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Total Phenolic, Flavonoid contents and Antioxidant assays in leaf extracts of *Senna alata* (L.) Roxb.

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Abstract

Senna alata (L.) Roxb. (Syn. Cassia alata) is an ethnomedicinal plant belonging to the family Fabaceae. A number of bioactive constituents which contribute to a wide range of medicinal properties are attributed to the species. In the present investigation, an attempt has been made to determine the total phenolic, flavonoid contents and also to study the in vitro antioxidant activity associated with leaf extracts of S. alata. According to our investigations, the highest amount of phenolic content was observed in aqueous extract (52.3 ± 0.03) followed by methanolic (41.6±0.41) extract. Similarly the concentration of flavonoids was also found to be higher in aqueous extract (41.6±0.34) followed by methanolic (31.9±0.63) extracts. In vitro antioxidant assays of aqueous and methanolic leaf extracts of S. alata have been carried out by DPPH assay, Reducing power assay, Phosphomolybdenum and Hydroxy radical scavenging activities. From the combined results of all the activities, it can be inferred that the extracts of S. alata exhibited potent antioxidant activities. Hence, they can be used as natural antioxidants like red wine, green tea, cocoa and can be replaced in place of harmful synthetic antioxidants.

Key Words: Antioxidant assays, Aqueous extract, Methanolic extract, Senna alata, Total Flavonoid content, Total Phenolic content.

INTRODUCTION:

Phenolics are secondary metabolites that are omnipresent in plants. They are a large group of bioactive compounds comprising about 8000 compounds. Phenolics show a wide range of biochemical activities such as antioxidant, antimutagenic and anticancerous activities. They have also played a major role in modifying gene expression [1]. More than 4000 flavonoids of plant origin have been identified till-to-date [2]. High intake of flavonoids helps in reducing cardiovascular and carcinogenic risks [3].

Antioxidants are the substances which present in low concentrations, compared to oxidisable substances, delay or prevent the oxidation of those substances. Oxidative stress arises due to the generation of reactive oxygen species (ROS) beyond the antioxidant capacity of the biological system during metabolism and other activities [4]. Most common ROS include super oxide anion (O_2^*), Hydroxy radical, Hydrogen Radical (OH^*), Hydrogen Peroxide (H_2O_2), Nitrogen derived free radicals such as Nitric Oxide (NO^*) and Peroxy nitrite anion ($ONOO^*$) [5].

High intake of foods containing antioxidants, lowers the risk of chronic diseases like neuro degenerative diseases, cardiovascular diseases, aging process, cataracts, cancer and brain dysfunction and other age related degenerative diseases by reacting with free radicals and also by acting as oxygen scavengers [6].

To meet the increasing demand for anti oxidants, a number of synthetic antioxidants such as butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), propyl gallate (PG) are used to slow down the oxidation process and their safety has long been questioned [7,8] due to their unwanted side effects and potential health hazards. Plants and plant based products are a rich source of natural antioxidants such as carotenoids, β-carotene, flavonoids, folic acid, ascorbic acid, tocopherols *etc*.

Senna alata (L.) Roxb. (Syn. *Cassia alata*) is an important medicinal plant belonging to Fabaceae family. The species is attributed to a wide range of medicinal properties [9-11]. Even though a large number of reports have been published on other medicinal properties of the species [12], very few reports are available on the *in vitro* antioxidant assays of *S. alata* [13,14]. Hence in the present study, we determine the antioxidant activity of aqueous and methanolic leaf extracts of *S. alata* through various *in vitro* techniques available.

MATERIALS AND METHODS

Plant material

Mature leaves of *S.alata* were collected from the medicinal garden, Department of Biotechnology, Kakatiya University, Warangal, TS, India. The samples were authenticated by Prof. N. Rama Swamy, Department of Biotechnology, Kakatiya University, Warangal TS, India. The leaves were shade dried, powered using a mechanical blender. The obtained coarse powder was further sieved to obtain a fine powder.

Preparation of extracts

15gm of sample was extracted by macerating overnight with 150ml of the respective solvents (chloroform, benzene, water, methanol, petroleum ether). The extracts were then filtered using whatman no.1 filter paper. The flow through was evaporated to dryness at room temperature, collected and stored for further use.

