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RESEARCH ARTICLE

Comparative analysis of antioxidant potential in different species of genus *Thunbergia* via phytochemical screening

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Abstract

Thunbergia, a genus under the family Acanthaceae, is known for important medicinal plants that, in general, exhibit antibacterial, antifungal, antioxidant, anti-inflammatory, and hepatoprotective activities. The present study aims to investigate antioxidant activities in alcoholic and aqueous plant extracts using ABTS (2,2 -Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric ion reducing antioxidant power) for free radical scavenging activity and evaluation of total content of phenolics, flavonoids and ascorbate. The current study is performed in the leaves and stems of *Thunbergia alata*, *Thunbergia erecta*, and *Thunbergia mysorensis* due to their easy availability in the Indian subcontinent and unexplored nature. The highest amount of phenolics and flavonoids was recorded in the leaves and stem of *T. alata*. In contrast, the highest ascorbate was recorded in *T. erecta* leaves and *T. mysorensis* stem. Interestingly, in aqueous extracts, *T. erecta* performed the best in FRAP assay in both leaves and stem segments; *T. alata* stem and *T. mysorensis* leaves performed well in ethanolic extract. Ethanolic extracts of *T. alata* and *T. mysorensis* leaves performed better for DPPH assay, while *T. erecta* stems performed better in aqueous extract. ABTS assay was exceedingly high in the ethanolic extract of *T. alata* stem and contrastingly low in *T. erecta* aqueous and ethanolic extract. Looking at the values, FRAP appears sensitive in depicting stark differences in leaves and stem parts in aqueous or ethanolic extracts. Thus, the present investigation suggests that the studied plant has an accessible source of antioxidants for medicinal and commercial purposes.

Keywords: ABTS, DPPH, FRAP, Ascorbate, Flavonoids and Phenolics

Introduction

Medicinal plants are commonly rich in phenolics, flavonoids, tannins, alkaloids, proteins and amino acids. These compounds have multiple biological effects, including antioxidant activity. Regarding the antioxidant assays, it has been found that molecules with hydrogen donating capabilities such as ascorbic acid, tocopherol, carotenoids, and phenolic compounds can reduce the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to its non-radical form 2,2-diphenyl-1-picrylhydrazine (Arya *et al*., 2011; Blois, 1958; Liu, 2009; Nunes *et al*., 2012). Due to its simplicity and efficiency, the DPPH method has become one of the most used in evaluating antioxidant activity (Nunes *et al*., 2012). Antioxidants are highly able to retard or prevent oxidation of main substances through free radical scavenging (C. Sanchez-Moreno, 2002 and H.Y. Chang, 2007). Several phytochemicals, particularly polyphenols like phenolic acids, flavonoids, and anthocyanins, are familiar and liable for free radical scavenging and antioxidant activities. Generally, the antioxidant capacity of phenols in plant extracts is effective at low concentrations, and in humans, it is associated with the prevention of cardiovascular disease and cancer (Duthie *et al.,* 2000; Li *et al.,* 2014 and Balmus *et al.,* 2016). Selection of these three methods were done based on their feasibility to perform experiments of samples with wide pH range, suitability for both hydrophobic and hydrophilic molecules, fast, stable, and reliable result producing nature.

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It is well known that reactive oxygen species (ROS) such as superoxide anion (O.), hydroxyl radicals, singlet oxygen (1/2 O_2) and hydrogen peroxide (H_2O_2) play a significant role in the development of oxidative stress that can lead to many health problems including cardiovascular disorders, diabetes, edema, degenerative disorders, cancer, anemia and ischemia etc. (Cai *et al.,* 2004). Plant based antioxidant compounds prevent the generation of free radicals, which helps alleviate diseases caused by oxidative stress. (Akinmoladun *et al.,* 2010)

Thunbergia, a genus of the family Acanthaceae, is native to tropical regions of Africa, Madagascar, Australia and South Asia. Species of the genus *Thunbergia* exhibit antifungal, antibacterial, antioxidant, detoxification, antipyretic and hepatoprotective activities.

