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RESEARCH ARTICLE

Evaluation of antioxidant potential and quantification of caffeic acid in root bark of *Tecomella undulata* (Sm.) Seem.

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Abstract

Tecomella undulata (Sm.) Seem. an endangered species of the family Bignoniaceae is native to India, Pakistan and Oman. Traditionally, it is used in the treatment of leucorrhea, fever, cough, digestive disorders, skin infections, sexual disorders and pain. The plant is also known for its diverse pharmacological properties, including antioxidant, antidiabetic, anticancer, hepatoprotective, antiobesity and antimicrobial properties. In the current study total phenolic content (TPC) and total flavonoid content (TFC) along with antioxidant potential of methanolic extract of root bark of *T. undulata* was evaluated, an important metabolite, caffeic acid, was identified and quantified using high performance thin layer chromatography (HPTLC). The extract exhibited comparatively higher TFC (3.20 mg QE g^{-1} dry weight of extract) as compared to TPC (2.02 mg GAE g^{-1} dry weight of extract). A significant antioxidant activity was also observed viz., 2,2-diphenyl-1-picrylhydrazyl (IC_{50} -151.53 µg mL^{-1}), nitric oxide scavenging assay (IC_{50}- 771.2 µg/mL), deoxyribose degradation assay (IC_{50}- 120.1 µg mL^{-1}) and ferric reducing antioxidant power assay (109.22µmol Fe^{2+} g^{-1}dry weight of extract). The presence of caffeic acid was confirmed by HPTLC profiling, with linear regression R^2 = 0.99848 and R*f* = 0.62. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 41.92 and 75.79 ng mL^{-1}, respectively, marking the first report of this compound in the root bark of *T. undulata*. These data highlight the importance of the tree as a medicinal and antioxidant agent, which makes it an important candidate to be further explored for its bioactive and therapeutic activities.

Keywords: antioxidant activity; caffeic acid; flavonoid content; high performance thin layer chromatography; phenolic content; *Tecomella undulata*

Introduction

Plants have been essential part of human life since time immemorial and have played a pivotal role in the well-being of humans (1). The doctrines of medicine and health management written in the past have been plant-based systems of therapeutics. Ayurveda, Unani, Siddha and Chinese systems of medicine are some of the major traditional medicinal systems, which are based on plant remedies (2).

Free radicals, also known as reactive oxygen species (ROS), are molecules that exist on their own and have a non-paired electron in an atomic orbital. These species are generated during various metabolic processes and under pathological conditions, their rate of synthesis enhances (3). The ROS damage the cell by initiating the chain reactions. Antioxidants regulate the concentration of ROS within cells by scavenging free radicals, chelating catalytic metal ions and preventing lipid peroxidation (4). The search for novel natural antioxidants is essential because phyto-antioxidants have fewer adverse effects compared with synthetic antioxidants (5).

Tecomella undulata (Sm.) Seem. belongs to the family Bignoniaceae and is commonly known as Rohida. It is an endangered deciduous tree found in different parts of India including Rajasthan, Gujarat, Punjab, Haryana and Maharashtra (6). *Tecomella undulata* has gained interest in both classical and folk streams of ancient medicinal systems because of its therapeutic value (7), which has also been mentioned in ancient Samhita's of the Ayurveda (8). The bark of rohida is used for making various Ayurvedic formulations like rohitakaghrita, rohitakaloha, rohitakarishta and rohitakadya choorna etc. (9).

Very few previous studies have attempted to elucidate the chemical composition of *T. undulata* especially flavonoids in leaves and flowers of *T. undulata* (10). Various compounds belonging to different groups including phenolics (Vanillic acid and its derivatives, n-Eicosanyl cinnamate) were isolated from the methanolic extract of stem bark using column chromatography followed by thin layer chromatography (11). Alkaloid extract when analysed through Gas chromatography-mass spectrometry yielded some phenolic compounds including veratric acid, benzoic acid, phthalic acid in different plant parts (12).

