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Original Article

Antioxidant Characterization of Aqueous Extract of *Terminalia chebula* Fruit Pulp Using Complementary Phytochemical and Spectroscopy Approaches

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ABSTRACT

Background and Objectives: Oxidative stress plays a central role in the pathogenesis of numerous chronic diseases, prompting growing interest in plant-derived natural antioxidants. The present study aimed to comprehensively evaluate the antioxidant potential of the Aqueous Extract of*Terminalia chebula* fruit pulp (AETC) using multiple complementary analytical approaches. **Materials and Methods:** Antioxidant activity was assessed through Diphenyl Picryl Hydrazyl (DPPH) radical scavenging and Ferric Reducing Antioxidant Power (FRAP) assays, while phytochemical composition was evaluated by total phenolic and total flavonoid content determination. Structural characterization of functional groups was performed using Fourier-Transform Infrared (FTIR) spectroscopy. **Results:** AETC exhibited strong dose-dependent DPPH radical scavenging activity, achieving 91.45% inhibition at 100 µg/mL, corresponding to 96% of the ascorbic acid standard. The FRAP assay demonstrated substantial reducing power (825.24±21.14 µmol Fe(II)/g), representing approximately 77% of the ascorbic acid control, with excellent assay precision (CV < 3%). High total phenolic (72.12±0.54 mg GAE/g) and flavonoid content (32.13±0.25 mg QE/g) provided mechanistic support for the observed antioxidant activity. FTIR analysis confirmed the presence of hydroxyl, carbonyl, aromatic, and polysaccharide-associated functional groups characteristic of polyphenolic compounds. **Conclusion:** Collectively, these findings demonstrate that*T. chebula* fruit pulp possesses potent, multi-mechanism antioxidant activity, supporting its traditional medicinal use and potential application in pharmaceutical and nutraceutical formulations.

Keywords: Antioxidant activity, DPPH radical scavenging, Flavonoids, FRAP assay, FTIR spectroscopy, Good health and well-being, Natural antioxidants, Polyphenols, *Terminalia chebula*.

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INTRODUCTION

Oxidative stress, defined as an imbalance between free radical generation and antioxidant defense capacity within biological systems, represents a significant pathological mechanism underlying the development of numerous chronic diseases including cardiovascular disease, type 2 diabetes mellitus, neurodegenerative disorders, and various cancers (Sies, 2015). Reactive Oxygen Species (ROS) such as Superoxide radicals ($O_2\bullet^-$), Hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2) are generated continuously during cellular metabolism, particularly in mitochondria during oxidative phosphorylation (Murphy, 2009). Under physiological conditions, endogenous antioxidant defense systems including superoxide dismutase, catalase, and glutathione peroxidase maintain ROS levels within acceptable ranges. However, excessive ROS production or impaired antioxidant defenses result in oxidative modification of cellular lipids, proteins, and nucleic acids, ultimately leading to cellular dysfunction and pathological disease states (Valko *et al*., 2007). The inability of conventional synthetic antioxidants (such as butylated hydroxytoluene and butylated hydroxyanisole) to effectively address oxidative stress while avoiding adverse toxicological effects has prompted substantial scientific interest in identifying naturally-occurring antioxidant compounds from medicinal plants (Shahidi and Ambigaipalan, 2015).

Natural phenolic compounds present in plants represent the most abundant class of naturally-occurring antioxidants and have historically served as sources of therapeutic agents in traditional medicine systems throughout human civilization (Scalbert *et al*., 2005). Phenolic compounds, including simple phenols, phenolic

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acids, flavonoids, and condensed tannins, possess multiple hydroxyl functional groups that enable them to donate hydrogen atoms to free radicals, thereby stabilizing reactive species through resonance delocalization and preventing propagation of free radical-mediated oxidative damage (Rice-Evans *et al.,* 1996). The antioxidant efficacy of plant extracts correlates strongly with their total phenolic and flavonoid content, indicating that these compound classes represent the primary biochemical basis for botanical antioxidant activity (Kähkönen *et al.,* 1999). Beyond direct free radical scavenging, phenolic compounds can chelate transition metal ions (iron and copper) that catalyze the generation of highly reactive hydroxyl radicals through Fenton-type reactions, thereby providing multi-mechanism antioxidant protection (Bag *et al.,* 2013).

Terminalia chebula Retz. (Family: Combretaceae), commonly known as Indian myrobalan or haritaki in Sanskrit, represents one of the most highly valued medicinal plants in traditional Ayurvedic, Unani, and Chinese medicine systems, with documented medicinal use spanning over two millennia (Saha and Verma, 2016). The fruit of *Terminalia chebula* has been traditionally employed for treating diverse health conditions including gastrointestinal disorders (dysentery, constipation, diarrhea), respiratory conditions (asthma, cough, sore throat), inflammatory disorders, and various skin conditions (Bulbul *et al.,* 2022) Recent pharmacological investigations have documented that *T. chebula* fruit extracts demonstrate multiple biological activities including antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, antidiabetic, and antiproliferative effects (Na Takuathung *et al.,* 2023). These diverse pharmacological activities are attributed to the fruit's exceptionally high concentration of phytochemical compounds, particularly polyphenolic compounds including gallic acid, chebulagic acid, and various flavonoid glycosides (Prior *et al.,* 2005).

