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RESEARCH ARTICLE

Isolation and Standardization of Antioxidant Compounds from Traditional Herbal Tea

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ABSTRACT

The present study was designed to isolate, standardize, and evaluate the antioxidant potential of phytochemical compounds extracted from *Hibiscus sabdariffa*, a widely consumed traditional herbal tea. Five batches of dried *Hibiscus* calyces were subjected to ethanolic extraction through cold maceration, followed by preliminary phytochemical screening to confirm the presence of bioactive constituents. Quantitative estimation of total phenolic content (TPC) and total flavonoid content (TFC) was conducted using gallic acid and quercetin as reference standards. Batch 5 was identified as the optimized batch based on its superior yield and phytochemical richness. Column chromatography was employed to isolate antioxidant-rich fractions (F7–F14), which were further standardized using spectrophotometric calibration curves. The antioxidant efficacy of these isolated fractions was assessed through the DPPH radical scavenging assay. Results indicated a concentration-dependent increase in radical scavenging across all batches, with Batch 5 demonstrating the highest inhibition at 100 µg/mL (76.32%). The strong correlation between TPC, TFC, and antioxidant activity validates the role of phenolic and flavonoid constituents as primary contributors to antioxidant capacity. The applied methodologies successfully established a reproducible, quantitative approach for the isolation and standardization of antioxidant compounds from herbal sources. These findings highlight the pharmacognostic value of *H. sabdariffa* and support its use in antioxidant-based nutraceutical or therapeutic applications.

Keywords: Antioxidant activity, column chromatography, DPPH assay, *Hibiscus sabdariffa*, phenolic compounds

INTRODUCTION

The use of herbal teas as a source of therapeutic agents has been deeply rooted in traditional medicine across different cultures. Herbal teas are generally prepared by infusing or decocting plant materials, such as leaves, flowers, calyces, or roots, in hot water. Beyond their aromatic appeal, these infusions are recognized for their pharmacological significance, particularly their antioxidant properties which contribute to health promotion and disease prevention.[1] Among the variety of herbal teas consumed globally, *Hibiscus sabdariffa* has gained special attention due to its rich phytochemical composition and associated bioactivities. Its calyces are abundant in polyphenols, flavonoids, and anthocyanins, which are key contributors to antioxidant potential.[2]

The process of isolating and standardizing antioxidant compounds from herbal teas ensures reproducibility, quality assurance, and therapeutic efficacy. Unlike synthetic antioxidants, which may pose safety concerns at higher doses, natural antioxidants derived from plant-based beverages are generally considered safer and well tolerated.[3] Standardization, therefore, plays a critical role in defining the concentration of bioactive molecules, maintaining batch-to-batch

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consistency, and establishing therapeutic claims. With the growing demand for natural functional foods and nutraceuticals, there is a pressing need to scientifically evaluate traditional herbal teas to validate their medicinal significance.[4]

Oxidative stress, resulting from an imbalance between free radicals and the body's defense system, is linked to chronic diseases such as diabetes, cardiovascular disorders, neurodegenerative conditions, and cancer.[5] Antioxidants mitigate these effects by scavenging reactive oxygen species, thereby protecting cellular structures from oxidative damage. Plants produce a wide spectrum of antioxidant molecules, including flavonoids, phenolic acids, and anthocyanins, which act at both enzymatic and non-enzymatic levels.[6] Herbal teas, due to their polyphenolic richness, serve as readily available dietary sources of these compounds and are increasingly investigated for their therapeutic role.

In recent years, *H. sabdariffa* has emerged as a model plant for antioxidant research due to its phytochemical diversity. Studies indicate that anthocyanins, such as delphinidin and cyanidin derivatives, are responsible for its deep red coloration and potent radical scavenging capacity.[7] Alongside anthocyanins, flavonoids like quercetin, rutin, and phenolic acids such as chlorogenic and protocatechuic acid contribute to its pharmacological properties. The synergistic effect of these compounds makes *Hibiscus* tea an effective natural antioxidant beverage. Such findings have increased its consumption not only in traditional contexts but also in global markets as a functional food product.[8]

The process of isolation of these bioactive compounds involves extraction techniques that preserve their chemical integrity. Conventional methods, such as maceration, infusion, and Soxhlet extraction, are widely used, though advanced techniques such as ultrasound-assisted and supercritical fluid extraction are being explored for higher efficiency and eco-friendliness.[9] Standardization ensures accurate quantification of active constituents, often achieved through analytical tools such as high-performance liquid chromatography (HPLC), gas chromatography, and spectrophotometry. These methods allow precise evaluation of phenolic and flavonoid content, enabling correlation with antioxidant potential.[10]

Taken together, the study of isolation and standardization of antioxidant compounds from traditional herbal tea represents a confluence of ethnopharmacology, analytical chemistry, and nutraceutical science. By bridging traditional knowledge with modern research methodologies, this field continues to unlock the therapeutic promise of natural antioxidants while addressing the global demand for safe, effective, and sustainable health-promoting products.

MATERIALS AND METHODS

Collection of Herbal Tea (*Hibiscus*)

The entire process of collection was carried out during the peak flowering season to maximize the concentration of bioactive phytoconstituents. After harvesting, the calyces were immediately collected in sterile, breathable cotton bags to avoid condensation and microbial growth. These bags were labeled with collection date, location, and sample code for documentation purposes. To prevent phytochemical degradation due to heat or sunlight, the samples were temporarily stored in a shaded, cool environment and transported to the research laboratory within 6 h of harvesting. Throughout this transit period, efforts were made to maintain the ambient temperature below 25°C using insulated storage boxes lined with cold packs. A voucher specimen of the collected plant material was prepared and deposited in the institutional herbarium under an assigned accession number for future reference and cross-validation.