In India and Pakistan, the root bark is traditionally utilised in the form of powder or decoction, often mixed with milk, sugar, or rice water, for the treatment of leucorrhea, as well as for managing hepatic disorders and skin diseases such as eczema (13). However, there are scanty reports on phytochemical investigation on the roots of the tree as compared to the aerial plant parts. Furthermore, considering that metabolite accumulation is often tissue-specific, the root bark may serve as a potential source of unique bioactive compounds. Viewing the research gap, the present study aims to evaluate the antioxidant potential of root bark together with the amount of caffeic acid in the methanolic extract of *T. undulata*.

Materials and Methods

Extraction

Tecomella undulata root bark samples were collected in November to March, 2025 from the Banasthali Vidyapith campus, Rajasthan. A voucher specimen (Specimen no. BURI-5914/2025) was deposited in the herbarium of the department of Bioscience and Biotechnology at Banasthali Vidyapith. The collected root bark was shade dried and then oven dried at 35–40 °C and showed a fresh-to-dry ratio of approximately 3:1 (w/w). A particle size of approximately < 2 mm was used, as finer particles enhance extraction efficiency by improving solvent penetration and solute diffusion (14). Powdered sample (5 g) was extracted in 50 mL methanol in an orbital shaker at 37 °C and 100 rpm for 24 hr. Thereafter, it was centrifuged at 5000 rpm for 20 min. The supernatant of the sample was collected and kept for further analysis at 4 °C (15). Sample was extracted twice to ensure complete extraction.

Methanol was selected as the extraction solvent due to its high efficiency in extracting phenolic compounds because of its polar nature. The conditions (37 °C and 100 rpm) were maintained to enhance extraction while preventing degradation of heat-sensitive metabolites (16)

Quantitative analysis of total phenol content

The concentration of total phenol content (TPC) in the extract was evaluated using Folin-ciocalteu reagent and results were expressed as mg of gallic acid equivalents (GAE) per gram of dry weight of sample (17).

Quantitative analysis of total flavonoid content

The assay was performed according to the method illustrated by Vats (18), The total flavonoid content (TFC) of the extract was determined using $AlCl_3$ spectrophotometric method and the amount was expressed as quercetin equivalent (QE) per gram of dry weight of sample.

Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

In this assay, 1 mL of plant extract with 1 mL of 0.3 mM 2,2-diphenyl-1 -picrylhydrazyl (DPPH) was mixed and allowed to stand at room temperature for 30mins. After measuring the absorbance at 517 nm and IC_{50} (µg mL^{-1}) was calculated (18).

Nitric oxide scavenging assay

Nitric oxide scavenging assay (NOSA) was performed by mixing phosphate-buffered saline (PBS, 0.5 mL) containing the extract with sodium nitroprusside (SNP, 10 mM; 2 mL) and incubating the mixture for 150 min at 25 °C. After incubation, 0.5 mL of the reaction mixture was withdrawn and mixed with sulphanilic acid reagent (1 mL). Then, N-(1-naphthyl)ethylenediamine dihydrochloride (NED, 0.1%; 1 mL) was added, followed by incubation at room temperature for 30 min. The absorbance was measured spectrophotometrically at 540 nm (19).

Deoxyribose degradation assay

Deoxyribose degradation assay (DDA) was carried out using a reaction mixture (1.0 mL) containing 2-deoxy-2-ribose (2-DR, 28 mM; 100 µL), various concentrations of the test sample in potassium phosphate buffer (50 mM, pH 7.4; 500 µL), EDTA (1.04 mM) and $FeCl_3$ (200 µM) solution (1:1, v/v; 200 µL), hydrogen peroxide (H_2O_2, 1 mM; 100 µL) and ascorbic acid (100 µL). The mixture was incubated at 37 ° C for 1 hr. Thereafter, thiobarbituric acid (TBA, 1 %; 1 mL) and trichloroacetic acid (TCA, 2.8 %; 1 mL) were added and the reaction tubes were incubated at 100 °C for 20 min. After cooling, absorbance was recorded at 532 nm. Results were expressed as IC_{50} (µg mL^{-1}) (20).

