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STUDY OF ANTIOXIDANT PROPERTIES AND PHYTOCHEMICAL CONSTITUENTS OF *SPHAGNETICOLA TRILOBATA* L. LEAVES EXTRACT

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Keywords:

Sphagneticola trilobata L., Antioxidant activity, Phytochemical analysis, DPPH radical scavenging Assay and FRAP radical scavenging assay

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ABSTRACT: *Sphagneticola trilobata* L. has been used to treat various diseases in worldwide. This study investigated the antioxidant activity and phytochemical constituents in chloroform and ethanol extract of *Sphagneticola trilobata* L. The extract of *Sphagneticola trilobata* L. was screened for antioxidant potential through DPPH assay, FRAP radical scavenging assay and phytochemical analysis was carried out for the detection of active secondary metabolite or different constituents such as alkaloids, flavonoids, proteins, carbohydrates and phenolic compound. Different concentrations of extract were analyzed and ascorbic acid was used as a standard antioxidant. IC_{50} and % inhibition were evaluated. *S. trilobata* L. leaves extracts of methanol and chloroform was found to be highly significant with the least IC_{50} value ranging from 68.0 to 75.0 µg/ml in DPPH. Chloroform and methanol extracts of *S. trilobata* L. leaves showed excellent FRAP free radical scavenging activity with IC_{50} value ranging from 0.55 ± 0.042 and 0.55 ± 0.03 respectively and ascorbic acid 0.63 ± 0.01 and the extract was found to contain large amounts of secondary metabolites such as alkaloids, flavonoids and carbohydrates. These findings suggested that extract of *S. trilobata* L. leaves has a potent antioxidant ability, which could be the reason for its use in oxidative stress-related diseases.

INTRODUCTION: Antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property. Antioxidants can decrease oxidative stress-induced carcinogenesis by direct scavenging of ROS and or by inhibiting cell proliferation secondary to the protein phospho-rylation. During excessive metabolism and oxidative stress-free radicals are generated within our cells cause extensive damage to nearby cells, mitochondria, DNA that leads to age-related degenerative diseases, cancer, diabetes, and other human diseases.

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Most antioxidants come from plants and include vitamins A, C, and E and carotenoids such as beta-carotene, minerals, phenolic compounds and other natural chemicals with antioxidant properties. Wedelia leaves are much used in soaking various sorts of powders for the preparation of pills in many countries where this plant is unavailable as a medicinal herb. The pills are used for many common ailments as a serious disease like Liver problems, Wound healing stimulation, Brain toxic, mental functions enhancer, stress reliever, Infections, Digestive tract ailments, Inflammations and pains and Respiratory tract diseases.

MATERIALS AND METHODS:

Plant Collection and Identification: *Sphagneticola trilobata* L. is commonly known as Wedelia. The plant of *S. trilobata* L. was collected from Sri Siddhartha medical college Heggere, Tumakuru

district, Karnataka, India and plant were botanically identified as *Sphagneticola trilobata* L. belongs to Asteraceae family with the help of regional Floras and taxonomists and finally confirmed with the herbarium of Atlas of Florida plants (ISB), Accession No. 240046.

Preparation of Extract: The collected plants were brought to the laboratory washed with tap water and distilled water. The washed material was shade dried and the dried samples were then ground to a fine powder using a mixer grinder. Solvent extraction was carried out using the Soxhlet apparatus. Powdered materials (20gm) were placed in a porous thimble in the upper chamber. In the lower chamber boiling flask, the extracting solvent such as methanol and chloroform (250 ml) was added; the flask was heated by thermostat-controlled heating mantle. The temperature was adjusted based on the boiling point of the solvent. The solvent was heated to reflux and extracted. The sample filled in the thimble was extracted with the non-polar solvents successively till colorless, then extract was weighed and preserved in a brown airtight bottle at 5 degrees Celsius until further use.

Screening for Phytochemicals: Phytochemical analysis of methanol and chloroform extract was carried out for the detection of active secondary metabolites or different constituents such as alkaloids, flavonoids, proteins, carbohydrates and phenolic compounds. Phytochemical analysis of *S. trilobata* L. was carried out by adopting standard methods.

Test for Alkaloids: To determine alkaloids in plant extract sample using Harborne gravimetric method.

