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Pharmacognostic Investigation and Antioxidant Activity of *Sphagneticola trilobata*

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Sphagneticola trilobata (L.) or Pruski is a medicinal plant and recognized for the treatment of a variety of ailments and partially explored for their pharmacological activity. The main objective of this present study is to find out the pharmacognosy and antioxidant activity of parts of plant *S. trilobata viz.* leaves, stem and aerial part. Pharmacognostic investigations for morphological characteristics, determination of plant content, ash values, extractive values, total tannin, flavonoid, alkaloid and phenol contents and preliminary phytochemical study. Antioxidant activity of the leaves, stem and aerial part of the plant and isolated products of *S. trilobata*, through different methods also included in present work. The morphology of this plant revealed that the leaves are green in colour with white coarse hairs and stem are rounded, green or reddish and maybe coarsely hairy. The antioxidant activity of the methanolic extracts of leaves stem and aerial part of the plant also revealed that the leaves containing higher antioxidant potential compared to other parts. The result of this study could be useful in the identification and preparation of monographs of the plant and also useful in findings the antioxidant potentiality of the plant *S. trilobata*.

Keywords: *Sphagneticola trilobata* (L.), Antioxidant activity, Phytochemical screening, HPLC analysis.

INTRODUCTION

Sphagneticola trilobata (L.) belongs to the Asteraceae family is commonly known as Pruski. *S. trilobata* is native from South America but it also introduced in India and commonly found in wet parts of India [1]. *S. trilobata* is an herbaceous perennial shrub, up to 70 cm tall, forms dense mounded mats over the ground [2]. In an Indian system of medicine, this plant is used for prevention and treatment of various disease as backache, muscle cramp, rheumatism, stubborn wounds, sores, swelling and arthritic pain, fever and malaria [3]. Quantitative determination of some pharmacogenetic parameters is useful to establish standers for crude drugs. Physical continuous assessment is an important parameter in detecting adulteration or improper handling of drugs. Different ash values are important to determine the purity of the drug, the presence and the absence of foreign presence [4]. Since the plant *S. trilobata* is useful in traditional medicine for the treatment of some diseases, it is important to standardize it for use [5]. The drug pharmacogenetic constants, clinical microscopic features and numerical standards stated in this work may be useful for the compilation of a suitable monograph for its proper identification [6]. Numerous pharmacological activities of *S. trilobata* offers been reported such as antimicrobial [7], anti-inflammatory [8], wound healing [9], anthelmintic [10] and anticancer [11].

Free radicals can cause degenerative diseases including cancer. Currently, study has increased interest in discovering therapeutic medicinal plants that have high antioxidant activity to reduce oxidative stress tissue injury [12-14]. In this study, the antioxidant activity of methanolic extract of *S. trilobata* and its isolated compounds are measured by different antioxidant assays. Hence, the objective of the present study is to evaluate various pharmacogenetic parameters such as macroscopy, physico-chemical, phytochemical and antioxidant studies of different parts of *Sphagneticola trilobata* plant.

EXPERIMENTAL

Sphagneticola trilobata grows as weed were collected from Noida Institute of Engineering and Technology (Pharmacy Institute), Greater Noida, India in October 2020. The parts of plant *i.e.* leaves, stem, aerial parts washed with water and shade dried at ambient temperature for 4-5 days. Dried leaves, stem and aerial parts of the plant were powdered for disintegration

using a grinder and stored in an air-dried container in a cool and dry place to prevent the sample from any potential contamination. A voucher plant specimen (NIET/Pharmacy Institute/R&D/07) was preserved and authenticated by the Botanical Garden of the Indian Republic, Noida, India.

Extraction: The dried powder of parts of plant *S. trilobata* was extracted by Soxhlet apparatus using 100 g per thimble. The solvents used for extraction were distilled water, methanol, chloroform and benzene. The extraction was conducted in 800 mL of each solvent for 15 h/solvent. Each crude extract was filtered and dried using a rotatory evaporator at 60 ºC and stored in a cooled and dry place until further use in the experiments [16].

