



World Journal of Pharmaceutical Science & Technology

Journal homepage: www.wjpst.com

Original Research Article

"Effect of Seasonal Variation on the Chemical Composition and Antioxidant–Anti-inflammatory Activities of *Bauhinia variegata* Bark Extract"

Ninad U. Bhatt^{1*}, Naval P. Kapuriya², Atul H. Bapodra¹

1. Ph.D. scholar, Department of chemistry and forensic science, Bhakt Kavi Narsih Mehta university, Junagadh.
2. Professor and Head, Department of chemistry and forensic science, Bhakt kavi Narsih mehta university, Junagadh.
3. Former Professor and Head, Department of chemistry and forensic science, Bhakt kavi Narsih mehta university, Junagadh

Address for correspondence:

Ninad U. Bhatt, MD Hom Department of chemistry and forensic science, bhakt kavi narsih mehta university, Junagadh.

Email Id: ninad.bht@gmail.com

Received: 15-04-2026, Revised: 22-05-2026, Accepted: 28-06-2026

ABSTRACT

Background: Seasonal climatic variations significantly influence the biosynthesis and accumulation of secondary metabolites in medicinal plants. *Bauhinia variegata* Linn. bark is an important traditional medicinal resource, but the optimal harvesting season for maximum phytochemical yield remains unclear.

Objective: To investigate the effect of seasonal variation on the physicochemical properties, phytochemical composition, antioxidant, and anti-inflammatory activities of *B. variegata* stem bark.

Methods: Bark samples were collected during monsoon (S1), winter (S2), and summer (S3). Physicochemical parameters, total phenolic, flavonoid, and tannin contents were evaluated. Phytochemical profiling was performed using TLC and HPTLC. Antioxidant activity was assessed by DPPH and FRAP assays, while anti-inflammatory activity was determined using HRBC membrane stabilization and BSA protein denaturation assays.

Results: Significant seasonal variations were observed. The winter sample (S2) showed the highest alcohol-soluble extractive value (7.5%), total phenolics (15.6 µg/mL), flavonoids (25.6 µg/mL), and tannins (5.40%). HPTLC profiling demonstrated distinct seasonal chemical variations. The S2 extract also exhibited the strongest antioxidant and anti-inflammatory activities, with 75.45% HRBC membrane stabilization and 37.03% inhibition of BSA denaturation at 400 µg/mL.

Conclusion: Seasonal variation markedly affects the phytochemical composition and biological activity of *B. variegata* bark. Winter is the optimal harvesting season, providing maximum bioactive constituents and superior antioxidant and anti-inflammatory potential, thereby supporting its standardization for phytopharmaceutical applications.

Keywords: *Bauhinia variegata* Linn.; Kanchanar; seasonal variation; phytochemical standardization; HPTLC fingerprinting; antioxidant activity; anti-inflammatory activity.

1. Introduction

Climate serves as a primary environmental driver that directly modulates the structural and physiological dynamics of plant ecosystems. These macro-climatic shifts govern essential processes including photosynthesis, transpiration, nutrient cycling, and the biosynthesis of both primary and secondary metabolites^{1,2}. Secondary metabolites encompass a diverse array of chemical compounds that, while not strictly necessary for basic vegetative survival, are synthesized to confer resistance against environmental stressors, pathogen incursions, and nutritional deficiencies³. The fundamental roles of these secondary products are largely rooted in protective antioxidant activities and defensive countermeasures, acting as natural antimicrobial agents, insecticides, and herbicides against external pressures⁴. Consequently, natural extracts derived from plant matrices function as intricate complexes of bioactive compound classes—such as polyphenols, terpenoids, alkaloids, and carotenoids—with phenolic compounds frequently representing one of the most pharmacologically active groups^{5,6}.

The foundational medicinal value of botanical species is inextricably linked to these inherent phytochemical constituents, which are capable of inducing specific physiological actions within mammalian systems⁷. Systematic phytochemical screening facilitates the characterization of critical chemical structures that can serve as lead templates for contemporary drug discovery and development⁸. Comprehensive phytochemical investigations have consistently demonstrated the ubiquitous presence of alkaloids, flavonoids, steroids, phenols, glycosides, and saponins across diverse plant taxa, thereby establishing plants as essential reservoirs for pharmaceuticals, agrochemicals, and nutraceuticals⁹. Notably, a substantial proportion of known natural products originate within the plant kingdom, highlighting their ongoing importance in medicinal chemistry¹⁰. Crucially, seasonal successions directly impact the bioavailability, concentration, and structural diversity of these active principles¹¹. In alignment with the principles of traditional herbal medicine and modern pharmacognosy, the therapeutic efficacy of plant material fluctuates across different seasons. Efficacy typically reaches optimal levels when the material is harvested at peak maturity and maximum chemical concentration¹². The specific spatial and temporal distribution of particular phenolic and flavonoid compounds within plant tissues changes continuously throughout distinct phases of growth, development, and macro-climatic transitions¹³. Therefore, quantifying these seasonal variations provides the empirical foundation necessary to establish standardized, evidence-based collection windows for raw materials¹⁴.