Thunbergia species have some bioactive compounds exhibiting pharmacological properties. Various species of this genus contain alkaloids, glycosides and phenolic compounds such as flavonoids, tannins, phenolic acid, rosmarinic acid, feruloylmalic acid, grandifloric acids and apigenin. These compounds are antioxidant in nature and helps to fight oxidative stress. *T. alata, T. erecta* and *T. mysorensis* are the three different species of the genus *Thunbergia* which have been selected for the comparative analysis of antioxidant potential. The selection of these three species was done on the basis of their easy availability in the Indian subcontinent. Moreover, these three species of *Thunbergia* have been least explored by the researchers but traditional usage of related species suggested strong potential of being these plant species are major source of medicinal principles.

The goal of the present work is to provide experimental and theoretical evidence on the radical scavenging behaviour of selected species of *Thunbergia* plant extract. In order to accomplish this, the phytoconstituents from *Thunbergia* species are extracted from two plant parts, the leaf and stem.

Material and Methods

Collection of plant material

The plant material was collected from the garden of the Botany department, C. C. S. University, Meerut (U.P.). The collected plant samples were rinsed with tap water twice or thrice to remove any dust particles. Then, washed samples were left completely dry for 8-10 days through the air drying method. After the complete drying of plant samples, they are converted to powder by using the Grinder. The rest of the dried plant samples were stored in air-tight polythene bags to protect them from other foreign materials for further use.

Preparation of plant extracts

The fine dry powder of plant sample (1gm) was taken in a thimble made up of Whatman filter paper and exposed to soxhlet using 250 ml of various polar solvents such as D. W. and ethanol for about 24 hours. After that, the extracted plant samples were kept in a water bath to obtain a concentrated solution of approximately 50 ml of the extracts for further use.

Preliminary Phytochemical Screening

Different solvent extracts from the stem and leaves of *T. alata, T. erecta* and *T. mysorensis* were subjected to different phytochemical tests for the presence or absence of various phytoconstituents by using given phytochemical tests (Sofowora 1993; Trease and Evans 1989 and Harborne 1998).

Test for Tannins

5 ml of plant extract was taken in a test tube. A few drops of 0.1% ferric chloride solution were added to the extract, and blue-black or greenish-brown colouration was observed.

Test for steroids

2 ml of plant extract from the stem and leaves of the plant was taken in a test tube, followed by the addition of 2 ml of acetic anhydride along with 2 ml of sulphuric acid. After a few seconds, if the colour changes from violet to blue or green, it shows the presence of steroids.

Test for flavonoids

5 ml of plant extracts was treated with 1.5 ml of 50% methanol solution. Add 8-10 drops of HCL and a pinch of magnesium in the solution. After boiling for 10-15 min, a red colouration indicates the presence of flavonoids.

Test for saponins

10 ml of distilled water was added to 5 ml of plant extracts in a boiling test tube and shaken vigorously. After boiling the extract in the water bath for a minute, frothing was observed, confirming the presence of saponins.

Test for alkaloids

If a white precipitate was shown after adding Mayer's reagent in the 1 ml of extract of leaf and stem, it showed a positive result for the alkaloids.

Test for glycosides

A solution of glacial acetic acid (4.0 ml) with one drop of 2.0% $FeCl_3$ mixture was mixed with the 10 ml extract and 1 ml concentrated H_2SO_4. A brown ring formed between the layers, which showed the entity of cardiac steroidal glycosides.

Test for phenolics

To 1 ml of extract, 2 ml of ferric chloride was added. A violet-blue precipitate settles down in the test tube, which indicates the presence of phenolics.

Test for Terpenoids

5 ml of extract was added to 2 ml of chloroform, and 3 ml of concentrated H_2SO_4 was poured into the mixture to form a layer. The interface layer of reddish-brown is indicative of the presence of terpenoids.