Quantitative Test for Presence of Alkaloids: Take 2gm of powder sample in 10 ml of ethanol. Shake vigorously for a few minutes. Filter the mixture using Whatman no. 40 filter paper. Take 2 ml of filtrate into a test tube and add 3 drops of picric acid and mix thoroughly. The formation of light green coloration indicates presence alkaloids.

Quantitative Determination of Alkaloids: Take 5gm. of plant powder sample into 250 ml of the beaker and add 200 ml of 20% acetic acid. Keep the mixture at room temperature of 4 h. Filter the suspension using filter paper. Concentrate the filter by evaporating over a steam bath to 1/4 of its original volume. After cooling, add dropwise Ammonium hydroxide to precipitate the alkaloid in an extract solution. Filter the precipitate alkaloids using preweighed filter paper. After filtration, wash the precipitate with 9% ammonium solution and dry in the oven at 60 °C for 30 min, cool in a desiccator and reweigh.

Test for Flavonoids: To determine flavonoids in plant extract sample using Harborne 1973 gravimetric method.

Quantitative Test for Presence of Flavonoids: Take 2 ml of aqueous extract into a test tube and few drops of bench concentrated ammonia. The formation of a yellow coloration shows the presence of flavonoids.

Confirmation Test: Add a few drops of concentrated HCl into the yellow solution, which turned colorless.

Quantitative Determination of Flavonoids: weigh 5 gm of plant powdered sample into a conical flask, add 50 ml distilled water, add 2 ml of HCl solution and boil for 30 min in a water bath and allow for cooling. The mixture is filtered through filter paper. Add 10 ml of ethyl Acetate and shake vigorously for 2 min; let layers separate. Drain out the lower aqueous player. Filter the solvent extract into a preweighed filter paper. Confirm the complete filtration and remove the filter paper carefully with the help of a needle. Place the filter paper for drying in an oven at 60 °C for at least 30 min. After drying, cooled by placing it in a desiccator for constant temperature and then weighed immediately.

Test for Carbohydrates: To determine total carbohydrates in plant samples using DNS method.

Weigh 100 mg of sample into a boiling tube. Hydroloids by keeping it in boiling water bath for 3 h with 5 ml of 2.5 N HCl and cool to room temperature. Neutralize it with solid sodium carbonite until the effervescence ceases. Make up the volume to 100 ml and centrifuge. Collect the supernatant and take 0.5 and 1 ml aliquots for analysis. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard "0" serves as blank. Make up the volume of 1 ml in all the tubes including the sample tubes by adding

distilled water. Then add 1 ml of dinitro salicylic acid reagent and heat for 8 min in boiling water bath. Cool rapidly and add 5 ml of distilled water. Read the observance at 540 nm. The standard graph by plotting concentration of standard on the "X" axis verses absorbance on "Y" axis. Calculate the amount of carbohydrate presenting sample tube.

Test for Phenolic Compound: To determine phenol in plant extract sample using folin clocalteu spectrophotometric method.

Preparation of Reagent A: 75 ml of folin clocalteu reagent is made up to 750 ml with deionised water.

Preparation of Reagent B: 57.5 gm of sodium carbonate is dissolved in deionized water and filled up to 500 ml with deionized water.

Standard Solution: Dissolve 0.500gm of dry gallic acid in 10 ml of ethanol and dilute to volume with water in a 100 ml volumetric flask can be opened daily, but to store keep closed in a refrigerator up two weeks. Take and then dilute to volume with water. These solutions will have phenol concentrations of 0, 50, 100, 150, 250 and 500 mg/l gallic acid, the effective range of the assay.

Extraction: 50 to 100 mg of dry plant material is extracted with 5 ml of 80% ethanol in a centrifugation tube. 500 mg of fresh plant material with extracted with 4 ml 80% of ethanol in a centrifugation tube. Put the tubes in a water bath for 30 min at 30 °C. Remove and preserve the supernatant after centrifugation at 4500 rpm (2649 gm.) for 10 min. Pipette 1, 2, 5, and 10 ml of above phenol stock solution into 100 ml volumetric flask.