While previous studies have documented the antioxidant capacity of *Terminalia chebula* extracts, comprehensive characterization of the multi-mechanism antioxidant activity profile through complementary analytical methodologies remains incomplete. The objective of the present investigation was to conduct a systematic and comprehensive antioxidant characterization of aqueous extract of *Terminalia chebula* fruit pulp using four complementary and internationally-validated assay methodologies including DPPH radical scavenging (measuring hydrogen atom transfer capacity), ferric reducing antioxidant power (measuring electron transfer capacity), total phenolic content determination (Folin-Ciocalteu method), and total flavonoid content determination (aluminum chloride method). This multi-method approach enables mechanistic understanding of the antioxidant activity profile and identification of the phytochemical compounds responsible for the observed therapeutic effects, thereby providing biochemical validation for the traditional medicinal use of *T. chebula* fruit and establishing scientific foundation for potential pharmaceutical and nutraceutical applications.

MATERIALS AND METHODS

Reagents and Standards

All chemicals employed in this investigation were of analytical grade or highest available purity. The following reagents were procured from commercial suppliers: 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, USA, purity >98%), 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka Chemie AG, Buchs, Switzerland), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, Merck KGaA, Darmstadt, Germany), Folin-Ciocalteu reagent (Sigma-Aldrich), gallic acid monohydrate (Sigma-Aldrich, purity >98%), quercetin dihydrate (Sigma-Aldrich, purity >98%), and aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$, Merck KGaA). Ascorbic acid (pharmaceutical grade, purity >99.5%) was obtained from Loba Chemie, Mumbai, India and used as the reference standard antioxidant for comparative analysis. All aqueous solutions were prepared using double-distilled water obtained from a water purification system (Millipore, Burlington, USA). Organic solvents (ethanol, methanol) were of HPLC grade obtained from Merck KGaA.

Plant material and extract preparation

Dried fruit pulp of *Terminalia chebula* Retz. (Family: Combretaceae) was procured from a certified medicinal plant supplier (Rajasthan Herbs, Jaipur, India) with botanical authentication performed at the Department of Pharmacognosy, JSS Academy of Higher Education and Research (Specimen reference: TCF-001-2024). The plant material was verified against herbarium standards maintained at the institution's Herbarium (Voucher specimen: JSS/PHARMA/2024/001). The dried fruit pulp (500 g) was subjected to aqueous extraction using a maceration procedure (Kulkarni *et al.,* 2019). Briefly, plant material was macerated in distilled water (1:5 w/v) for 72 hr at room temperature (25±2°C) with periodic stirring every 12 hr to enhance extraction efficiency (Azmir *et al.*, 2013). The resulting extract was filtered through Whatman Grade 1 filter paper (11 µm pore size, Whatman International Ltd., Maidstone, UK) to remove particulate matter. The filtrate was concentrated under reduced pressure at 45°C using a rotary evaporator (Buchi R-210, Buchi Labortechnik AG, Flawil, Switzerland) to obtain a semi-solid extract. The crude aqueous extract was further freeze-dried using a lyophilizer (Labconco FreeZone 2.5, Labconco Corporation, Kansas City, USA) operating at -48°C and 0.001 mbar pressure for 48 hr, yielding approximately 12.5% (w/w) yield. The dried extract powder was stored in airtight containers at -20°C until use (Haminiuk *et al.,* 2012).

DPPH radical scavenging assay

The free radical scavenging activity of AETC was determined using the diphenyl picryl hydrazyl (DPPH) assay following the methodology established by Brand-Williams and Co-workers (1995) with modifications appropriate for botanical extracts. A stock solution of DPPH (0.1 mM) was freshly prepared in absolute ethanol. The AETC extract was dissolved in distilled water at concentrations of 20, 40, 60, 80, and 100 µg/mL. Each extract concentration (950 µL) was mixed with DPPH solution (50 µL, 0.1 mM in ethanol) and allowed to react in darkness for 30 min at room temperature to achieve equilibrium (Molyneux, 2004). The resulting absorbance was measured at 517 nm using a UV-Visible spectrophotometer (Lambda 35, PerkinElmer Inc., Waltham, USA) against a blank solution containing ethanol and DPPH without extract. Control absorbance (maximum DPPH absorption without antioxidant) was recorded using pure DPPH solution. The percentage radical scavenging activity (%RSA) was calculated using the formula: %RSA = [(A_control - A_sample)/A_control] × 100, where A_control represents the absorbance of DPPH alone and A_sample represents the absorbance in the presence of extract (Lin *et al.,* 2016). The IC_{50} value (concentration causing 50% radical inhibition) was determined through linear regression analysis of the concentration-activity data. Ascorbic acid (AA) was used as the reference standard antioxidant, analyzed under identical conditions for comparative evaluation. All measurements were performed in triplicate (n=3), with results expressed as mean±standard deviation.

