole plant possessed 126.32 % scavenging activity on hydroxyl radical. Results showed the percentage of inhibition in a dependent manner. The concentration of *A. echioides* whole plant methanol extract needed for 50 % inhibition (IC_{50}) was found to be 36.18 µg/ml, whereas 29.57 µg/ml (table 3) needed for ascorbic acid.

Superoxide radical scavenging activity

The different solvent extracts of *A. echioides* whole plant were subjected to superoxide radical scavenging activity, and the results were shown in fig. 3. It indicates that methanol extract of *A. echioides* whole plant (800 µg/ml) exhibited the maximum superoxide radical scavenging activity of 134.68 % which is higher than the standard ascorbic acid whose scavenging activity is 118.16 %. The IC_{50} value

of methanol extract of *A. echioides* whole plant on superoxide radical was found to be 37.13 $\mu\text{g/ml}$ and 30.16 $\mu\text{g/ml}$ for ascorbic acid, respectively. (table 3).

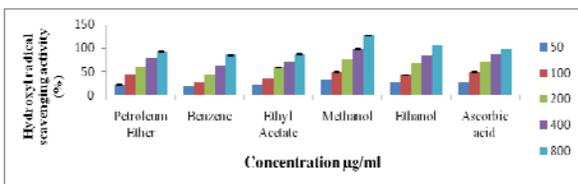


Fig. 3: Hydroxyl radical scavenging activity of different extracts of *A. echioides*

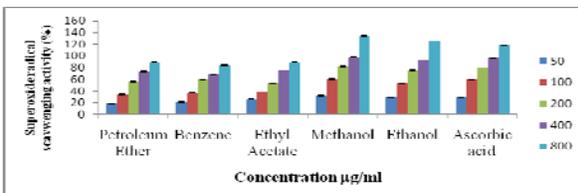


Fig. 4: Superoxide radical scavenging activity of different extracts of *A. echioides*

ABTS radical cation scavenging activity

The different solvent extracts of *A. echioides* the whole plant were subjected to ABTS radical cation scavenging activity, and the result were shown in the fig 4. The methanol extract exhibited potent ABTS radical cation scavenging activity in a concentration-dependent manner. At 800 $\mu\text{g/ml}$ concentration, 126.34 % scavenging activity on ABTS which was higher than the standard trolox whose scavenging activity was 102.16 %. The quantity of *A. echioides* methanol extract required to produce 50% inhibition of ABTS radical 38.15 $\mu\text{g/ml}$ whereas 28.41 $\mu\text{g/ml}$ (table 3) needed for trolox.

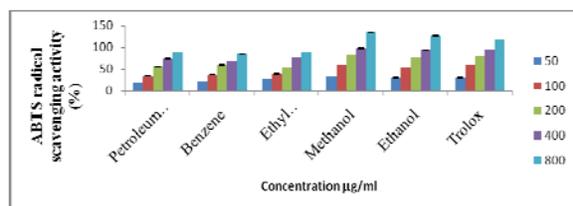


Fig. 5: ABTS radical scavenging activity of different extracts of *A. echioides*

Reducing power

Fig. 5 revealed the reducing ability of different solvent extracts of *A. echioides* the whole plant compared to ascorbic acid. Absorbances of the solvent extracts were increased as the concentration is increased. A higher absorbance indicates a higher reducing ability. Among the solvents treated, methanol extract exhibited higher reducing ability.

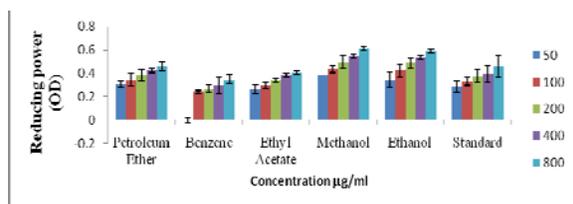


Fig. 6: Reducing power activity of different extracts of *A. echioides*

DISCUSSION

Phytochemicals are chemical compounds formed that are in the processes of plant's normal metabolic activities. These chemicals are often called 'secondary metabolites' and there are several classes including alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids [27]. In the present study, the qualitative phytochemicals investigation showed that methanol and ethanol extracts contained some phytoconstituents. Alkaloid, coumarin, catechin, saponin, steroid, flavonoid, terpenoid, tannin and phenol were found in both the extracts.