Quantitative analysis of total phenolics, flavonoids and ascorbic acid

Determination of total phenolic content

The total phenolic contents in medicinal plants was determined spectrophotometrically using the Folin-Ciocalteu method (Bray and Thorpe, 1954). For this procedure, 50 mg of fresh plant material, dried over filter paper, was homogenised using a mortar and pestle with 5 ml of 80% ethanol. The resulting supernatant was re-extracted with five volumes of 80% ethanol. The combined supernatant was evaporated to dryness, and the residue was dissolved in distilled water (DW) to a final volume of 5 ml. Varying volumes of aliquots (0.2–1 ml) were taken and diluted with DW to make a total volume of 1 ml. To this, 0.5 ml of Folin ciocalteu's reagent was added. After 3 minutes, 0.5 ml of 20% sodium carbonate (Na_2CO_3) solution was added, mixed thoroughly, and heated in boiling water for exactly one minute. The mixture was then cooled, and the absorbance was measured at 650 nm against a blank solution. The blank solution consisted of 3.0 ml DW, 0.5 ml Folin ciocalteu's reagent, and 2.0 ml of 20% Na_2CO_3. Gallic acid was used to establish the standard curve. The phenolic content of the samples was expressed as gallic acid equivalents (GAE) in mg per gram of dry weight. All samples were analysed in triplicate to ensure accuracy.

Determination of total flavonoid content

The $AlCl_3$ method (Ordon *et al.,* 2006) was used to quantify the total flavonoid content of the methanolic plant extracts. 20 µl of the sample extract was added to a solution of 2% $AlCl_3.6H_2O$. The mixture was vigorously shaken and diluted with double distilled water to make the total volume of solution 10 ml. The absorbance of the reaction mixture was measured after 10 min incubation at 440 nm. Flavonoid content was expressed as quercetin equivalents in mg per gram of dry material. All the determinations were performed in triplicates.

Determination of Ascorbic Acid

Ascorbic acid was extracted in 5% (m/v) meta-phosphoric acid with quartz sand at 4^0 C. The homogenate was then centrifuged at 3000 rpm for 20 min at 4^0 C. Ascorbic acid content was quantified in the supernatant as described by Shukla *et al*., 1979. In brief, an aliquot of 1 cm^3 of the supernatant was mixed with 2.5 cm^3 of 1% (v/v) freshly diluted Folin-ciocalteu's regent. The reaction mixture was allowed to stand at room temperature for 40 minutes. The absorbance was recorded at 730 nm, using Ascorbic acid as a Standard.

Determination of antioxidant activity

By using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method

DPPH-free radical scavenging activity of different species of genus *Thunbergia* extract was measured following Yen and Duh, 1994 using ascorbic acid as a standard with some slight modification. To calculate DPPH activity, in a test tube 5 µl of methanolic plant extract solution (mg/ml) was added to 3.95 ml of methanolic DPPH solution. DPPH-free radical scavenging activity was calculated as percentage inhibition (PI) according to the formula: PI= {(AC-AT)/AC} ×100 where: AC= Absorbance of the control at t=0 min, AT=Absorbance of the sample + DPPH at t=16min. DPPH-free radical scavenging activity was calculated and expressed as % Inhibition of plant extract solution. A smaller value indicates a better antioxidant property of the extract.

By using the Ferric reducing Antioxidant power (FRAP) Method

This method is based on the ability of the sample to reduce Fe^{3+} ions to Fe^{2+} ions. At low pH, in the presence of TPTZ (Sigma Aldrich, India), the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}-TPTZ) form, producing an intense blue colour with a maximum absorption at 593 nm. The procedure followed was as described by Benzie and Strain (1996).

For the assay, 2.3 ml of FRAP reagent was mixed with 0.7 ml of aqueous extracts (mg/ml). The reaction mixture was incubated at 37°C for 30 minutes in the dark. Absorbance was measured at 593 nm using a spectrophotometer against a blank containing all reagents except the sample. Increased absorbance of the reaction mixture indicated a higher reduction capability. All measurements were conducted in triplicate. Ascorbic acid was used as the standard. A standard curve of ascorbic acid solution was prepared using the same procedure. Results were expressed as milligrams of ascorbic acid equivalents (AAE) per ml of extract.

By using ABTS Free radical Scavenging Method

This assay was performed using a modified method of Re *et al.* (1999). The $ABTS^+$ stock solution was prepared by reacting a 7 mM aqueous solution of ABTS with a 2.45 mM aqueous solution of potassium persulfate in equal volumes. The mixture was kept in the dark at room temperature for 12–16 hours before use to allow the formation of the $ABTS^+$ radical cation.

The working solution of $ABTS^+$ was obtained by diluting the stock solution with methanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 2.0 ml of the $ABTS^+$ solution was mixed with 1.0 ml of the aqueous extract (µg/ml). The mixture was incubated at room temperature for 10 minutes in the dark.

