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PRELIMINARY PHYTOCHEMICAL SCREENING, QUANTITATIVE ESTIMATION OF FLAVONOIDS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF SELECTED TRADITIONAL MEDICINAL PLANTS

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ABSTRACT

The potential utility of safer and cheaper herbal medicines has been increased in the recent past due to no side effects from the herbal medicine. Herbal plants have bioactive compounds which are used in curing of various ailments.

In the present investigation different medicinal plants *Entada rheedii* and *Sarcostemma acidum* were studied.

In this study petroleum ether, ethyl acetate and ethanol were used as solvents to get extracts from bark of *Entada rheedii* and stem of *Sarcostemma acidum*. The obtained extracts were subjected to qualitative phytochemical screening and quantitative estimation of total flavonoids using standard procedures.

Phytochemical screening showed the presence of large variety of pharmacologically active compounds such as alkaloids, carbohydrates, saponins, little phenolic compounds, steroids and especially flavonoids were present in the crude extracts. However, glycosides, fats and oils were absent in both plants. This study also showed that flavonoid content was 18.0 mg/g and 13.6 mg/g of rutin equivalent flavonoid in ethyl acetate extract of *Entada rheedii* (EAER) and ethanolic extract of *Sarcostemma acidum* (EESA) respectively.

At the same time EAER and EESA showed that they have high in vitro antioxidant activity and powerful oxygen free radical scavenging abilities as well as the IC50 for the plants was almost equivalent to the reference standard antioxidant ascorbic acid (vitamin C) which justified its uses in the traditional medicine and could be a good candidate for further biological and chemical analysis. EAER and EESA can be further subjected for isolation of the therapeutically active compounds for further pharmacological evaluations and anti-stress activity.

INTRODUCTION

The importance of medicinal plants in drug development is known to us and humans are using them for different diseases from the beginning of human history [1]. Traditional folk treatment from wild plants has always guided researchers to search for novel medications to develop healthy life for humans and animals [2]. Some medicinal plants are still obscured within the plant kingdom which need to be scientifically evaluated.

Entada rheedii Spreng (Fam. Mimosaceae) is a woody climbing shrub that grows naturally in Africa, tropical Asia, Australia and a small part of the pacific islands [3,4]. It has been

reported for usefulness in the treatment of jaundice, diarrhea, musculo-skeltal problems and mumps. They are also used as remedy for cerebral hemorrhage and oral contraceptive [5]. Bark of *Entada rheedii* contains saponin, which is used as a substitute of soap [6].

Sarcostemma acidum is an Indian traditional therapeutic herb. It has been considered as aspirant of Soma plants by numerous authors. It was said that Soma (Somlata) was used to make 'Som ras' (Rejuvenating drink) by Aryans. The original source of 'Soma' plant is a mystery that has been discussed by the vedic and botanical researchers for more than two and a half centuries [7]. It is available wildly in India, Pakistan and Europe etc. It is spread in numerous parts of India. It is originating in dry rocky places in Andhra Pradesh, Bihar, Konkan, Tamil Nadu, Bengal, Deccan, Madhya Pradesh, Maharashtra, Andhra Pradesh and Kerala. The plant is unpleasant, acrid, chilling, alternate, sedative, emetic, antiviral and revitalizing. Several chemical constituents present in the plant like succinic acid, malic acid, reducing sugar - sucrose, traces of tannin, alkaloids, phytosterols, alpha and beta amyrins, lupeol and lupeol acetate and beta sitosterol. Antifertility, anti-microbial and i*n vitro* anti-inflammatory actions have been described on this plant [7,8].

According to the World Health Organization (WHO, 1978), about 80% of the world's rural population currently depends on medicinal plants as their complementary or alternative source of health care [9,10]. Medicinal plants contain bioactive non-nutrient and biologically active compounds known as phytochemicals which contain a broad spectrum of chemical structures and protective/disease preventative properties. Thus, identification of phytochemicals through preliminary phytochemical screening of plants is important in determining the chemical constituents in plant crude extracts. Preliminary phytochemical screening of plant extracts is also necessary for the discovery and development of novel therapeutic agents with improved efficacy [11,12].

The present study reports on the phytochemical screening, total flavonoid contents as well as antioxidant activity of crude extracts of *Entada rheedii* and *Sarcostemma acidum*.

2. MATERIALS AND METHODS:

Plant material and preparation of extracts

The bark of *Entada rheedi* and stem of *Sarcostemma acidum* were collected from the Tirumala forests, Tirupati, A.P, India in the month of Feb 2016 and was authenticated by Dr. K. Madhava Chetty, Professor and Head, Department of Botany, S. V. University, Tirupati and the voucher sample number was lodged (plant specimen number: **1101 and 1102**) and conserved in the herbarium, which was reserved in our lab for future reference.