(i) Determination of total phenolic content (TPC)

Total phenolic content (TPC) was determined by the method described by Jia *et al.* (1998) with some modifications. To 1 ml of the test extract, 1ml of Folin-Ciocalteu reagent was added and allowed to stand for 2 min. After 2 min, 1 ml of 35% Na_2CO_3 solution was added to the mixture and the final volume was made up to10ml with distilled water. The reaction mixture was incubated in dark for 90 min at room temperature and absorbance was measured against reagent blank.

The total phenolic content was expressed in terms of mg of gallic acid equivalents (GAE) per gram of extract.

(ii) Determination of total flavonoid content (TFC)

The total falvonoid content of the plant extracts was determined by the method described by Jia *et al.* (1998). 0.25ml of the plant extracts were made upto 1.5 ml using distilled water and to this 75µl of 5% $NaNO_2$ solution was added. The reaction mixture was allowed to stand for 5 min and 150 µl of 10% $AlCl_3$ was added to it. The reaction mixture was mixed well and incubated for 5 min at room temperature. 0.5ml of 1M NaOH was added and absorbance was measured against reagent blank at OD=510nm. The results were expressed as mg of rutin equivalents (RE) per gram of extract.

(iii) DPPH- Free radical scavenging activity

DPPH- Free radical scavenging activity of dried crude aqueous and methanolic leaf extracts of *S. alata* were examined following the procedure given by Sudha *et al.* (2011).

DPPH solution was prepared by dissolving 4.3 mg of DPPH in 3.3 ml of methanol. The solution was covered with aluminium foil to protect it from light until use. 150 µl of DPPH solution was added to 3 ml of methanol which serves as control. The absorbance of the control was taken immediately at OD=517 nm. Different concentrations of aqueous and methanolic leaf extracts (0.2, 0.4, 0.6, 0.8, 1 mg/ml) were prepared in distilled water. Then each sample was made up to 3ml with methanol and 150µl of DPPH was added to each tube. Later the tubes were incubated in dark for 15 min and absorbance was taken at OD=517 nm using methanol as blank on UV-Visible spectrometer. Ascorbic acid was taken as standard. The IC_{50} values for aqueous and methanolic extracts were calculated. The DPPH free radical scavenging activity was calculated by using the formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

(iv) Determination of reducing power

The reducing power of aqueous and methanolic leaf extracts of *S. alata* was determined according to the method of Sudha *et al.* (2011). 2.5 ml of different concentrations of leaf extracts (0.4, 0.8, 1.2, 1.6, 2 mg/ml) and 2.5 ml of phosphate buffer (0.2 M, pH 6.6) were mixed with 2.5 ml of potassium ferricyanide and incubated at 50^0c for 20 min. To this, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 5000g for 10 min. To 2.5 ml of supernatant, 2.5 ml deionised water and 0.5 ml of 0.1% ferric chloride were added and incubated for 10 min at room temperature. Absorbance at OD=700 nm was measured against blank.

(v) Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of aqueous and methanolic leaf extracts of *S. alata* was determined using the method given by Sudha *et al.* (2011). Different concentrations of leaf extracts (250, 500, 750, 1000 µg/ml), 1 ml of 1.5mM $FeSo_4$, 0.3 ml of 20mM sodium salicylate, 0.7 ml of 6mM hydrogen peroxide were made up to 3 ml with distilled water. The reaction mixture was incubated in a water bath at 37^0c for 1h. After incubation, the absorbance of the solution was measured at OD=562 nm. The hydroxyl radical scavenging activity of the extracts was calculated by the following formula:

$$(1-(A_1-A_2) / A_0) \times 100$$

(A_1 = Absorbance in the presence of extract, A_2 = Absorbance without sodium salicylate and A_0 = Absorbance of the control).

(vi) Phosphomolybdenum assay

Phosphomolybdenum activities of the acetone, aqueous, ethanol, ethyl acetate and chloroform leaf extracts of *S. alata* were determined by the method given by Sudha *et al.*(2011). 1 ml of reagent solution containing 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate was mixed with 0.1 ml aliquot of sample solution. The samples were incubated at 95^0c for 90 min by covering them with aluminium foil. Later the samples were cooled to room temperature and absorbance was measured at OD=695 nm against blank. Total antioxidant activity was expressed nM gallic acid equivalents (GAE) per gram of dry extract and ascorbic acid was used as standard.