A control solution was prepared by mixing 2.0 ml of $ABTS^+$ solution with 1.0 ml of double-distilled water. The absorbance of the reaction mixture was measured at 734 nm using a spectrophotometer against a blank. Butylated hydroxytoluene was used as the standard. All samples were prepared and analysed in triplicate. The percentage of scavenging activity of each extract on $ABTS^+$ was calculated using the following formula:-

{(Ao-As)/Ao}×100

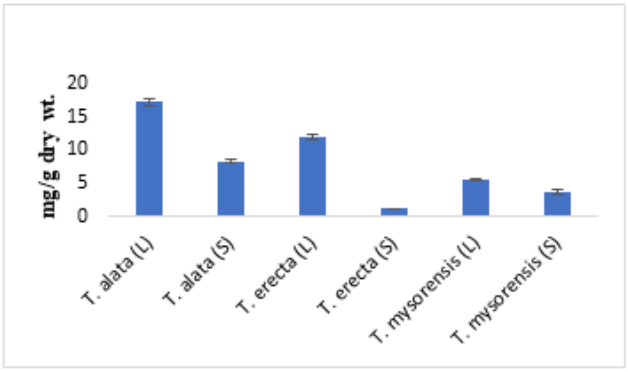
Where: Ao is the absorbance of the control
As is the absorbance of the test extract solution.

Results and Discussion

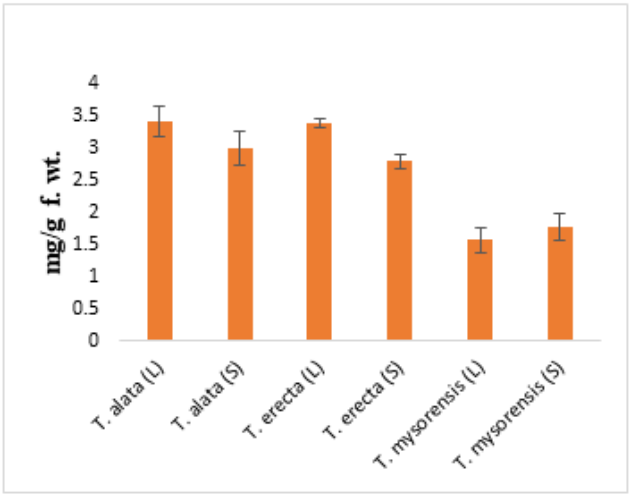
The findings of the present studies are described as follows.

Preliminary Phytochemical Screening

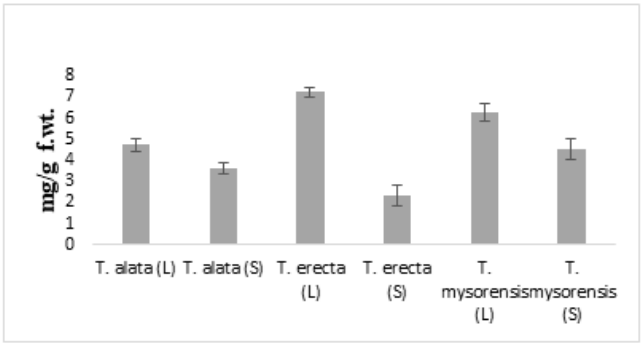
The ethanolic extract of *T. alata*, *T. erecta* and *T. mysorensis* were investigated for some major phytoconstituents, e.g., alkaloids, flavonoids, cardiac glycosides, saponins, steroids, tannins and terpenoids, phenolics by using some spot test. The obtained results are tabulated in the following table 1, 2 and 3.



