RESEARCH PAPER

Antioxidant Capacity of Ethanolic Root Extract of *Rotula Aquatica* Lour. (Boraginaceae): A DPPH-Based in Vitro Free Radical Scavenging Evaluation

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ABSTRACT

The present work aimed to determine the antioxidant capacity of an ethanolic root extract derived from *Rotula aquatica* Lour. (Boraginaceae)—an aquatic medicinal herb recognized in Ayurveda as Pasanabheda and historically used to manage urinary calculi and inflammatory disorders. The extract, hereafter referred to as ERERA, was assessed through the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging method for in vitro antioxidant activity, while its chemical profile was characterized using UV-Visible (UV-Vis) and Fourier Transform Infrared (FTIR) spectroscopy. Root material was extracted by cold maceration in 95% ethanol over 72 hours. Qualitative phytochemical tests detected alkaloids, tannins, flavonoids, saponins, phenolic compounds, terpenoids, and glycosides as principal secondary metabolite groups. UV-Vis scanning revealed absorbance peaks at 268.50 nm and 671.50 nm, attributable to aromatic polyphenolic chromophores. FTIR spectral data confirmed the presence of O–H, C–H, C=O, and C=C functional groups, consistent with a predominantly polyphenolic molecular framework. DPPH radical scavenging activity increased in a concentration-dependent manner across the 20–100 µg/mL range. The ethanolic root extract exhibited an IC_{50} value of 52.6 µg/mL, whereas standard ascorbic acid showed a lower IC_{50} value of 21.4 µg/mL, confirming the comparatively higher antioxidant potency of the standard compound. Despite this difference, ERERA demonstrated appreciable free radical scavenging activity, supporting the presence of bioactive phytoconstituents with antioxidant potential. These findings scientifically support the traditional medicinal use of *Rotula aquatica* roots and suggest their possible application as a natural antioxidant source.

Keywords: *Rotula aquatica*, DPPH, antioxidant, ethanolic extract, free radical scavenging, UV-Vis, FTIR, Boraginaceae, IC_{50}, phytochemical screening

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1. INTRODUCTION

Aerobic metabolism continuously produces free radicals and reactive oxygen species (ROS) as unavoidable by-products of mitochondrial electron transport, enzyme-mediated oxidative reactions, and immune-driven inflammatory responses.[1] Under healthy physiological conditions, a coordinated antioxidant defense system—encompassing enzymatic components such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, together with various non-enzymatic molecules—maintains redox balance by counteracting ROS accumulation. When this equilibrium is disrupted and ROS production exceeds the neutralizing capacity of endogenous defenses, oxidative stress ensues, which has been causally linked to the pathogenesis of numerous non-communicable diseases including cardiovascular disorders, type 2 diabetes, malignancies, neurodegenerative conditions, and premature cellular senescence.[2-4]

Concerns over the safety and potential pro-oxidant behavior of certain synthetic antioxidants under specific conditions have fueled growing scientific interest in plant-derived alternatives that may combine a favorable safety profile with broad biological activity.[5] Medicinal plants produce a diverse array of secondary metabolites—polyphenols, flavonoids, tannins, terpenoids, alkaloids, and vitamins—that exert antioxidant effects through several mechanistic routes: hydrogen atom transfer (HAT), single-electron transfer (SET), chelation of transition metal ions, and termination of radical chain propagation.[6,7]

Rotula aquatica Lour. (Boraginaceae), referred to as Pasanabheda in Sanskrit and Ratan-purus in Hindi, is an obligate aquatic herb growing on submerged rocky substrates in rapidly flowing river systems. Its native range encompasses the Western Ghats and Deccan plateau of India, extending into Sri Lanka and Myanmar.[8,9] Classical Ayurvedic texts, including the Charaka Samhita and Sushruta Samhita, record its therapeutic utility in conditions such as urolithiasis, dysuria, hematuria, dermatological

complaints, joint inflammation, and snake envenomation.[10,11]

Phytochemical characterization of *Rotula aquatica* root material has previously identified rotundifoline, alkaloids, tannins, flavonoids, saponins, and terpenoids—several of which are well-characterized antioxidant constituents.[12,13] Pharmacological studies have attributed anti-inflammatory, diuretic, nephroprotective, antibacterial, and antifungal activities to different extract preparations of this species.[14,15] However, a systematically documented antioxidant study of a maceration-derived ethanolic root extract, supported by combined UV-Vis and FTIR spectroscopic characterization, has not yet been reported.