Data Analysis

All the experiments were repeated thrice and the data in the form of graphs were plotted by using MS Excel ® software 2010. The values of correlation coefficient, intercept, slope and standard errors were obtained by non linear and linear regression analysis applying this program.

RESULTS

In the present investigation, the total phenolic and total flavonoid contents in the leaf extracts of *S.alata* were determined. The different leaf extracts were also analysed for various antioxidant activities.

(i) Total phenolic content

The total phenolic content (TPC) of the leaf extract was determined using Folin- Ciocalteu's reagent and the concentration of total phenolic content is given in terms of mg GAE/g of the extract (Table-1). Highest amount of phenolic content was observed in aqueous extract (52.3 ± 0.03) followed by methanol (41.6±0.41), chloroform (32.4±0.32), benzene (31.4±0.71) and petroleum ether (21.6±0.09) extracts respectively (Table-1).

(ii) Total flavonoid content

The concentration of flavonoids in various leaf extracts expressed in terms of rutin equivalents per gram of extract is given in Table-1. The concentration of flavonoids ranged from 20.1±0.19 in chloroform extract to 41.6±0.34 in aqueous extract (Table-1). The concentration of flavonoids was found highest for aqueous extract (41.6±0.34) followed by methanol (31.9±0.63), benzene (30.6±0.66), petroleum ether (24.5±0.63) and chloroform (20.1±0.19) extracts respectively (Table-1).

Table-1: Total Phenolic and Flavonoid contents in leaf extracts of *S. alata*

Type of Extract	Total phenolic content (mg GAE /gm of extract)±SE[a]	Total flavonoid content (mg RE /gm of extract)±SE [a]
Aqueous	52.3±0.03	41.6±0.34
Benzene	31.4±0.71	30.6±0.66
Chloroform	32.4±0.32	20.1±0.19
Methanol	41.6±0.41	31.9±0.63
Petroleum ether	21.6±0.09	24.5±0.63

[a]Mean±Standard Error

(iii) DPPH scavenging activity

The antioxidant activity of aqueous and methanolic leaf extracts of *S. alata* was determined by DPPH activity using ascorbic acid as standard. DPPH shows a strong absorption band in the visible spectrum at OD=517nm. The percentage of inhibition of free radicals was found to be increasing on an increase in the concentration of test extract (Figs. 1 & 2). The IC_{50} values of aqueous and methanolic extracts were found to be 0.619 and 0.662 respectively. The scavenging activity of aqueous extracts was found to be higher than the methanolic extracts.

(iv) Reducing power assay

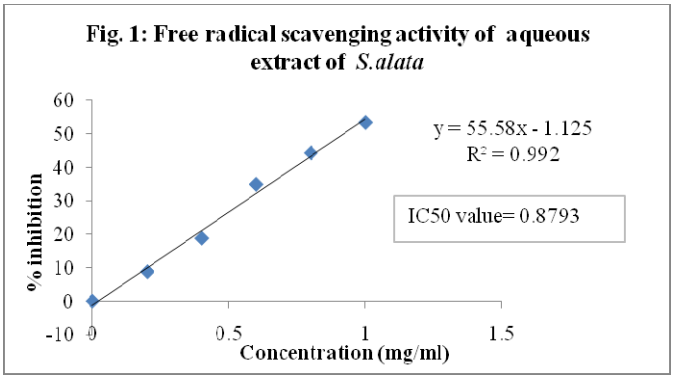
The reducing ability of aqueous and methanolic leaf extracts of *S. alata* are given in Fig 3. An increase in the absorbance at OD=700 nm with an increase in the concentration of the test extract, suggests the increased reducing ability of the extract. The IC_{50} values of aqueous and methanolic extracts were found to be 56.68 and 42.45 respectively. The reducing power of aqueous extract was found to be lower than the methanolic extract (Fig.3). The reduction of a compound in the reducing power assay can be related to its electron transferring ability and may serve as a significant indicator of its potentiality to serve as an antioxidant. Greater the absorbance of a compound at OD=700 nm, greater is its reducing ability.