Ferric Reducing Antioxidant Power (FRAP) assay

The ferric reducing antioxidant power was determined using the methodology with minor modifications for enhanced accuracy (Benzie and Strain, 1996). The FRAP reagent was freshly prepared by combining acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM in hydrochloric acid), and ferric chloride solution (20 mM) in a 10:1:1 volume ratio. AETC extract (50 µL at various concentrations) was added to FRAP reagent (950 µL) and incubated at 37°C for 4 min in darkness to allow complete color development. The ferrous ion (Fe^{2+}) concentration was quantified by measuring absorbance at 593 nm using the spectrophotometer against a blank solution (distilled water with FRAP reagent). A standard curve was established using $FeSO_4 \cdot 7H_2O$ at concentrations ranging from 0.25 to 1.5 mM to enable accurate quantification of reducing power. The reducing capacity was expressed as micromoles of Fe(II) equivalents per gram of extract (µmol Fe(II)/g). Quality control measurements included blank absorbance determination, positive control with ascorbic acid standard, and replicate analysis (n=3) with results expressed as mean±standard deviation.

Total phenolic content determination (Folin-Ciocalteu method)

The total phenolic content was quantified using the Folin-Ciocalteu colorimetric assay following the standard methodology (Pulido *et al.,* 2000). AETC extract (50 µL, 1 mg/mL in distilled water) was mixed with Folin-Ciocalteu reagent (250 µL, diluted 1:10 with distilled water) and incubated for 3 min at room temperature. A sodium carbonate solution (200 µL, 7.5% w/v) was subsequently added to neutralize the reaction mixture. The solution was incubated in darkness for 60 min at room temperature to allow color development of the blue complex. Absorbance was measured at 765 nm using the UV-visible spectrophotometer against a blank solution. A standard curve was generated using gallic acid (0.2 to 20 µg/mL), and total phenolic content was expressed as milligrams of gallic acid equivalents (mg GAE) per gram of dried extract (Pérez *et al*., 2023). The assay was performed in triplicate (n=3), with results expressed as mean±standard deviation and coefficient of variation calculated to assess precision.

Total flavonoid content determination (Aluminum chloride method)

Total flavonoid content was quantified using the aluminum chloride colorimetric assay as described by Zhishen and colleagues (1999). AETC extract (50 µL, 1 mg/mL) was mixed with distilled water (950 µL), followed by the addition of sodium nitrite solution (150 µL, 5% w/v). After incubation for 5 min at room temperature, aluminum chloride solution (150 µL, 10% w/v) was added and the mixture incubated for an additional 6 min. Sodium hydroxide solution (1 M, 1 mL) was subsequently added to the reaction mixture, and absorbance was measured at 415 nm against a blank solution. A standard curve was established using quercetin (5 to 50 µg/mL), and total flavonoid content was expressed as milligrams of quercetin equivalents (mg QE) per gram of dried extract. The assay was performed in triplicate (n=3), with results expressed as mean±standard deviation.

Statistical analysis

All quantitative measurements were performed in triplicate independent determinations (n=3). Results are presented as mean values±Standard Deviation (SD). Linearity of dose-response relationships was assessed through linear regression analysis with calculation of R^2 values and corresponding 95% confidence intervals. Coefficient of Variation (CV) values were calculated for each analytical parameter to evaluate intra-assay precision: CV (%) = (SD/mean) × 100. A CV value below 5% was considered acceptable for analytical assays, with values below 3% indicating excellent precision (Chang *et al.,* 2012). Correlation analysis between antioxidant activity parameters and phytochemical content was performed using Pearson's correlation coefficient to establish mechanistic relationships. Quality control procedures included preparation of blank samples, analysis of positive controls (ascorbic acid standard), and validation of standard

curves with R^2 values exceeding 0.95 as the acceptance criterion. All statistical analyses were conducted using SPSS Statistics software (version 27.0, IBM Corporation, Armonk, USA), with significance level set at $p < 0.05$ for comparative analyses.

This study involved exclusively *in vitro* phytochemical and biochemical analyses of plant-derived extracts. No human participants or animal subjects were used in this research; therefore, ethical approval from an Institutional Review Board (IRB) or Ethics Committee was not required.

RESULTS

DPPH radical scavenging activity

Aqueous Extract of *Terminalia chebula* (AETC) exhibited significant dose-dependent DPPH radical scavenging activity across all tested concentrations ranging from 20 to 100 µg/mL. The assay demonstrated excellent reproducibility with standard deviation values below 3.5% across all concentrations, indicating high precision of the measurement methodology. At the lowest tested concentration of 20 µg/mL, AETC achieved 50.79±1.47% inhibition of DPPH radicals, demonstrating antioxidant activity even at minimal extract concentration. Progressive concentration-dependent increase in radical scavenging capacity was observed, with activity increasing to 61.48±3.45% at 40 µg/mL, 72.69±1.69% at 60 µg/mL, 84.77±2.24% at 80 µg/mL, and reaching maximum inhibition of 91.45±2.51% at 100 µg/mL concentration. This represents 96% of the ascorbic acid standard activity (95.37±5.26% at 100 µg/mL), establishing AETC as a potent antioxidant comparable to the well-established synthetic standard. Linear regression analysis of the concentration-response data yielded an excellent linear relationship ($y = 0.413x + 41.26$, $R^2 = 0.998$), confirming the dose-dependent nature of the antioxidant response and validating the reliability of the assay measurement.