The DPPH (1,1-Diphenyl-2-Picrylhydrazyl) radical scavenging assay is among the most widely adopted methods for preliminary in vitro antioxidant screening, valued for its technical simplicity, good reproducibility, and adaptability to routine laboratory settings. The DPPH radical is a stable, deep purple chromophore absorbing near 510–517 nm; reduction by an antioxidant—through hydrogen atom donation or single-electron transfer—converts it to the yellow-colored diphenylpicrylhydrazine, and the resulting decrease in absorbance is directly proportional to the antioxidant activity of the test compound.[16,17]

Against this background, the current study was designed to: (i) prepare ERERA by room-temperature maceration; (ii) conduct qualitative phytochemical screening; (iii) record UV-Vis spectra over the 200–800 nm range; (iv) obtain FTIR spectral data using a Bruker ATR instrument; and (v) evaluate in vitro antioxidant activity by DPPH assay, with ascorbic acid serving as the reference standard.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Material

Fresh root material of *Rotula aquatica* Lour. was obtained from rocky riverbeds in Mullaringanadu, Idukki District, Kerala, India, during the post-monsoon period (October–November). The plant identity was authenticated by a certified taxonomist at [Science College of Warana Nagar], Maharashtra. Collected roots were cleaned under running tap water, then dried in shade at 25 ± 2°C for 15–20 days until moisture was fully eliminated. The dried material was size-reduced using a mechanical grinder and passed through sieve No. 40 to produce a uniform coarse powder, which was stored in sealed airtight containers at room temperature until further use.[18,19]

2.2 Extract Preparation by Cold Maceration

Cold maceration was selected for extraction in order to preserve thermolabile secondary metabolites—particularly tannins and flavonoids—that may undergo structural degradation when exposed to elevated temperatures.[20] Precisely 100 g of root powder was submerged in 95% ethanol (500 mL) in a wide-mouthed glass vessel and allowed to macerate at 25 ± 2°C for 72 hours. The mixture was mechanically stirred at 8-hour intervals to ensure effective solute diffusion. After filtration through Whatman No. 1 filter paper, the spent marc was re-extracted with an additional 250 mL of ethanol for 24 hours. The two filtrates were pooled and then concentrated to a semisolid consistency on a rotary evaporator (40°C, reduced pressure), yielding ERERA. Percentage yield was calculated on a w/w basis. The extract was stored in a sealed amber vial at 4°C until analysis.[21]

2.3 Phytochemical Screening

Standard chemical tests were used to detect major classes of secondary metabolites in ERERA: alkaloids (Dragendorff's and Mayer's reagents); tannins and phenolic compounds (ferric chloride test); flavonoids (Shinoda/magnesium-HCl reduction test); saponins (froth test); terpenoids (Salkowski test); glycosides (Keller–Killiani test); and carbohydrates (Molisch's test). All procedures were carried out as per the standardized protocols of Trease and Evans (2002) and Harborne (1998).[22,23]

2.4 UV-Visible Spectroscopic Analysis

The UV-Vis absorption spectrum of ERERA (10 ppm solution in ethanol) was acquired on a UV-1800 Series double-beam spectrophotometer (Shimadzu Corporation, Japan) across the 200–800 nm range in absorbance mode. Instrument parameters: slit width 1.0 nm, light source changeover at 340 nm, fast scan speed, sampling interval 0.5 nm, S/R exchange in Normal mode. Baseline correction was performed using a pure ethanol blank. Characteristic absorption peaks were identified using the built-in peak-pick function (threshold: 0.001).[24]

2.5 FTIR Spectroscopic Analysis

FTIR spectra of ERERA were acquired on a Bruker FTIR spectrometer (Bruker Corporation, Germany) operating in Attenuated Total Reflectance (ATR) mode over the 400–4000 cm^{-1} wavenumber range. Functional group identification was accomplished by comparing observed spectral bands against published infrared correlation tables.[25]

2.6 DPPH Free Radical Scavenging Assay

The DPPH method, originally described by Brand-Williams et al. (1995) and adapted with minor procedural changes, was used to assess the in vitro antioxidant activity of ERERA.[26,27]

DPPH stock solution: A 0.1% (w/v) DPPH working solution was prepared by dissolving 3.94 mg of DPPH (Sigma-Aldrich, ≥95% purity) in 100 mL of analytical-grade methanol. The solution was stored at 4°C in a light-protected container and used within 24 hours of preparation.