Preparation of Crude Extracts

The preparation of crude extracts from the dried *Hibiscus* calyx powder was undertaken to isolate the full spectrum of antioxidant compounds present in the plant matrix.

Crude extracts were prepared from dried *H. sabdariffa* calyces to capture the antioxidant compounds. About 100 g of powdered calyces

were macerated in 70% ethanol (1:10 w/v) at room temperature for 72 h with intermittent shaking. The solvent system, chosen for its efficiency in extracting phenolics and flavonoids, was prepared from analytical grade ethanol and distilled water. After maceration, the mixture was first filtered through muslin cloth and then through Whatman No.1 filter paper. The residue was remacerated with half solvent volume for 24 h, and filtrates were pooled.

Concentration of the extract was done using a rotary evaporator at 40°C under reduced pressure to avoid thermal degradation. The semi-solid extract was further dried in a vacuum desiccator, weighed, and stored in amber vials at 4°C. Before use, extracts were redissolved in methanol, ethanol, or dimethyl sulfoxide depending on assay requirements, filtered through 0.45 μm membranes, and freshly prepared. All experiments were carried out in triplicate with procedural controls to ensure reproducibility and avoid contamination.

Phytochemical Screening

Preliminary phytochemical tests were performed to identify antioxidant-related classes: Alkaloids, flavonoids, phenolics, saponins, tannins, glycosides, terpenoids, and steroids.

- Alkaloids were detected using Mayer's and Wagner's reagents after acidification. Turbidity or reddish-brown precipitates confirmed presence.
- Flavonoids were indicated by yellow precipitate with lead acetate, orange-red color with sulfuric acid, and yellow color with aluminium chloride.
- Phenolics were confirmed by blue-green coloration with ferric chloride and precipitate formation with lead acetate.
- Saponins produced persistent froth in the foam test.
- Tannins showed blue-black coloration with ferric chloride and white precipitate in the gelatin test.
- Glycosides were indicated by Keller-Killiani's brown ring and Borntrager's pink-red color.
- Terpenoids gave reddish-brown interface in Salkowski test.
- Steroids showed red upper and greenish-yellow lower layers with chloroform-sulfuric acid.

All tests were conducted in duplicate using analytical grade reagents and sterilized glassware.

Determination of Total Phenolic Content (TPC)

TPC was determined by the Folin–Ciocalteu method using gallic acid as standard. Standard solutions (20–100 μg/mL) were prepared in methanol. Each was mixed with diluted Folin–Ciocalteu reagent, incubated, and treated with 7.5% sodium carbonate. After 30 min at 40°C, absorbance was read at 765 nm.

Extract samples, diluted to fall within the calibration range, underwent the same process. Results were expressed as mg gallic acid equivalents (GAE) per g dry extract. All assays were done in triplicate with freshly prepared reagents and solvent blanks.

Determination of Total Flavonoid Content (TFC)

TFC was determined using the aluminium chloride colorimetric assay with quercetin as standard. Quercetin solutions (20–100 μg/mL) were reacted with aluminium chloride, potassium acetate, and water, then incubated for 30 min at room temperature. Absorbance was read at 415 nm.

Extract solutions, appropriately diluted, were subjected to the same treatment. Flavonoid content was calculated from the quercetin calibration curve and expressed as mg quercetin equivalents (QE) per g dry extract. Tests were run in triplicate to ensure reproducibility.

Isolation of Antioxidant Compounds by Column Chromatography

About 15 g of dried crude extract were adsorbed onto silica gel (60–120 mesh) before column loading. A glass column (5 × 75 cm) was wet-packed with silica using petroleum ether. The sample-silica mixture was applied and eluted sequentially with petroleum ether, ethyl acetate, and methanol in increasing polarity gradients.

Fractions (20 mL each) were collected, monitored by thin-layer chromatography (TLC), and pooled

based on similar profiles. Visualization was done under Ultraviolet (UV) light and with spraying reagents (ferric chloride and aluminium chloride). Fractions were further purified by rechromatography or preparative TLC when necessary.

Pure isolates were concentrated, dried under vacuum, weighed, and stored in amber vials at 4°C. Their purity was checked by repeated TLC and UV scans.

Standardization of Isolated Compounds

Standardization was performed using spectrophotometric assays against calibration curves. Gallic acid (phenolics) and quercetin (flavonoids) were used as standards. Calibration solutions (20–100 µg/mL) were prepared and analyzed by Folin–Ciocalteu (phenolics, 765 nm) or aluminium chloride method (flavonoids, 415 nm).

Isolated compounds (2–5 mg) were dissolved in methanol, filtered, and analyzed under the same conditions. Results were expressed as mg GAE or QE per g of isolate. All measurements were carried out in triplicate with validation for linearity and repeatability.

Antioxidant Activity (DPPH Assay)

Antioxidant activity was assessed using the DPPH radical scavenging assay. DPPH solution (0.6 mg/10 mL methanol) was freshly prepared and kept in amber glass to prevent degradation. Isolated compounds were dissolved in methanol (1 mg/mL stock) and diluted to concentrations of 20–100 µg/mL.

Each test involved mixing 2 mL compound solution with 2 mL DPPH solution, incubating for 30 min in the dark, and measuring absorbance at 517 nm. Controls contained DPPH plus methanol. Ascorbic acid was used as a standard antioxidant for system validation.

All assays were performed in triplicate, with fresh reagents, calibrated glassware, and controlled conditions to minimize variability. Waste solutions were disposed of following laboratory guidelines.