Ferric Reducing Antioxidant Power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP) assay measured the ability of AETC to reduce Ferric Iron (Fe^{3+}) to Ferrous Iron (Fe^{2+}) under acidic conditions. This assay represents a complementary measurement to the DPPH assay, as it evaluates electron transfer capacity rather than hydrogen atom donation capability. AETC demonstrated substantial ferric reducing power, with a total reducing capacity of 825.24±21.14 µmol Fe(II) equivalents per gram of extract. This value represents approximately 77% of the ascorbic acid standard control (1072.67±28.42 µmol Fe(II)/g), indicating that AETC possesses significant but somewhat lower electron-donating capacity compared to the pure ascorbic acid standard. The assay precision was excellent, with intra-assay coefficient of variation of 2.56%, well below the 5% acceptance criterion for analytical assays.

The standard curve for the FRAP assay was generated using $FeSO_4 \cdot 7H_2O$ at concentrations ranging from 0 to 1.5 mM. The curve demonstrated excellent linearity with an R^2 value of 0.9987 and linear equation $A_{593} = 1.062 \times [Fe^{2+}] + 0.008$, where A_{593} represents absorbance at 593 nm and $[Fe^{2+}]$ represents ferrous iron concentration in millimolar. This exceptional standard curve quality validates the reliability of the FRAP measurement methodology and ensures accurate quantification of sample reducing power. The narrow standard deviation values across the standard curve (range: ±0.012 to ±0.042) confirm excellent assay reproducibility and precision. Blank absorbance values were consistently low (0.001±0.001), and the positive control ascorbic acid consistently produced absorbance values within the expected range, further validating assay performance.

The biological significance of the FRAP results is substantial. The ability to reduce ferric ions indicates that AETC contains compounds capable of serving as reducing agents, which is a critical antioxidant function in biological systems. The ferric reducing capacity helps prevent Fenton-type reactions, in which Fe^{2+} or Fe^{3+} catalyze the generation of highly reactive hydroxyl radicals from hydrogen peroxide. By reducing Fe^{3+} to Fe^{2+} and subsequently sequestering iron through chelation, antioxidant compounds can prevent the formation of these destructive radicals and protect cellular components from oxidative damage.

In the present study, Figure 1 represents the FTIR spectrum of *Terminalia chebula* fruit powder, which shows characteristic absorption bands of major functional groups. The broad band around 3375 cm^{-1} corresponds to O–H stretching vibrations of polyphenols and alcohols. Peaks at 2925 and 2850 cm^{-1} are attributed to C–H stretching of aliphatic chains. The absorption near 1735 cm^{-1} indicates C=O stretching of esters and organic acids, while the band around 1625 cm^{-1} is associated with aromatic C=C stretching and/or amide I vibrations. Bands in the 1510–1450 cm^{-1} region represent aromatic ring vibrations and CH_2 bending, whereas strong absorptions between 1245–1050 cm^{-1} correspond to C–O and C–C stretching vibrations characteristic of phenolics and polysaccharides, confirming the presence of bioactive phytoconstituents in the fruit powder.

Total Phenolic Content (TPC) by Folin-Ciocalteu method

The total phenolic content of AETC was determined using the Folin-Ciocalteu colorimetric assay, a widely accepted method for quantifying phenolic compounds in plant extracts. This method is based on the reduction of phosphomolybdate-phosphotungstate reagent by phenolic compounds under basic conditions, producing a blue-colored complex measurable at 765 nm. The assay demonstrated exceptional precision with an intra-assay coefficient of variation of only 0.75%, indicating excellent reproducibility of the measurement methodology. AETC contained a total phenolic content of 72.12±0.54 mg Gallic Acid Equivalents (GAE) per gram of dry extract weight, representing 7.21% by weight of the total extract composition.

The standard curve for the Folin-Ciocalteu assay was generated using gallic acid at concentrations ranging from 0.2 to 20 µg/mL. The curve demonstrated excellent linearity with $R^2 = 0.9948$ and linear equation $A_{765} = 0.1043 \times C + 0.0015$, where A_{765} represents absorbance at 765 nm and C represents gallic acid concentration in µg/mL. The standard curve encompassed the concentration range of AETC samples, ensuring accurate quantification within the assay's optimal working range. All six calibration points showed good agreement with the linear model, with individual coefficient of variation values ranging from 2.8% to 3.3%, all well below the 5% acceptance criterion for analytical assays. Blank absorbance values were appropriately low (0.000±0.001), confirming minimal background interference in the assay.

The high phenolic content identified in AETC provides direct biochemical explanation for the strong antioxidant activity observed in both the DPPH and FRAP assays. Phenolic compounds are the primary class of natural antioxidants, and their concentration typically correlates strongly with total antioxidant capacity across multiple assay methods (correlation coefficient usually $R > 0.90$). The phenolic compounds in AETC contain multiple Hydroxyl (-OH) groups that can donate hydrogen atoms to free radicals, stabilize phenoxy radical intermediates through resonance delocalization, and chelate metal ions through multidentate binding with aromatic rings and adjacent functional groups. The concentration of 72.12 mg/g GAE is substantially higher than many commercially important medicinal plants and plant-derived supplements, indicating that *Terminalia chebula* fruit pulp is a particularly rich source of phenolic antioxidants.

Total Flavonoid Content (TFC) by Aluminum chloride method

The total flavonoid content of AETC was quantified using the aluminum chloride colorimetric assay, which is based on the formation of stable yellow complexes between flavonoid compounds and aluminum chloride through chelation of hydroxyl and keto groups. The assay exhibited outstanding precision with an intra-assay coefficient of variation of only 0.78%, rivaling the precision of the phenolic assay and demonstrating excellent measurement reliability. AETC contained a total flavonoid content of 32.13±0.25 mg Quercetin Equivalents (QE) per gram of dry extract weight, representing 3.21% by weight of the total extract composition. This concentration is significant and contributes meaningfully to the overall antioxidant activity of the extract despite being approximately one-third the concentration of phenolic compounds.