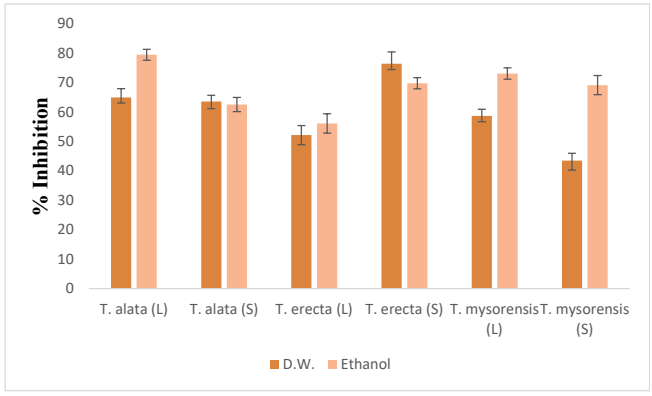
A: Estimation of Total Phenolics



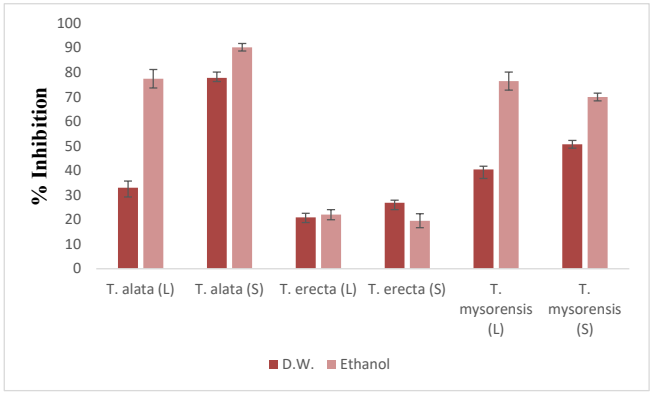
B: Estimation of Flavonoids



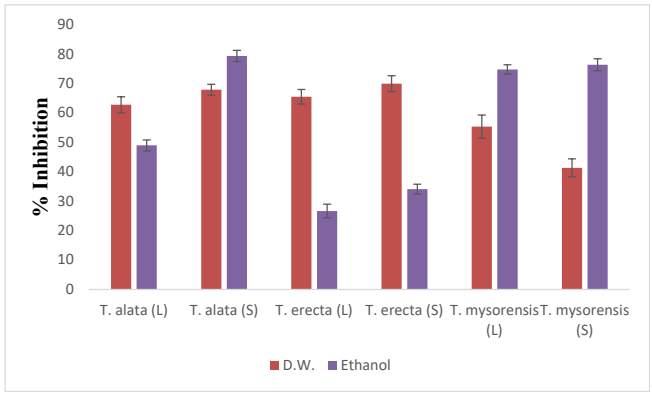
C: Estimation of Ascorbic Acid



D: Radical scavenging capacity of DPPH (% Inhibition)



E: ABTS Activity



F: FRAP Activity

Figure 1 (A to F): Showing antioxidant activity via DPPH assay, FRAP assay and ABTS assay; phenolics, Flavonoids and Ascorbic acid content in *T. alata*, *T. erecta* and *T. mysorensis*

Quantification of total phenolics, flavonoids and ascorbic acid

The total phenolic content of different species of *Thunbergia* was determined by the Folin–Ciocalteu reagent method; it was expressed as Gallic acid equivalent (Table 2), while total flavonoid content was determined by the aluminium chloride colourimetric methods. It was expressed as quercetin equivalents (Table 2). The ascorbic acid was determined by Shukla *et al*. (1979) (Table 2).

Phenolic Content Analysis

The phenolic content across the leaves and stems of three *Thunbergia* species (*T. alata*, *T. erecta*, and *T. mysorensis*) exhibited significant variation. The highest phenolic content was recorded in the leaves of *T. alata* (17.27 mg/g of GAE), followed by the leaves of *T. erecta* (11.9 mg/g of GAE) and *T. mysorensis* (5.57 mg/g of GAE). In contrast, the stems contained lower phenolic levels, with *T. alata* stems showing 8.25 mg/g, *T. mysorensis* stems 3.72 mg/g, and *T. erecta* stems exhibiting the lowest value at 1.1 mg/g. (Fig.1)

Across all species, the leaves consistently exhibited higher phenolic content than the stems. This can be attributed to their involvement in photosynthesis and exposure to environmental stressors such as UV radiation, which stimulate phenolic biosynthesis Ilbong *et al.* (2024). Among the species, *T. alata* emerged as the richest source of phenolics, highlighting its potential pharmacological relevance and traditional medicinal applications. Conversely, *T. erecta* and *T. mysorensis* showed relatively moderate to low phenolic levels, suggesting variations in their biosynthetic capacities or ecological adaptations.

