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COMPARATIVE ANALYSIS OF ANTIOXIDANT POTENTIAL AND ANDROGRAPHOLIDE CONTENTS IN *ANDROGRAPHIS PANICULATA* (L.) NEES AND *ANDROGRAPHIS ECHIOIDES* (L.) NEES

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ABSTRACT

Andrographis paniculata and *Andrographis echioides* are well-known species of Indian and south Asian traditional medicines. *Andrographis paniculata* (AP) commonly known as Nilavembu is recently, attracted as a valuable therapeutic plants for treating Dengue fever. *Andrographis echioides* (AE) is a closely related species of *Andrographis paniculata*. Hence, the phytochemical contents was screened and compared between the two species. In the present investigation, protocols for methanolic extract of i*n vivo* plant parts like leaf, stem, root, have been standardized. Maximum percentage of Andrographolide was obtained in both the species. *Andrographis echioides*. Methanolic extracts of both plant parts (leaf, stem, and root) have showed antioxidant activity in DPPH, FRAP, ABTS, H_2O_2, reducing power, metal chelating efficiency with little variation. Similarly, Andrographolide contents were also varied among the plant parts of two species where maximum content was present in leaf (381.72 mg/g DW) of AP and 347.93mg/g DW in stems of AE.

KEYWORDS: *Andrographis paniculata* and *Andrographis echioides.*

INTRODUCTION

Andrographis paniculata Nees and *Andrographis echioides* (L) have received much attention in recent years because of its therapeutics, pharmaceutical and health protective value and recently valued as a potential plants for treating the Dengue fever. Hence has heavy demand in India as well as international markets. The main bioactive compounds present in these plants are Andrographolide, dehydroandrographolide, neoanderographolide and deoxyandrographolide.[1] In addition, main phytocompounds present in *Andrographis paniculata Nees and Andrographis echioides* (L) includes flavonoids, phenolics, terpenoids, alkaloids and tannins.[2] Therefore, these plant extracts have been used to treat various treatments like as anticancer, anti-inflammatory, angiogenic, antivenom, antidiabetic, antimalarial. In addition, used for cold, diarrhea, fever due to several infective causes, jaundice, as a health tonic for the liver and cardiovascular health, and various investigations around the globe in India. Recently the demand for natural Andrographolide has increased in the pharmaceutical industry, which has led us to search for explored sources of Andrographolide from different parts of the plant sources. Hence in the present study the phytochemical screening of *Andrographis echioides* and *Andrographis paniculata* were analyzed and a simple methanolic extraction method for quantification of Andrographolide using HPLC containing C18 column. The results showed both the species has similar bioactive compounds with the varied concentrations and suggest both the species can be used for extraction of Andrographolide.

MATERIALS AND METHODS

Plant material

A. paniculata and *A.echioides* were obtained from TNAU (Tamilnadu agriculture university) and Bharathiar university campus. The samples were washed thoroughly in distilled water and the surface water was removed by air-drying under shade. The samples (root, stem, leaf) were powdered by using liquid nitrogen and used for extraction. The extraction is done by serial maceration method.

Free radical scavenging activity test (DPPH)

In order to evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH (1, 1-diphenyl-2-picrylhydrazyl) radicals is monitored. The sample extract (0.1mL) is diluted with methanol and 1mL of DPPH solution (0.5 mM) is added. After 30 min, the absorbance is measured at 517 nm.[3] The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = $(A_0 – A_1) /A_0 \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Ferric reducing-antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the method.[4] The FRAP reagent was generated by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM (Tripyridyl Triazine) TPTZ solution and 20.0 mM $FeCl_3.6H_2O_2$ solution in a ratio of 10:1:1 in volume. Samples at different concentrations (100,200,300,400 and 500 µg/ml) was then added to 3 ml of FRAP reagent and the reaction mixture was incubated at 37 °C for 30 min. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mM $FeSO_4$ equivalents per gram of sample (DW).

Hydrogen peroxide scavenging (H_2O_2) assay

The hydrogen peroxide scavenging assay was carried out following the procedure.[5] A solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). The extract at different concentrations in 3.4 ml phosphate buffer was added to 0.6 ml of H_2O_2 solution (0.6 ml, 43 mM). The absorbance value of the reaction mixture was recorded at 230 nm.