Extract the pellet again with 2.5 ml of 80 % ethanol put the tubes in a water bath for 30 min at 30 °C; after centrifugation, the supernatant is added to the previous supernatant. To remove other compounds such as chlorophyll and lipids because that can disturb the color reaction. The extract solution with 5 ml chloroform and 2.5 ml deionized water. Remove the chloroform by centrifugation for 10 min at 4500 rpm. The ethanol-water fraction is ready to be used for the determination. The residue can be used for the extraction of insoluble sugar (fructones and starch).

Determination: Transfer 0.1 ml of the sample or standard solution to a test tube. Add 5 ml of folin ciocaltue reagent mix well. After 0.5 to 8 min, 3.5 ml of sodium carbonate reagent B is added mix well incubate the tubes either 2 h at 20 degrees or 1 hour at 40 °C in a water bath. Measure the absorbance at 765 nm with a spectrophotometer. The colour stable for several hours.

Test for Proteins:

To Determine Proteins in Plant Sample using LOWRY'S Method:

Extraction of Proteins from Sample: Extraction is usually carried out with buffers used for the enzyme assay weigh 500 mg of sample and grained well with pestle and mortar in a 5 to 10 ml of buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of Proteins: Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standards into a series of test tubes. Pipette out 0.5 ml and 0.2 ml of sample extract in two other test tubes. To the test tubes, makeup to 1 ml distilled water. A tube with 1 ml of water server as the blank add 5 ml of reagent C to each test tubes, including the blank and mixed well and allow standing for 10 min. And 0.5 ml of reagent D mix well and incubate at room temperature in the dark for 30 min. The blue color is developed. Take the readings at 660 nm. Standard graphs are drawn and calculate the amount of proteins in the sample.

Determination of Antioxidant Activity:

DPPH Radical Scavenging Assay: Free radical scavenging activity of different extracts were measured in terms of hydrogen donating or radical scavenging ability using stable radical DPPH as described by Blois method. 3 ml of methanol is taken in all test tubes in different concentrations and solutions (20, 40, 60, 80 and 100 mg) were made using the stock solution (0.01gm of extract dissolved in 1 ml of methanol). The standard ascorbic acid solution was prepared by dissolving 0.001gm of ascorbic acid in 1 ml methanol. For Negative standard solution 3 ml of methanol was taken in another test tube then 1 ml DPDH was added to all the solution. The mixtures were vigorously shaken. Now all the mixture were kept for incubation in the dark for one hour after incubation, observance was measured for all the

mixture, including negative controlled and standard ascorbic solution. The DPPH scavenging activity was calculated using the formula,

DPPH scavenging Effect (%) = Ac - Ab / Ac ×100

Ac-Absorbance of Negative control
Ab-Absorbance of sample

FRAP Radical Scavenging Assay: Ferric reducing antioxidant power (FRAP) assay.

Principle: FRAP assay stands for Ferric Reducing Antioxidant Power Assay. The FRAP assay is high throughout, adaptable and can detect antioxidant capacities as low as 0.2 Mm Fe^{2+} equivalents. The assay measures the antioxidant potential in samples through the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants present in the samples. Following the reduction of the ferric iron, a blue color develops that is read colorimetrically at 593 nm.

Materials:

1. Acetate buffer (300 Mm, pH 3.6).
2. 10 Mm TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (# T1253, Sigma- Aldrich).
3. 40 Mm HCl.
4. 20 Mm $FeCl_3$.
5. Adjustable multichannel pipettes and a pipettor (Benchtop, USA).
6. 96-well plate (From Corning, USA).
7. 1.5 ml centrifuge tubes (TORSON).
8. 10 ml serological pipettes (TORSON).
9. 10 to 1000 ul tips (TORSON).
10. 96-well ELISA plate reader or spectrophotometer capable of measuring the absorbance (ELX-800 Biotek).
11. 37 °C incubator (Healforce, China).

Procedure:

1. The FRAP reagent was prepared by mixing acetate buffer (300 Mm, pH 3.6), a solution of 10 Mm TPTZ in 40 Mm HCl and 20 Mm $FeCl_3$ at 10:1:1 (v/v/v).
2. The reagent (3.400 µl) and 100 µl of test sample/standard solutions with different concentrations (100 to 500 µg/ml) were added and mixed thoroughly and incubated for 30 min.
3. 200µl of each mixture was taken into 96 well plates and the absorbance was taken at 593 nm.
4. All solutions were used on the day of preparation.

