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PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITIES OF *IN VITRO* AND *IN VIVO* EXTRACTS OF *SPERMACOCE ARTICULARIS* L.f.

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ABSTRACT

The plant is traditionally used by various tribes for curing a wide range of diseases. A 50% methanolic extract of the In vitro leaf, In vitro root, In vivo leaf and In vivo Roots were subjected to phytochemical studies and further investigated for *in vitro* antioxidant and antibacterial activities. *In vitro* antioxidant activity was determined by DPPH free radical scavenging assay, superoxide radical scavenging activity, Nitric oxide radical scavenging activity, hydroxyl radical scavenging activity and reducing power assay. Antibacterial activity was studied by agar well diffusion method. The free radical scavenging activity of the extract was concentration dependent and IC50 was observed at a concentration of 172.62µg/ml for DPPH free radical scavenging activity, and 80.79µg/ml for hydroxyl radical scavenging activity. The extract showed significant total antioxidant activity and reducing power. The results obtained from current study demonstrate that the leaf, root extract of *Spermacoce articularis* L.f. possess significant antioxidant Presence of various classes of phytocompounds e.g. Phenols, flavonoids, saponins, alkaloids etc. contribute highly to its medicinal values.

KEYWORDS: Spermacoce Articularis, DPPH, Antioxidant, Reducing Power

INTRODUCTION

Medicinal plants used by traditional healers have long served mankind as the primary source of therapeutic aids. In spite of the great march of synthetic products in to modern medicine, half of the world's medicinal compounds are still derived or obtained from plants. Mostly herbs contain secondary metabolites like polyphenols, flavonoids, triterpenoids etc. which have significant antioxidant and antibacterial properties. About 17000-18000 flowering plant species are known in India, out of which about 6000-7000 have been known to be used as folk medicines. Some of the most important drugs which have revolutionized the modern medicare systems have been isolated first from the medicinal plants used by primitive or ancient societies. These wonder drugs include the curare alkaloids, penicillin and other antibiotics, cortisone, reserpine, podophyllotoxin and other therapeutic agents. Drugs or pharmaceutical products isolated from plants are at times found to be less costly than synthetic drugs. Well known examples of such products are atropine, digoxin and morphine. Natural products may also be used as building blocks for the synthesis of 'semi synthetic' drugs; this is the case with plant saponins that can be extracted and easily altered chemically to produce sapogenins for the manufacture of steroidal drugs. Furthermore, the medicinal plants may give the chemical blueprints for the development of related synthetic drugs; for example cocaine from *Erythroxylum coca* which provides the chemical structure for the synthesis of procaine and other related local anesthetics.

MATERIALS AND METHODS

Sample Collection and Extraction

Whole plant of *Spermacoce articularis* L.f. were collected from Karur district, Tamil Nadu, (India) during the month of January, 2012. The voucher specimen was identified, authenticated and submitted at Botanical Survey of India (BSI/ SRC/ 5/23/2013-14/Tech, 1643) Coimbatore. *In vivo* and *In vitro* leaves and roots of the collected plants were washed thoroughly with distilled water and shade dried for ten days. A 1000 g dried leaves were ground to a fine powder using mixer grinder and subjected to extraction thrice in 50% methanol using cold maceration technique. The extract was concentrated in rotary vacuum evaporator and stored at 40C until further use (yield = 8.24%).

PHYTOCHEMICAL ANALYSIS

Qualitative Analysis of Phytochemicals

The *Spermacoce articularis* L.f. *In vitro, In vivo* plant leaves and roots extracts were subjected to preliminary Phyto chemical screening. Presence of alkaloids (Mayer's test), flavonoids (alkaline reagent test), tannins (Braymer's test) carbohydrates (Molischs test), glycosides (Liebermann's test), saponins (Salkowski test), triterpenoids (Liebermann Burchard test), proteins and amino acids (Ninhydrin test) were tested.

