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IN VITRO ANTIOXIDANT STUDIES ON ETHANOLIC EXTRACTS OF LEAF, STEM AND ROOT OF *Sida rhombifolia* L.

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ABSTRACT

Antioxidant activity of the ethanol extract of *Sida rhombifolia was* determined by DPPH free radical, hydrogen peroxide scavenging assays, reducing power, total antioxidant capacity using phosphomolybdenum method, and rapid radical scavenging screening methods. Preliminary phytochemical screening revealed that the extract of *S. rhombifolia* leaves (SR-L), stem (SR-S) and roots (SR-R) possesses phenols, flavonoids, steroids, glycosides, saponins, tannins, and terpenoids. The extract showed significant activities in all antioxidant assays compared to the standard antioxidant (ascorbic acid) in a dose dependent manner and remarkable activities to scavenge reactive oxygen species (ROS) may be attributed by the presence of the above active compounds in SR-L, SR-S and SR-R.

KEYWORDS

DPPH, free radical, H_2O_2 scavenging, phytochemical, *Sida rhombifolia*, total antioxidant.

INTRODUCTION

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are often generated as by-products of biological reactions, or from exogenous factors [1]. *In vivo*, some of these ROS play positive roles such as energy production, phagocytosis, regulation of cell growth and intercellular signalling, or synthesis of biologically important compounds [2]. However, ROS may also be very damaging as they can induce oxidation of lipids, causing membrane damage, decreasing membrane fluidity, and leading to cancer via DNA mutation [3]. A potent scavenger of these ROS may serve as a possible preventative against free radical mediated diseases [4].

Sida rhombifolia is an Ayurvedic herb, used in the treatment of a variety of conditions including female infertility. Maha bala or mahabala is the Hindi-Sanskrit name of Kurunthotti (or kurumthotti Â– Malayalam word for sida). It is a very small perennial plant. Leaves, stem, bark and roots are usable parts. The stems are rich in mucilage and are employed internally as an emollient, [5] diuretic, [6] and as a

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febrifuge with pepper. The roots are bitter, cooling, and anthelmintic.

Ayurvedic physicians prescribe the infusion made from the root of this plant for the treatment of rheumatism and neurological complaints, including epilepsy [7]. The ethyl acetate extract of *S. rhombifolia* leaves showed potent cytotoxicity [8]. The alcohol extract of the whole plant showed anti-microbial activity [9]. Fatty acids from the leaves of the plant exhibited anti-bacterial activity [10]. Aerial parts of *S.rhombifolia* were screened for various parameters of anti-arthritic activity, such as adjuvant-induced arthritis, motor performance, mean distance travelled, and histopathological study.

In the present study, we have evaluated the *in vitro* antioxidant activity of ethanolic extract along with phenolic content determination.

MATERIALS AND METHODS

(i) Plant collection

Leaves, stem, and root parts of *Sida rhombifolia* (L.) were collected from Thrissur, Kerala, India during the month of September, 2009. The plants were identified by Dr.V.S Ramachandran, Reader, Department of Botany, Bharathiar University, Coimbatore, India.

(ii) Extraction and phytochemical analysis

Leaves, stems and roots of the plant were shade-dried, powdered and named it as SR-L, SR-S, SR-R respectively. They were extracted successively with each of petroleum ether, chloroform, ethyl acetate, and ethanol in a soxhlet extractor for 18-20 hrs. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50ºC) in a rotavapor. All the extracts were subjected to qualitative chemical tests for the identification of various phytoconstituents [11]. The total phenol content was determined using Folin-ciocalteau reagent, [12] and the total flavanoid content was estimated using aluminium chloride method [13].

(iii) DPPH scavenging assay [14, 15]

Different concentration of substrate, 1.0 ml of (0.1mM) DPPH in ethanol, 550 µl of 50 mM Tris-HCl buffer (pH 7.4) were added and the mixture was incubated for 30 min at room temperature. After 30 min, absorbance of the mixture was measured using spectrophotometer at 517 nm. Mixture without substrate served as control.

(iv) Hydrogen peroxide scavenging assay

Hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the extracts in distilled water was added to 0.6 ml of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage inhibition of different concentrations of the extracts was determined and compared with the standard, ascorbic acid [16].