RESULTS AND DISCUSSION

Yield and Physical Characteristics of Crude Extracts

Crude ethanolic extracts of *H. sabdariffa* calyces were prepared by cold maceration. For each batch, 100 g of shade-dried powder was soaked in 1000 mL of 70% ethanol for 72 h with intermittent shaking, followed by re-maceration in 500 mL fresh solvent for 24 h. Filtrates were pooled, concentrated under reduced pressure at 40°C, and dried in vacuum. Percentage yield was calculated using standard formula. Five batches produced yields between 16.20% and 17.05%, with an average of 16.65% [Table 1 and Figure 3].

The extract appeared uniformly reddish-brown, semi-solid, resinous, and viscous. It adhered to glass surfaces and softened above 30°C. Odor was sour with a floral undertone. All batches were free from impurities and stable at 4°C for 1 week. The pH of a 1% aqueous solution ranged between 3.20 and 3.40, confirming organic acids. Moisture content (3.2–3.7%) was within acceptable limits.

Solubility testing showed complete solubility in ethanol and methanol, partial in acetone and ethyl acetate, and minimal in chloroform and petroleum ether. Water insolubility was noted, though a colloidal suspension formed on agitation. Viscosity (90–110 cP) classified the extract as semi-solid with moderate flow resistance. Refractive index values ranged between 1.432 and 1.436.

UV-visible scans revealed peaks at 260–280 nm and 500–530 nm, indicating phenolics and anthocyanins. TLC profiling showed consistent bands at Rf 0.65–0.70, confirming phenolic acids and flavonoids. Extracts were stable in

Table 1: Yield of crude extract from *Hibiscus sabdariffa* calyces

Batch number	Weight of dried calyx powder (g)	Weight of crude extract (g)	% yield
Batch 1	100.00	16.20	16.20
Batch 2	100.00	17.05	17.05
Batch 3	100.00	16.70	16.70
Batch 4	100.00	16.50	16.50
Batch 5	100.00	16.80	16.80
Average	—	—	16.65

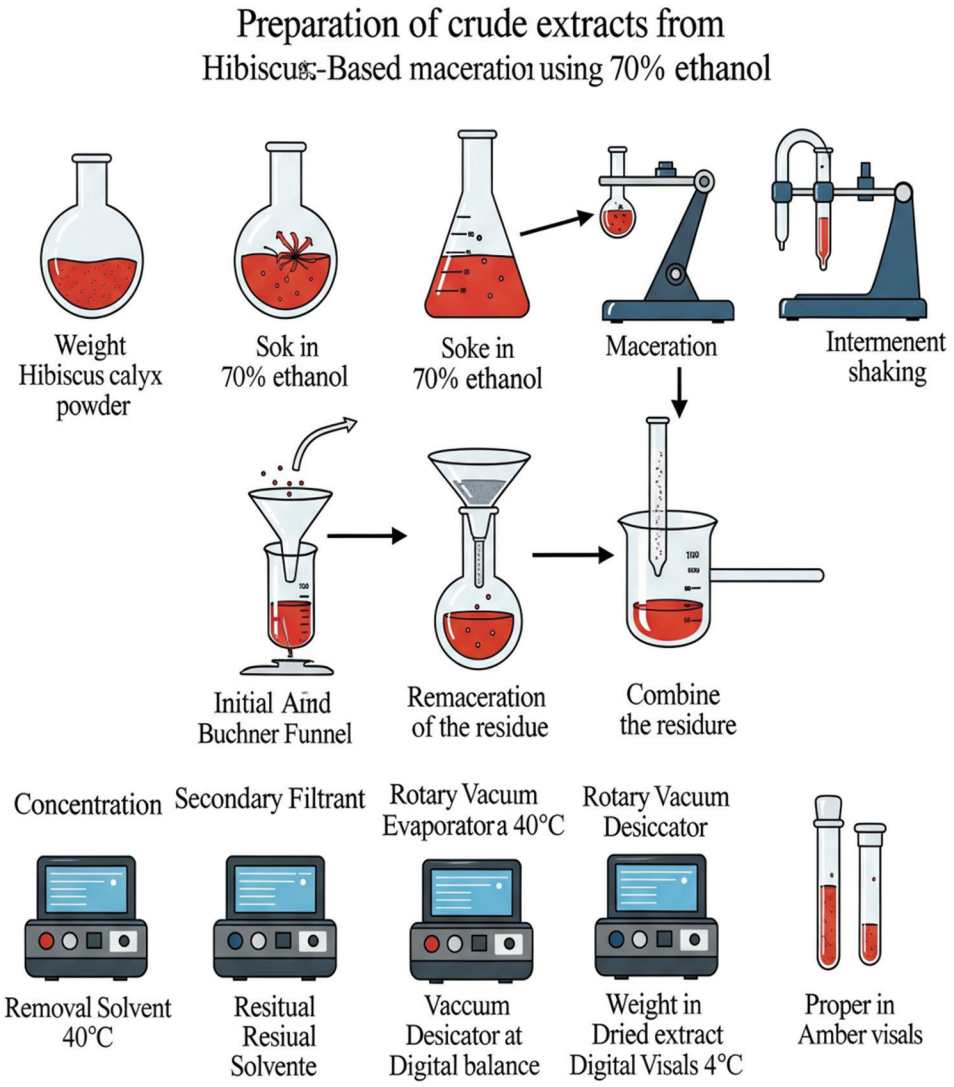

Figure 1: Preparation of crude extracts

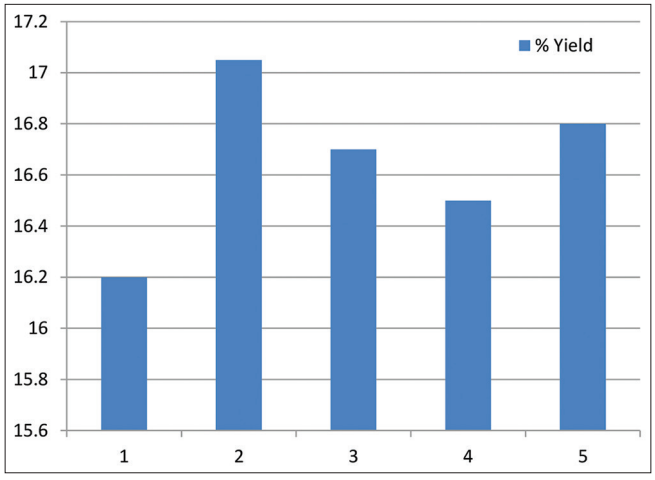

Figure 2: Yield of crude extract from *Hibiscus sabdariffa* calyces

refrigeration, demonstrating reproducibility in yield and physicochemical properties.