Ferric reducing antioxidant power assay

2, 4, 6-tripyridylstriazine (10 mM), acetate buffer (300 mM, pH 3.6) and ferric chloride hexahydrate (20 mM), were prepared. All the reagents were mixed as per Benzie and Strain (21), to make a working solution. The extract and ferric reducing antioxidant power assay (FRAP) reagent were mixed and absorbance was taken at 593 nm after 5mins.

High performance thin liquid chromatography analysis

High performance thin liquid chromatography analysis (HPTLC) analysis was carried out as per the methods of Cirumarnean et al., (22). The stock solutions were prepared by dissolving 2 mg of caffeic acid standard in methanol. The different concentrations of 50, 100, 150, 200, 250 and 300 ng were prepared from the stock solution. Pre-coated aluminum TLC plates (20 × 10 cm) with a 0.2 mm silica gel layer (60 F_{254} by MERCK, India) were used for HPTLC analysis. Using an automatic TLC sampler, the standard and root bark extract (R*f* equivalent to standard caffeic acid on TLC plates) were applied to the silica-coated plate for HPTLC analysis. The plate was then developed using methanol: formic acid: ethyl acetate and toluene (5:10:15:10) as a mobile phase within the twin chamber and the mobile phase was used for 15 min of pre-saturation. After the gel run, the plate was dried and scanned at 254 nm using the CAMAG TLC Scanner 3.

A TLC plate visualisation was utilised for the photo documentation of the plate. The method was validated by using International Conference on Harmonization (ICH) guidelines (23). The peak area was plotted against the amount applied to determine the calibration curve. According to ICH guidelines, the equation was used to determine the limit of detection (LOD) and limit of quantification (LOQ). The regression equation obtained from the standard curve was used to quantify the amount of caffeic acid present in the sample.

Statistical analysis

The experiments were repeated 3 times and the data were reported as mean ±standard deviation (n = 3). The data were subjected to analysis of variance (ANOVA), a statistically robust method used to evaluate the significance of observed variations, followed by Duncan's multiple range test (DMRT). was employed as a post-hoc analysis. The statistical software package SPSS Inc., Version 20.0, was utilised for executing these analyses, with a significance threshold set at $p \leq 0.05$, ensuring that only results meeting this criterion were deemed statistically meaningful. The graphical representation of the experimental findings was meticulously executed utilising OriginPro 2021.

Results

Total phenolic content

A calibration curve prepared with gallic acid exhibited excellent linearity, with the regression equation y = 0.0031x + 0.011 and $R^2 = 0.9974$, indicating high precision of the method. The TPC of the methanolic root bark extract was recorded as 2.02 mgGAE/g dry weight (DW) is shown in Fig. 1. which was significantly high ($p \leq 0.05$).

Total flavonoid content

The calibration curve for quercetin exhibited strong linearity with a coefficient of determination (R^2 = 0.99), confirming the reliability of the assay. The methanolic root bark extract of the experimental plant recorded a TFC of 3.20 mg QE g^{-1}DW, which was statistically significant ($p \leq 0.05$) and is shown in Fig. 1.

Antioxidant assays

Tecomella undulata root bark methanolic extracts have an IC50 value of 151.53±1.72 µg mL^{-1} (Fig. 2), demonstrating significant strong scavenging activity. The standard antioxidant, ascorbic acid, showed a significantly lower IC_{50} = 15.35±1.34 µg mL^{-1}), reflecting its higher antioxidant potency. On the other hand, the IC_{50} value with respect to deoxyribose degradation was found to be 120.1±2.01 µg mL^{-1} as shown in Fig. 2, indicating significant hydroxyl radical scavenging activity. This inhibition of deoxyribose degradation was found to be 120.1±2.01 µg mL^{-1} as shown in Fig. 2, indicating significant hydroxyl radical scavenging activity. This inhibition of deoxyribose degradation may be due to the appreciable content of phenolic and flavonoids. Quercetin showed strong NO scavenging activity with an IC_{50} of 17.01 ± 0.03 µg mL^{-1}, while the root bark extract exhibited comparatively higher IC_{50} values (24). The NOS activity of root bark IC_{50} is 771.2±21.41µg mL^{-1} and is shown in (Fig. 2). The fact that the scavenging process works with various types of molecules might be the reason for the results. The sequential extraction method was not found to be conducive to NO scavenging activity. The FRAP activity was shown to be significantly higher (Fig. 2). The maximum reduction in terms was the formation of ferrous sulphate which was 109.22±1.24 µmol Fe^{2+} g^{-1}(DW). The FRAP assay demonstrated that ascorbic acid exhibited strong reducing power with a value of 0.98 ± 0.05, indicating its high electron-donating capacity (25).