The bark of *Entada rheedi* and stem of *Sarcostemma acidum* were shade dried and coarsely powdered. The 500 g of the powdered plant material was defatted with petroleum ether (60-80°C) using a Soxhlet extractor and then it was successively extracted with ethyl acetate and 70% aqueous ethanol for 72 hours. The extract obtained from the solvents was filtered and concentrated using rota evaporator (Medika Instrument). The yield of the extracts was found to be 10.8% and 12.3% respectively.

Preliminary phytochemical screening:

EAER and EESA were tested for the existence of various phytoconstituents [13].

High performance thin layer chromatography:

The EAER and EESA were then subjected to high performance thin layer chromatography (HPTLC) for identification of specific phytoconstituents [13]. The extract was dissolved in HPTLC grade chloroform: methanol (6:4), which was used for sample application on precoated silica gel GF 254 aluminum sheets. Many solvent systems were tried, but the satisfactory resolution was obtained in the solvent chloroform: methanol (6:4). The samples (5 µL-10 mg/5 ml) were spotted in the form of bands of width 8 mm with a 100 µL sample using a Hamilton syringe on silica gel, which was precoated on aluminum plate GF 254 plates (5 × 10 cm-E. MERCK KGaA) with the help of Linomat five applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software, CAMAG USA. 15 ml of mobile phase was used for chromatography run. The linear ascending development was carried out in a (5 × 10 cm) twin through the glass chamber saturated with the mobile phase.

The developed plate was dried in oven at 60°C to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images under ultraviolet (UV) light at 254 and 366 nm, respectively. The Rf values and finger print data were recorded by WIN CATS software.

Estimation of Total Flavonoids:

The flavonoid content of EAER and EESA was determined by the method of Helmja et al [14]. Briefly, an aliquot of the sample was pipetted out in a test tube and the volume was made up to 0.5 ml with distilled water. Sodium nitrite (5%; 0.03 ml) was added to the tube and incubated for 5 min. at room temperature. Aluminum chloride solution (10%; 0.06 ml) was added and incubated for 5 min. at room temperature. Sodium hydroxide solution (1 M; 0.2 ml) was added and the total volume was made up to 1 ml with distilled water. Absorbance was measured at 510 nm against a reagent blank. Standard curve using different concentrations of rutin was prepared. From the standard curve, the concentration of flavonoids in the test sample was determined and expressed as mg of rutin equivalent.

In vitro antioxidant activity:

Determination of reducing power [15]

The reducing power of EAER and EESA was studied by adopting the method of Oyaizu. About 2.5 ml of different concentrations of the plant extract (10-500 μg/ml) was mixed with 2.5 ml each of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, then rapidly cooled and then mixed with 2.5 ml of 10% Trichloroacetic acid and centrifuged at 3000 rpm for 10 min. About 2.5 ml of the supernatant was taken to this 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride was added and mixed well then allowed to stand for 10 min and the absorbance was measured at 700 nm. Vitamin-C was used as standard.

2,2-diphenyl-1-picrylhydrazyl assay (DPPH) [16]

The free radical scavenging activity of EAER and EESA was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. A 0.004% DPPH solution in methanol was prepared and 4 ml of this solution was added to 1 ml of plant extract of EESA/EAER solution in water at different concentrations (10-500 mg/ml). It was left for 30 min at room temperature for the reduction of the DPPH free radicals and the absorbance was measured at 517 nm. The procedure was repeated for vitamin-C, which was used as standard. The antioxidant activity of the extract was expressed as IC50, which is the inhibitory concentration required to scavenge 50% of DPPH free radicals. The percentage inhibition was calculated from the following equation:

% inhibition = Ablank − (Asample/Ablank) ×100

Peroxide radical scavenging activity [17]

The peroxide radical scavenging activity of EAER and EESA was carried out by adapting the method of Ruch *et al*. 4 mM of H_2O_2 solution in phosphate buffered saline was prepared. 0.6 ml of this solution was added to 1 ml of plant extract solution at different concentrations (10-500 μg/ml). This solution was incubated for 10 min at room temperature and the absorbance was measured at 230 nm. Vitamin-C was used as standard. The antioxidant activity of the extract was expressed as IC50, which is the inhibitory concentration required to scavenge 50% of peroxide free radicals. The percentage inhibition was calculated from the following equation:

% inhibition = Ablank− (Asample/Ablank) ×100

Nitric oxide radical scavenging activity [18]

Nitric oxide (NO) was spontaneously generated from sodium nitroprusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. To 1 ml of plant extract solution at different concentrations (10-500 μg/ml), 1 ml of 10 mM sodium nitroprusside was added. It was incubated for 150 min at room temperature and 0.5 ml of Griess reagent was added and the absorbance of the chromophore formed during the deionization of nitrite with sulfanilamide and subsequent coupling with napthyl ethylene diamine dihydrochloride was measured at 546 nm. Vitamin-C was used as

standard. The antioxidant activity of the extract was expressed as IC50, which is the inhibitory concentration required to scavenge 50% of NO radicals. The percentage inhibition was calculated from the following equation:
% inhibition = Ablank − (Asample/Ablank) ×100