The FT-IR spectrum was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. From the spectral data, the presence of C-I, C-F, SO_2 , SO_3 , C=C, C=O, C=C, C-H and O-H were identified. These bonding structures are responsible for the presence of aliphatic iodo, aliphatic fluoro, sulphur sulphonic acid, carboxylic, aromatic, alkyl and hydroxyl compounds. The more intense bands occurring at 574.02 cm^{-1} , 1021.20 cm^{-1} , 1155.29 cm^{-1} , 1243.73 cm^{-1} , 1384.33 cm^{-1} , 1652.31 cm^{-1} , 2924.16 cm^{-1} and 3416.71 cm^{-1} corresponds to C-I stretching, aliphatic iodo compound, C-F stretching, aliphatic fluoro compound, SO_2 sym. Stretching sulphur compound, SO_3 asymmetric stretching, sulphonic acid compound, C=O stretching, carboxylic, carbonyl compounds, C=C stretching, an aromatic compound, C-H stretching, alkyl compound, O-H stretching, hydroxyl compound respectively. Carboxylic acids present in the medicinal plant serves as a main pharmaceutical product in curing ulcers, jaundice, headache, stomatitis, hemicranias, pain in liver, treatment of edema and rheumatic joint pains. Sulphur derivative compounds were needed as disinfectant dermal cream. Therefore, the FT-IR analysis of *A. echioides* the whole plant displayed novel phytochemicals marker as a useful analytical tool to check not only the quality of the powder but also to identify the important medicinal plants.

Phenolic compounds are considered to be the most important antioxidants of plant materials. They contribute one of the major groups and compounds acting as primary antioxidants or free radical terminators. Antioxidant activity of a phenolic compound is based on their ability to donate hydrogen atoms to free radicals. In addition, they possess ideal structural properties for free radical scavenging properties [28]. Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis, and carcinogenesis. It has been confirmed that pharmacological effects of flavonoids is correlating with their antioxidant activity [29]. The presence of these compounds such as total phenolics and flavonoids in *A. echioides* whole plant extract may give credence to its local usage for the management of oxidative stress induced ailments.

In the present study, *in vitro* antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. echioides* the whole plant were investigated using DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities. These methods have established the efficacy of the extracts when compared to that of the reference standard antioxidant, ascorbic and trolox.

DPPH are extensively used in model systems to analyse the radicals are scavenging activities of several natural compounds. When DPPH radicals are scavenged, the color of the reaction mixture changed from purple to yellow as there is a decrease in absorbance at wavelength 517 nm. It was reduced to a yellow colored product, diphenyl picrylhydrazine, with the addition of *A. echioides* the whole plant extracts in a concentration-dependent manner. Among the solvent tested, the methanol extract of *A. echioides* the whole plant exhibited more DPPH radical scavenging activity.

The hydroxyl radical is one of the potent reactive oxygen species in the biological systems. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [30]. Hydroxyl radical scavenging capacity of *A. echioides* extract is

directly related to its antioxidant activity. This method involves *in vitro* generation of hydroxyl radicals using Fe^{3+} /ascorbate/EDTA/ H_2O_2 system using Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid [31]. When *A. echioides* extracts were added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. Among the solvent tested, methanol extract possessed more hydroxyl radical scavenging activity when compared with standard ascorbic acid.

Superoxide anion is a weak oxidant, but it generates potent and dangerous hydroxyl radicals and singlet oxygen, and both promote oxidative stress [32]. Numerous biological reactions generate superoxide anions which are highly toxic species. In the PMS/NADH-NBT system, the superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The decrease in absorbance at 560 nm, *A. echioides* the whole plant extracts indicated the ability of quench superoxide radicals in the reaction mixture. The present study showed potent superoxide radical scavenging activity for *A. echioides* whole plant extracts. Methanol extract showed potent superoxide radical scavenging activity.

The ABTS assay is founded on the inhibition of the absorbance of the radical cation ABTS with a typical long wavelength spectrum [33]. The scavenging activity of ABTS radical is relatively new. It involves a more drastic radical that is chemically produced and it is used more for screening complex antioxidants mixtures such as plant extracts, beverages, and biological fluids. This ability is effective in both the organic and aqueous media and its stability in a wide range of pH have enhanced the interest in the use of ABTS for estimating antioxidant activity [25]. The present study, methanol extract of the whole plant of *A. echioides* was fast and effective scavengers of ABTS radical, and this activity was higher than that of trolox standard. Proton radical scavenging is an important attribute of antioxidants. ABTS a protonated radical has characteristic absorbance maxima at 734 nm which decreases with scavenging of the proton radicals.

Several reports indicated that the reducing power of bioactive compounds was associated with antioxidant activity [34]. Therefore, it is necessary to determine the reducing power of phenolic constituents contained in the plant extracts to elucidate the relationship between their antioxidant effect and their reducing power. In the present study, an increase in absorbance of the reaction mixture indicates the reductive capabilities of *A. echioides* extract in concentration dependent manner when compared to the standard ascorbic acid.

CONCLUSION

The present study reveals that the whole plant of *Andrographis echioides* exhibited a satisfactory scavenging effect in all the radical scavenging assays. The results obtained by these methods provide some insight into the important factors responsible for the antioxidant potential and the mechanism of action. However, the methanol extract of the whole plant possessed good antioxidant activity. Further studies on isolating and characterizing the antioxidant substances and their potential as pharmacological agents are in progress.

CONFLICT OF INTERESTS

Declared none

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