Identification of caffeic acid using

A preliminary investigation of the root bark HPTLC of *T. undulata* revealed that the extract is rich in total phenolic and flavonoid content; and exhibits strong antioxidant activity. Quantitative screening of root bark extracts revealed the presence of phenolic and flavonoid compounds. Many studies have reported that phytochemicals, particularly phenolics and flavonoids, exhibit prominent glucose and blood pressure lowering activities (22).

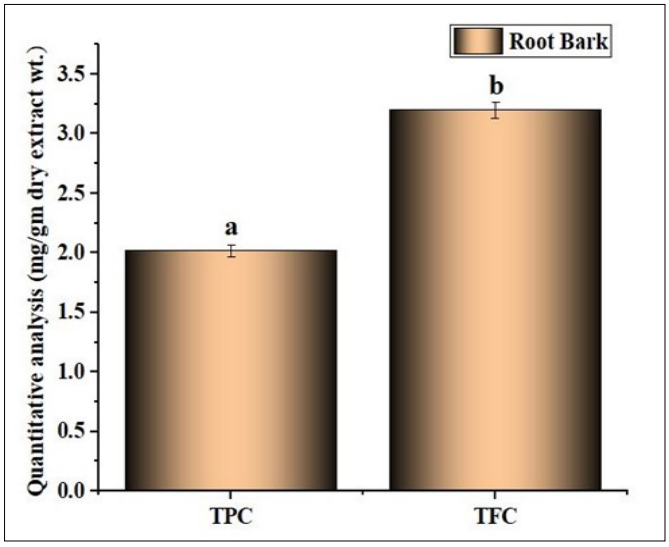


Fig. 1. Total phenolic content (TPC) and total flavonoid content (TFC) of root bark extract of *Tecomella undulata*.

Caffeic acid in the root bark extract was analysed both qualitatively and quantitatively using HPTLC. The method showed a highly reliable and accurate tool for the bioactive compound quantification. The mobile phase consisting of methanol: formic acid: ethyl acetate and toluene (5:10:15:10) acceptable resolution and separation of the component from the root bark extract (Fig.3). Detection was carried out at 254 nm in the visible range, producing sharp and well-resolved spots for caffeic acid with an R*f* value of 0.62 by superimposing the UV spectra of the sample and standard (Table 1). Linearity for the caffeic acid was recorded with a correlation coefficient (R^2) of 0.99848. The average equation for calibration curves is Y= 255.610+14.512*X. The chromatographs of the standard and samples are shown in Fig. 3. The specificity and selectivity were monitored and no peak was detected close to the standard, which shows a high level of specificity and selectivity of the method. The LOD and LOQ were found to be 41.92 ng mL^{-1} and 75.79 ng mL^{-1}, respectively (Table 2). The quantitative estimation of caffeic acid in the root bark extract ranged between 50 to 300 ng and the 3D HPTLC chromatogram demonstrated peak purity, underscoring the method's accuracy and reliability (Fig.3; Table 3).