Isolation: Literature review of the plant shows that the methanolic extracts of aerial parts of *Sphagneticola trilobata* were determined as highly active compared to other extracts, this extract was processed further to isolate more compounds [16]. The crude extract was fractionated using silica gel column chromatography and the elute with a gradually increasing polarity (10% increment) of petroleum ether-chloroform, ethyl acetate-chloroform and ethyl acetate-methanol, respectively. All the fractions were subjected to thin-layer chromatography (TLC) and those with similar components were combined. Using this procedure two fractions were obtained. Active fraction A (90% chloroform-10% petroleum ether) having, R_f value of 0.63 and it appears as reddish-brown semi-solid and active fraction B (40% ethyl acetate-60% chloroform) having R_f value 0.51 and it appears as dark greenish semi-solid.

The isolated compound was subsequently subjected to structural analysis using ^{1}H NMR, ^{13}C NMR, IR and LC-MS.

Macroscopy: The leaf and stem parts were separated from the other parts, washed, rinsed, cleaned and dried for further use. The following macroscopic character of fresh leaves and stem were noted: colour, odor, taste, shape, size, surface, vein, appearance or absence of petiole, apex, margin base, lamina, texture, *etc*. [17].

Fluorescence analysis: Crude drugs show their characteristics of fluorescence when exposed to ultraviolet radiation and rely on their chemical components. This analysis is useful to identify adulteration during crude drug evaluation. In the present study, 5 g of crude drug was taken in a watch glass and fluorescent analysis was performed after treatment with various such reagents [18].

Determination of extractive values: Considering the variety and chemical nature of the drug, various solvents *viz.* distilled water, petroleum ether, chloroform, methanol, ethyl acetate and benzene were used to determine the values of extracts. About 5 g of powdered material was subjected to continuous Soxhlet extraction with 100 mL distilled water, petroleum ether, chloroform, methanol, ethyl acetate, benzene as solvents. Wherever the evaluation of chemical composition is applicable, determining the preventive value of the crude drug is beneficial in the evaluation process. After extraction, the extracts are concentrated in a rotatory evaporator and dried in vacuum desiccators. Then the expected values are calculated as the percent weight by the weight of the solvent-soluble extractor for the air-dried drug [19].

Primary phytochemical analysis: A proper primary phytochemical screening of plant material (leaves, stem and aerial part) is the first step toward identifying the secondary metabolites of the plant and establishing a chemical profile of crude drug for its proper evaluation. Extracts obtained by continuous Soxhlet by using different solvents *viz.* distilled water, methanol, chloroform, benzene, petroleum ether, ethyle acetate was subjected to a standard qualitative test to identify the presence of chemical compounds like alkaloid [20], glycoside [21], tannins [22], flavonoids [23], sterols [24], fats [25], oils [26], phenols [27] and saponins [28] present in the parts of plants.

Determination of ash values: For the determination of the ash content of the drug, approximately 5 g of powder was dispersed in a pre-ignited and loaded silica crucible. The crucible was then stirred slowly to make the crucible free of carbon. After cooling the crucible was weighted to obtain the total ash content and then the ash was subjected to determination of acid-insoluble [29], water-soluble [30] and sulfated ash [31] content. Percent total ash was calculated by using an air-dried sample as the standard.

Determination of total flavonoid content: The total flavonoid content of methanol of leaves, stem and the aerial portion of *Sphagnaticola trilobata* was measured by aluminum chloride colourimetry assay. For this process, 1 mL of each extract was mixed with 2 mL of distilled water and taken into a flask with a volume of 10 mL. In a volumetric flask, 0.3 mL of 5% sodium nitrate was treated and after 5 min, 0.3 mL of 10% $AlCl_3$ was mixed. After 5 min, 2 mL of 1 M NaOH was treated and diluted to 10 mL with distilled water. A set of reference standard solution of quercetin (10, 20, 30, 40 and 100 µg/mL) was prepared in the same manner as previously described. The absorbance of the test and standard solutions was determined with a UV visible spectrophotometer against reagent blank at 510 nm. The total flavonoid content was expressed as milligrams of quercetin equivalent/g extract [32].