H_2O_2 scavenging activity (%) = $(A_0 – A_1) /A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

Reducing power method (RP)

Reducing power was determined by the method.[6] The sample in 1ml of methanol at various concentrations was mixed with a phosphate buffer (5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5 ml, 1%), and the mixture was incubated at 50°C for 20 min. Next, 5ml of trichloroaceticacid (10%) were added to the reaction mixture, which was then centrifuged at 3000 RPM for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5ml) and ferricchloride (1 ml, 1%), and the absorbance was measured at 700 nm. A stronger absorbance will indicate increased reducing power.

ABTS radical cation decolorization assay

ABTS radical-scavenging activity of the extract was determined.[7] The ABTS**,** cation radical was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 h. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. The plant extract at various concentrations with 1ml of ABTS solution was homogenized and its absorbance was recorded at 734 nm. The inhibition percentage of ABTS radical was calculated using the following formula:

ABTS scavenging activity (%) = $(A_0 – A_1) /A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

Metal chelating activity

The chelating activity of the extracts was determined using ferrous ions.[8] The reaction mixture was prepared using 0.1mL of extract dissolved in DMSO at different concentrations (10, 20, 30, 40, and 50 mg/mL) followed by the addition of 0.1mL of (0.2 mM) ferrous chloride and 0.2 mL (5 mM) of ferrozine solution. The solution was mixed thoroughly and incubated in the dark for 30 min. The absorbance was measured at 562 nm.

Quantification of Andrographolide by HPLC analysis

Solvents used for chromatographic analysis were HPLC grade Methanol and HPLC grade water. The standard solution was prepared by dissolving 1 mg of standard andrographolide (98%, pure, Sigma) in 1 ml methanol 100% (v/v) purchased from Sigma Aldrich (US) was used as standard. Solvents (HPLC grade) like methanol used for the analysis and extraction was obtained from Merck (Mumbai). All other chemicals used for *in vitro* culture preparation and Polyvinylidene difloride (PVDF) syringe filter with pore size 0.45 µm with highest purity were purchased from Himedia (Mumbai).

Sample preparation

The powder (0.1g) of plant material was refluxed for 1 hour with methanol (10 ml) on a water bath. The mare was filtered and subjected for another two cycles of refluxes (1 hr each) with methanol (10 ml). The combined filtrates were evaporated under vacuum to dryness. The residue was dissolved in methanol (1 ml) and filtered through a 0.45 µm (Nylon) filter into HPLC vials.

Analysis

HPLC analysis was performed on a Waters HPLC system, equipped with a 2996 photodiode array detector (Waters Pvt. Ltd). For all separation X Bridge TM C18 column (4.6 mm ×250 mm, 5.0 µm particle size) was used. The mobile phase consisted of water (A) and a mixture of MeOH(Sigma) and reagent alcohol in ratio of 1:1. Each run was followed by a 5 min wash with 100 B and an equilibration period of 10 min. The flow rate and sample volumes were monitored at 223 nm. Peaks were assigned by spiking the sample with authentic sample followed by comparison of UV spectra and retention time. The amount of Andrographolide present in each sample was calculated by comparing the standard area with sample area. The standard and sample solution was injected in triplicate,

Concentration of the sample (µg/ml) = Peak area of the sample Peak area of the standard X 1000

RESULTS

Scavenging activity of plant extracts in DPPH radical

All the plant parts showed scavenging activity (**Table. 1**). The percentage of inhibition in different samples were varied in *A. echioides* viz., 51.22% was observed for the root, 58.28% for the stem, 42.63% for the leaf and 25.46% similarly 68.40% was observed for stem, 51.84% in leaf, and 33.12% in *A. paniculata* (**Fig.1**). *A. paniculata* has highest amount scavenging activity when compared to the *A. echioides*. Especially the stem sample showed the highest percentage of scavenging activity

(**Fig.1**).

Ferric reducing-antioxidant power (FRAP) assay
The ferric reducing activity of different samples of *A.echioides* and *A. paniculata*. The percentage of ferric reducing property was obtained in all the samples tested. The percentage of inhibition in different samples were recorded in *A. echioides* as 43.65% for the root, 64.44% for the stem, 47.74% for the leaf and 46.63% in root, 65.37% in stem, 23.44% in leaf of *A. paniculata*. (**Table.1**). Based on these data, the *A. paniculata* has highest amount scavenging activity when compared to the *A. echioides* (**Fig.2**).