These findings underscore the species-specific and tissue-specific distribution of phenolics, emphasising the importance of selecting appropriate plant parts to extract bioactive compounds. The significant phenolic content in *T. alata* supports its potential as a promising candidate for antioxidant and therapeutic research. In contrast, the relatively lower phenolic levels in *T. erecta* and *T. mysorensis* indicate the need for further investigation into other bioactive compounds that may contribute to their medicinal properties.

Flavonoid Content analysis

The flavonoid content (expressed as mg/g quercetin equivalent) also varied significantly across *T. alata*, *T. erecta*, and *T. mysorensis* leaves and stems. The highest level of ascorbic acid was recorded in the leaves of *T. alata* (3.41 mg/g), followed closely by the leaves of *T. erecta* (3.39 mg/g). The lowest content was observed in the stems of *T. mysorensis* (1.57 mg/g). (Fig.:1)

In all species, leaves consistently exhibited higher flavonoid levels than stems, likely due to their active biosynthetic processes and exposure to environmental factors that stimulate flavonoid accumulation. Among the species, *T. alata* demonstrated the highest overall

Table 1: Spot test for qualitative analysis of phytoconstituents in different species of genus *Thunbergia*

S.N.	*Phytoconstituents*	*Test for confirmation*	*T. alata*		*T. erecta*		*T. mysorensis*	
			Leaf	Stem	Leaf	Stem	Leaf	Stem
1.	Alkaloids	Wagner's/Dragendorff's Test	+	+	+	+	+	+
2.	Phenolic	Ferric Chloride Test	+++	++	++	+	+++	++
3.	Flavonoids	Shinoda/Alkaline Reagent Test	+++	++	++	+	+++	++
4.	Cardiac glycosides	Keller-Killiani Test	+	+	+	+	+	+
5.	Saponins	Foam Test	++	+	+	-	++	+
6.	Terpenoids	Salkowski Test	++	+	+	+	++	+
7.	Tannins	Ferric Chloride Test	+++	++	++	+	++	++
8.	Coumarins	UV fluorescence Test	+	+	+	+	+	+

Note: '+++' shows very high, '++' shows high, '+' shows moderate and '-' shows nill

Table 2: Estimation of phenolics, flavonoids and ascorbic acid in leaf and stem of *T. alata*, *T. erecta* and *T. mysorensis*

S. No.	*Plant Name*	*Plant Parts*	*Phenolics (GAE mg/gdw)*	*Flavonoids (QE mg/gfw)*	*Ascorbic Acid (mg/gfw)*
1.	*T. alata*	Leaf	17.27	3.41	4.72
		Stem	8.25	2.99	3.59
2.	*T. erecta*	Leaf	11.9	3.39	7.23
		Stem	1.1	2.8	2.31
3.	*T. mysorensis*	Leaf	5.57	1.57	6.26
		Stem	3.72	1.78	4.55

Table 3: Assay for DPPH, FRAP and ABTS to detect antioxidant activity in different plant parts of *T. alata*, *T. erecta* and *T. mysorensis* by using aqueous and ethanolic solvent system

S. No.	Solvent System	Assay for Antioxidant Activity	% Inhibition in different plant parts (leaf and stem) of different species of genus *Thunbergia*					
			TAL	TAS	TEL	TES	TML	TMS
1.	D. W.	DPPH	64.91	63.53	52.19	76.38	58.64	43.5
		FRAP	62.71	67.83	65.45	69.9	55.3	41.28
		ABTS	33.00	77.8	20.95	26.93	40.44	50.73
2.	Ethanol	DPPH	79.43	62.54	56.09	69.75	73.07	69.13
		FRAP	48.92	79.29	26.63	34.09	74.75	76.32
		ABTS	77.43	90.27	22.04	19.58	76.48	69.98

(TAL: *T. alata* leaf, TAS: *T. alata* stem, TEL: *T. erecta* leaf, TES: *T. erecta* stem, TML: *T. mysorensis* leaf and TMS: *T. mysorensis* stem)

flavonoid content, highlighting its pharmacological potential, particularly for antioxidant and anti-inflammatory applications. The moderate levels observed in *T. erecta* and the relatively low levels in *T. mysorensis* suggest species-specific variations influenced by genetic and ecological factors. These results underscore *T. alata* as a key candidate for further phytochemical and pharmacological investigations.