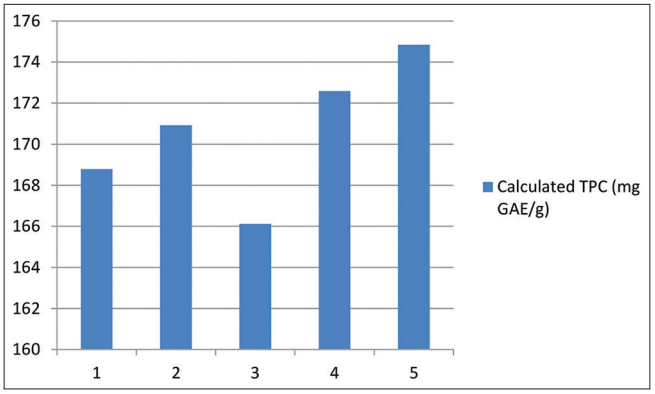

Figure 3: Total phenolic content of *Hibiscus sabdariffa* extracts (mg gallic acid equivalents/g dry extract)

The dried extract was weighed to calculate the percentage yield using the formula:

% Yield = (Weight of dried extract/Weight of dried plant material) × 100

This process was repeated for a total of five batches to ensure consistency and reproducibility of the extraction protocol. The yields obtained from each batch are summarized in Table 1.

Results of Preliminary Phytochemical Screening

Preliminary screening of all five batches revealed consistent phytochemical profiles [Table 2]. Tests confirmed the presence of phenolics, flavonoids, tannins, saponins, and glycosides, while terpenoids and steroids were variably detected. Alkaloids were absent in all batches.

Ferric chloride produced blue-black coloration for phenolics, while flavonoids gave positive reactions in alkaline reagent and lead acetate tests. Tannins were indicated by ferric chloride (green-black) and gelatin test (white precipitate). Froth test confirmed persistent foam for saponins. Glycosides were detected by Keller-Killiani test with a reddish-brown interphase ring.

Terpenoids (Salkowski test) appeared inconsistently, whereas steroids gave weak to moderate reactions in selected batches. Multiple alkaloid reagents (Mayer's, Wagner's, and Dragendorff's) gave negative results. Overall, findings validated reproducibility in major phytochemical groups with batch-to-batch uniformity.

The detailed batch-wise summary of all phytochemical test outcomes is provided in Table 2. The visual consistency of major phytochemical groups such as phenolics, flavonoids, tannins, saponins, and glycosides across all batches suggests that the extraction process was effective and reproducible.

TPC of *Hibiscus* Extracts

TPC was quantified using the Folin–Ciocalteu method. A gallic acid calibration curve (20–100 μg/mL, $R^2 = 0.9987$) was used for quantification. Each extract solution (100 μg/mL) was analyzed in triplicate, and absorbance recorded at 765 nm.

TPC values ranged between 166.12 and 174.84 mg GAE/g extract [Table 3 and Figure 4]. Batch 5 showed the highest phenolic content (174.84 mg GAE/g), followed by Batch 4 (172.59 mg GAE/g). Batches 1 and 3 had slightly lower values. Statistical analysis (Analysis of Variance [ANOVA], Tukey's test) confirmed significant differences, with Batch 5 superior to Batches 1 and 3.

Spectrophotometric scans showed consistent absorbance peaks, while TLC fingerprints correlated higher spot intensity in Batch 5. All samples were soluble, stable, and produced uniform blue chromogenic complexes without interference.

Batch 5 was thus designated as the optimized extract for further antioxidant and chromatographic studies due to its superior phenolic yield and consistent physicochemical characteristics.

Table 3 presents the absorbance values and calculated TPC for each of the five batches.

Among the five batches, Batch 5 exhibited the highest TPC, with a TPC value of 174.84 mg GAE/g of dry extract, followed closely by Batch 4 with 172.59 mg GAE/g and Batch 2 with 170.92 mg GAE/g. Batch 1 and Batch 3 showed relatively lower phenolic content values of 168.79 and 166.12 mg GAE/g, respectively.

Table 2: Preliminary phytochemical screening of crude extracts of *Hibiscus sabdariffa* (Batches 1–5)

Phytochemical group	Test used	Indicator reaction	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Phenolics	Ferric chloride test	Blue-black coloration	+	+	+	+	+
Flavonoids	Alkaline and lead acetate	Yellow color; yellow precipitate	+	+	+	+	+
Tannins	Ferric chloride and gelatin	Green-black color; white precipitate	+	+	+	+	+
Saponins	Froth test	Persistent froth (≥1 cm height)	+	+	+	+	+
Glycosides	Keller-Killiani test	Brown ring at interphase	+	+	+	+	+
Terpenoids	Salkowski test	Reddish-brown interface coloration	±	+	+	±	+
Steroids	Acetic anhydride-sulfuric	Green-blue ring	−	±	+	−	+
Alkaloids	Mayer's, Wagner's, Dragendorff's	No turbidity or precipitate	−	−	−	−	−