(v)Assay of Reducing Power, Makari et al. [17], Koleva et al. [18]

Different concentrations of plant extract solution (final concentration 100- 500 µg/ml) was mixed with 2.5 ml phosphate buffer(0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide $[K_3Fe(CN_6)]$ (10g/l), then mixture was incubated at 50º C for 20 minutes. Two and one-half, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1g/l) and absorbance measured at 700nm in UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of five parallel experiments was expressed as mean ±

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standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

(vi)Total Antioxidant Capacity

The total antioxidant capacity assay was followed by the method of Preito et al. [19]. 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in an eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

(vii)Rapid radical scavenging screening

The method of Mensor et al. [20] as modified by Burits et al. [21] was followed in screening for the antioxidant property of the extracts. With the aid of capillary tube, stock solutions (1 mg/ml) of extracts were spotted on silica gel thin layer chromatographic (TLC) plate and developed with a solvent system of ethanol: methanol (90:10). After development, the chromatograms were dried and sprayed with a 0.3 mM solution of the stable radical DPPH. Purple spot formed were taken as positive results. The duration for the development of yellow colour indicated whether the antioxidant activity was strong or not. Ascorbic acid was used as a reference standard in all the above assays.

(viii)Statistical analysis

All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. *P* values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean ± S.D. for five experiments in each.

RESULTS

In this present study the antioxidant activity of the ethanol extracts of the leaves, stems, and roots of *Sida rhombifolia* were investigated by using DPPH scavenging assay, H_2O_2 assay, reducing power, of the extract and by determining total antioxidant capacity of the extract. All the methods have proven the effectiveness of the ethanol extract compared to standard ascorbic acid.

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Table .1
Phytochemical screening of Sida rhombifolia ethanol extracts

Extract	Phytochemical analysis		
	SR-L	SR-S	SR-R
Petroleum ether extract	Steroids, terpenoids	Steroids, terpenoids	Steroids, terpenoids
Chloroform extract	Alkaloids, phenol	Alkaloids, phenol	Alkaloids, phenol
Ethyl acetate extract	saponins, glycosides, tannins	saponins, glycosides, tannins	saponins, glycosides, tannins
Ethanol Extract	Alkaloids, glycosides, tannins, saponins, terpenoids	Alkaloids, glycosides, tannins, saponins, terpenoids	Alkaloids, glycosides, tannins, saponins, terpenoids

Preliminary phytochemical screening of the extract of *S.rhombifolia* revealed the presence of various bioactive components which are steroids, alkaloids, saponins, glycosides, tannins and terpenoids. The **results of phytochemical test have** been summarized in Table 1.

Table .2
Phenol and Flavanoid content in the studied plant parts

	Total phenol (mg/g)		Total flavanoid (mg/g)
Leaf	91.97 ± 2.24	Leaf	28.69 ± 0.21
Stem	95.48 ± 1.22	Stem	30.45 ± 0.7
Root	99..67 ± 0.56	Root	33.39 ± 1.05

(values are mean ± SD of five determinants)

Table 2 explains the amount of total phenol and flavanoids presents in SR-L, SR-S, and SR-R. SR-R extract has the higher amount of phenol and flavanoid among these three extracts.

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Table .3

Radical Scavenging activities of the ethonolic extracts from the leaf, stem and root of Sida rhombifolia using rapid DPPH TLC screening.

Plant part	Reaction speed	Intensity of spots
SR-L	Intermediate	++
SR-S	Fast	+++
SR-R	Fast	+++

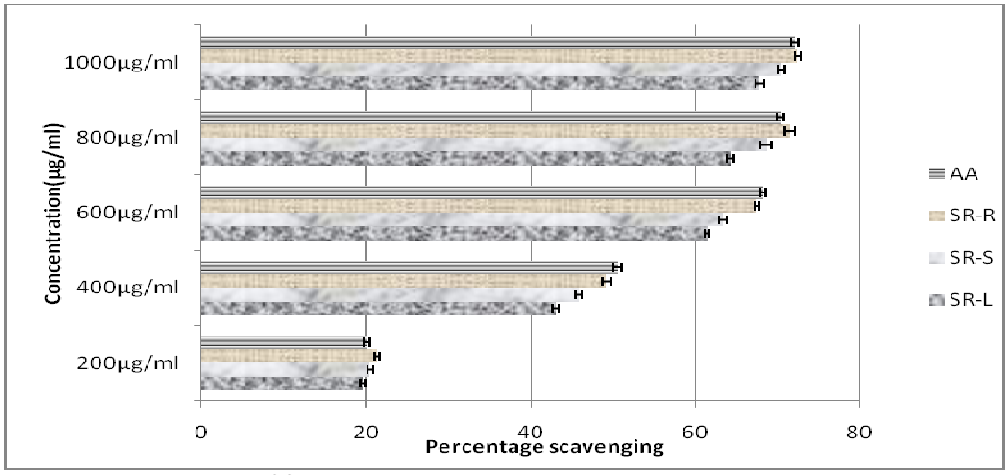
+ + + = Strong intensity (immediate reaction).

The result of the rapid radical scavenging screening of the plant parts confirmed their high radical scavenging activity (Table 3). The reaction is immediate and the intensity of spots is high in roots when compared with leaves and stems.