Ascorbic Acid Content Analysis

The ascorbic acid content varied significantly among the leaves and stems of *T. alata*, *T. erecta*, and *T. mysorensis*. The highest ascorbic acid content was observed in the leaves of *T. erecta* (7.23 mg/g), followed by the leaves of *T. mysorensis* (6.26 mg/g) and the leaves of *T. alata* (4.72 mg/g). In contrast, *T. erecta* stem exhibited the lowest ascorbic acid levels (2.31 mg/g), while *T. alata* and *T. mysorensis* stem containing 3.59 mg/g and 4.55 mg/g, respectively (Fig.:1).

Leaves consistently exhibited higher ascorbic acid levels than stems across all species, reflecting their role as active sites for ascorbate biosynthesis and storage. The elevated ascorbic acid levels in the leaves of *T. alata* suggest their potential as rich sources of antioxidants. Conversely, the moderate levels in *T. mysorensis* highlight species-specific differences in ascorbate metabolism. These findings establish the potential of *Thunbergia* species as sources of ascorbic acid, with the leaves of *T. erecta* emerging as the most promising candidate for further antioxidant and nutritional research.

Antioxidant activity Analysis

The antioxidant activity of *Thunbergia* species was evaluated using DPPH, FRAP, and ABTS assays in water (DW) and ethanol extracts (Table-3). Ethanol extracts showed higher antioxidant activity compared to water extracts in most cases. Among the species, *T. alata* leaves (TAL) exhibited the highest DPPH inhibition in ethanol (79.43%), while *T. alata* stems (TAS) showed the highest ABTS inhibition in ethanol (90.27%) (Fig.-4). In contrast, *T. erecta* leaves (TEL) and stems (TES) had relatively lower antioxidant activity in all assays, particularly in ABTS. *T. mysorensis* leaves (TML) and stems (TMS) displayed moderate activity across assays, with ethanol extracts consistently outperforming water extracts (Fig.-5). *T. alata* (TAS) stems exhibited the highest reducing power in ethanol (79.29 %), suggesting a rich presence of electron-donating antioxidants(Fig.-6). In comparison, *T. erecta* (TEL) leaves showed moderate activity (26.63 %) in ethanol, highlighting species-specific differences in antioxidant capacity.

The results indicate that ethanol is a more efficient solvent for extracting antioxidant compounds from *Thunbergia* species. *T. alata* leaves demonstrated the most potent antioxidant activity, emphasising their pharmacological potential, while *T. erecta* exhibited the lowest activity, suggesting species-specific differences in secondary metabolite profiles. These observations confirm that *T. alata* leaves possess the most substantial antioxidant potential, particularly in ethanol extracts, making them a promising candidate for therapeutic applications.

Conclusion

The study highlights significant variations in phenolic, flavonoid, and ascorbic acid content, as well as antioxidant activity (DPPH, FRAP, and ABTS assays) across the leaves and stems of *Thunbergia* species (*T. alata*, *T. erecta*, and *T. mysorensis*). Among these species, the leaves of *T. alata* consistently exhibited the highest phenolic and flavonoid content, coupled with superior antioxidant activity, particularly in ethanol extracts. This was evidenced by potent DPPH inhibition and FRAP activity. In contrast, *T. erecta* stems displayed the lowest levels of bioactive compounds and antioxidant potential across most assays. Meanwhile, *T. mysorensis* leaves and stems demonstrated moderate activity, with ethanol extracts outperforming aqueous ones.

The findings establish that *T. alata* leaves are the most potent source of bioactive compounds with significant antioxidant potential, emphasising their pharmacological

and therapeutic relevance. The type of solvent used for extraction significantly influenced the yield, with ethanol proving more effective than water. These results underscore the importance of species selection, solvent optimisation, and plant part specificity in maximising the recovery of bioactive compounds for potential medicinal applications. These studies suggest that *T. alata* is a promising source of natural raw materials for phytopharmaceutical preparations, supporting its traditional medicinal uses and highlighting its potential for future therapeutic development.

The use of two different solvents i.e. aqueous and ethanol helps to understand nature of extracted metabolites in respect to their solubility in water or alcohol. This suggests that all the hydrophilic metabolites would be extracted in aqueous medium and all hydrophobic metabolites would be extracted in alcoholic medium. This separation of metabolites will help in large scale production of active medicinal principle as the protocols can be developed accordingly.

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