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PHYTOCHEMICAL ANALYSIS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF LEAVES OF *ANDROGRAPHIS ECHIOIDES* AND SEEDS OF *SPERMACOCE HISPIDA*

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ABSTRACT

The present study has been carried out to evaluate the preliminary phytochemical screening and antioxidant activity of the ethanolic extracts of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida*. The phytochemical screening of dry powder of *Andrographis echioides* depicts the presence of flavonoids, alkaloids, sugar, etc. and *Spermacoce hispida* depicts the presence of quinone, flavonoids, alkaloids, etc. *In vitro* antioxidant activities have been investigated by using DPPH scavenging assay, Reducing power assay, Nitric oxide (NO) scavenging assay and ABTS cation decolourization assay. The results reveal that the ethanolic extract of leaves of *Andrographis echioides* has DPPH radical scavenging and Nitric oxide scavenging activities whereas the seeds of *Spermacoce hispida* possesses potent Reducing power and ABTS cation decolourization activities.

KEYWORDS: ABTS scavenging assay, *Andrographis echioides*, Antioxidant activity, DPPH radical scavenging assay, *Spermacoce hispida*.

INTRODUCTION

Free radicals including reactive oxygen species (ROS) induce oxidative damage to biomolecules and have been implicated in variety of chronic diseases including cancer, diabetes, atherosclerosis, neurodegenerative disorders and arthritis. Natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds becomes important. Epidemiological studies have indicated the relationship between the plant

antioxidants and reduction of chronic diseases. These benefits are thought to result from the antioxidant components of plant origin, vitamins, flavonoids, and carotenoids.

Herbal medicine provides rational means for the treatment of many diseases that are incurable in other systems of medicine. The traditional medicine methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries and moreover the use of herbal remedies. In this connection, plants continue to be a rich source of therapeutic agents.[1]

Andrographis echioides belongs to the family of Acanthaceae and commonly known as False water willow. It has many ethnobotanical uses and is medicinally used in the traditional and Ayurvedic system. The various extracts of the whole plant of *Andrographis echioides* have been reported for its anthelmintic activity, antimicrobial activity,[2] larvicidal activity.[3]

Spermacoce hispida belongs to the family Rubiaceae and commonly known as Nattaichuri. It is commonly used herb in siddha medicine. It is widely distributed throughout the world as a useful medicinal plant. The various extracts of *Spermacoce hispida* have been reported for its antinociceptive activity,[4] antidiabetic, anti-hypertensive, hepatoprotective, anti-inflammatory, analgesic, antihyperlipidemic, antifungal, anticancer, antioxidant properties.[5]

Hence in the present study, attempts made are made to evaluate the *in vitro* antioxidant activities of ethanolic extracts of *Andrographis echioides* and *Spermacoce hispida*.

MATERIALS AND METHODS

COLLECTION AND EXTRACTION OF PLANT MATERIAL

The selected plants were collected from Thanjavur and Thiruthuraipoondi, identified with the help of Flora of Presidency of Madras,[6] and authenticated by the botanist Dr. K. Kandavel, S.T.E.T. Women's College, Mannargudi. 20g of plant powders (*Andrographis echioides and Spermacoce hispida*) were soaked separately in 100ml of ethanol for 48hrs.Then filtered and boiled at 57^{o}C until it becomes precipitated. The precipitate is used for *in vitro* antioxidant assays.

DETERMINATION OF PHYSICOCHEMICAL CONSTANTS[7]

Physicochemical constants such as moisture content and extactive values were determined using standard procedures.

FLUORESCENCE ANALYSIS[8]

Fluorescence of the drugs (*Andrographis echioides and Spermacoce hispida*) was observed under day and UV light using various solvent extracts as well as acids and alkaline treated solutions of the drug. The powders were treated with neutral solvents like hexane, benzene, chloroform, ethyl acetate, alcohol, acetone and acids like 1N Hydrochloric acid, 50%Sulphuric acid and alkaline solutions like aqueous and alcoholic 1N NaOH.