Comparison of the antioxidant activity of the extracts and ascorbic acid by DPPH method is shown in Figure 1. The ethanol extract of SR-L, SR-S and SR-R exhibited significant dose dependent inhibition of DPPH activity. **The scavenging activities of the extracts were very potent and the power of the extracts increased with** increasing concentration. Among these, SR-R showed the highest potential to scavenge DPPH, followed by SR-S and SR-L.

Fig. 1

Free radical scavenging activity of various amounts of ethanol extracts of Sida rhombifolia leaf, stem, and roots.



Values are mean ± SD of five determinations.

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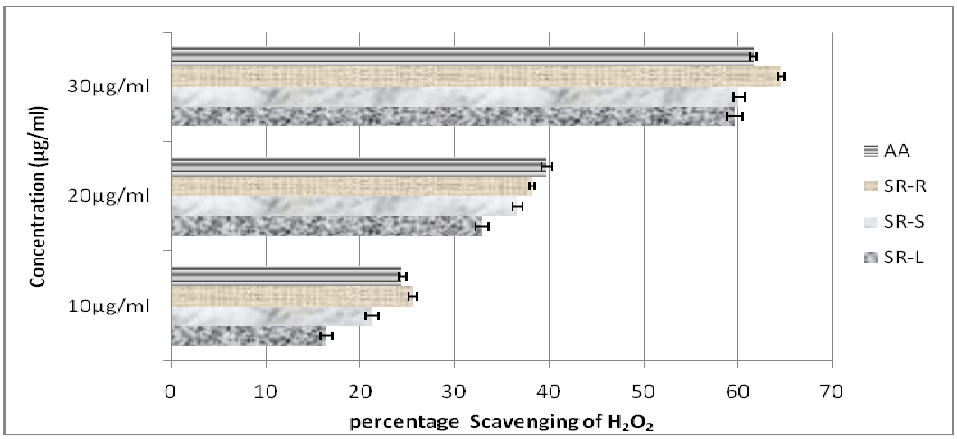
Ability of the investigated SR-L, SR-S, and SR-R extracts to scavenge hydrogen peroxide is shown in figure 2. SR-L showed low scavenging activity, whereas SR-S and SR-R extracts showed higher activity respectively.

Fig. 2

Hydrogen peroxide radical scavenging activity of Sida rhombifolia leaf, stem, root.

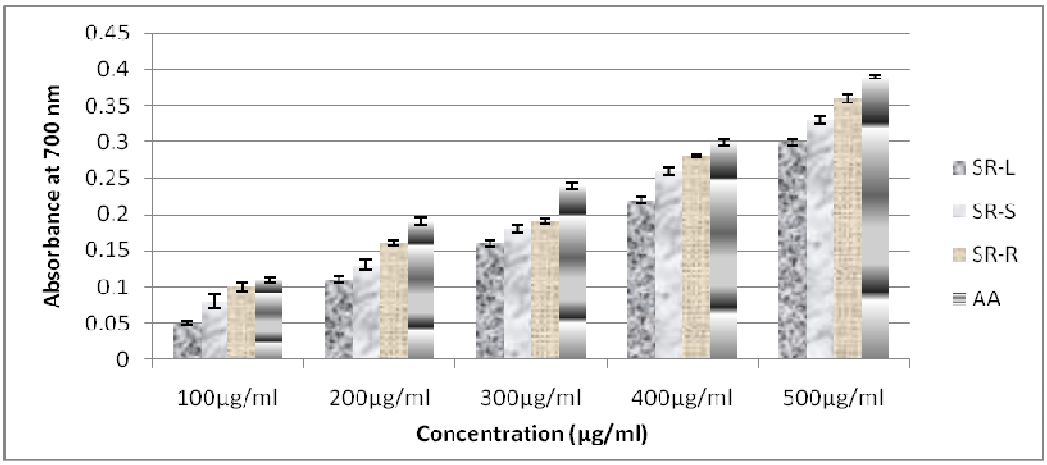


Values are mean ± SD of five determinations.

Figure 3 shows the reductive capabilities of SR-L, SR-S, SR-R extracts along with ascorbic acid. The reducing power of extracts was very potent and the power of the extract increased with increasing concentration.

Fig .3

Reducing power of the leaves of Sida rhombifolia extract compared to ascorbic acid.



Values are mean ± SD of five determinations.