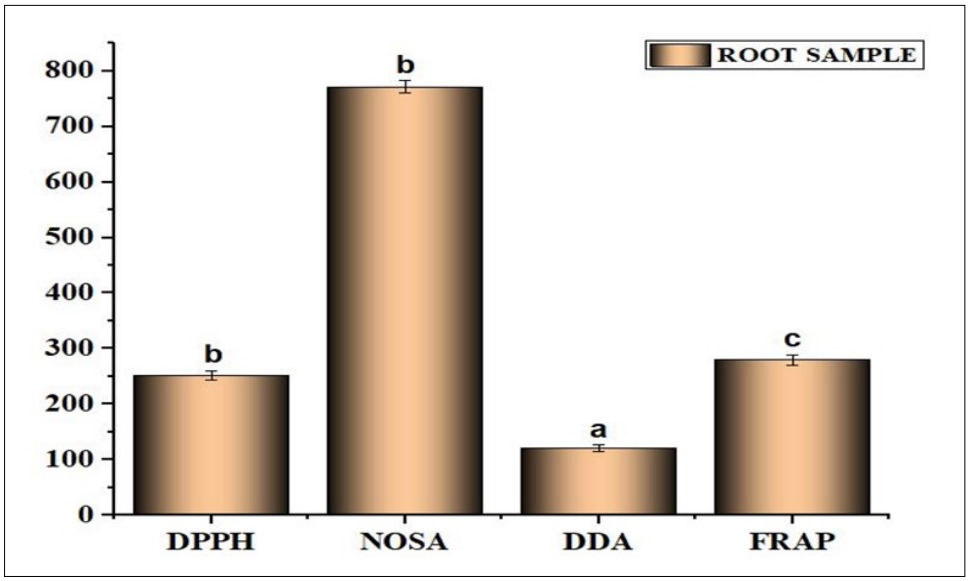


Fig. 2. Antioxidant potential of root bark extract of *Tecomella undulata* evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide scavenging and deoxyribose degradation assays (µg mL^{-1}) and ferric reducing antioxidant power (FRAP) assay (µM).