Determination of total phenolic content: The concentration of phenolics in methanolic extracts of leaves, stem and aerial parts was determined using the spectrophotometric method. The Folin-Ciocalteu assay method was used for the determination of the total phenolic content. For this procedure, 1 mL of extract of 9 mL of distilled water is taken in 25 mL of volumetric flask. Folin-Ciocalteu phenol reagent (1 mL) was treated to the mixture and shake well. After 5 min, 10 mL of 7 % Na_2CO_3 solution was treated to the mixture. The volume was made up of 25 mL. A set of a standard solution of gallic acid (10, 20, 30, 40 and 100 µg/mL) was prepared in the same manner as described earlier. Incubated for 90 min at room temperature and the absorbance for the test and the standard solution was determined against the reagent blank at 550 nm with a UV/visible spectrophotometer. Total phenolic content was expressed as mg of GAE/g of the extract [33].

Determination of tannin content: Tannin was determined by the Folin-Ciocalteu method. Approximately 0.1 mL of methanol extracts of leaves, stem and aerial portion were added to a 10 mL volumetric flask containing 7.5 mL distilled water and 0.5 mL of Folin-Ciocalteu phenol reagent, 1 mL of 35% Na_2CO_3 solution and diluted by 10 times in distilled water.

The mixture was stirred well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (10, 20, 30, 40 and 100 µg/mL) was prepared in the same manner as previously described. Absorbance for the test and standard solutions was measured against a blank at 725 nm with a UV/visible spectrophotometer. The tannin content was expressed as milligram gallic acid/g of extracts [34].

Determination of alkaloid content: Methanolic extract of the stem and aerial part of the leaves (1 mL) was dissolved on DMSO, 1 mL of 2N HCl was added and filtered. This solution was added to a separate funnel, 5 mL bromocresol green solution and 5 mL phosphate buffer. The mixture was shaken with vigorous shaking with 1, 2, 3 and 4 mL chloroform and assembled on a 10 mL volumetric flask and diluted to volume with chloroform. A set of reference standard solutions of atropine (10, 20, 30, 40 and 100 µg/mL) was prepared in the same manner as described earlier. The absorbance of the test and standard solutions was determined with a UV/visible spectrophotometer against reagent blank at 470 nm. Total alkaloid content was expressed as equal to milligram/extract of atropine [35].

HPLC analysis: A literature survey of the plant shows that the methanolic extract of the aerial parts of *Sphagneticola trilobata* was determined as highly effective as compared to other extracts. The methanolic extract of the aerial part (50 mg) is taken and used for the HPLC analysis. The column used for analysis is C18 250 × 4.6, 5 µm. The wavelength of 254 nm was set and the sample was injected. The chromatographic separation was accomplished using solvent system (A) consisting of acetonitrile and solvent system (B) 0.1% [5:95:CH_3CN:H_2O] buffer, filtered through a filter using value stag vacuum pump. The mobile phase was pumped at a flow of 1.5 mL/min at room temperature [36].

Determination of antioxidant activity

DPPH radical scavenging assay: Antioxidant activity was evaluated by Bliss's method using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. The reaction mixture consisted of 100 µM DPPH in methanol and various concentrations of compounds (0.5-2.5 µg/mL). The absorbance at 517 nm was determined after 30 min at room temperature and the scavenging activity was calculated as a percentage of radical reduction. Each experiment was performed in triplicate. BHT was used as a reference compound [37].