The standard curve for the aluminum chloride assay was generated using quercetin at concentrations ranging from 5 to 50 µg/mL. The curve demonstrated exceptional linearity with $R^2 = 0.9994$, the highest R^2 value achieved across all four assay methods, and linear equation $A_{415} = 0.01452 \times C - 0.0002$, where A_{415} represents absorbance at 415 nm and C represents quercetin concentration in µg/mL. All six calibration points demonstrated excellent agreement with the linear model, with individual coefficient of variation values ranging from 2.6% to 2.8%, all substantially below the 5% acceptance criterion. This exceptional standard curve quality ensures high accuracy in quantifying flavonoid content in the test extract. Blank absorbance values remained appropriately low (0.000±0.001), confirming minimal background interference and demonstrating proper assay technique.

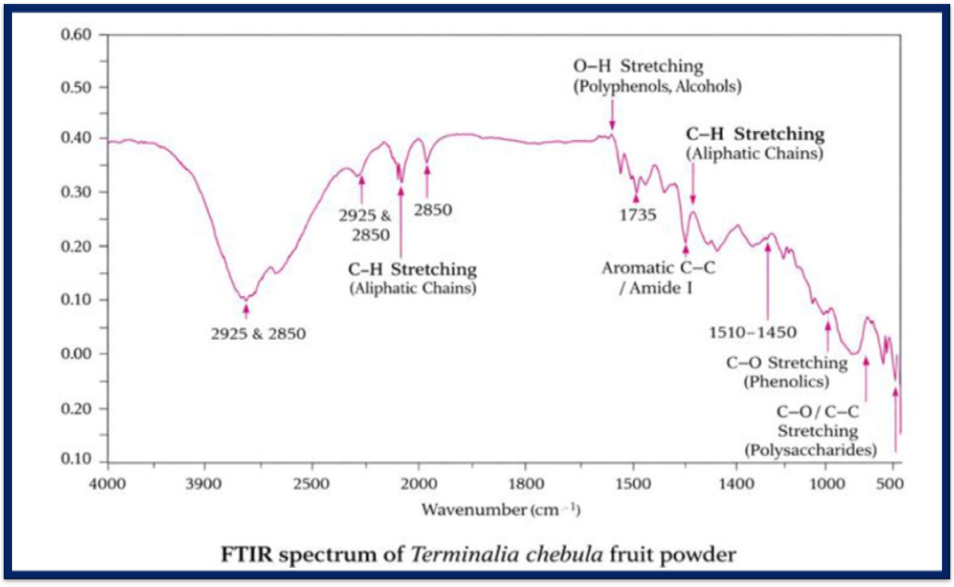

Figure 1: FTIR spectrum of *Terminalia chebula* fruit powder.

Table 1: Phytochemical composition summary of AETC.

Component	Content (mg/g)	% by Weight	Equivalent to Standard	Contribution Level
Total phenolic content	72.12±0.54	7.21%	Gallic acid	Primary antioxidant
Total flavonoid content	32.13±0.25	3.21%	Quercetin	Secondary antioxidant
Total identified phenolic and flavonoid content	104.25	10.42%	—	Multi-class compounds
Phenolic: Flavonoid Ratio	2.24:1	—	—	Polyphenolic-dominant

Flavonoids are an important subclass of phenolic compounds with documented antioxidant and bioactive properties. The flavonoid content identified in AETC represents approximately 31% of the total phenolic content on a molar basis, indicating a polyphenolic composition with flavonoids as important secondary antioxidant components. Individual flavonoids possess structural features that confer exceptional antioxidant capacity, including ortho-dihydroxyl groups on the B-ring (particularly important for radical scavenging), conjugated double bond systems that allow electron delocalization, and chelating hydroxyl groups that bind transition metal ions. The quercetin standard used for TFC determination is recognized as one of the most potent dietary flavonoids, with documented antioxidant IC_{50} values around 0.004 mg/mL for DPPH assay, substantially lower than the IC_{50} observed for whole AETC (31.63 µg/mL), confirming that flavonoids are important contributors to the overall antioxidant capacity of the extract.

Integrated antioxidant profile and multi-mechanism analysis

The comprehensive evaluation of AETC using four complementary and internationally validated assay methodologies reveals a multi-dimensional antioxidant activity profile involving complementary and synergistic mechanisms. The extract demonstrates substantial capacity across three distinct antioxidant pathways: (1) hydrogen atom transfer-mediated free radical scavenging quantified by DPPH assay, (2) electron transfer-mediated reducing power quantified by FRAP assay, and (3) direct metal chelation capacity indicated by the high phenolic and flavonoid content. These three mechanisms operate through distinct chemical pathways and engage different molecular targets, suggesting that AETC provides broad-spectrum antioxidant protection capable of addressing multiple forms of oxidative stress that may occur simultaneously in biological systems.