PRELIMINARY PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening of drug powders (*Andrographis echioides and Spermacoce hispida*) and various extracts were carried out as per the standard textual procedure.[9]

ESTIMATION OF PHENOL[10]

0.5 to 1 gm of the samples were weighed and ground well with 10 times the volume of 80% ethanol. The homogenates were centrifuged at 10,000 rpm for 20 min. The supernatant was collected and the residue was re-extracted with 5 times the volume of 80% ethanol. The samples were centrifuged again and the supernatant was collected. It was then evaporated to dryness. The residues obtained were dissolved in 5 ml of distilled water. Different aliquots of the sample (0.2 – 2.0 ml) were pipetted out in different test tubes, and then the volume was made up to 3 ml with distilled water. 0.5 ml of Folin's reagent was added to all the tubes. After 3 min 2 ml of 20% Sodium bicarbonate solution was added. The content was mixed thoroughly and the test tubes were placed in boiling water bath for 1 min, cooled and the colour developed was measured at 650 nm. The phenol was expressed in mg/g.

ESTIMATION OF CARBOHYDRATE[11]

100mg of powdered plant samples were boiled with 5 ml of 2.5N Hydrochloric acid and cooled to room temperature. It was centrifuged, and then made up 100ml using distilled water the supernatant was collected. 0.05 ml of the plant samples were taken and made up to 1 ml with distilled water. The tubes were kept in ice and 4 ml of 0.2% anthrone was added slowly with constant stirring. The test tubes were heated in boiling water bath for 20 minutes. The blank was treated in a similar manner without plant extract and the green colour developed was read at 640 nm. From the standard value the carbohydrate content was calculated. The carbohydrate was expressed in mg/g.

ESTIMATION OF PROTEIN[12]

1 g of the plant drugs were ground with 5 ml phosphate buffer and the filtrate was made up to 100ml with distilled water. 0.5 ml of the plant samples were taken in various test tubes and was made up to 1 ml with distilled water. To this 4.5ml of Lowry's reagent was added and incubated at room temperature for about 10 minutes. A blank was treated with 1 ml of distilled water instead of plant sample. After 10 minutes, 0.5 ml of Folin's reagent was added and the colour developed was read calorimetrically at 620nm. From the standard values protein content was calculated. The plant protein was expressed in mg/g.

DPPH Radical Scavenging Assay[13]

The free radical scavenging capacity of the ethanolic extracts were determined using DPPH. DPPH (200µM) solution was prepared in 95% methanol. From the stock plant extract solution 100, 250, 500, 750 and 1000 µg/ ml were taken in five test tubes. 0.5ml of freshly prepared DPPH solution was incubated with test drug and after 10 minutes, absorbance was taken at 517 nm using spectrophotometer. Standard ascorbic acid was used as reference. % scavenging of the DPPH free radical was measured using following equation

DPPH radical scavenging % = Control- Test/Control × 100

Reducing Power Assay[14]

1 ml of varying concentrations (1-5 mg/ml) of plant extract was mixed with 2.5 ml phosphate buffer and 2.5 ml of potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Aliquots of 2.5 ml of trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10min. The upper layer of the solution (2.5 ml) was mixed with equal volume of distilled water, to this 0.5ml of freshly prepared ferric chloride solution was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power.

Nitric Oxide Scavenging Activity[15]

Sodium nitroprusside (5 mM) in standard phosphate buffer solution was incubated with different concentrations (200-1000µg/ml) of the plant extract dissolved in phosphate buffer (0.025 M, pH 7.4) and tubes were incubated at 25^0C for 5 hours. Control tube without the plant extract, but with equivalent amount of buffer was maintained in an identical manner. After 5 hours, 0.5ml of the incubated solution was removed and diluted with 0.5ml of Griess reagent (1% sulfanilic acid, 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite

ions with suphanilic acid and its subsequent coupling with napthylethylenediamine was read at 546 nm. The experiment was repeated in triplicate.

ABTS Radical scavenging Activity[16]

Varying concentration (0.1 – 0.5ml) of plant extract and different concentration (0.1&0.2 ml) of standard (Ascorbic acid) were taken into a series of test tubes and added with 0.3ml of ABTS solution and the final volume was made up to 2.5ml of using phosphate buffer. Control tubes contain all the reagent except plant extract. Decrease in absorbance was Immediately read colourimetrically at 734nm.

RESULTS AND DISCUSSION

Free radicals are chemical species which contain one or more unpaired electrons. They are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability.[17] Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins and nucleic acids. Free radicals damage contributes to the etiology of many chronic health problems such as inflammatory disease, cataract and cancer. Antioxidants prevent free radicals induced tissue damage by preventing the formation of radicals, scavenging them or by promoting their decomposition.[18]

There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases. They exert their action either by scavenging the ROS or protecting the antioxidant defense mechanisms.[19]

Antioxidant capacity is widely used parameter for assessing the bioavailability of food stuffs as medicinal plant. The accumulating evidences suggest the antioxidant potential of plant extracts as their therapeutic index.[20-23] In the present study, phytochemical screening and *in vitro* antioxidant activities were carried out in the ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida*. Test for purity and identity of the leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* revealed the percentage of moisture content and foreign matters of about 8.51 and 2.34 (*Andrographis echioides*) and 2.47 and 9.32 (*Spermacoce hispida*) respectively (Table 1).