Nitric oxide scavenging assay: The interaction of methanolic extracts of *Sphagneticola trilobata* with nitric oxide (NO) was evaluated by the nitrite detection method. The chemical source of NO was sodium nitroprusside (10 mM) in 0.5M phosphate buffer, pH 7.4, which spontaneously produces NO in an aqueous solution. The NO interacted with oxygen to produce stable products, producing nitrites. After 60 min of incubation at 37 ºC, Griess reagent (naphthyl-ethylenediamine 0.1% in water and sulphanilic acid 1% in H_3PO_4) was added. The same reaction mixture without extracts of a sample but with equal amounts of distilled water served as a control. Ascorbic acid was used as a positive control [38].

Hydrogen peroxide radical scavenging assay: The ability of extracts to discard off H_2O_2 was determined according to Navi's method. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer, pH 7.4. The concentration of hydrogen peroxide was assessed by absorbance at 230 nm, after 10 min against an empty solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard [39].

Ferric reducing antioxidant power (FRAP) assay: The FRAP procedure was followed as described by Benzie & Strain [40]. Briefly, FRAP reagent containing 5 mmol of $FeCl_3$ (20 mmol/L) and 50 mL of (10 mmol/L) TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40 mmol/L of HCl. Acetate buffer, (0.3 mmol/L, pH = 3.6, 5 mL) and freshly prepared and heated at 37 ºC. The sample extract (0.5-2.5 µg/mL) was mixed with 3 mL of FRAP reagent and the absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37 ºC for 10 min [40].

Phosphomolybidnum method: Methanolic extracts of leaves, stem and and aerial part of the plant were studied at the concentrations of 50-500 mg/mL. The methanolic extract of *S. trilobata* (0.5 mL) was mixed with a phosphomolybdenum reagent in a combination of 2 mM sodium phosphate and 4 mM ammonium molybdate. Sulfuric acid (0.6 M) was added in a capped test tube. The reaction mixture was then incubated on a water bath for 100 min at 95 ºC. After incubation, allow the test tube to cool to room temperature and absorbance of dilution was measured at 695 nm. The blank contains 0.3 mL of methanol in place of the drug with phosphomolybdenum reagent [41].

RESULTS AND DISCUSSION

The macroscopic studies of leaves revealed that the leaves are glossy green in colour. Polar green below, with simple coarse white hairs, serrated margins. The fresh leaves have an agreeable odor, the taste is slightly acrid. And the macroscopic studies of the stem show that the stem was rounded, green or reddish and maybe coarsely hairy. They develop roots at their nodes. Short, semi-upright, flowering branches were produced of their stems.

Phytochemical screening: Preliminary phytochemical screening of leaves, stem and aerial parts of *Sphagneticola trilobata* mainly revealed the presence and absence of different phytochemicals in different solvents (Table-1). While the different extractive values are shown in Table-2.

Fluorescence analysis: The fluorescence analysis of leaves, stem and aerial parts are shown in Table-3, respectively.

Total flavonoid content: The concentration of flavonoids of methanolic extracts of leaves, stem and aerial parts was found using a spectrophotometric method with $AlCl_3$. The contents of flavonoids were expressed in terms of quercetin equivalents (standard curve equation: $y = 0.0005x + 0.0034$, $R^2 = 0.9981$). The concentration was found to be mg of quercetin equivalent/g; shown in Table-4.

Total phenolic content: The total phenolic contents of methanolic extracts of leaves, stem and aerial parts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: $y = 0.0004x + 0.0116$, $R^2 = 0.9921$). The values obtained of total phenols are expressed as mg of GAE/g of extract (Table-4).

TABLE-1
PHYTOCHEMICAL SCREENING OF LEAVES, STEM AND AERIAL PART OF *Sphagneticola trilobata*

Type of constituents	Distilled water			Petroleum ether			Chloroform			Methanol			Ethyl acetate			Benzene		
	L	S	AP	L	S	AP	L	S	AP	L	S	AP	L	S	AP	L	S	AP
Alkaloids	–	–	+	–	–	–	+	+	+	–	–	–	–	+	–	–	–	–
Glycosides	+	–	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
Carbohydrate	+	–	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
Reducing sugar	+	–	+	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
Flavonoids	–	+		–	–	–	–	–	–	–	+	–	+	+	+	–	–	–
Tannins and phenolic	–	+	+	–	–	–	–	–	–	+	+	+	–	–	–	–	–	+
Protein and amino acids	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	+	–
Gum and resin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
triterpenoids	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–
Saponins	+	+	+	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
Steroids and sterols	–	–	+	+	+	+	–	–	–	+	–	+	–	–	–	–	–	–