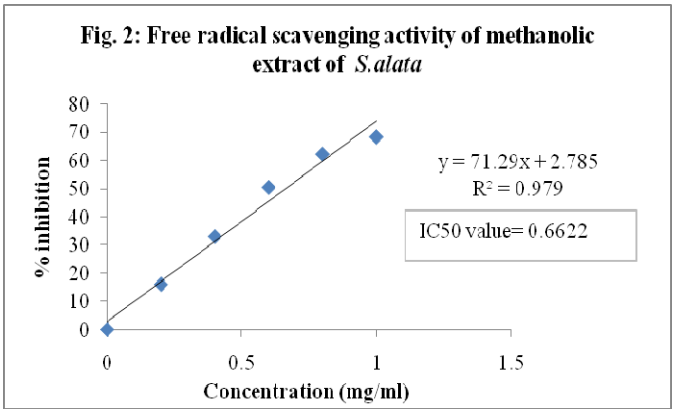
(v) Hydroxy radical scavenging activity

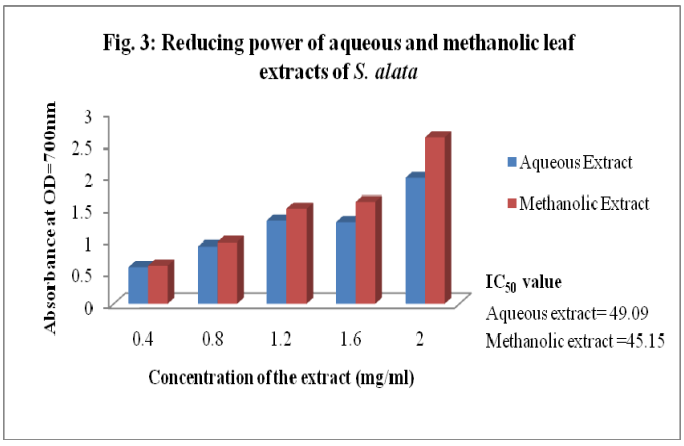
The scavenging activity of aqueous and methanolic leaf extracts of *S. alata* against the free radicals is shown in Fig. 4. The percentage of inhibition of hydroxyl radicals increased with an increase in the concentration of the extracts in both aqueous and methanolic extracts. The scavenging activity of aqueous extract (IC_{50}- 49.09) was found to be higher than the methanolic extract (IC_{50}- 45.15).

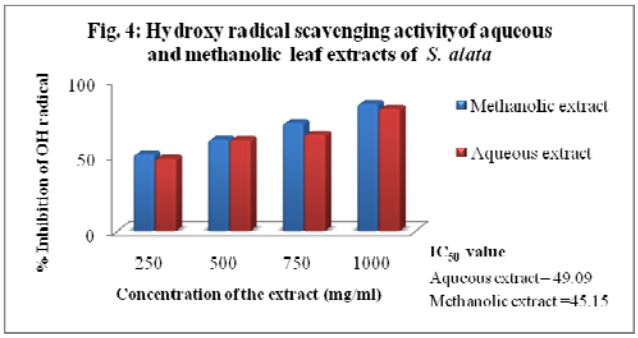
(vi) Phosphomolybdenum activity

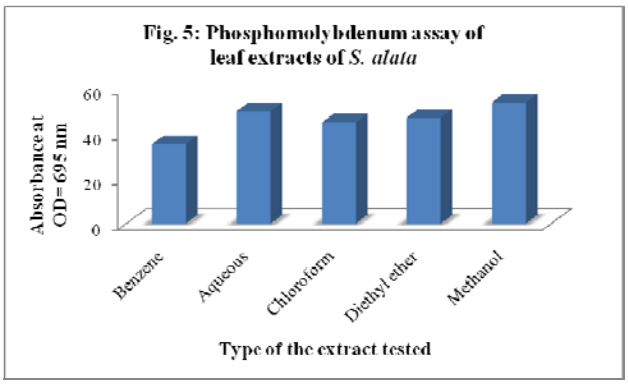
Phosphomolybdenum activity of aqueous, benzene, chloroform, diethyl ether and methanolic leaf extracts was determined (Fig. 5). The activity was found to be highest for methanolic extract followed by aqueous, di ethyl ether, chloroform and benzene respectively.











DISCUSSION:

The total phenolic and flavonoid contents of various solvent leaf extracts of *S. alata* have been assayed. Since both TPC and TFC were found to be high for aqueous and methanolic extracts, antioxidant activity of only these two extracts has been carried out using various assays such as DPPH assay, Reducing power assay, Hydroxy radical scavenging activity and Phosphomolybdenum activity. Although a few reports on the *in vitro* antioxidant assays of *S. alata* by DPPH assay have already been reported, so far no work has been carried out on the aqueous and methanolic leaf extracts of the species.