ABTS radical cation decolorization assay
ABTS radical cation decolorization assay of the *A. echioides* and *A. paniculata* samples were analyzed and compared the standard. The decolorization of the extracts was 39.74% for the root, 35.56%, for the stem and 48.53% for the leaf of *A.paniculata* whereas A.*echioides* showed 30.54 for the root, 56.25% and 42.25% in stem and leaf respectively (**Fig.3**). These data show that the *A.paniculata* has less ABTS radical cation decolorization than the *A.echioides* (**Table.1**).

Metal chelating activity
Iron is essential for life as it is required for oxygen transport, respiration and for activity of many enzymes. Chelating agents inhibit lipid peroxidation by stabilizing the transition metals. The metal chelating activity of different samples were recorded as 9.89% for the root, 17.28% for stem, and 8.71% for leaf in *A.echiodies*, whereas the *A. paniculata* contains 8.71% for the root, 9.53% for the stem and 5.16% in leaf (**Table.1**). These data showed that the *A.echioides* had the highest chelating activity (**Fig.4**).

Reducing power assay
The reducing ability of a compound generally depends on the presence of reductones, which exert their antioxidant activity by donating a hydrogen atom and breaking the free radical chain. The reductive abilities of the *A. echioides* and *A. paniculata* samples were analyzed and compared with standard. As the concentration of the extract increased the reducing power increased. The reducing power of the extracts was 3.8% for the root, 2.4% for the stem and 5.1% for the leaf in *A.echioides. A.paniculata* contains 4.6% for the root, 3.4% for the stem and 4.9% in the leaf. These data show that the *A.paniculata* (root) sample had good reducing power then the *A.echioides*. (**Fig.4; Table.1**).

Hydrogen peroxide (H_2O_2) scavenging activity
The radical scavenging property of a compound, which may serve as a significant indicator of its potential antioxidant activity, was evaluated using the H_2O_2 radical scavenging ability of *A. echioides* and *A. paniculata* compared with ascorbic acid as a standard. The percentage of inhibition in *A. echioides* and *A. paniculata* were recorded as 6.35%, 2.99%, 2.42% for the root, stem and leaf respectively, and 6.91%, 4.11%, 1.68% in root, stem and leaf (**Figure 6**). The data from this assay also showed that the *A, paniculata* contains more amount of scavenging activity compared with the *A.echiodies* specifically root sample had the highest scavenging activities.

Analysis of Andrographolide by HPLC
The conditions for chromatography, in particular, mobile phase composition, temperature and flow rate were optimized to accomplish a good resolution and symmetrically shaped peak for Andrographolide in less run time. Symmetry® C18 column (4.6 mm x 250 mm, 5 µm) exhibited good chromatographic peak for investigation of Andrographolide when compared to other conventional C18 column. Further, the isocratic mobile phase composition like, methanol: water (70:30 v/v) with 0.2% ortho-phosphoric acid at a flow rate of 1 ml/min and temperature at 30 °C was pertinent for the effective separation of Andrographolide. The wavelength for the detection of Andrographolide was set at 223 nm wherein signal-to-noise (S/N) ratio was low.

In the present study various part of the plans such as root, stem, leaf were used for HPLC quantification. The **Fig 7.** shows that stem contains 34.17 mg/g(DW), leaf contains 381.72 mg/g(DW) and the root sample has 33.64mg/g(DW) of Andrographolide in *A.paniculata*. The leaf sample of *A.echioides* contains 79.52mg/g(DW) and stem contains 347.93 mg/g(DW) of Andrographolide were present but the root sample of *A.echioides* shows the absence of Andrographolide. In the present study, andrographolide content were significantly higher in methanolic extracts of *A.paniculata* when compared to *A.echioides*.

Table 1: Quantitative Determination of antioxidant Constituents of *A.paniculata* and *A.echioides*.