In Vitro Antioxidant Activity

DPPH● Radical Scavenging Activity

The antioxidant activity of the sample was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois (1958).The sample extracts at various concentrations (100-500 µg/mL) was taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1 mM methanolic solution of DPPH● was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Different concentrations (4 -20 µg/mL) of standard antioxidant Butylated hydroxyanisole (BHA) was used as positive control. Percentage radical scavenging activity of the sample was calculated as follows % DPPH radical scavenging activity = (control OD-sample OD / control OD) × 100. The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Superoxide Radical Scavenging Activity

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich (1971). The assay was based on the capacity of the sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1mg NBT and various concentrations of sample extracts (100-500 µg/ml). Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with reaction mixture kept in dark served as blank. Different concentrations (10 -50 µg/ml) standard antioxidant Butylated hydroxyanisole

(BHA) was used as positive control. The percentage inhibition of superoxide anion generation was calculated as follows:

% Inhibition = (control OD-sample OD / control OD) x 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Nitric Oxide Radical Scavenging Activity

The nitric oxide scavenging activity of the sample was measured according to the method of Sreejayan and Rao (1997). 3ml of 10 mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations of the samples (100-500 µg/ml) and incubated at room temperature for 150 min. After incubation time, 0.5 ml of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO4) was added. The absorbance of the chromophore formed was read at 546 nm. Different concentrations (10 -50 µg/ml) standard antioxidant Butylated hydroxyanisole (BHA) was used as positive control. Percentage radical scavenging activity of the sample was calculated as follows:

% Nitricoxide radical scavenging activity = (control OD-sample OD / control OD) x 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Reducing Power

The reducing power of the sample extract was determined by the method reported by Siddhuraju *et al*. (2002). 100-500 µg/mL of extract was taken in 1 ml of phosphate buffer and 5 ml of 0.2M phosphate buffer (pH 6.6) was added. To this, 5 ml of 1% potassium ferricyanide solution was added and the mixture was incubated at 50°C for 20 min. After the incubation, 5ml of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5ml) was mixed with 5ml of distilled water and 0.5ml of 0.1% ferric chloride. The absorbance of the reaction mixture was read spectroscopically at 700 nm. Different concentrations (20-100 µg/ml) standard antioxidant Butylated hydroxyanisole (BHA) was used as positive control.

Hydroxyl Radical Scavenging Activity

The scavenging activity of the sample on hydroxyl radical was measured according to the method of Klein *et al*. (1991). Different concentrations of the extract (100-500 µg/ml) were added with 1ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5ml of EDTA solution (0.018%), and 1ml of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 Mphosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. Different concentrations (4 -20 µg/ml) standard antioxidant Butylated hydroxyanisole (BHA) was used as positive control. The % hydroxyl radical scavenging activity was calculated as follows

% hydroxyl radical scavenging activity = (control OD-sample OD / control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Statistical Analysis

All the data were presented as mean ± SEM and analyzed by Wilcoxon Sum Rank Test and unpaired Student's t-test for the possible significant interrelation between the various groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Phytochemical Studies

Preliminary phytochemical estimation showed the presence of sugars, proteins, alkaloids, tannins, saponins, glycosides, triter penoids, flavonoids and phenols.

Solvent Extraction

20 g of air dried, coarsely powdered sample was successively extracted with methanol using soxhlet apparatus. The solvent extract was concentrated to dryness under reduced pressure using rotary vacuum evaporator, and weighed. The percentage yield (recovery) of evaporated extract was calculated as follows:

$$\text{Yield (\%)} = \frac{[\textit{Extract + container (g)}] - [\textit{Empty container (g)}]}{\text{Sample weight (g)}} \times 100$$

The percentage yields were expressed in terms of the air dried drug.

Qualitative Analysis

Phytochemical screening was carried out following the methods of Horbone, 1984 and

Kokate *et al*., 1995

Test for Alkaloids

Wagner's Test

To 1 ml of the extract, a few drops of Wagner's reagent were added and the formation of a reddish brown precipitate indicates the presence of alkaloids.

Test for Flavanoids

Shinoda Test

To 1 ml of the extract, magnesium turnings and 1-2 drops of concentrated hydrochloric acid were added. Formation of pink color indicates the presence of Flavanoids.