Phenolics are the important secondary metabolites in plants which are known to possess therapeutic uses such as antioxidant, anticarcinogenic, antimutagenic activities. They are also known to decrease cardiovascular risks [17]. A highest amount of total phenolic content was recorded in aqueous extract while the lowest in the petroleum ether extract (Table-1) in leaf extracts of *S. alata*. The difference in the concentration of phenolics in the respective solvents is due to the polarity of the solvents used for extraction. Higher solubility of the phenols in polar solvents provides higher concentration of these compounds in the extracts obtained by using the respective solvent during extraction [18].

Flavonoids are a class of polyphenolic compounds, which exhibit several potent bioactivities such as anti-cancer, anti-inflammatory, anti-ulcer, anti-hepatotoxic, anti-allergic and anti-viral activities. They are potent antioxidants as they are capable of effectively scavenging the ROS due to their phenolic hydroxyl groups [19]. Higher amounts of flavonoids were recorded in the aqueous extract while lower amounts were recorded in chloroform extract (Table-1). The total flavonoid content in plant extracts depends on the extraction process and the polarity of the solvents used [20]. The presence of high phenolic and flavonoid contents in the extracts may have contributed directly to the antioxidant activity of the species *S. alata*.

DPPH is the most widely used method for evaluating antioxidant activity of natural antioxidants. The rate of reaction of DPPH differs with various antioxidants [21] which results in the difference in the free radical scavenging activity of different compounds.

The presence of scavenging activity of aqueous and methanolic extracts of *S. alata* may be due to the availability of free hydroxyl groups. Our results also suggest that the number of free hydroxyl groups are more in aqueous extracts than in methanolic extracts as the DPPH free radical scavenging activity is more in aqueous extract than in methanolic extracts Figs. 1 and 2).

In general, lower the IC_{50} values, higher will be the antioxidant activity. The scavenging activity of methanolic extract is lower than that of aqueous extract which can be learned from the difference in their IC_{50} values. The difference in their scavenging activities may be due to the difference in the polarities and the dissolution capacities of the extract in respective solvents. Antioxidant activity of *S. alata* by DPPH assay was carried out earlier by Panichayupakaranant and Kaewsuwan (2004) in methanolic extracts. They have reported that Kaempferol present in the leaf extract was a major contributor for the antioxidant activity of leaf extracts of *S. alata*.

Reducing power ability of a compound is the indicator of the potential antioxidant activity of a compound. The reducing ability of a compound depends on the number of reductones present in the compound. These reductones inhibit free radicals chain by donating a hydrogen ion. The reducing power ability of both aqueous and methanolic extracts increased with an increase in the concentration of the test extract indicating the presence of higher concentrations of reductones in leaf extracts of *S. alata*.

Generation of OH^* radicals is crucial for the irreversible damage imposed by oxidative stress [6]. In biological systems, hydroxyl radicals are highly reactive and are generated by Fenton reaction. Hydroxyl radical scavenging activity of a test compound can be calculated by determining the inhibition of degradation of deoxyribose by free radicals [22].

In our present investigation both aqueous and methanolic leaf extracts exhibited a potent antioxidant activity. The hydroxyl radical scavenging activity of aqueous extract was found to be higher than the methanolic extract which can be inferred from their IC_{50} values in *S. alata*. From the above results it is clear that both the leaf extracts were capable of removing hydroxyl radicals from the deoxyribose sugar and forbid their degradation.

Phosphomolybdenum assay is used for determining the total antioxidant capacity of a compound [23]. Phosmomolybdenum assay is dependent on the principle of reducing MO (VI) to phosphomolybdenum (MO (V) by the test compound which can be detected by the formation of Mo (V) green coloured complex which shows maximum absorption at OD= 695nm. All the extracts tested were capable of reducing Mo (VI) complex to a green coloured phosphomolybdenum (Mo (V) complex indicating the strong antioxidant ability of the test extract of *S. alata*.

CONCLUSIONS:

Thus, it can be inferred that methanolic extracts of *S. alata* have higher antioxidant activity compared to the aqueous extracts and can be used as natural antioxidants like red wine, green tea and cocoa.

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