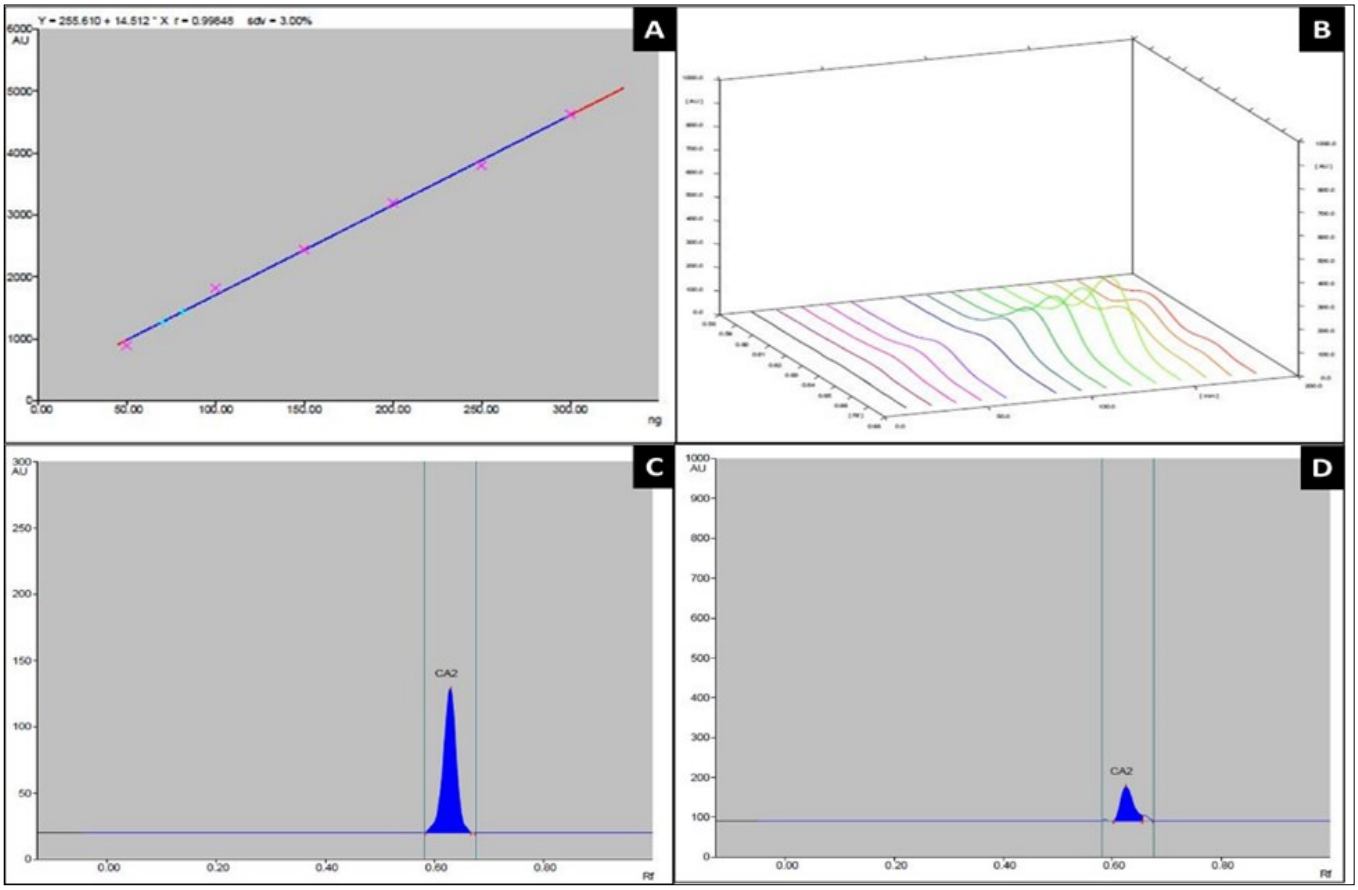


Fig. 3. High performance thin-layer chromatography (HPTLC) analyses: (A) Standard calibration curve; (B) Three-dimensional densitogram (3D); (C) Chromatogram of caffeic acid standard; (D) Chromatogram of root bark extract of *Tecomella undulata.* (ng: Nanograms; AU: Absorbance Units; R*f*: Retention factor).

Table 1. High performance thin-layer chromatography (HPTLC) profile of the methanolic extract of root bark of *Tecomella undulata* for caffeic acid as standard

Track	Assigned substance	Applied amount	Maximum R*f*	Maximum height	Area
1	Standard (Caffeic acid)	1µl	0.63	50.1	892.3
2		2 µl	0.63	108.7	1814.8
3		3 µl	0.62	141.8	2440.1
4		4 µl	0.62	184.2	3193.7
5		5 µl	0.63	216.6	3800.8
6		6 µl	0.63	255.8	4629.1
7	Root bark extract	5 µl	0.63	58.3	782.5
8		10 µl	0.62	80.5	1274.8
9		20 µl	0.63	86.1	1436.0

Table 2. Method validation and system suitability parameters for the quantitation of β-carotene from methanolic extract of root bark of *Tecomella undulata* by high performance thin-layer chromatography (HPTLC)

Parameter	Value
Method validation	
Wavelength	254 nm
Linearity range (ng spot^{-1})	50–300 ng spot^{-1}
Correlation coefficient (r^2)	0.99848
R*f* (n=3)	0.62
LOD (ng spot^{-1})	41.92 ng mL^{-1}
LOQ (ng spot^{-1})	75.79 ng mL^{-1}
Specificity	Confirmed by matching R*f* of standard and sample peak (Caffeic acid at ~0.62-0.63)
Selectivity	Method shows selective detection of caffeic acid without major interference
System suitability of caffeic acid	
Ratio	Linear regression (Y = 255.6 + 14.51X)
Area ± SD (ng/band)	Standard peak areas range from 892.3 to 4629.1 AU
% RSD	41.924 %

SD: standard deviation; RSD: relative standard deviation.