Antioxidant activity	***A.paniculata***			***A.echioides***		
Samples	**Root (%)**	**Stem (%)**	**Leaf (%)**	**Root (%)**	**Stem (%)**	**leaf (%)**
DPPH	58.28	51.84	68.40	51.22	58.28	42.63
FRAP	46.63	23.44	65.37	43.65	64.44	47.74
ABTS	39.74	35.56	48.53	30.54	56.25	42.25
Metal chelating activity	5.16	7.53	8.71	9.89	17.28	8.7
Reducing power	4.6	3.4	4.9	3.8	2.4	5.1
Hydrogen peroxide	1.68	4.11	6.91	6.35	2.99	2.42

Mean values of three independent experiments (N=3/replicate) with standard deviations and standard errors.

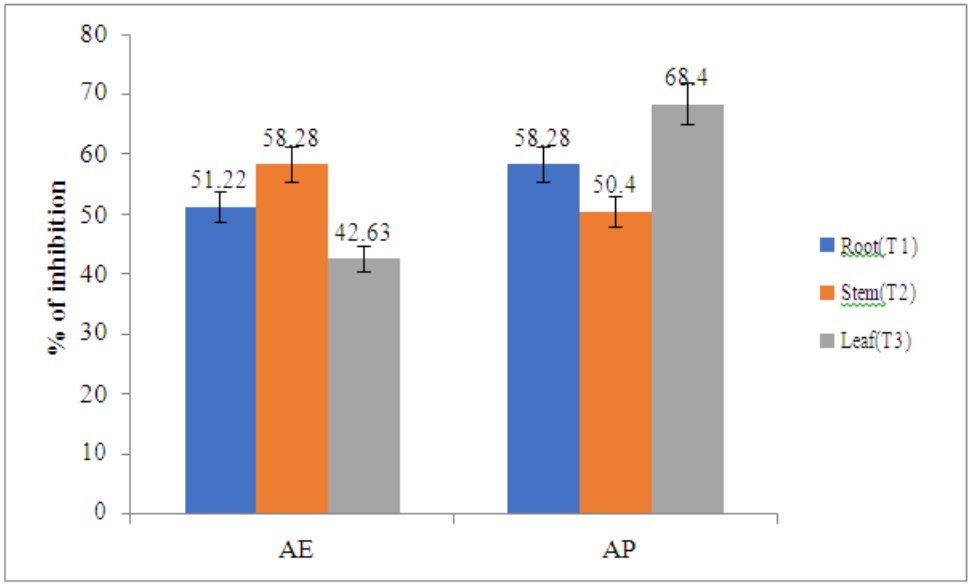

Figure No: 1: Scavenging activity on DPPH radical (DPPH method) of *A.paniculata* and *A. echioides.*
Mean values of three independent experiments (N=3/replicate) with standard deviations and standard errors.

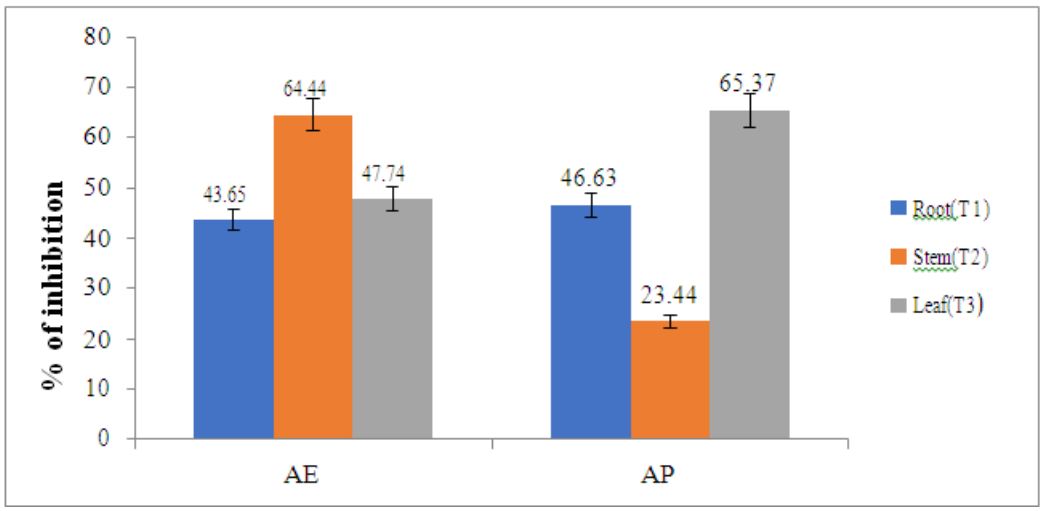

Figure No: 2: Ferric reducing-antioxidant power (FRAP) assay of *A.paniculata* and *A.echioides.*
Mean values of three independent experiments (N=3/replicate) with standard deviations and standard errors.