L = Leaves, S = Stem, AP = Aerial part

TABLE-2
EXTRACTIVE VALUES OF LEAVES, STEM AND AERIAL PARTS OF *Sphagneticola trilobata*

Type of solvents	% Extractive value mean ± SD		
	Leaves	Stem	Aerial part
Distilled water	7.128 ± 0.68	5.234 ± 0.23	6.328 ± 0.59
Petroleum ether	2.632 ± 0.63	1.235 ± 0.56	2.056 ± 0.58
Chloroform	1.523 ± 0.56	0.965 ± 0.45	1.235 ± 0.85
Methanol	5.923 ± 0.23	4.118 ± 0.23	4.956 ± 0.78
Ethyl acetate	3.328 ± 0.89	2.568 ± 0.58	3.235 ± 0.25
Benzene	3.956 ± 0.23	2.963 ± 0.45	3.629 ± 0.52

TABLE-4
TOTAL FLAVONOID, TOTAL PHENOLIC, TOTAL TANNIN AND TOTAL ALKALOID CONTENTS OF LEAVES, STEM AND THE AERIAL PART OF *Sphagneticola trilobata*

Part of plant	Total flavonoid content	Total phenolic content	Total tannin content	Total alkaloid content
Leaves	5.82 mg	10.78 mg	7.81 mg	1.22 mg
Stem	2.63 mg	6.23 mg	8.67 mg	0.95 mg
Aerial	4.97 mg	8.65 mg	6.24 mg	1.02 mg

Total tannin content: The tannins contents were examined in methanolic extracts of leaves, stem and aerial parts using Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalents (the standard curve equation: $y = 0.0006x + 0.0019$, $R^2 = 0.9958$). The values obtained for the concentration of tannin are expressed as mg of GA equivalent/g (Table-4).

Total alkaloid content: The alkaloid contents were examined and expressed in terms of atropine equivalent/g of extract (the standard curve equation: $y = 0.0007x + 0.0083$, $R^2 = 0.9985$). The values obtained for the concentration of alkaloid are expressed as mg of atropine equivalent/g (Table-4).

Ash and extractive values: The estimated values of ash are also given in Table-5.

HPLC analysis: The extract solution was injected and chromatograms were recorded. Two peaks were observed at the retention time of 11.20 min and 11.84 min. The chromatogram of extract at 254 nm in acetonitrile solvent system (A) and solvent system (B) are shown in Fig. 1.

Antioxidant activity: Antioxidant activity of the methanolic extracts of *S. trilobata* leaves, stem, aerial parts and two

TABLE-3
FLUORESCENCE ANALYSIS OF DIFFERENT EXTRACTS AND POWDER OF LEAVES, STEM AND AERIAL PART OF *Sphagneticola trilobata* WITH THE VARIOUS REAGENT