Table 3. Robustness study for the high performance thin-layer chromatography (HPTLC) method

Parameters	Changes	Amount of caffeic caid spotted (ng)	Amount of caffeic caid detected (ng)	% RSD
Application repeatability	Multiple spotting (n = 3)	50	81.34	41.92
		100	70.23	
		150	<45.00	

RSD: relative standard deviation.

Discussion

Phenolics are found in all plants and are essential for neutralising free radicals by giving electrons or hydrogen atoms, which prevent lipid peroxidation and the oxidative stress after it (26). The Folin–Ciocalteu method, which is frequently used because of its simplicity, rapidity and repeatability, was used in this investigation to determine the TPC of *T. undulata* root bark extract. The molybdotungstate reagent in Folin-Ciocalteu solution oxidises phenolics in this analysis, producing a blue complex that can be measured at 765 nm. The phenolic content obtained in this study is consistent with earlier findings. Previous researchers analysed the methanolic extract of different plant parts of *T. undulata* for its antioxidant activity and reported that the total phenolic content was found to be maximum in the stem followed by leaves, bark and root (12.70, 10.25 and 7 mg GAE g^{-1} DW, respectively) (27). The relatively lower phenolic content observed in the present study may be attributed to variations in the polarity of the extraction solvent. Flavonoids are essential polyphenolic antioxidants known for their ability to scavenge free radicals and chelate metal ions. The methanolic root bark extracts observed high TFC content, which may enhance the antioxidant activity. The precision of the aluminum chloride method used for quantification. The quercetin standard curve has strong linearity. Similarly, previous study also show variable flavonoid content among different parts of *T. undulata*, in the order: leaves (71.87 ± 18.39 mg QE g-1 DW) > stem > bark > root, indicating that roots generally contain lower flavonoid levels than aerial parts (27). The differences observed between the present work and previous reports may be attributed to biological, environmental and methodological factors. Variations in plant metabolism due to developmental stage, plant part and genetic variability can influence the accumulation of secondary metabolites. Additionally, extraction conditions, solvent type and assay sensitivity may affect the quantification of phenolics and flavonoids. Environmental factors such as geographical location, climate, soil composition, temperature and stress conditions also play a significant role in regulating the biosynthesis of bioactive compounds (28–31).

The DPPH is a free radical and due to antioxidants scavenging radicals by donating hydrogen to form the stable DPPH-H molecule, its absorbance lowers and its color changes from purple to yellow (yellow from the picryl group still present). The low IC_{50} value of the methanolic extract indicates strong antioxidant activity (15). The IC_{50} value of the root bark extract was found to be higher than ascorbic acid showing a comparatively lower antioxidant potential than the standard compound. This potent activity supports its potential as a natural source of antioxidants and is consistent with its high phenolic and flavonoid content. In this study, the finding of *T. undulata* ability to scavenge free radicals is consistent with previous reports. The free radical scavenging activity (DPPH assay) showed the lowest IC_{50} value in the stem, followed by bark, root and leaves (32). Similarly, previous researchers, reported that the leaves and flowers collected from various parts of Pakistan showed better DPPH scavenging activity (range- 1.7 mg $L^{-1}$and 2.2 mg L^{-1}) (10). Earlier researchers reported comparatively reduced scavenging activity of the methanolic extract of the leaves of *T. undulata* procured from Coimbatore, Tamil Nadu (33). This variation in the content may be attributed to different geographical locations, which affect the production of phytochemicals in a plant.

The observed OH• radical scavenging activity of *T. undulata* root bark extract suggests the presence of potent antioxidants, which could be useful in protecting against oxidative damage.