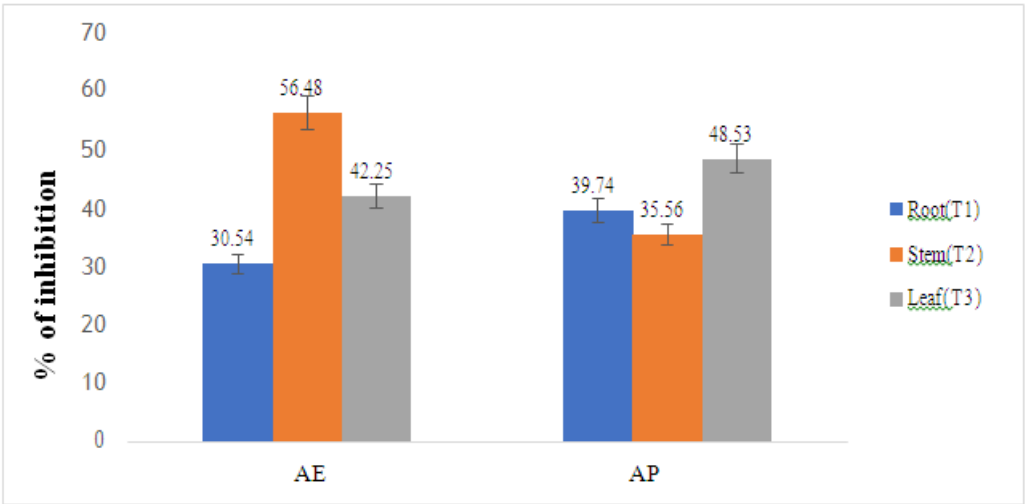

Figure No: 3: ABTS radical cation decolorization assay of *A.paniculata* and *A.echioides.*
Mean values of three independent experiments (N=3/replicate) with standard deviations and standard errors.

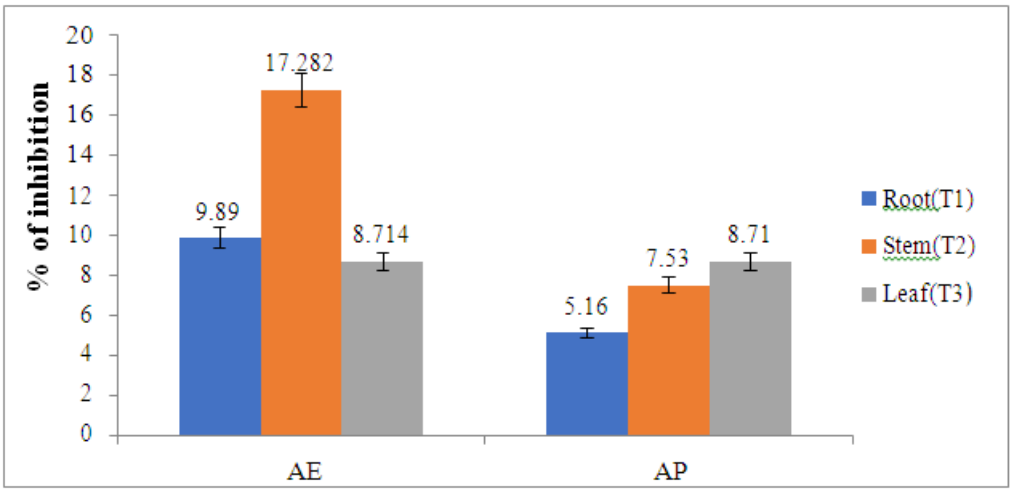

Figure No: 4: Metal chelating activity of *A.paniculata* and *A.echioides.*
Mean values of three independent experiments (N=3/replicate) with standard deviations and standard errors.