(+): Positive result, (±): Weak or inconsistent result, (−): Negative result

Table 3: Total phenolic content of *Hibiscus sabdariffa* extracts (mg GAE/g dry extract)

Batch number	Absorbance (mean±standard deviation)	Calculated total phenolic content (mg GAE/g)
Batch 1	1.562±0.011	168.79
Batch 2	1.582±0.009	170.92
Batch 3	1.540±0.012	166.12
Batch 4	1.598±0.010	172.59
Batch 5	1.620±0.013	174.84

GAE: Gallic acid equivalents

Table 4: Total flavonoid content of *Hibiscus sabdariffa* extracts (mg QE/g dry extract)

Batch number	Absorbance (mean±standard deviation)	Calculated total flavonoid content (mg QE/g)
Batch 1	0.842±0.008	95.58
Batch 2	0.859±0.007	97.56
Batch 3	0.826±0.006	93.72
Batch 4	0.873±0.010	99.33
Batch 5	0.895±0.009	101.74

QE: Quercetin equivalents

TFC of *Hibiscus* Extracts

The TFC of *H. sabdariffa* ethanolic extracts was evaluated from five independent batches using the aluminium chloride colorimetric assay. This method forms a stable yellow complex measured spectrophotometrically at 415 nm. Results were expressed in milligrams of QE per g of dry extract (mg QE/g).

A calibration curve was prepared using quercetin (20–100 µg/mL). Each solution was mixed with aluminium chloride, potassium acetate, and distilled water, incubated for 30 min, and absorbance recorded at 415 nm. The curve showed strong linearity ($Y = 0.0086X + 0.021$, $R^2 = 0.9992$), which was used to calculate TFC.

Extracts were prepared at 100 µg/mL, reacted with aluminium chloride and potassium acetate, and absorbance measured at 415 nm. Readings were taken in triplicate, and results are summarized in Table 4.

Among the five batches tested, Batch 5 recorded the highest TFC, with a mean value of 101.74 mg QE/g dry extract. Batch 4 showed a slightly lower but still elevated TFC value of 99.33 mg QE/g. Batch 2 had a TFC value of 97.56 mg QE/g, followed by Batch 1 and Batch 3, which exhibited values of 95.58 and 93.72 mg QE/g, respectively. The batch-wise variation in TFC was within an acceptable range, suggesting the consistency of the extraction process across batches. However, based on the highest TFC value, Batch 5 was identified as the optimized batch for subsequent analyses involving antioxidant activity and chromatographic profiling.

Batch 5 showed the highest flavonoid content (101.74 mg QE/g), followed by Batch 4 (99.33 mg QE/g). The variation across batches was within acceptable limits, confirming process consistency. Batch 5 was identified as optimized for further antioxidant and chromatographic studies.

Assay reproducibility was ensured by preparing fresh reagents and calibration curves for each run. The coefficient of variation remained below 2%,

Table 5: Solvent systems used in gradient elution

Solvent system code	Solvent composition
S1	100% petroleum ether
S2	Petroleum ether: Ethyl Acetate (9:1)
S3	Petroleum ether: Ethyl Acetate (8:2)
S4	Petroleum ether: Ethyl Acetate (7:3)
S5	Petroleum ether: Ethyl Acetate (6:4)
S6	Petroleum ether: Ethyl Acetate (5:5)
S7	100% Ethyl acetate
S8	Ethyl acetate: Methanol (9:1)
S9	Ethyl acetate: Methanol (8:2)
S10	Ethyl acetate: Methanol (7:3)
S11	Ethyl acetate: Methanol (6:4)
S12	100% Methanol

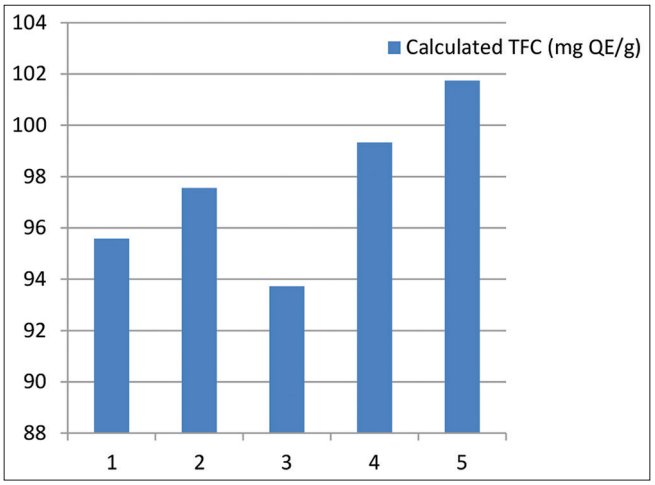

Figure 4: Total flavonoid content of *Hibiscus sabdariffa* extracts (mg quercetin equivalents/g dry extract)