Lead Acetate Test

To 1 ml of the extract, few drops of 10% Lead acetate solution were added. Appearance of yellow colour precipitate indicates the presence of flavonoids.

Test for Phenols and Tannins

Lead Acetate Test

To 1 ml of the extract, few ml of 1% lead acetate solution was added and the formation of precipitate indicates the presence of tannins and phenolic compounds.

Ferric Chloride Test

To 1 ml of the extract, few ml of 5% ferric chloride was added. The development of dark bluish black color indicates the presence of tannins.

Sodium Hydroxide Test

A small quantity of extract was dissolved in 0.5ml of 20%sulphuric acid solution. Followed by addition of few drops of aqueous sodium hydroxide solution, it turns blue presence of phenols

Test for Steroids and Sterols

Salkowski's Test

The extract was dissolved in 2ml of chloroform and equal volume of concentrated sulphuric acid was added along the sides of the test tube. The upper layer turns red and lower layer turns yellow with green fluorescence, indicating the presence of the steroids and sterols compound, in the extract.

Test for Saponins

Honey Comb Test

5 ml of the extract was taken in a test tube and few drops of 5% sodium bicarbonate solution were added. The mixture was shaken vigorously and kept for 3 minutes. Formation of honey comb like froth shows the presence of saponins.

Foam Test

About 1 ml of the extract was diluted with 20ml distilled water and shaken well in a graduated cylinder for 15min. The formation of foam to a length of 1cm indicated the presence of Saponins and steroids.

Test for Glycosides

Legal Test

The extract was dissolved in pyridine and freshly prepared sodium nitroprusside solution was added. The formation of pink to red color indicates the presence of glycosides.

Test for Protein & Amino Acids

Biuret Test

To 1 ml of extract, equal volume of 40% NaOH solution and two drops of 1% Copper sulphate solution were added. The appearance of violet colour indicates the presence of protein.

Ninhydrin Test

To 1 ml of extract, 2drops of freshly prepared 0.2% ninhydrin reagent was added and heated. The appearance of pink or purple colour indicates the presence of proteins, peptides or amino acids.

Test for Carbohydrates

Fehling's Test

Five ml of Fehling's solution was added to 2 ml of the extract and boiled in a water bath. The formation of yellow or red precipitate indicates the presence of reducing power.

Benedict's Test

Five ml of Benedict's solution was added to 2 ml of the extract boiled in water bath. The appearance of red or yellow or green precipitate indicates the presence of reducing sugars.

Table 1: Preliminary Phytochemical Screening

Phytoconstituents	Name of the Test	*In Vitro* Root	*In Vitro* Leaf	*In Vivo* Root	*In Vivo* Leaf
Alkaloids	Wagner's test	+	+	+	+
Flavonoids	Shinoda	−	+	+	+
	Lead acetate	+	+	+	−
Phenolics & tannins	Ferric chloride	−	−	−	−
	Lead acetate	+	+	+	+
	Sodium hydroxide	−	−	−	−
Steroids & sterols	Salkowski's test	−	−	−	−
Saponins	Honey comb test	+	+	+	+
	Foam test	−	−	−	−
Glycosides	Glycoside test	+	+	+	+
Protein	Protein	+	−	−	−
	Biuret test	+	+	−	−
	Ninhydrin test				
Carbohydrates	Fehling's test	+	+	+	+
	Benedict's test	+	+	+	+

Note: '+' – presence of compounds; '−' – absence of compounds

***In Vitro* Antioxidant Activity**

At present, the study of ethno-medicinal plants has become increasingly valuable in the development of health care and conservation programs in different parts of the world. Medicinal plants used by traditional healers have long served mankind as the primary source of therapeutic aids. The herbal extracts are known to exert their medicinal effect by synergistic action of their several constituents acting at single or several target sites. This study provided bases to the folkloric use of *Spermacoce articularis* L.*f* leaves for cure diseases.