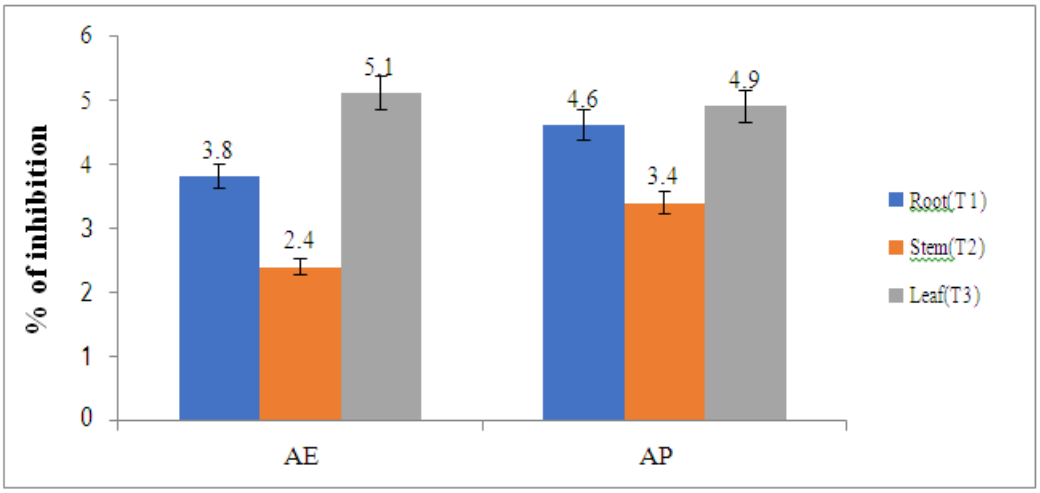

Figure No: 5: Reducing power assay of A.paniculata and A.echioides.
Mean values of three independent experiments (N=3/replicate) with standard deviations and standard error.

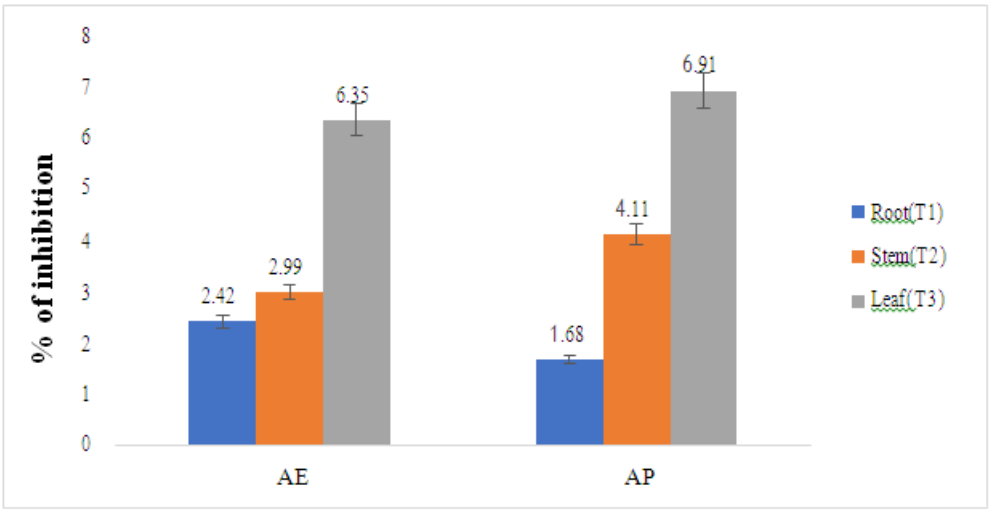

Figure No: 6: Hydrogen peroxide (H_2O_2) scavenging activity of *A.paniculata* and *A.echioides.*
Mean values of three independent experiments (N=3/replicate) with standard deviations and standard errors.