Extract/Powder	Colour								
	Daylight			Short UV (254nm)			Long UV (365 nm)		
	Leaves	Stem	Aerial part	Leaves	Stem	Aerial part	Leaves	Stem	Aerial part
Petroleum ether extract	Light green	Light green	Light green	Green	Green	Green	Dark green	Yellowish green	Dark green
Chloroform extract	Pale brown	Light brown	Dark green	Dark brown	Pale brown	Dark brown	Greenish brown	Greenish brown	Greenish brown
Ethyl acetate extract	Brown	Yellowish-brown	Brown	Yellowish-brown	Yellowish-brown	Yellowish-brown	Greenish brown	Greenish brown	Greenish Brown
Methanolic extract	Greenish black	Yellowish green	Greenish brown	Black	Dark green	Black	Black	Dark green	Black
Powder	Greenish brown	Yellowish green	Green	Greenish brown	Yellowish green	Brown	Greenish brown	Yellowish green	Brown
Powder + sodium hydroxide in methanol	Greenish black	Greenish brown	Greenish black	Greenish black	Greenish brown	Greenish black	Greenish black	Greenish brown	Greenish black
Powder + sodium hydroxide in water	Dark brown	Pale brown	Dark brown	Brown	Dark green	Brown	Dark brown	Dark green	Dark brown
Powder + 1 N hydrochloric acid	Dark brown	Pale brown	Dark brown	Fluorescent green	Light green	Green	Dark brown	Dark green	Dark brown

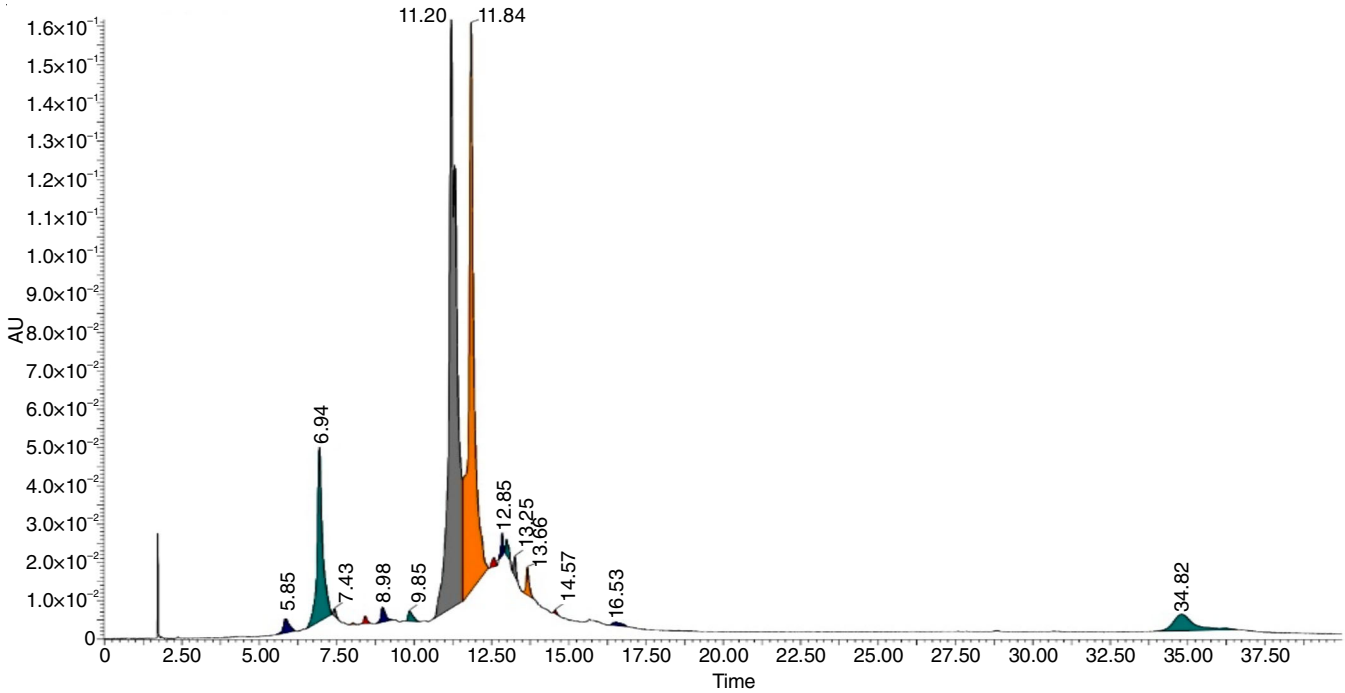

Fig. 1. HPLC profile of extracts from aerial parts of *Sphagneticola trilobata* at 254 nm on SUNFIRE C18 250 × 4.6, 5 µM column. The peak at the retention time of 11.20 and 11.84 min