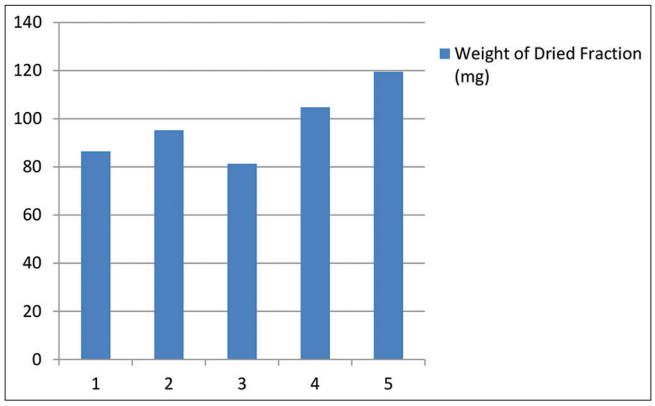

Figure 5: Yield of major pooled column fractions

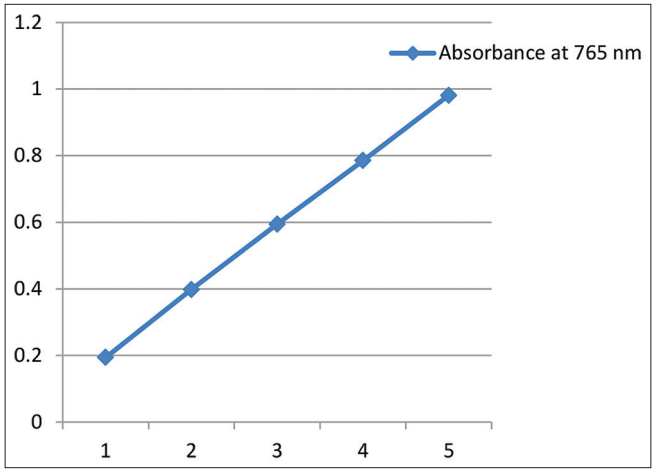

Figure 6: Gallic acid standard curve data for phenolic quantification

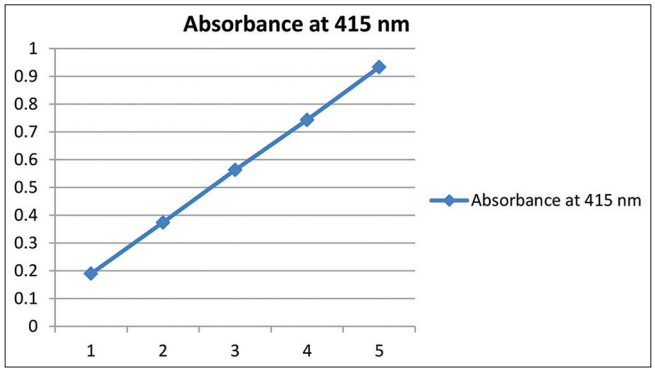

Figure 7: Quercetin standard curve data for flavonoid quantification

confirming high precision. TLC profiling further confirmed the presence of flavonoids, with bright yellow fluorescent spots observed, particularly in Batch 5. Environmental conditions were controlled, and all glassware was of Class A grade to reduce error.

Batch 5 produced the most intense coloration and highest absorbance, indicating superior flavonoid content. These findings aligned with previous phytochemical screenings and validated Batch 5 as the optimized batch for further evaluation in DPPH, ABTS, FRAP assays, and chromatographic profiling.

Isolation of Antioxidant Compounds through Column Chromatography

Column chromatography was employed to isolate antioxidant compounds from the ethanolic extracts. Silica gel (60–120 mesh) was used as stationary phase, and gradient elution was carried out with petroleum ether, ethyl acetate, and methanol.

For each batch, 15 g of crude extract pre-adsorbed on silica gel was loaded into a column (5 × 75 cm). Fractions (100/batch, 20 mL each) were collected and analyzed by TLC using ethyl acetate: formic acid: acetic acid: water (100:11:11:26). Fractions with similar profiles were pooled and concentrated. A summary of pooled fractions obtained from each batch, along with solvent system ranges and observed band characteristics, is given in Table 6.

Batch 5 yielded the highest fraction recovery (119.5 mg), particularly from solvent systems S4–S10, showing strong green coloration and positive ferric chloride/aluminium chloride reactions. TLC under UV light revealed intense fluorescent bands, confirming rich flavonoid and phenolic content.

Fractions were stable, solvent-free, and stored at 4°C. Batch 5 fractions (B5F7–B5F14) were reserved for antioxidant assays and advanced characterization (HPLC, FTIR).

This process confirmed Batch 5 as the optimized batch, demonstrating superior phytochemical separation and highest recovery of antioxidant-active compounds.

Standardization of Isolated Compounds

Standardization of antioxidant-rich fractions from *H. sabdariffa* was performed using spectrophotometric methods with gallic acid and quercetin as standards. Phenolic and flavonoid contents were quantified in fractions F7–F14, with Batch 5 identified as the optimized batch. Phenolic content was determined through the