DPPH Free Radical Scavenging Method

Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging and other degenerative diseases in human. The *in vivo* generations of free radicals are done by addition of DPPH that acts as source of free radicals. The extracts having hydrogen donor group will scavenge this free radicals by binding to them, hence in the present investigation the plant extracts. Among four extracts and standard tested for the in vitro antioxidant activity using the DPPH method, the crude methanolic extracts of *In vitro* root, *In vitro* leaf, In vivo root and In vivo leaf showed antioxidant activity, with IC_{50} values of 194.81 ± 1.20, 188.09 ± 1.13, 495.95 ±7.88, **172.62 ± 0.94** µg/ml, respectively (Table 2). DPPH Activity standard value BHA 4.42 ± 0.04.*In vivo* leaf has showed hyper antioxidant activities which are evident by DPPH assay method.

Table 2: DPPH Radical Scavenging Activity of Methanolic Extracts of the Samples

Sample	Concentration (Mg)	Percentage Activity (%)	IC_{50} (Mg/Ml)
In vitro root	100	10.21 ± 0.75	194.81 ± 1.20
	200	21.29 ± 0.39	
	300	31.30 ± 0.39	
	400	37.17 ± 0.15	
	500	43.99 ± 0.78	
In vitro leaf	100	10.14 ± 0.35	188.09 ± 1.13
	200	23.29 ± 0.84	
	300	28.57 ± 0.06	
	400	39.94 ± 0.64	
	500	46.73 ± 0.20	
In vivo root	100	3.16 ± 0.66	495.95 ± 7.88
	200	7.99 ± 0.20	
	300	10.92 ± 0.29	
	400	14.46 ± 0.31	
	500	18.62 ± 0.78	
In vivo leaf	100	9.50 ± 0.49	172.62 ± 0.94
	200	21.61 ± 1.22	
	300	31.21 ± 0.10	
	400	40.55 ± 0.30	
	500	54.75 ± 0.37	
BHA	4	29.56 ± 0.47	4.42 ± 0.04
	8	40.27 ± 0.70	
	12	53.45 ± 0.54	
	16	71.50 ± 1.09	
	20	88.00 ± 0.41	

Values are means of three independent analysis of the ± standard deviation (n=3)

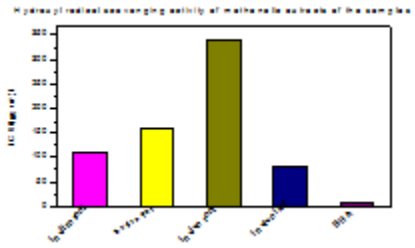

Figure 1

Nitric Oxide Neutralizing Method

The NO^o on reaction with oxygen gives ONOO- which can react and denature the biomolecules present in our body leads to disease progression. Hence the NO^o can be scavenged by hydrogen donating extracts by binding with them will be an effective antioxidant. Among four extracts and standard tested for the in vitro antioxidant activity using the Nitric oxide method, the crude methanolic extracts of In vitro root, In vitro leaf, In vivo root and In vivo leaf showed antioxidant activity, with IC_{50} values of **109.13 ± 2.45**, 166.02 ± 7.66, 259.78 ± 3.71, 407.23 ± 10.37 µg/ml, respectively (Table 3).Nitric oxide Activity The standard value BHA 17.25 ± 0.23. *In vivo* leaf has showed hyper antioxidant activities which are evident by Nitric oxide assay method.

Table 3: Nitric Oxide Radical Scavenging Activity of Methanolic Extracts of the Samples

Sample	Concentration (Mg)	Percentage Activity (%)	IC_{50} (Mg/Ml)
In vitro root	100 200 300 400 500	37.17 ± 2.29 45.39 ± 1.91 55.02 ± 2.23 64.31 ± 1.57 73.47 ± 1.23	109.13 ± 2.45
In vitro leaf	100 200 300 400 500	12.26 ± 2.23 21.41 ± 2.67 32.39 ± 2.34 42.63 ± 2.72 56.63 ± 0.84	166.02 ± 7.66
In vivo root	100 200 300 400 500	7.61 ± 1.30 13.33 ± 0.61 20.27 ± 0.31 28.35 ± 1.49 35.89 ± 1.11	259.78 ± 3.71
In vivo leaf	100 200 300 400 500	5.72 ± 0.62 8.55 ± 0.82 31.21 ± 0.10 17.31 ± 0.62 23.57 ± 0.71	407.23 ±10.37
BHA	10 20 30 40 50	13.55 ± 2.03 22.47 ± 1.29 33.58 ± 0.92 48.11 ± 0.56 57.26 ± 0.76	17.25 ± 0.23