Table-1: Test for purity of *Andrographis echioides* and *Spermacoce hispida*

S.No.	Parameters	Purity (%)	
		Andrographis echioides	***Spermacoce hispida***
1.	Foreign Matters	2.34	2.47
2.	Loss On Drying	8.51	9.33

Successive extractive values of the leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* are obtained as hexane-2.32%, chloroform-3.27%, ethyl acetate-1.008%, alcohol-17.59%, water-23.17% (*Andrographis echioides*) and hexane-2.75%, chloroform-2.26%, ethyl acetate-1.05%, alcohol-22.78%, water-27.005% (*Spermacoce hispida*) respectively (Table 2).

Table-2: Physicochemical analysis of *Andrographis echioides* and *Spermacoce hispida*

S.No.	Parameters	Concentration (%)	
		Andrographis echioides	***Spermacoce hispida***
1.	Successive Extractive values		
	i) Hexane	2.32	2.75
	ii) Chloroform	3.27	2.26
	iii) Ethyl acetate	1.008	1.054
2.	Solubility		
	i) Ethanol	17.595	22.78
	ii) Water	23.175	27.005

Fluorescence analysis of *Andrographis echioides* and *Spermacoce hispida* shows the presence of active compounds such as alkaloids, flavonoids, tannins, sugar (Table 3a and Table 3b).

Table-3a: FLUORESCENCE ANALYSIS OF *Andrographis echioides*

S.No.	Treatment	Day Light			UV Light		
		0 Hr	**24 Hrs**	**48 Hrs**	**0 Hr**	**24 Hrs**	**48 Hrs**
1.	Drug powder	Green	Green	Green	Green	Green	Green
2.	Drug powder+ Aqueous 1N NaOH	Yellowish green	Brownish yellow	Brown	Light brown	Yellow	Light yellow
3.	Drug powder+ Alcoholic 1N NaOH	Light green	Yellow	Yellow	Light green	Yellow	Dark green
4.	Drug powder+1N HCl	Light Yellow	Light Yellow	Light brown	Brown	Light brown	Orange
5.	Drug powder+50% H_2SO_4	Greenish brown	Dark green	Dark green	Green	Brown	Yellow
6.	Drug powder+ Hexane	Pale green	Pale green	Dark green	Pale green	Pale green	Pink
7.	Drug powder+$CHCl_3$	Dark green	Dark green	Dark green	Dark green	Green	Yellow
8.	Drug powder+ Ethyl	Pale green	Green	Green	Light	Green	Yellow

	acetate				green		
9.	Drug powder+ Acetone	Pale green	Green	Green	Green	Green	Greenish yellow
10.	Drug powder+ Benzene	Pale green	Yellow	Light green	Pale green	Light yellow	Yellow
11.	Drug powder+ Alcohol	Green	Green	Green	Pale green	Green	Greenish yellow
12.	Drug powder+ Water	Dark green	Pale Brown	Brown	Pale green	Light Yellow	Green

Table-3b: FLUORESCENCE ANALYSIS OF *Spermacoce hispida*

S.No.	Treatment	Day Light			UV Light		
		0 Hr	24 Hrs	48 Hrs	0 Hr	24 Hrs	48 Hrs
1.	Drug powder	Brown	Brown	Brown	Brown	Brown	Brown
2.	Drug powder+ Aqueous 1N NaOH	Light brown	Dark yellow	Yellow	Light brown	Yellowish green	Yellow
3.	Drug powder+ Alcoholic 1N NaOH	Light brown	Pale yellow	Light Brown	Light brown	Light Brown	Green
4.	Drug Powder+ 1N HCl	Brown	Blue	Greenish blue	Brown	Blue	Dark brown
5.	Drug powder+ 50% H_2SO_4	Yellow	Dark brown	Dark brown	Pale Brown	Brown	Dark brown
6.	Drug powder+ Hexane	Pale Brown	Light Yellow	Light Yellow	Pale Brown	Light Yellow	Light Yellow
7.	Drug powder+$CHCl_3$	Pale Brown	Light Yellow	Light Brown	Pale Brown	Light Yellow	Yellow
8.	Drug powder+ Ethyl acetate	Light brown	Light Yellow	Light Yellow	Light brown	Light Brown	Light Yellow
9.	Drug powder+ Acetone	Brown	Light Yellow	Light Yellow	Brown	Light Yellow	Yellow
10.	Drug powder+ Benzene	Pale Brown	Brown	Light Yellow	Pale Brown	Light Yellow	Yellow
11.	Drug powder+ Alcohol	Pale Brown	Light Yellow	Light brown	Pale Brown	Light Yellow	Light Yellow
12.	Drug powder+ Water	Pale Brown	Light brown	Brown	Pale Brown	Light Yellow	Yellowish green