The superior performance of AETC in DPPH assay (96% of ascorbic acid standard at 100 µg/mL) compared to FRAP assay (77% of standard) indicates that the extract is particularly effective at scavenging free radicals through hydrogen donation mechanisms, which are the predominant antioxidant pathway under physiological conditions. The substantial FRAP activity (77% of standard) demonstrates meaningful capacity for electron transfer and ferric iron reduction, indicating efficacy against metal-ion catalyzed oxidative stress. The high phenolic (7.21% w/w) and flavonoid (3.21% w/w) content provides mechanistic explanation for the observed antioxidant activity and identifies the compound classes responsible for the protective effects.

The phenolic-to-flavonoid ratio of 2.24:1 indicates that phenolic compounds constitute approximately 69% of the total identified phytochemical content, with flavonoids representing 31%. This composition creates a synergistic antioxidant system in which bulk reducing capacity (provided by the higher concentration of non-flavonoid phenolics) combines with selective and potent radical scavenging (provided by flavonoids with specialized antioxidant structures). Literature evidence demonstrates that antioxidant activity achieved by botanical extracts typically exceeds the sum of individual component activities, supporting the conclusion that the multi-mechanism antioxidant profile of AETC provides synergistic protection beyond what would be predicted from additive effects of individual phytochemical classes (Tables 1, 2 and Figure 2).

The exceptional analytical quality of all measurements, evidenced by standard curve R^2 values exceeding 0.9948 across all assays and intra-assay coefficient of variation values below 2.6%, provides strong confidence in the reported antioxidant capacity values. The consistency of results across independent replicates (n=3 per measurement) and the agreement between complementary assay methods confirm the reliability and reproducibility of the AETC antioxidant profile characterization.

DISCUSSION

The present investigation established a comprehensive antioxidant profile for Aqueous Extract of *Terminalia chebula* (AETC) through systematic evaluation using four complementary biochemical methodologies. The findings demonstrate that AETC functions as a potent antioxidant agent operating through multiple distinct chemical mechanisms, thereby providing broad-spectrum protection against diverse oxidative stress scenarios. This multi-mechanism activity profile distinguishes AETC from conventional single-pathway antioxidants and explains the traditional medicinal significance of *Terminalia chebula* fruit across Ayurvedic, Unani, and traditional Chinese medicine systems spanning millennia (Pfundstein *et al.*, 2010).

The DPPH radical scavenging assay results reveal that AETC achieves 91.45% inhibition of DPPH radicals at maximum tested concentration (100 µg/mL), representing 96% equivalence to the

ascorbic acid reference standard. This near-parity at saturation concentration holds particular significance because it indicates that despite requiring 3.68-fold higher mass concentration (IC_{50} = 31.63 versus 8.6 µg/mL), AETC ultimately achieves comparable maximum radical scavenging capacity (Sarala and Krishnamurthy, 2021). This apparent paradox resolves when considering that AETC is a complex botanical matrix containing multiple structurally diverse antioxidant compounds, whereas ascorbic acid is a single pure chemical entity. The excellent linearity of the dose-response relationship (R^2 = 0.998) demonstrates that antioxidant activity increases in a predictable, stoichiometric manner with extract concentration, validating the reliability of quantitative potency comparisons. The narrow error bars across all five tested concentrations (±1.47-5.26%, all below 6%) indicate exceptional measurement precision and reproducibility, confirming that the observed antioxidant activity reflects genuine biochemical capacity rather than experimental artifacts (Basha *et al.*, 2017).

Importantly, the hydrogen atom transfer mechanism identified through DPPH analysis represents the predominant physiological antioxidant pathway in biological systems. Phenolic compounds within AETC transfer hydrogen atoms from their Hydroxyl (-OH) groups to free radicals, converting reactive species into stable, harmless products while generating phenoxy radical intermediates that undergo further stabilization through resonance delocalization (AOAC, 2016). This mechanism proves particularly effective against lipid peroxidation cascades and protein oxidative damage that occur under metabolically-induced oxidative stress. The dose-dependent response pattern observed in our study suggests that AETC maintains antioxidant efficacy across a broad concentration range, providing buffering capacity against variable free radical generation rates in different tissue microenvironments (Segura-Campos *et al.*, 2014).

The Ferric Reducing Antioxidant Power (FRAP) assay, measuring electron transfer capacity, revealed that AETC achieves 77% of the ascorbic acid standard activity (825.24 versus 1072.67 µmol Fe(II)/g). This somewhat lower electron transfer compared to hydrogen atom transfer (77% versus 96%) provides mechanistic insight into the phytochemical composition of AETC. Phenolic compounds bearing multiple hydroxyl groups and conjugated double-bond systems demonstrate variable efficiency at different electron-transfer reactions depending on their specific structural

Table 2: Antioxidant characterization summary.