Superoxide radical scavenging activity [19]
This activity was determined by the NBT reduction method. To 1 ml of EAER and EESA solution at different concentrations (10-500 µg/ml), 1 ml each of 60 µM phenazine metho sulfate and 450 µM NADH was added and it was incubated for 5 min at 25°C and the percentage inhibition of superoxide generation was evaluated by measuring the absorbance values. Vitamin-C was used as standard. The antioxidant activity of the extract was expressed as IC50, which is the inhibitory concentration required to scavenge 50% of superoxide radicals. The percentage inhibition was calculated from the following equation:
% inhibition = Ablank − (Asample/Ablank) ×100

RESULTS AND DISCUSSION

3.1 Result of Phytochemical Tests

The priliminary phytochemical analysis revealed the presence of alkaloids, carbohydrates, saponins, little phenolic compounds and especially flavonoids.

Table 1: Results of phytochemical screening of bark *Entada rheedi*

Chemical test	Petroleum ether extract	Ethyl acetate extract	Ethanolic extract
Carbohydrates	+	+	+
Alkaloids	+	–	+
Flavanoids	+	+	+
Phenolic and Tannin compounds	–	+	–
Glycosides	–	–	–
Steroids	+	–	+
Test for fats and oils	–	–	–
Lignins and Terpenoids	–	+	–

(+ve) – Presence (-ve) – Absence

Table 2: Results of phytochemical screening of stem of *Sarcostemma acidum*

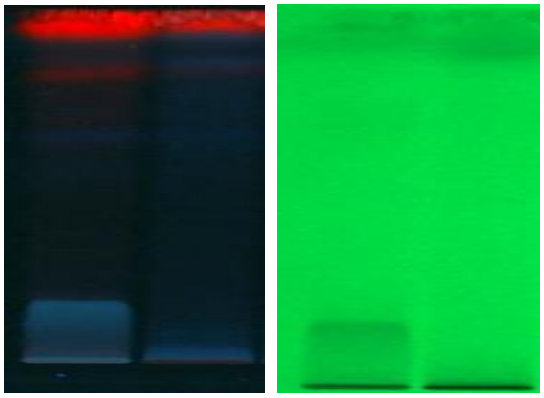
Chemical test	Petroleum ether extract	Ethyl acetate extract	Ethanolic extract
Carbohydrates	+	+	+
Alkaloids	+	–	–
Flavanoids	+	+	+
Phenolic and Tannin compounds	–	+	–
Glycosides	–	–	–
Steroids	+	–	+
Test for fats and oils	–	–	–
Lignins and Terpenoids	–	+	+

(+ve) – Presence (-ve) – Absence

3.2 High performance thin layer chromatography

Finger print analysis of EAER

High performance thin layer chromatography finger printing analysis of EAER revealed several peaks and were recorded. HPTLC profile under UV 366 and 254 nm was recorded in the Figure 1 and 2. The Rf value of spots observed were 0.13, 0.80 and 0.92. Appearance of blue and orange color under UV examination confirmed the presence of flavone and flavonoid components in the extract.



366 nm 254 nm

Figure 1. HPTLC finger print analysis of EAER at 366 and 254 nm

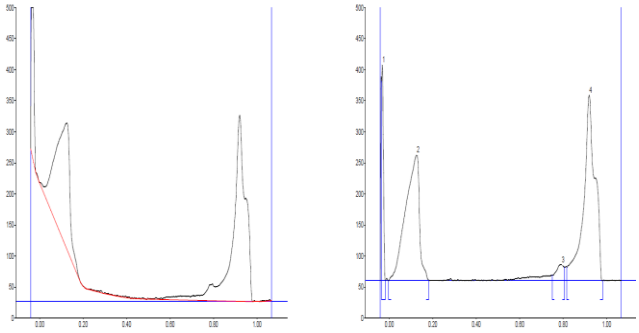
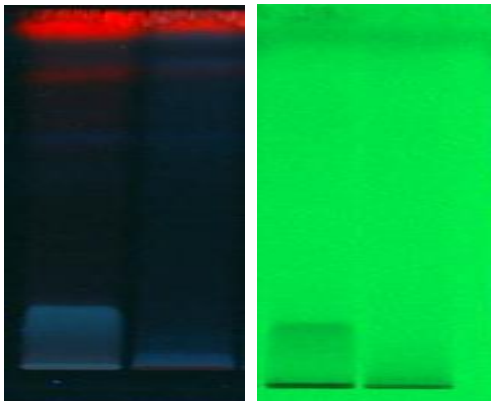


Figure 2. HPTLC finger print analysis of EAER at 366 and 254 nm

Finger print analysis of EESA

High performance thin layer chromatography finger printing analysis of EESA revealed several peaks and was recorded. HPTLC profile under UV 366 and 254 nm was recorded in the Figure 3 and 4. The Rf value of spots observed was 0.12, 0.81 and 0.93. Appearance of blue and orange color under UV examination confirmed the presence of flavone and flavonoids components in the extract.