Values are means of three independent analysis of the ± standard deviation (n=3)

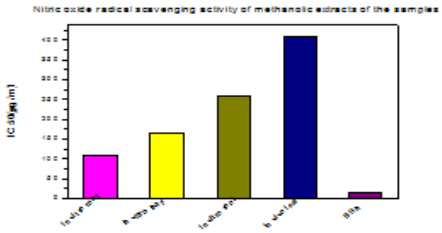

Figure 2

Superoxide Radical Scavenging Activity

Among four extracts and standard tested for the in vitro antioxidant activity using the Nitric oxide method, the crude methanolic extracts of In vitro root, In vitro leaf, In vivo root and In vivo leaf showed antioxidant activity, with IC_{50} values of 262.71 ± 13.47, 285.37 ± 14.33, **180.92 ± 0.12**, 395.17 ± 20.29 µg/ml, respectively (Table 4). Nitric oxide Activity The standard value BHA 9.03 ± 0.07. *In vivo* leaf has showed hyper antioxidant activities which are evident by Nitric oxide assay method.

Table 4: Superoxide Radical Scavenging Activity of Methanolic Extracts of the Samples

Sample	Concentration (Mg)	Percentage Activity (%)	IC50 (Mg/Ml)
In vitro root	100	9.93 ± 0.30	262.71 ± 13.47
	200	12.10 ± 0.59	
	300	17.66 ± 0.40	
	400	23.06 ± 0.69	
	500	28.81 ± 2.83	
In vitro leaf	100	4.34 ± 0.82	285.37 ± 14.33
	200	6.62 ± 0.30	
	300	12.71 ± 1.44	
	400	22.83 ± 0.20	
	500	30.52 ± 3.71	
In vivo root	100	10.39 ± 2.85	180.92 ± 0.12
	200	20.13 ± 0.24	
	300	28.81 ± 0.57	
	400	35.39 ± 0.41	
	500	38.36 ± 0.11	
In vivo leaf	100	2.78 ± 0.26	395.17 ± 20.29
	200	7.23 ± 1.67	
	300	12.21 ± 1.30	
	400	16.51 ± 0.57	
	500	19.33 ± 0.56	
BHA	10	32.27 ± 0.46	9.03 ± 0.07
	20	42.56 ± 0.64	
	30	55.24 ± 0.52	
	40	72.60 ± 1.04	
	50	88.46 ± 0.39	

Values are means of three independent analysis of the ± standard deviation (n=3)

Reducing Power Assay Hydrogen Peroxide Radical Inhibition Assay

Many degenerative diseases are induced due to the formation of peroxides and free radical chain reactions in human system. An effective antioxidant will serve as reductones. Reductones are molecules responsible for the reduction of peroxides and free radical chain reactions by donating hydrogen atoms. Among four extracts and standard tested for the in vitro antioxidant activity using the Reducing power method, the crude methanolic extracts of *In vitro* root, leaf and *In vivo* root, leaf showed antioxidant activity. *In vitro* root, leaf and *In vivo* root, leaf extracts are uniformly absorbance (Table 5).