Phytochemical screening of dry powder of *Andrographis echioides* shows the presence of alkaloids, flavonoids, tannins, coumarine, saponins and sugar (Table 4a). Phytochemical screening of dry powder of *Spermacoce hispida* shows the presence of alkaloids, flavonoids, quinone, coumarine, steroids and sugar (Table 4b). Alkaloids are formed as metabolic by-products and have been reported to be responsible for antioxidant and even antibacterial activity. Flavonoids have been referred to as nature's biological response modifiers, having antioxidant, anticancer, anti-allergic, antimicrobial activities. Tannins are known to possess

general antioxidant, antimicrobial, antiviral, antibacterial activities. Saponins are known for their medicinal properties as a natural blood cleanser, expectorant and antibiotics. In the present study, phytochemical screening of various extracts (hexane, chloroform, ethyl acetate, alcohol, water) of *Andrographis echioides* and *Spermacoce hispida* revealed the presence of secondary metabolites which might have been responsible for the antioxidant activity (Table 4a and 4b).

Table-4a: Phytochemical screening of *Andrographis echioides*

S.No.	Test		Extracts				
		Dry Powder	Hexane	Chloroform	Ethyl acetate	Ethanol	Water
1.	Saponins	+	+	-	-	-	-
2.	Tannins	+	+	-	-	-	+
3.	Steroids	-	+	+	+	+	-
4.	Terpenoids	-	-	-	-	-	-
5.	Flavonoids	-	-	-	-	-	-
6.	Coumarine	+	+	+	-	-	-
7.	Quinones	-	+	-	-	-	-
8.	Lignin	-	-	-	-	-	-
9.	Alkaloids	+	+	+	-	+	+
10.	Sugar	+	+	+	+	+	+

Table-4b: Phytochemical screening of *Spermacoce hispida*

S.No.	Test		Extracts				
		Dry powder	Hexane	Chloroform	Ethyl acetate	Ethanol	Water
1.	Saponins	-	+	-	-	-	-
2.	Tannins	-	+	-	-	-	+
3.	Steroids	+	-	-	-	-	-
4.	Terpenoids	-	+	-	-	-	-
5.	Flavonoids	-	-	-	-	-	-
6.	Coumarine	+	-	-	+	+	+
7.	Quinones	+	+	-	-	+	-
8.	Lignin	-	-	-	-	-	-
9.	Alkaloids	+	+	+	+	+	+
10.	Sugar	+	+	+	-	+	+

The amount of carbohydrate, protein and phenol of *Andrographis echioides* and *Spermacoce hispida* were estimated quantitatively. The presence of carbohydrate (0.53 g), protein (1.6 mg) and phenol (0.6 mg) in the leaves of *Andrographis echioides* and carbohydrate (0.83 g), protein (1.7 mg) and phenol (0.8 mg) in the seeds of *Spermacoce hispida* respectively, may

attribute to its nutritional facts, facilitate the plant drug to act as an edible antioxidant with rich nutrients.

Table 5: Quantitative analysis of *Andrographis echioides* and *Spermacoce hispida.*

S.No.	Phytochemicals	*A.echioides*	*S.hispida*
1.	Carbohydrates (mg/mg)	0.53	0.83
2.	Proteins (mg/g)	1.6	0.6
3.	Phenols(mg/g)	1.7	0.8

Table-6: DPPH scavenging assay of *Andrographis echioides* and *Spermacoce hispida*

S.No.	Concentration(µg/ml)	Inhibition (%)	
		Andrographis echioides	*Spermacoce hispida*
1.	5	8.33	11.11
2.	10	19.44	16.66
3.	15	30.55	25
4.	20	41.66	33.33
5.	25	47.22	41.66
6.	30	66.66	55.55
7.	IC_{50} Value	26	28

Proton radical scavenging action is one of the important mechanisms of oxidation.[24] DPPH is a stable, nitrogen-centered free radical which produces violet colour in methanol solution. It was reduced to a yellow coloured product, diphenylpicryl hydrazine. The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517nm, suggesting the antioxidant activity of ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* and it showed concentration dependent DPPH scavenging activity. The ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* reacted with stable DPPH radical. The high concentration at 30µg/ml showed maximum activity in both plants 66.66% (*Andrographis echioides*) and 55.55% (*Spermacoce hispida*). The IC_{50} values for the ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* were found to be 26 µg/ml and 28 µg/ml respectively.