Sample and standard solutions: ERERA was dissolved in DMSO (≤0.1% v/v final concentration) and serially diluted with methanol to yield concentrations of 20, 40, 60, 80, and 100 µg/mL. Ascorbic acid (HiMedia Laboratories, Mumbai) was prepared at identical concentrations as the positive reference.

Assay procedure: In a 96-well microplate, 100 µL of each test or standard solution was dispensed in triplicate. An

equal volume (100 µL) of 0.1 mM methanolic DPPH was then added to each well. After gentle mixing, the plate was incubated for 30 minutes at ambient temperature in the dark. Absorbance was subsequently measured at 517 nm using a microplate reader. Control wells contained DPPH solution with methanol (no sample); blank wells contained methanol only (no DPPH).[28]

Calculation: DPPH radical scavenging activity (% inhibition) was derived as follows:

$$\% \text{ Scavenging} = [(A_0 - A_t) / A_0] \times 100$$

where A_0 = absorbance of DPPH control (no sample) and A_t = absorbance of the test or standard well. The IC_{50}—the extract concentration producing 50% inhibition of DPPH radicals—was derived by non-linear regression of the concentration–inhibition curve. All measurements were performed in triplicate; data are reported as mean ± SD.[29,30]

2.7 Statistical Analysis

All results are expressed as mean ± SD (n = 3). IC_{50} values were calculated using GraphPad Prism v8.0 (GraphPad Software, USA). Intergroup differences were evaluated by one-way ANOVA with Tukey's post hoc test; statistical significance was defined at $p < 0.05$.

3. RESULTS

3.1 Extraction Yield and Macroscopic Properties

Maceration of 100 g dry root powder with 95% ethanol produced a dark brown semisolid extract (ERERA) carrying a characteristic earthy odor and a pronounced bitter taste—sensory attributes consistent with the co-extraction of tannins and alkaloids. The gravimetric yield was 8.6% w/w, in line with yield values documented for analogous root extract preparations of this species.[12]

3.2 Phytochemical Screening

Qualitative chemical analysis of ERERA detected a broad spectrum of secondary metabolite classes, as shown in Table 1.

Table 1: Results of qualitative phytochemical screening of ERERA

Phytochemical Class	Test Applied	Outcome
Alkaloids	Dragendorff's / Mayer's reagent	+++ (Present)
Tannins	Ferric chloride ($FeCl_3$) test	+++ (Present)
Flavonoids	Shinoda (Mg-HCl) test	+++ (Present)
Saponins	Froth test	++ (Present)
Terpenoids	Salkowski test	++ (Present)
Phenolic compounds	$FeCl_3$ test	+++ (Present)
Glycosides	Keller–Killiani test	+ (Present)
Carbohydrates	Molisch's test	++ (Present)
Proteins	Biuret test	− (Absent)
Fixed oils and fats	Spot test	− (Absent)

(+++) Abundantly present; (++) Moderately present; (+) Trace amounts; (−) Absent

3.3 UV-Visible Spectroscopic Analysis

UV-Vis scanning of ERERA (10 ppm, 200–800 nm) identified two absorption features (Table 2). The dominant peak at 268.50 nm (Abs. = 1.088) lies within the spectral window (250–290 nm) characteristic of π→π* electronic transitions in flavonoid and hydroxycinnamic acid chromophores—compound classes well represented in *Rotula aquatica* roots.[13] A weaker feature at 671.50 nm (Abs. = 0.028) is tentatively attributed to n→π* transitions originating from extended conjugated systems or charge-transfer interactions within the complex extract matrix.[24]

Table 2: UV-Vis absorption peak data for ERERA (10 ppm)

Peak No.	Type	Wavelength (nm)	Absorbance	Probable Assignment
1	Valley (V)	671.50	0.028	n→π* conjugated / charge-transfer
2	Peak (P)	268.50	1.088	π→π* polyphenolics / flavonoids

Instrument: UV-1800 Series (Shimadzu, Japan); Range: 200–800 nm; Slit: 1.0 nm.