FRAP (Mm) = (ΔA_{593nm} of sample from 0 to 4min) × FRAP value of standard (1000mM) / (ΔA_{593nm} of standard from 0 to 4min)

RESULTS:

Qualitative Screening for Phytochemicals: The phytochemicals were extracted from *S. trilobata* L. methanolic and chloroform solvent. The preliminary qualitative analysis indicates the presence of alkaloids, flavonoids, carbohydrates, phenolic compounds and protein was shown in **Table 1**. Compare to other extracts; the methanolic extract was abundant with secondary metabolites.

TABLE 1: QUALITATIVE SCREENING FOR PHYTO-CHEMICALS IN *S. TRILOBATA* L.

S. no.	Phytochemical constituents	Methanolic extract	Chloroform extract
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Carbohydrates	+	+
4	Phenolic compound	+	+
5	Protein	+	+

Quantitative Screening for Phytochemicals: The methanol extract showed a high % of Flavonoids (39.5%) followed by Protein (5.2%), Alkaloids (4.5%), Phenolic compounds (1.45%) and Carbohydrates (0.5%)..

The chloroform extract showed a high % of Carbohydrates (38.3%) followed by flavonoids (29.3%), Alkaloids (3.7%), Proteins (3.5%), and Phenolic compounds (0.95%) was shown in

TABLE 2: QUANTITATIVE SCREENING FOR PHYTOCHEMICALS IN *S. TRILOBATA* L.

Sample	Alkaloids (%)	Flavonoids (%)	Phenolic compounds (%)	Proteins (%)	Carbohydrates (%)
Methanol extract	4.5±0.01	39.5±0.1	1.45±0.02	5.2±0.41	05±0.1
Chloroform extract	3.7±0.3	29.3±0	0.95±0	3.5±0.5	38.3±0.03

Antioxidant Activity: The free radical scavenging activity of the plant leaves extract was evaluated by hydrogen stable radical DPPH. The ability of the extracts to neutralize donating ability using hydroxyl radical was expressed as 50% inhibition concentration in µg/ml.

The Methanol extract of *S. trilobata* L. leaves was most active with IC_{50} value at 68.0 ± 0.36 µg/ml. Chloroform extract showed with IC_{50} value at 75.0 ± 0.31 µg/ml. The methanol extract of *S. trilobata* L. leaves showed the maximum radical scavenging activity compared to standard ascorbic acid 92.0 ± 0.5 µg/ml.

In FRAP radical scavenging assay, Antioxidant capacity of *S. trilobata* L. leaves showed the least IC_{50} value for Methanol 0.55 ± 0.03 µg/ml compared to standard ascorbic acid 0.63 ± 0.01 µg/ml. The antioxidant efficiency of *S. trilobata* L. leaves extract by DPPH and FRAP methods increases with an increase in concentration, and the data are depicted in **Fig. 1A, B** & **C**.

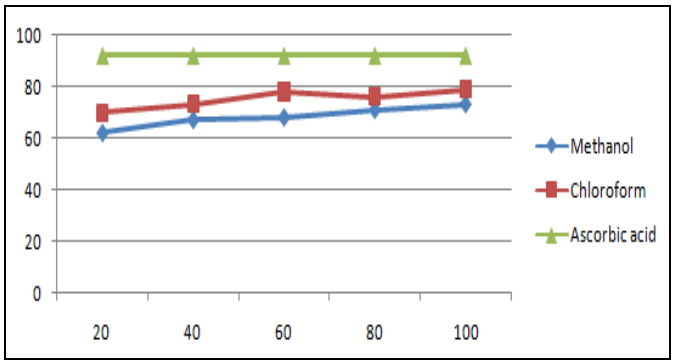

FIG. 1A: DPPH FREE RADICAL SCAVENGING ACTIVITY OF DIFFERENT EXTRACT OF *S. TRILOBATA* L. LEAVES AND STANDARD ASCORBIC ACID AT DIFFERENT CONCENTRATION (20-100 µg/ml)