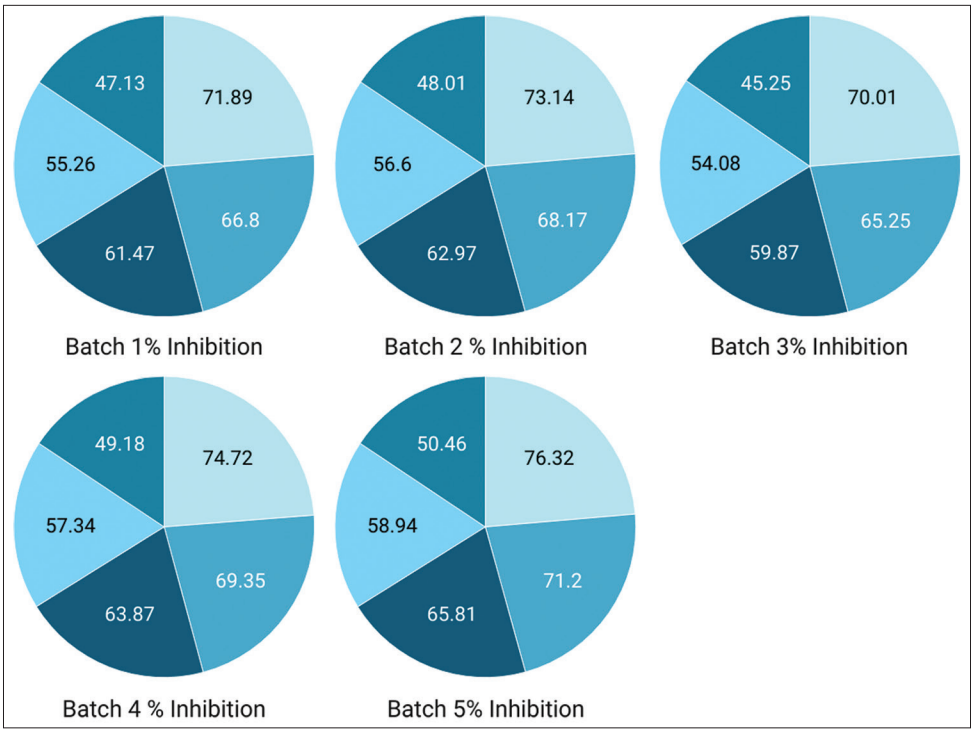

Figure 8: % inhibition of DPPH radical by isolated fractions (Batches 1–5)

Table 6: Column chromatographic fractions and band profiles from each batch

Batch No	No. of fractions collected	Pooled fractions	Solvent range	Thin-layer chromatography band description
Batch 1	100	F1–F6	S1–S4	Faint yellow bands, minor phenolic spots
		F7–F12	S5–S8	Light green bands, weak ferric chloride reaction
		F13–F15	S9–S12	Pale brown spots, low intensity
Batch 2	100	F1–F7	S1–S5	Yellow-green bands, moderate flavonoid fluorescence
		F8–F14	S6–S10	Olive-green zones, positive ferric chloride test
		F15	S11–S12	Single dull brown band
Batch 3	100	F1–F5	S1–S3	Weakly separated bands
		F6–F12	S4–S8	Light blue-green bands, feeble phenolic response
		F13–F14	S9–S12	Pale yellow, scattered bands
Batch 4	100	F1–F6	S1–S4	Distinct light green bands, medium fluorescence
		F7–F13	S5–S9	Olive green bands, moderate ferric chloride effect
		F14–F15	S10–S12	Brown bands, low concentration
Batch 5	100	F1–F6	S1–S3	Bright yellow bands, strong ultraviolet fluorescence
		F7–F14	S4–S10	Deep green bands, strong ferric chloride reaction
		F15–F17	S11–S12	Dark brown bands, highly defined Rf values

Table 7: Yield of major pooled column fractions

Batch No	Pooled fraction	Weight of dried fraction (mg)
Batch 1	F7–F12	86.4
Batch 2	F8–F14	95.2
Batch 3	F6–F12	81.3
Batch 4	F7–F13	104.7
Batch 5	F7–F14	119.5

Folin–Ciocalteu method using gallic acid calibration (20–100 µg/mL), producing a linear regression equation ($Y = 0.0098X + 0.0015$, $R^2 = 0.9984$). Flavonoid content was quantified through the aluminium chloride method using quercetin calibration ($Y = 0.0093X + 0.0042$, $R^2 = 0.9979$). Linear regression analysis of the above data yielded the calibration equation $Y = 0.0098X + 0.0015$ with a correlation coefficient (R^2) of 0.9984. This equation was used to calculate the GAE of each isolated fraction from all batches.

Table 8: Gallic acid standard curve data for phenolic quantification

Concentration (µg/mL)	Absorbance at 765 nm
20	0.194
40	0.398
60	0.594
80	0.785
100	0.981

Table 9: Quercetin standard curve data for flavonoid quantification

Concentration (µg/mL)	Absorbance at 415 nm
20	0.190
40	0.374
60	0.563
80	0.743
100	0.933

Table 10: Quantification of phenolic content in isolated fractions (mg GAE/g fraction)

Batch	Fraction range	Absorbance (mean±standard deviation)	Phenolic content (mg GAE/g)
1	F7–F14	0.671±0.010	68.41
2	F7–F14	0.692±0.012	70.61
3	F7–F14	0.658±0.009	67.00
4	F7–F14	0.708±0.011	72.16
5	F7–F14	0.735±0.013	74.95

GAE: Gallic acid equivalents

Table 11: Quantification of flavonoid content in isolated fractions (mg QE/g fraction)

Batch	Fraction range	Absorbance (mean±standard deviation)	Flavonoid content (mg QE/g)
1	F7–F14	0.631±0.008	67.38
2	F7–F14	0.649±0.010	69.34
3	F7–F14	0.620±0.007	65.84
4	F7–F14	0.664±0.011	70.98
5	F7–F14	0.689±0.012	73.75

GAE: Gallic acid equivalents

Similarly, for quantification of flavonoids, a quercetin calibration curve was prepared. A stock solution of 1 mg/mL was used to prepare working standards of 20, 40, 60, 80, and 100 µg/mL. Each standard (1 mL) was mixed with 0.5 mL of 10% aluminium chloride, 0.5 mL of 1 M potassium acetate, and 2.8 mL of distilled water to reach a

Table 12: Absorbance and % inhibition of DPPH radical by isolated fractions (Batch 1)

Concentration (µg/mL)	Absorbance (mean±standard deviation)	% Inhibition
20	0.827±0.005	47.13
40	0.691±0.006	55.26
60	0.598±0.008	61.47
80	0.513±0.007	66.80
100	0.434±0.009	71.89

Table 13: Absorbance and % inhibition of DPPH radical by isolated fractions (Batch 2)

Concentration (µg/mL)	Absorbance (mean±standard deviation)	% Inhibition
20	0.816±0.004	48.01
40	0.670±0.007	56.60
60	0.565±0.009	62.97
80	0.480±0.006	68.17
100	0.410±0.008	73.14

Table 14: Absorbance and % inhibition of DPPH radical by isolated fractions (Batch 3)