TABLE-5
ASH VALUES OF LEAVES, STEM AND AERIAL PART OF *Sphagneticola trilobata*

Ash values	% w/w (mean ± SD)		
	Leaves	Stem	Aerial parts
Total ash	6.42 ± 0.52	5.23 ± 0.46	6.95 ± 0.25
Acid insoluble ash	2.21 ± 0.78	2.04 ± 0.80	2.33 ± 0.66
Water insoluble ash	2.92 ± 0.55	2.13 ± 0.59	3.46 ± 0.28
Sulphated ash	0.97 ± 0.58	0.76 ± 0.24	1.12 ± 0.36

isolated compounds (compound A and B) was measured by DPPH method, H_2O_2 method, nitric oxide method, phosphomolybdinum method and ferric antioxidant method.

Ferric reducing antioxidant assay: The results revealed that the methanolic extract of leaves, stem and aerial parts possess IC_{50} values of 19.05, 18.68 and 19.77 µg/mL, respectively. These values were higher than the standard values of ascorbic acid (12.63 µg/mL). Whereas, the IC_{50} values of the isolated compounds A & B were 38.44 and 31.62 µg/mL, respectively. This shows that the leaves of *S. trilobata* possess high antioxidant activity as compared to stem and aerial parts of the plant. These results also showed that isolated compound B show higher antioxidant activity as compared to isolated compound A.

DPPH assay: The DPPH free radical scavenging method results revealed that the methanolic extract of leaves, stem and aerial parts possess IC_{50} values of 18.64, 39.07 and 31.6 µg/mL, respectively. Whereas, the IC_{50} values of the isolated compounds A & B were 22.61 and 32.09 µg/mL, respectively. This shows that the leaves of *S. trilobata* possess high antioxidant activity as compared to stem and aerial parts of the plant. However, using DPPH method, isolated compound A shows the higher antioxidant activity as compared to isolated compound B.

Hydrogen peroxide assay: In the H_2O_2 free radical scavenging method, the results revealed that the methanolic extract of leaves, stem and aerial part possess IC_{50} values of 27.04, 63.89 and 47.43 µg/mL, respectively. Again, these values were higher than the standard values of ascorbic acid, (18.61 µg/mL). The IC_{50} values of the isolated compounds A & B were 25.52 and 27.08 µg/mL. Similarly, using H_2O_2 free radical scavenging method, isolated compound A shows the higher antioxidant activity as compared to isolated compound B.

Nitric oxide assay: Using nitric oxide free radical scavenging method, the methanolic extract of leaves, stem and aerial part possess IC_{50} values of 21.57, 31.23 and 22.60 µg/mL, respectively. Whereas, the IC_{50} values of isolated compounds A & B were 31.16 and 44.41 µg/mL, respectively. Thus, again isolated compound A shows the higher antioxidant activity as compared to isolated compound B.

Phosphomolybdenum assay: Applying the phosphomolybdenum free radical scavenging method, the results revealed that the methanolic extract of leaves, stem and aerial parts possess IC_{50} values of 33.95, 32.05 and 44.31 µg/mL, respectively. The IC_{50} values of isolated compounds A & B were found to be 45.26 and 60.42 µg/mL. This shows that the stem of *S. trilobata* possesses high antioxidant activity as compared to leaves and aerial parts of the plant. Moreover, isolated compound A shows the higher antioxidant activity as compared to isolated compound B.

Conclusion

In present investigation, various standardized parameters such as macroscopic, pharmacognostic and phytochemical screening was carried out, which could be helpful in authentication of *Sphagneticola trilobata* (L.). Also based on the above experiments of the antioxidant assay in different models, it is

concluded that the methanolic extracts and isolated compounds from *Sphagneticola trilobata* exhibit high antioxidant activity. The results of present study will also serve as reference material in the preparation of monograph.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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