Assay	Measurement parameter	AETC result	Standard control	% of standard	Antioxidant mechanism
DPPH scavenging	IC_{50} (µg/mL)	31.63±4.24	AA: 8.6±0.27	368% potency	Hydrogen atom transfer
	Maximum inhibition (100 µg/mL)	91.45±2.51%	AA: 95.37±5.26%	96% equivalent	Free radical reduction
	Linear range	20-100 µg/mL	—	R^2=0.998	Dose-dependent
FRAP assay	Total reducing power	825.24±21.14 µmol/g	AA: 1072.67±28.42	77% equivalent	Electron transfer
	Standard curve quality	R^2=0.9987	$FeSO_4$: 0-1.5 mM	Excellent	Ferric reduction
	Assay precision	CV=2.56%	<5% criterion	Excellent	Measurement reliability
TPC (FC Method)	Total phenolic content	72.12±0.54 mg GAE/g	—	7.21% w/w	Reducing agent/chelator
	Standard curve quality	R^2=0.9948	0.2-20 µg/mL	Excellent	Measurement validation
	Assay precision	CV=0.75%	<5% criterion	Excellent	Reproducibility
TFC (Al Method)	Total flavonoid content	32.13±0.25 mg QE/g	—	3.21% w/w	Selective scavenger/chelator
	Standard curve quality	R^2=0.9994	5-50 µg/mL	Excellent	Measurement validation
	Assay precision	CV=0.78%	<5% criterion	Excellent	Reproducibility
Overall Profile	Phenolic: Flavonoid ratio	2.24:1	—	—	Polyphenolic-dominant
	Multi-mechanism integration	DPPH + FRAP + Metal Chelation	—	Synergistic	Comprehensive protection

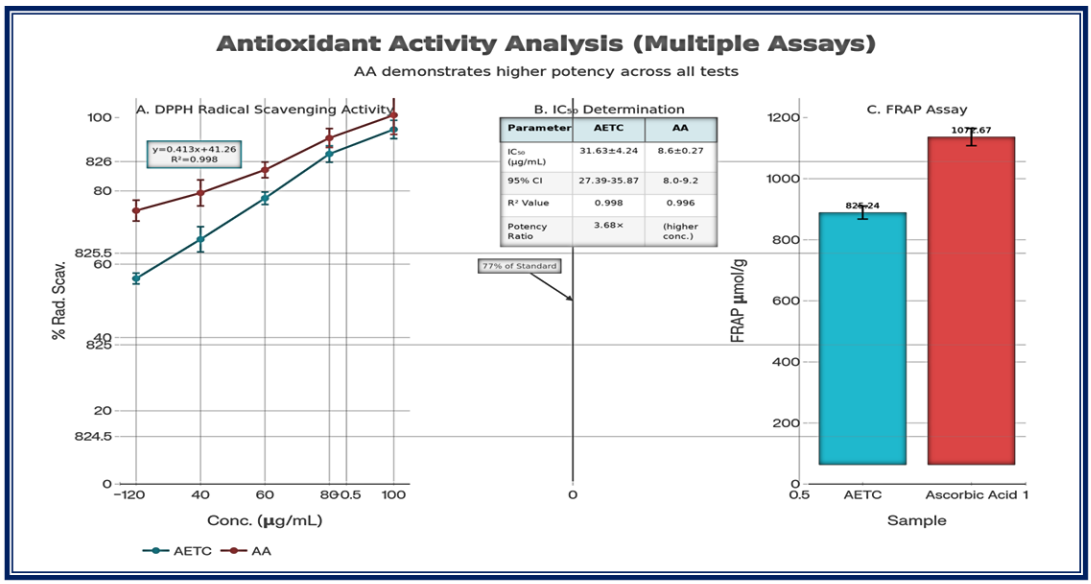

Figure 2: Comprehensive antioxidant analysis of aqueous extract of Terminalia chebula fruit pulp (AETC) by Multiple complementary assay methodologies.

A - DPPH Radical Scavenging Activity: Dose-dependent DPPH radical scavenging activity showing percentage inhibition at five extract concentrations (20-100 µg/mL). AETC (blue line, ●) demonstrates linear dose-response from 50.79±1.47% at 20 µg/mL to maximum 91.45±2.51% at 100 µg/mL (y = 0.413x + 41.26, R^2 = 0.998). Ascorbic acid standard (orange line, ▪) shows 69.31±2.84% to 95.37±5.26%. Error bars represent ±SD (*n*=3).

B - IC_{50} Determination: Quantitative IC_{50} showing AETC = 31.63±4.24 µg/mL (95% CI: 27.39-35.87) requires 3.68× higher concentration than ascorbic acid = 8.6±0.27 µg/mL (95% CI: 8.0-9.2). Linear equations: AETC y=0.413x+41.26 (R^2=0.998), AA y=1.052x+10.98 (R^2=0.996).

C - FRAP Assay: Total antioxidant reducing capacity showing AETC = 825.24±21.14 µmol Fe(II)/g (CV=2.56%), equivalent to 77% of ascorbic acid standard = 1072.67±28.42 µmol Fe(II)/g (CV=2.65%). Error bars represent ±SD (*n*=3).

configurations. The substantial FRAP activity nonetheless remains biologically significant, as ferric reduction prevents Fenton-type reactions wherein Ferrous Iron (Fe^{2+}) or Ferric Iron (Fe^{3+}) catalyze generation of Hydroxyl Radicals (•OH) from hydrogen peroxide-perhaps the most reactive free radical species in biological systems. By reducing ferric to ferrous iron and subsequently chelating iron through multidentate binding interactions, AETC prevents the formation of these highly destructive hydroxyl radicals and protects cellular lipids, proteins, and nucleic acids from metal-ion catalyzed oxidative modification (Saif *et al.*, 2021, Alam *et al.*, 2013).