Despite higher flavonoid content, the plant extract exhibited comparatively lower NO scavenging activity than quercetin, possibly due to the complexity of phytoconstituents, however, it still reflects appreciable antioxidant potential (24). The observed NO is used as a signalling molecule with a variety of physiological roles, such as smooth muscle relaxant, vasodilator and neuronal messenger. However, excessive production of this free radical increases the pathogenicity of numerous diseases including inflammation and cancer (34). The root bark extract of *T. undulata* has been observed to have NO scavenging activity, which suggests that it has the potential to neutralise reactive nitrogen species and lower nitrite formation. Moreover, NO easily reacts with the superoxide anion to produce peroxynitrite, a reactive intermediate that can produce toxic compounds such as nitric dioxide and hydroxyl radicals, which adversely affect the tissue systems (35). Therefore, albeit to a lesser extent compared to other radical scavenging pathways, the extract's moderate NO scavenging activity observed in the root bark extract may contribute to its overall antioxidant and protective benefits.

The root bark of *T. undulata* exhibits a strong ferric-reducing potential observed in demonstrating the presence of electron-donating antioxidants that reduce oxidised intermediates, thereby preventing oxidative damage. The FRAP activity of ascorbic acid reflects its efficient ability to reduce Fe^{3+} to Fe^{2-}, confirming its potent antioxidant nature. These findings are consistent with previous reports, where ascorbic acid has been identified as a strong ferric reducing agent due to its redox properties (25). Relatively higher scavenging activity of the ethyl acetate extract of the bark of *T. undulata* (178.32 ± 5.85 mg AAE g^{-1}) collected from Baroda (36). This variation may be due to the sample being extracted with a different solvent affecting the metabolite profile, especially for antioxidants. The plant parts of *T. undulata* for its antioxidant activity using different solvents was analysed in earlier studies. The best ferric reducing antioxidant power was observed in the methanolic extract of leaves (96.66±0.675 mM g^{-1}) followed by the bark (78.090±1.543 mM g^{-1}), stem (66.030±0.539 mM g^{-1}) and root (58.090±1.317 mM g^{-1}).

These findings are consistent with the extracted observed higher flavonoid content which supports its strong antioxidant potential through effective electron transfer and radical neutralization. There are a few studies on *T. undulata* DPPH and FRAP activities; there is no previous report on NOSA and DDA activity,

suggesting a research gap that needs more investigation.

A well-known phenolic compound exhibiting antioxidants and anti-inflammatory properties is caffeic acid. It is reported to effectively reduce inflammation, hyperglycaemia and oxidative stress while promoting IGF-1 signalling and exhibiting significant neuroprotective benefits in diabetic neuropathy (37). The presence of caffeic acid in *T. undulata* root bark supports its therapeutic potential, particularly in oxidative stress- related disorders like leucorrhea, hepatic disorders and skin diseases such as eczema as mentioned in its traditional uses. However, the therapeutic implications should be interpreted with caution, as the current findings are based on *in vitro* assays and do not provide direct *in vivo* or mechanistic evidence. Therefore, while the observed antioxidant activity indicates promising bioactivity, further studies involving *in vivo* models and detailed mechanistic investigations are necessary to substantiate its therapeutic efficacy.

Thus, the integration of traditional medicinal knowledge with advanced pharmaceutical analytical techniques like HPTLC ensures the safety, efficacy and consistency of herbal medicines, bridging traditional herbal use with modern pharmaceutical standards (38). The results of this study, which identify *T. undulata* as a promising source of natural antioxidants and bioactive compounds for pharmacological development for future therapeutic formulations, fill the research gap between ethnomedicinal and modern pharmaceutical sciences.

Conclusion

The present study demonstrates that *Tecomella undulata* root bark contains significant amounts of phenolic metabolites with notable *in vitro* antioxidant potential. Importantly, caffeic acid is reported for the first time in the root bark extract using a validated high performance thin-layer chromatography method, which also provided clear and reproducible chromatographic fingerprints suitable for quality evaluation. These findings highlight the root bark as a promising source of bioactive compounds. However, the study is limited to *in vitro* analyses and the identification of a single compound; comprehensive *in vivo* studies and isolation of additional active constituents are necessary to fully understand the pharmacological potential of this species. Future research should focus on characterising other bioactive metabolites and evaluating their therapeutic activities.

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Authors' contributions

NB carried out the experiments, collected the data and prepared the original draft. SV conceptualised the study and reviewed and edited the manuscript. Both authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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