3.4 FTIR Spectral Analysis

ATR-FTIR spectroscopy identified 13 distinct absorption bands in the ERERA spectrum (Table 3). The broad, high-intensity band at 3341.90 cm^{-1} is the diagnostic O–H stretching frequency of phenolic hydroxyl groups engaged in hydrogen bonding—a hallmark feature of tannin- and polyphenol-rich extracts. Aliphatic C–H stretching vibrations appear at 2973.41 cm^{-1} (asymmetric) and 2888.62 cm^{-1} (symmetric). The absorption at 1922.34 cm^{-1} may reflect conjugated C=O stretching from ester or aldehyde moieties, whereas the band at 1650.58 cm^{-1} corresponds to aromatic C=C ring stretching characteristic of polyphenolic scaffolds. A cluster of C–O stretching signals across 1043–1386 cm^{-1} points to ether, ester, and phenolic C–OH linkages. Bands at 879.33, 802.01, and 663.42 cm^{-1} indicate out-of-plane aromatic C–H bending, consistent with substituted aromatic rings.[25]

Table 3: FTIR peak assignments for ERERA (Bruker ATR-FTIR)

Peak No.	Wavenumber (cm^{-1})	Functional Group Assignment
1	3341.90	O–H stretch (broad) — phenolic / hydrogen-bonded hydroxyl
2	2973.41	C–H asymmetric stretch — aliphatic CH_2/CH_3
3	2888.62	C–H symmetric stretch — aliphatic methylene
4	1922.34	C=O stretch — conjugated carbonyl or ester
5	1650.58	C=C aromatic ring stretch — flavonoids / polyphenols
6	1385.91	C–O–H bending / C–H bending — phenolic OH
7	1326.37	C–O stretch — aromatic ethers / esters
8	1260.64	C–O–C asymmetric stretch — aryl ethers
9	1137.52	C–O stretch — secondary alcohols
10	1043.96	C–O stretch — primary alcohols / glycosidic C–O
11	879.33	Aromatic C–H out-of-plane bending
12	802.01	Aromatic C–H bending — para-substituted ring
13	663.42	C–H out-of-plane bending — aromatic

Instrument: Bruker FTIR Spectrometer; Mode: ATR.

3.5 DPPH Free Radical Scavenging Activity

Table 4 presents the complete triplicate absorbance data, mean absorbance values, percentage inhibition, and IC_{50} results for ERERA and ascorbic acid across all five tested concentrations.

Table 4: DPPH radical scavenging data for ERERA and ascorbic acid (standard)

S.No.	Sample	Conc. (µg/mL)	Test 1	Test 2	Test 3	Mean Abs.	% Inhibition	IC_{50} (µg/mL)
1	Control	—	1.93	1.93	1.93	1.93	—	—
2	Ascorbic Acid	20	1.05	1.03	1.02	1.03	46.5%	21.4
		40	0.70	0.68	0.69	0.69	64.5%	
		60	0.41	0.39	0.40	0.40	79.3%	
		80	0.20	0.19	0.19	0.19	90.1%	
		100	0.08	0.07	0.07	0.07	96.1%	
3	ERERA	20	1.47	1.45	1.44	1.45	24.5%	52.6
		40	1.18	1.16	1.17	1.17	39.2%	
		60	0.89	0.86	0.87	0.87	54.8%	
		80	0.62	0.60	0.59	0.60	68.6%	
		100	0.35	0.33	0.32	0.33	82.3%	

ERERA = Ethanolic Root Extract of Rotula aquatica; Absorbance at 517 nm; n = 3; IC_{50} by non-linear regression (GraphPad Prism 8.0)

ERERA exhibited a clear, progressive increase in radical scavenging activity with rising concentration. At 20 µg/mL the inhibition was 24.5%, climbing steadily to 82.3% at 100 µg/mL. The derived IC_{50} of ERERA (52.6 µg/mL) was slightly higher than that of ascorbic acid (21.4 µg/mL), indicating that although the extract possessed significant antioxidant activity, its radical scavenging potency was lower than the standard under the experimental conditions employed.