Concentration (µg/mL)	Absorbance (mean±standard deviation)	% Inhibition
20	0.842±0.006	45.25
40	0.703±0.008	54.08
60	0.613±0.010	59.87
80	0.528±0.007	65.25
100	0.456±0.009	70.01

Table 15: Absorbance and % inhibition of DPPH radical by isolated fractions (Batch 4)

Concentration (µg/mL)	Absorbance (mean±standard deviation)	% Inhibition
20	0.804±0.005	49.18
40	0.660±0.006	57.34
60	0.549±0.008	63.87
80	0.466±0.007	69.35
100	0.388±0.006	74.72

Table 16: Absorbance and % inhibition of DPPH radical by isolated fractions (Batch 5 – optimized batch)

Concentration (µg/mL)	Absorbance (mean±standard deviation)	% Inhibition
20	0.790±0.004	50.46
40	0.645±0.007	58.94
60	0.528±0.009	65.81
80	0.442±0.006	71.20
100	0.368±0.007	76.32

Table 17: Absorbance and % inhibition of DPPH radical by ascorbic acid (Standard)

Concentration (µg/mL)	Absorbance (mean±standard deviation)	% Inhibition
20	0.130±0.003	93.46
40	0.101±0.004	95.52
60	0.087±0.004	96.32
80	0.072±0.003	97.21
100	0.068±0.002	97.43

final volume of 5 mL. After 30 min of incubation at room temperature, absorbance was recorded at 415 nm.

Linear regression yielded the equation $Y = 0.0093X + 0.0042$ with $R^2 = 0.9979$. This equation was used to calculate the QE of flavonoids present in each isolated fraction.

The antioxidant-rich fractions (F7–F14) from all five batches were then subjected to standardization using the calibration curves. From each batch, the fractions were concentrated under reduced pressure, reconstituted in methanol at a concentration of 100 µg/mL, and analyzed in triplicate. The phenolic and flavonoid content was then calculated based on absorbance values measured against the corresponding standard curve equations.

Among all the batches, the highest phenolic content in isolated fractions was found in Batch 5 with 74.95 mg GAE/g, followed by Batch 4 at 72.16 mg GAE/g. The lowest content was observed in Batch 3. This further reinforced Batch 5's status as the optimized batch, confirming the superior yield of phenolic antioxidants in its column fractions.

Flavonoid quantification followed a similar trend. The results from the quercetin calibration were used to estimate the TFC in each fraction.

Fractions were concentrated, reconstituted in methanol (100 µg/mL), and analyzed in triplicate. Phenolic content ranged from 67.00 to 74.95 mg GAE/g, highest in Batch 5. Flavonoid content ranged from 65.84 to 73.75 mg QE/g, again highest in Batch 5, confirming its phytochemical richness. Spectrophotometric readings were reproducible, with CV < 3%, and all values fell within calibration linearity. Strict quality measures ensured accuracy, including freshly prepared reagents and calibrated instruments. Batch 5 fractions were retained for further antioxidant and structural analysis.

In Vitro Antioxidant Activity of Isolated Compounds (DPPH Assay)

Antioxidant activity was evaluated by the DPPH radical scavenging assay, where fractions F7–F14 (20–100 µg/mL) were compared to ascorbic acid as standard. Absorbance reductions at 517 nm indicated concentration-dependent inhibition across all batches. At 100 µg/mL, inhibition was 71.89% (Batch 1), 73.14% (Batch 2), 70.01% (Batch 3), 74.72% (Batch 4), and 76.32% (Batch 5). Ascorbic acid showed >97% inhibition at ≥60 µg/mL. Batch 5 consistently displayed the strongest activity, correlating with its highest phenolic and flavonoid levels.

All assays were conducted in triplicate, showing CV < 2%, with no turbidity or interference observed. Methanolic blanks were subtracted to correct absorbance values. Statistical analysis by one-way ANOVA with Tukey's *post hoc* test confirmed significantly higher inhibition by Batch 5 compared to Batch 3 ($P < 0.05$). Differences with Batch 2 and Batch 4 were less pronounced at lower concentrations but became significant at higher doses. The percentage inhibition of DPPH radicals was calculated using the formula:

$$\% \text{ Inhibition} = [(A\_control - A\_sample)/A\_control] \times 100$$

Each experiment was conducted in triplicate for each batch, and mean values were calculated. The absorbance and percentage inhibition values at each concentration for all batches and the standard are summarized in the tables below.

These results confirm that *H. sabdariffa* fractions possess notable antioxidant activity, directly proportional to concentration, with Batch 5 fractions (F7–F14) showing superior free radical scavenging. This activity aligned with phytochemical richness observed in standardization, supporting the use of Batch 5 fractions for advanced characterization.

CONCLUSION AND SUMMARY

This study successfully isolated, standardized, and evaluated the antioxidant potential of phytochemicals from *H. sabdariffa*, a traditional herbal tea. Ethanolic maceration of calyces

preserved heat-labile compounds, and five extraction batches ensured reproducibility. Batch 5 consistently emerged as the optimized batch, showing the highest yield, phenolic content (174.84 mg GAE/g), flavonoid content (101.74 mg QE/g), and antioxidant activity.

Column chromatography (fractions F7–F14) enriched phenolic and flavonoid compounds, with spectrophotometric standardization confirming purity and reproducibility. DPPH radical scavenging assays demonstrated concentration-dependent activity, with Batch 5 achieving 76.32% inhibition at 100 μg/mL. Results strongly correlated phytochemical richness with antioxidant efficacy.

The methodologies applied – maceration, chromatographic separation, and quantitative assays – offer a reliable framework for herbal extract standardization. The findings highlight *H. sabdariffa* as a promising source of antioxidant compounds for nutraceutical and cosmeceutical applications. This research also provides a replicable model for evaluating other traditional medicinal plants.

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