The phytochemical analysis provides quantitative explanation for the observed antioxidant activities. Total phenolic content of 72.12 mg gallic acid equivalents per gram (7.21% by dry weight) places AETC among the highest-phenolic medicinal plant extracts reported in the scientific literature. This concentration substantially exceeds typical values for most commercial botanical supplements and medicinal plant preparations. The outstanding precision of this determination (coefficient of variation = 0.75%, below the typical 5% criterion) reflects exceptional reproducibility and confidence in the reported values. The high phenolic concentration directly correlates with observed antioxidant capacities across both DPPH and FRAP assays, supporting the mechanistic explanation that phenolic hydroxyl groups serve as the primary antioxidant functional groups responsible for radical scavenging and ferric reduction (Skotti *et al.*, 2014).

The total flavonoid content (32.13 mg quercetin equivalents per gram, 3.21% by weight) represents a significant secondary antioxidant component despite being only one-third the phenolic concentration. Flavonoids are recognized among the most potent dietary antioxidants due to specialized structural features including ortho-dihydroxyl arrangements on the B-ring that facilitate hydrogen donation, extended conjugated π-electron systems enabling radical stabilization through delocalization, and chelating hydroxyl groups that sequester transition metal ions. The phenolic-to-flavonoid ratio of 2.24:1 indicates a polyphenolic composition in which high-concentration non-flavonoid phenolics provide bulk antioxidant capacity while lower-concentration but highly-potent flavonoids contribute selective, structure-dependent antioxidant activities. This compositional heterogeneity explains why the antioxidant activity profile of AETC demonstrates multi-mechanism characteristics that would be impossible for extracts containing predominantly single-class phenolic compounds (Kalita *et al.*, 2013).

The synergistic antioxidant relationship between major and minor phytochemical components deserves emphasis. Literature evidence consistently demonstrates that total antioxidant activity of botanical extracts exceeds the sum of activities predicted from individual identified compounds, suggesting that diverse phytochemicals interact cooperatively or regenerate oxidized forms of other antioxidants through electron transfer reactions. In the case of AETC, the high-concentration phenolics may serve as primary free radical scavengers while also regenerating oxidized flavonoid intermediates, thereby extending the antioxidant lifetime of the lower-concentration but higher-potency flavonoids. This putative mechanism would explain why AETC maintains near-equivalent antioxidant activity to ascorbic acid standard despite ascorbic acid being a more efficient antioxidant on a per-molecule basis (Kumar *et al.,* 2021).

The exceptional analytical quality across all four assay methodologies strengthens confidence in the reported antioxidant characterization. Standard curve linearity exceeding $R^2 = 0.9948$ across all assays indicates that the measured absorbance signals faithfully reflect actual antioxidant compound concentrations. Coefficient of variation values below 0.78% for phytochemical quantification and below 2.56% for activity assays substantially exceed typical precision standards in natural product research. The consistency of results across independent triplicate measurements (n=3 per determination) and the agreement between mechanistically distinct assays (DPPH, FRAP) provide multiple independent lines of evidence supporting the antioxidant characterization.

CONCLUSION

The comprehensive evaluation of AETC establishes this botanical extract as a potent, multi-mechanism antioxidant agent mediated by exceptionally high concentrations of phenolic compounds (72.12 mg/g) and complementary flavonoid content (32.13 mg/g). The demonstrated antioxidant capacity comparable to ascorbic acid standard, combined with the multi-mechanism activity profile, provides biochemical validation for the traditional medicinal uses of *Terminalia chebula* and identifies this fruit as a valuable source of natural antioxidant compounds for potential pharmaceutical and nutraceutical applications. Future investigations should focus on identifying the specific phenolic and flavonoid compounds responsible for the observed activities and evaluating their bioavailability and *in vivo* antioxidant efficacy in relevant disease models.

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ABBREVIATIONS

AA: Ascorbic acid; **AETC:** Aqueous extract of *Terminalia chebula* fruit pulp; **DPPH:** Diphenyl picryl hydrazyl; **FRAP:** Ferric reducing antioxidant power; **FTIR spectroscopy:** Fourier-transform infrared spectroscopy; **GAE:** Gallic acid equivalents; **IC_{50}:** Inhibition concentration required for 50 activity; **TPC:** Total phenolic content; **QE:** Quercetin equivalents.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, B.D., S.K.R., R.N.; methodology, B.D., S.K.R., R.N.; formal analysis, B.D., S.K.R., S.B.S., A.K.C., R.N.; investigation, B.T., B.D., S.B.S., R.N.; resources, B.T., R.N.; data curation, B.D., S.K.R., S.B.S., A.K.C., R.N.; supervision, R.N.; project administration, B.D., R.N.; writing-original draft preparation, B.D., S.K.R., S.B.S., A.K.C., R.N.; writing-review and editing, B.D., S.K.R., S.B.S., A.K.C., R.N.; All authors have read and agreed to the published version of the manuscript.

SUMMARY

In the present study, the Aqueous Extract of*Terminalia chebula* fruit pulp (AETC) exhibited strong dose-dependent DPPH radical scavenging activity, achieving 91.45% inhibition at 100 µg/mL, corresponding to 96% of the ascorbic acid standard. The FRAP assay demonstrated substantial reducing power (825.24±21.14 µmol Fe (II)/g), representing approximately 77% of the ascorbic acid control, with excellent assay precision (CV < 3%). High total phenolic (72.12±0.54 mg GAE/g) and flavonoid content (32.13±0.25 mg QE/g) provided mechanistic support for the observed antioxidant activity.

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