4. DISCUSSION

The outcomes of this study provide a systematic, evidence-based account of the antioxidant properties of ERERA and offer a mechanistic rationale grounded in the phytochemical and spectroscopic data collected.

Room-temperature maceration was deliberately chosen to prevent heat-induced degradation of sensitive secondary metabolites such as condensed tannins and flavonoids.[20] The use of 95% ethanol exploits its broad polarity window, facilitating simultaneous extraction of hydrophilic phenolics and relatively lipophilic alkaloids and glycosides. Ethanol's established GRAS status also supports pharmaceutical applications of the resulting extract.[21]

The phytochemical profile of ERERA—rich in tannins, flavonoids, alkaloids, phenolics, saponins, terpenoids, and glycosides—is broadly consistent with previously published data for root extracts of *Rotula aquatica*.[11-13] Tannins and polyphenols are well recognized as efficient hydrogen-atom donors capable of quenching multiple radical equivalents, while flavonoids serve dual roles as direct radical scavengers and metal chelators that suppress metal-catalyzed oxidative reactions.[6,7]

The UV-Vis absorption maximum at 268.50 nm is located within the 250–290 nm spectral range associated with $\pi \rightarrow \pi^*$ transitions of flavone, flavonol, and hydroxycinnamic acid chromophores. This observation aligns with UV profiles reported for phenolic constituents in *Rotula aquatica* and related Boraginaceae members.[13,24]

FTIR data reinforced the polyphenolic character of ERERA. The broad O–H stretching band at 3341.90 cm^{-1} is the

principal vibrational signature of phenolic hydroxyl groups—functional moieties directly implicated in hydrogen-atom donation to DPPH radicals as the dominant antioxidant mechanism.[25] The aromatic C=C absorption at 1650.58 cm^{-1} and the series of C–O bands between 1043 and 1386 cm^{-1} together corroborate an aromatic polyphenolic framework underpinning the observed radical-scavenging behavior.

The IC_{50} value of ERERA (52.6 µg/mL) indicated appreciable antioxidant activity, although it was lower in potency compared with the reference standard ascorbic acid (21.4 µg/mL). Earlier reports using Soxhlet-derived methanolic extracts have demonstrated lower IC_{50} values (e.g., 14.71 ± 0.03 µg/mL; Sathish et al., 2011), and the variation between those findings and the present study may reasonably be attributed to differences in extraction techniques, solvent polarity, geographical origin of the plant material, and seasonal fluctuations in phytochemical composition.[14,18,20]

The methodological conditions employed—0.1 mM methanolic DPPH, 30-minute dark incubation, and spectrophotometric reading at 517 nm—are consistent with widely validated antioxidant screening protocols.[16,17,26,27] The monotonic concentration-response relationship observed for ERERA reflects incremental radical saturation kinetics that are characteristic of polyphenol-rich extracts.[28,29] Taken together, the phytochemical, UV-Vis, and FTIR evidence point to phenolic hydroxyl-bearing polyphenols and flavonoids as the primary antioxidant-active constituents of *Rotula aquatica* roots.[6,30]

5. CONCLUSION

This study confirms that ERERA—an ethanolic root extract of *Rotula aquatica* Lour. prepared by cold maceration—possesses appreciable in vitro antioxidant activity. The extract demonstrated significant DPPH radical scavenging potential in a concentration-dependent manner, with an IC_{50} value of 52.6 µg/mL. Although the activity was lower than that of standard ascorbic acid (IC_{50} = 21.4 µg/mL), the findings suggest the presence of bioactive phytoconstituents capable of effectively neutralizing free radicals. Phytochemical screening identified tannins, flavonoids, alkaloids, saponins, terpenoids, and phenolic compounds as major constituents. UV-Vis analysis revealed a characteristic absorption at 268.50 nm attributable to polyphenolic chromophores, and Bruker ATR-FTIR spectroscopy confirmed the presence of O–H, C=C aromatic, and C–O functional groups consistent with a polyphenol-dominant extract composition. These findings collectively support a scientific rationale for the traditional use of *Rotula aquatica* in oxidative stress-linked disease management. Future work should focus on isolation and structural elucidation of individual bioactive constituents, in vivo antioxidant validation models, and investigation of potential synergistic activity among co-occurring phytochemicals.

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