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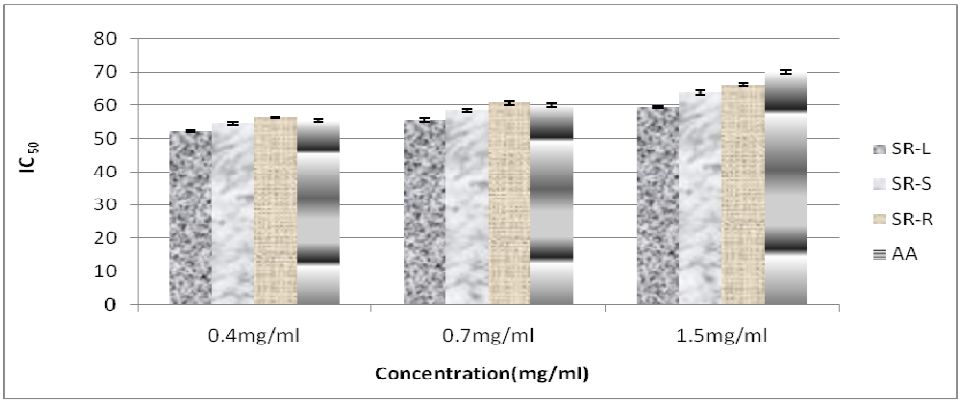
The total antioxidant capacity of the extracts were calculated based on the formation of the phosphomolybdenum complex and is shown in figure 4. The total antioxidant capacity of the SR-R was found higher next to SR-S and SR-L shows less activity compared with the above and ascorbic acid.

Fig .4

Total antioxidant activity of ethanol extract of Sida rhombifolia leaf, stem and root



Values are mean ± SD of five determinations.

DISCUSSION

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defenses. Potential antioxidant therapy therefore should include either natural free radical scavenging antioxidant enzymes or agents which are capable of augmenting the activity of these enzymes. Reactive oxygen species (ROS) has received considerable attention in the recent past because of its role in several pathological conditions including cancer, diabetes, arthritis, aging and atherosclerosis. ROS produced *in vivo* include superoxide radical O^{2-}, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) and H_2O_2 can interact in the presence of transition metal ions to yield a highly reactive oxidizing species, the hydroxy radical [22]. If human disease is believed to be due to the imbalance between oxidative stress and antioxidative defense, it is possible to limit oxidative tissue damage and hence prevent disease progression by antioxidant defense supplements.

DPPH is characterized as stable free radical by virtue of the delocalization of the spare electron where the molecule as a whole, so that the molecule do not dimerise, as would be the case with most other free radicals. The delocalization gives rise to the deep violet colour, characterized by an absorption band (517 nm) in ethanol solution. When a solution of DPPH is mixed with a substance of H donor, it gets reduced into non radical state [23]. Hence the ethanol extract of SR-R, SR-S and SR-L exhibited a significant dose dependant inhibition of DPPH activity. Comparatively, SR-R showed higher inhibition than SR-S and SR-L.

The reducing ability of a compound generally depends on the presence of reductants [24], which have been exhibited antioxidative potential by breaking the

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free radical chain, donating a hydrogen atom [25]. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. By measuring the formation of Perl's Prussian blue at 700 nm, it is possible to determine the Fe^{2+} concentration. The reducing power of the various extracts of *Sida rhombifolia* were very potent and increased with the concentration of the sample.

The measurement of H_2O_2 scavenging activity is one of the useful methods of determining the ability of antioxidants to decrease the level of pro-oxidants such as H_2O_2 [26]. It can cross membranes and may slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells [27]. The results showed that SR-R had an effective H_2O_2 scavenging activity.

Total antioxidant capacity of *Sida rhombifolia* is expressed as the number of equivalents of ascorbic acid. The phosphomolebdnum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The study reveals that the antioxidant activity of the extract exhibits increasing trend with the increasing concentration of the plant extract. High radical scavenging activity of the plant parts were confirmed by performing qualitative analysis using TLC method.

The quantitative estimation of the phytochemical constituents of the leaves, stems and roots of *S.rhombifolia* shows that the plant is rich in flavonoids, tannins, and phenols to some extent. Phenolic compounds have been recognized as antioxidant agents, which act as free radical terminators [28], and have been known to show medicinal activity as well as exhibiting physiological functions [29]. It has been reported that compounds such as flavonoids, which contain hydroxyls, are responsible for the radical scavenging effects of most plants [30]. The mechanisms of action of flavonoids are through scavenging or chelating process [31, 32]. The presence of these phytochemicals in *Sida rhombifolia* stem, leaf and root are significant finding in this present study. The results obtained indicate that *Sida rhombifolia* is a potential source of natural antioxidant.

CONCLUSION

This study suggests that ethanol extract of *S.rhombifolia* has potent antioxidant activity, achieved by scavenging abilities observed against DPPH, H_2O_2. Phytochemical studies show that it contains phenols, flavanoids, glycosides which are known antioxidants. Also the wide use of the plant in the indigenous system of medicine as anti-inflammatory may be in part due to the antioxidant potential of the extract. Since reactive oxygen species are important contributors to various serious ailments, the observed antioxidant property of the extract of *Sida rhombifolia* in the present study might be useful for the development of newer and more potent natural antioxidants. The plant merits further investigation in animal models to confirm its antioxidant activity and to isolate the active constituents, especially due to their non-polar nature.

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