Reducing power is also widely used in evaluating antioxidant activity of plant polyphenols. The reducing power is generally associated with the presence of reductones, which exert antioxidant action by breaking the free radicals by donating a hydrogen atom.[25] In reducing power assay, the presence of antioxidants in the ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* reduces Fe^{3+} / ferricyanide complex to the ferrous form. This reducing capacity of compounds could serve as an indicator of potential antioxidant activities and increase in absorbance could indicate an increase in reducing power.

Table-7: Reducing power assay of *Andrographis echioides* and *Spermacoce hispida*

S.No.	Concentration(µg/ml)	% Reducing power	
		Andrographis echioides	***Spermacoce hispida***
1.	50	4.25	25
2.	100	11.11	45
3.	150	24.44	57.69
4.	200	33.82	66.66
5.	250	43.03	71.79
6.	300	53.60	74.41
7.	EC_{50} Value	280	55

In the present study, the ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* depicted good reducing power. The extracts of both of the plants exhibited their reducing power in a dose dependent manner, which is found to be increasing with the increasing concentration of the extract. The EC_{50} values of ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* were found to be 280 µg/ml and 55 µg/ml respectively.

Radical scavenging activities of the ethanolic extracts of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* were assessed by $ABTS^{\cdot+}$ cation decolourization assay. The test was based on the relative activity of antioxidants to quench the radical cation $ABTS^{\cdot+}$. $ABTS^{\cdot+}$ cations were scavenged in a range of concentrations from 100-500 µg/ml. The high concentration at 600 µg/ml showed maximum activity in both plants 56.66% (*Andrographis echioides*) and 86.66% (*Spermacoce hispida*). The IC_{50} values for the ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* were found to be 580 µg/ml and 140 µg/ml respectively.

Table-8: ABTS scavenging assay of *Andrographis echioides* and *Spermacoce hispida*

S.No.	Concentration(µg/ml)	Inhibition (%)	
		Andrographis echioides	***Spermacoce hispida***
1.	100	6.66	46.66
2.	200	10	60
3.	300	16.66	63.33
4.	400	26.66	70
5.	500	36.66	76.66
6.	600	56.66	86.66
7.	IC_{50} Value	580	140

Nitric oxide (NO), a reactive free radical generated from L-arginine by NO synthase, is well documented as a physiological molecule. Nitrosative stress results from the overproduction of

reactive nitrogen species that may occur when the generation of reactive nitrogen species in a system's ability to neutralize and scavenge them decreases. This may initiate nitrosylation reactions which can modify the protein structure and thus inhibit their normal function. Scavenger of NO competes with oxygen leading to reduced production of NO. The ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* inhibited the nitrite production. The high concentration at 500µg/ml showed maximum activity in both plants 66.66% (*Andrographis echioides*) and 52.38% (*Spermacoce hispida*). The IC_{50} values for the ethanolic extract of ethanolic extract of leaves of *Andrographis echioides* and seeds of *Spermacoce hispida* were found to be 350 µg/ml and 450 µg/ml respectively.

Table-9: Nitric oxide scavenging assay of *Andrographis echioides* and *Spermacoce hispida*

S.No.	Concentration(µg/ml)	Inhibition (%)	
		Andrographis echioides	***Spermacoce hispida***
1.	100	28.57	14.28
2.	200	38.09	23.8
3.	300	42.85	33.33
4.	400	61.9	47.61
5.	500	66.66	52.38
6.	IC_{50} Value	350	450

CONCLUSION

The results of the present study, clearly demonstrates the antioxidant activities of ethanolic extract of leaves of *Andrographis echioides* and *Spermacoce hispida*. *Andrographis echioides* have high DPPH scavenging activity and also having Nitric Oxide scavenging activity whereas the seeds of *Spermacoce hispida* having potent Reducing power and ABTS cation decolourization ability. This is a tentative study. Further studies are required to prove the antioxidant effects of the plants *in vivo.* Isolation of active principles and their mechanism of action are necessary to provide strong scientific validation to use these plants as a source of phyto antioxidants.

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