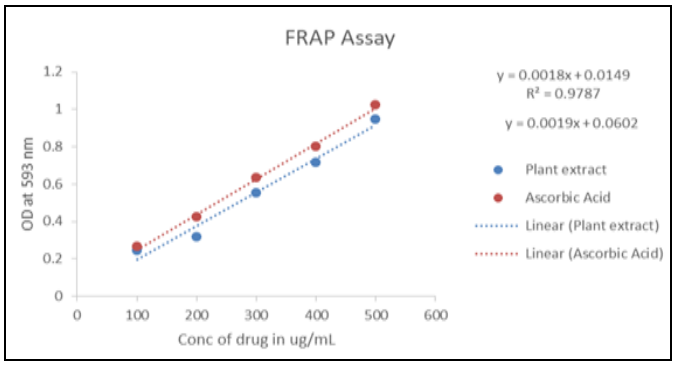

FIG. 1B: FRAP RADICAL SCAVENGING ACTIVITY OF METHANOL EXTRACT OF *S. TRILOBATA* L. LEAVES AND STANDARD ASCORBIC ACID AT DIFFERENT CONCENTRATION (100-600 µg/ml)

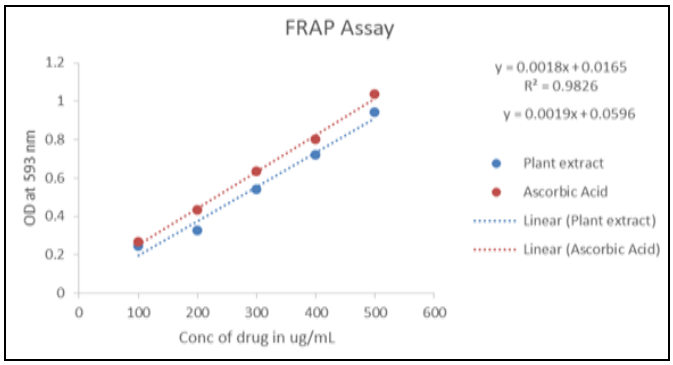

FIG. 1C: FRAP RADICAL SCAVENGING ACTIVITY OF CHLOROFORM EXTRACT OF *S. TRILOBATA* L. LEAVES AND STANDARD ASCORBIC ACID AT DIFFERENT CONCENTRATION (100-600 µg/ml)

TABLE 3: THE IC_{50} VALUES OF DPPH RADICAL AND FRAP RADICAL SCAVENGING ASSAY OF DIFFERENT EXTRACTS OF *S. TRILOBATA* L. LEAVES

Solvent extracts	IC_{50} (µg/ml)	
	DPPH	FRAP
Methanol	68.0±0.36	0.55±0.03
Chloroform	75.0 ± 0.31	0.55±0.042
Ascorbic acid	92.0±0.5	0.63±0.01

DISCUSSION AND CONCLUSION: Free radicals are reactive atoms or group of atoms that has one or more electrons, one that is produced in the body by natural biological processes or introduced from outside sources like tobacco smoke, toxins, pollutants and that can damage cells, proteins and DNA by altering their chemical structure. Several free radical scavenging methods are performed for the determination of antioxidant activity. The free radical scavenging ability of the sample is the measure of antioxidant activity. In the current study, DPPH scavenging assay and FRAP scavenging assay are selected for the determination of antioxidant activity. In our study showed that methanol and chloroform extracts of *S. trilobata* L. leaves were found to be highly significant with least IC_{50} value ranging from 68.0 to 75.0 µg/ml in DPPH.

Chloroform and methanol extracts of *S. trilobata* L. leaves were found to be a highly significant value ranging from 0.55 ± 0.042 and 0.55 ± 0.03 respectively and ascorbic acid 0.63 ± 0.01 in FRAP free radical scavenging activity.

The antioxidants of plant origin with free radical scavenging properties could have great importance as therapeutic agents in several diseases caused due to oxidative stress. Many synthetic antioxidants compounds have shown toxic or mutagenic effects, which have stimulated the interest of many investigators to search for natural antioxidants. The use of synthetic antioxidants is to be thought twice because WHO's. International agency for research on cancer has evaluated the carcinogenicity of BHA on animal models and suggested the possible role of the intake of synthetic antioxidants in cancer.

Thus, the results obtained in the present study indicate *S. trilobata* L. leaves extracts to have the potential to act as a source of useful drugs because of the presence of various phytochemical components such as carbohydrate, protein, flavonoids, phenolic compounds and alkaloids.

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CONFLICTS OF INTEREST: Nil

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