366 nm 254 nm

Figure 3. HPTLC finger print analysis of EESA at 366 and 254 nm

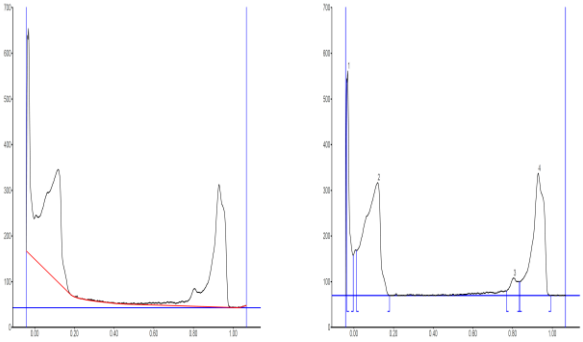


Figure 4. HPTLC finger print analysis of EESA at 366 and 254 nm

3.3 Flavonoid content of EAER and EESA

Flavonoid content of EAER and EESA was determined and the results indicated that EAER contains 18.0 mg/g of rutin equivalent flavonoid and EESA contains 13.6 mg/g of rutin equivalent flavonoid.

3.4 Results of *in-vitro* antioxidant activity of EAER and EESA

Table 3: Reducing power activity of EAER and EESA

Concentration µg/mL^{-1}	Reducing Power (OD)		
	EAER	AA	EESA
10	0.14	0.234	0.132
50	0.168	0.647	0.169
100	0.19	0.782	0.218
200	0.196	0.675	0.237
500	0.21	2.826	0.285
IC_{50}	---	---	---

Table 4: DPPH assay activity of EAER and EESA

Concentration µg/mL^{-1}	DPPH assay (%)		
	EAER	AA	EESA
10	40.31	49.74	29
50	41.77	90.19	37
100	52.3	90.34	56
200	53.18	92.09	72
500	75.35	92.32	94
IC_{50}	85	10	89

Table 5: Superoxide radical scavenging assay (%) activity of EAER and EESA

Concentration µg/mL^{-1}	Superoxide radical scavenging assay (%)		
	EAER	AA	EESA
10	35.31	26.74	34
50	49.05	39.23	56
100	67.3	72.16	68
200	82.18	93.09	89
500	95.35	98.32	100
IC_{50}	55	70	45

Table 6: H_2O_2 radical scavenging assay (%) of EAER and EESA

Concentration µg/mL^{-1}	H_2O_2 radical scavenging assay (%)		
	EAER	AA	EESA
10	86.68	25	18
50	88.33	33.33	37
100	90	41.68	59
200	94.17	50	72
500	97.5	58.33	91
IC_{50}	6	200	85

Table 7: Nitric oxide inhibition assay (%) of EAER and EESA

Concentration µg/mL^{-1}	Nitric oxide inhibition assay (%)		
	EAER	AA	EESA
10	16.23	39.1	23
50	28.19	52.08	29
100	41.98	63.21	48
200	77.26	88.3	76
500	89.28	97.9	88
IC_{50}	125	40	104

Table 8: Hydroxyl radical scavenging assay (%) of EAER and EESA

Concentration µg/mL^{-1}	Hydroxyl radical scavenging assay (%)		
	EAER	AA	EESA
10	42.19	49.38	29
50	58.23	57.15	35
100	69.45	72.87	65
200	88.23	83.43	82
500	96.14	99.12	94
IC_{50}	27.5	10	77

CONCLUSION

The phytochemical analysis showed that the EAER and EESA contains a mixture of phytochemicals such as reducing sugars, phenolic compounds, flavonoids, and alkaloids. The quantitative total flavonoids estimation and HPTLC indicated that among three extracts (Petroleum ether, ethanol and ethyl acetate) the ethyl acetate extract of *Entada rheedii* and ethanolic extract of *Sarcostemma acidum* has the highest contents of flavonoids which might be responsible for the antioxidant activity. *In vitro* antioxidant activity showed extracts has powerful oxygen free radical scavenging abilities which can be an excellent option for biological and chemical analysis and can be further subjected for the isolation of the therapeutically active compounds. These plant extracts (EAER and EESA) can be further subjected for the isolation of therapeutically active substances with antioxidant potency and anti-stress/adaptogenic activity.

Conflicts of interest

The authors declare no conflicts of interest.

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