Table 5: Reducing Power of Methanolic Extracts of the Samples

Sample	Concentration (Mg)	Absorbance At 700nm
In vitro root	100	0.067 ± 0.004
	200	0.122 ± 0.005
	300	0.168 ± 0.010
	400	0.211 ±0.006
	500	0.259 ± 0.005
In vitro leaf	100	0.071 ± 0.005
	200	0.109 ± 0.002
	300	0.158 ± 0.003
	400	0.216 ± 0.005
	500	0.241 ± 0.004
In vivo root	100	0.067 ± 0.003
	200	0.088 ± 0.002
	300	0.103 ± 0.006
	400	0.120 ± 0.003

	500	0.134 ± 0.002
In vivo leaf	100	0.123 ± 0.062
	200	0.131 ± 0.003
	300	0.175 ± 0.004
	400	0.220 ± 0.003
	500	0.259 ± 0.004
BHA	10	0.147 ± 0.005
	20	0.359 ± 0.012
	30	0.548 ± 0.024
	40	0.632 ± 0.023
	50	0.718 ± 0.019

Values are means of three independent analysis of the ± standard deviation (n=3)

Hydrogen Peroxide Radical Inhibition Assay

The extracts having hydrogen donor group will scavenge this free radicals by binding to them, hence in the present investigation the plant extracts. Among four extracts and standard tested for the in vitro antioxidant activity using the DPPH method, the crude methanolic extracts of In vitro root, In vitro leaf, In vivo root and In vivo leaf showed antioxidant activity, with IC_{50} values of 108.70 ± 0.51, 161.30 ± 1.13, 339.03 ± 4.93, **80.79 ± 0.03** µg/ml, respectively (Table 6). Hydrogen peroxide Activity standard value BHA 6.26 ± 0.18. *In vivo* leaf has showed hyper antioxidant activities which are evident by Hydrogen peroxide assay method.

Table 6: Hydroxyl Radical Scavenging Activity of Methanolic Extracts of the Samples

Sample	Concentration (Mg)	Percentage Activity (%)	IC_{50} (Mg/Ml)
In vitro root	100	40.81 ± 0.09	108.70±0.51
	200	42.05 ± 0.12	
	300	43.11 ± 0.20	
	400	44.95 ± 0.54	
	500	48.39 ± 0.12	
In vitro leaf	100	24.94 ± 0.24	161.30±1.13
	200	26.27 ± 0.49	
	300	27.62 ± 0.42	
	400	30.79 ± 1.44	
	500	34.90 ± 0.33	
In vivo root	100	3.09 ± 0.53	339.03±4.93
	200	4.44 ± 0.20	
	300	7.01 ± 0.47	
	400	11.26 ± 0.59	
	500	28.35 ± 0.35	
In vivo leaf	100	49..69 ± 0.33	80.79 ± 0.03
	200	52.78 ± 0.42	
	300	55.54 ± 0.19	
	400	64.23 ± 0.46	
	500	66.10 ± 0.20	
BHA	4	14.29 ± 2.99	6.26 ± 0.18
	8	21.29 ± 0.50	
	12	32.70 ± 4.10	
	16	36.32 ± 0.46	
	20	40.39 ± 0.95	

Values are means of three independent analysis of the ± standard deviation (n=3)

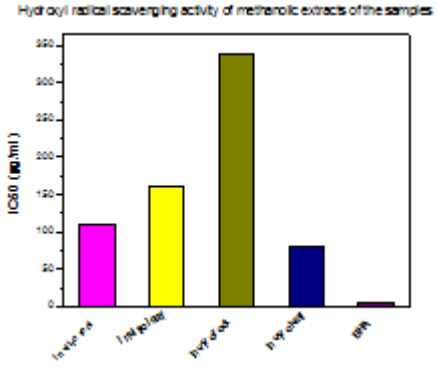

Figure 3

CONCLUSIONS

The results obtained from current study demonstrate that the leaf extract of *Spermacoce articularis* L.*f.* possess significant antioxidant and antibacterial properties. Presence of various classes of phytocompounds e.g. Phenols, flavonoids, saponins, alkaloids etc. contribute highly to its medicinal values, which could one of the reasons for its use in curing a wide range of diseases. The present study was done with an objective to provide scientific support to the traditional claims of various tribes regarding curative effects of *Spermacoce articularis* L.*f* leaves on headache, dysentry and it provides a lead for further exploring the herb for drug development.

CONSENTS

Not applicable.

ETHICAL APPROVAL

Not applicable.

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