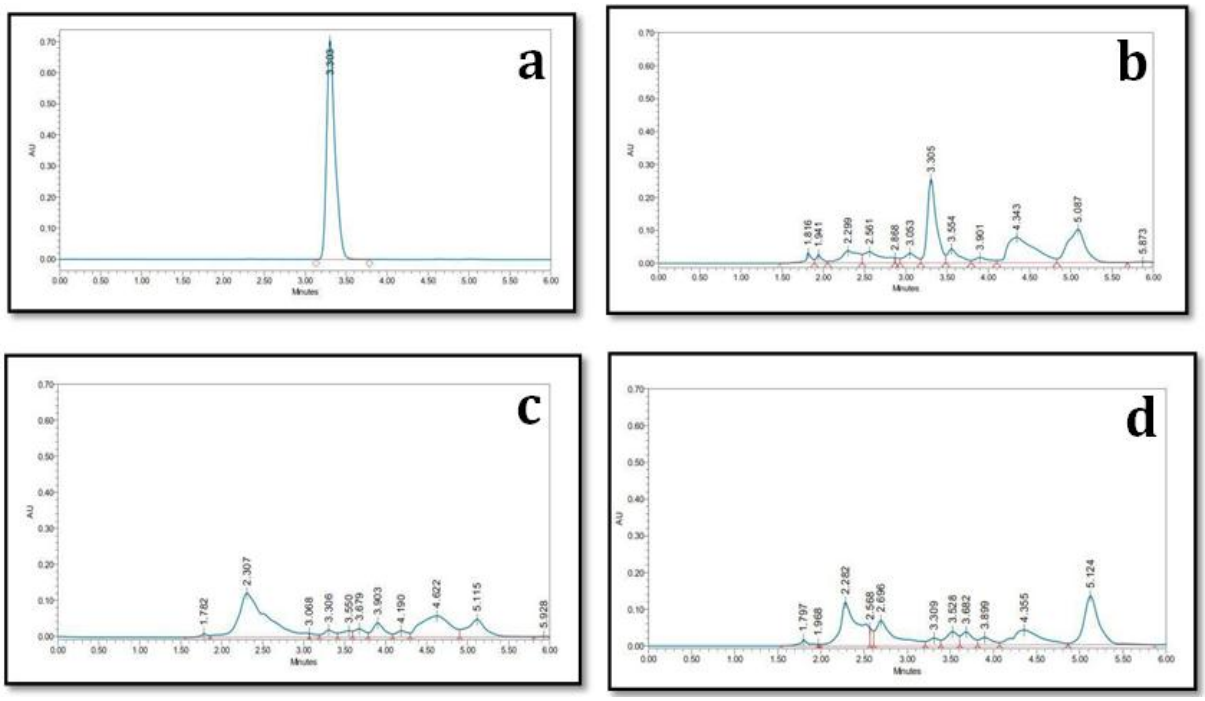

Figure No. 7: HPLC chromatograms of Andrographolide from different parts of *Andrographis paniculata* methanolic extract.

a. Standard of Andrographolide
b. Methanolic extract of leaves extract
c. Methanolic extract of stem.
d. Methanolic extract of root.

DISCUSSION

In the present work methanolic extraction were obtained in both the species from different plant parts. The antioxidant ability of the plants can be exploited for the potential pharmaceutical applications.[7] The antioxidant activity of all the extracts were analysed and found all the extracts found effective in free radical scavenging assay. Three main secondary metabolites were identified in the *A. paniculata* leaves were andrographolide, neo-andrographolide and deoxyandrographolide.[9],[10] The previous findings of *Andrographis paniculata*, aerial part of the plant contains higher amount of Andrographolide. The evaluated the anti-inflammatory, analgesic and antipyretic activity of ether, chloroform, and ethyl acetate extract of *Andrographis echioides* in rats and mice.[11] The present study is mainly based on the comparison of andrographolide compound present in *A.paniculata* and *A.echioides* To our knowledge; this is the first study on comparative analysis of both the species. Although no much deviation were observed in antioxidant potential of both the species the metal chelating activity of *A. echioides* were more compared to *A. paniculata*. The andrographolide content in both the plants did not had the significant difference. The present study attempts a modest comprehensive investigation of the whole plant (especially root, stem, and leaves) of *Andrographis* echioides and *Andrographis paniculata* for its andrographolides content quantity by using the *RP-HPLC* analysis technique. In addition, the antioxidant content and phytoconstituents were checked by employing biochemical assay methods, since the whole plant of Andrographis as the folklore claims has therapeutic qualities.

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