Within the traditional system of Indian medicine, Ayurveda & natural substances are comprehensively documented as potential therapeutic agents (*Aushadhi*). Within this extensive pharmacopeia, Kanchar (*Bauhinia variegata* Linn.; Family: Fabaceae) is recognized as a highly valued medicinal tree, traditionally utilized for the management of wounds, dermal afflictions, and glandular swellings, notably goitre^{15,16}. Historical documentation of *B. variegata* is extensive across classical Ayurvedic texts (*Samhitas* and *Nighantus*). The *Charaka Samhita* (~1000 BCE) details its application for bleeding disorders (*Raktapitta*), while the *Sushruta Samhita* (~1500 BCE) categorizes it within specific pharmacological cohorts (*Urdhwabhagahara* and *Kshayaghna*), underscoring its broad therapeutic utility¹⁷. In contemporary ethnopharmacological and clinical practice, the stem bark is frequently employed in the treatment of goitre, tumors, hemorrhoids, diarrhea, helminthic infestations, and various inflammatory skin disorders^{18,19}.

Taxonomically, the genus *Bauhinia* is named in honour of the 16th-century Swiss-French botanists, brothers Jean and Gaspard Bauhin, while the specific epithet *variegata* refers to the distinct, variegated pigmentation of its floral petals²⁰. Morphologically, the plant is a medium-sized deciduous tree characterized by dark brown bark exhibiting prominent vertical fissures¹⁶.

Despite the clear, long-term traditional utility of *B. variegata* bark, rigorous scientific evaluation tracking the seasonal fluctuations of its bioactive constituents remains insufficient. Given that environmental parameters significantly influence the biosynthetic equilibrium of secondary metabolites, elucidating these variations is a prerequisite for modern quality control and standardization of herbal formulations²¹. Therefore, the present investigation aims to systematically evaluate the stem bark of *Bauhinia variegata* collected across three distinct seasons. By employing a comprehensive suite of physicochemical, phytochemical, chromatographic, antioxidant, and anti-inflammatory methodologies, this study seeks to define the optimal harvesting window to maximize therapeutic yield and ensure commercial standardization.

2. Materials and Methods

2.1 Collection and Identification of Plant Material

Stem bark of *Bauhinia variegata* Linn. was collected from healthy mature plants growing in Jamnagar, Gujarat, India, during three distinct seasons: Rainy Season: Collected in August (S1) Winter Season: Collected in December (S2), Summer Season: Collected in April (S3) To minimize environmental variation, all samples were collected from the same geographical location and from plants of comparable maturity. Botanical identification and authentication were carried out by the Department of Pharmacognosy, Institute of Teaching and Research in Ayurveda (ITRA), Jamnagar, India.

2.2 Processing and Powder Preparation

Immediately following collection, the fresh bark samples were thoroughly cleaned to remove adhering dirt, epiphytes, and extraneous surface debris. The cleaned material was subsequently shade-dried at ambient room temperature ($25\pm 2^{\circ}\text{C}$) to minimize the thermal degradation of thermolabile secondary metabolites²². Upon achieving constant weight, the dried bark was comminuted into a coarse powder using a mechanical mill. The resultant powders were passed through a standard mesh sieve to ensure uniform particle size and were stored in amber-colored, airtight glass containers in a cool, dry environment to mitigate degradation induced by moisture and photo-oxidation prior to extraction and analysis²³.

2.3 Microscopic Studies

For microscopic analysis, thin transverse sections (TS) of the fresh bark were prepared manually. The sections were cleared utilizing a chloral hydrate solution to remove cellular contents and enhance structural clarity. Following clearing, the sections were stained with an equal-volume mixture of phloroglucinol and concentrated hydrochloric acid (HCl) to identify lignified tissues^{24 25}. The prepared sections were then mounted in glycerine and examined under a compound optical microscope (Olympus, Japan) equipped with a digital camera to document diagnostic anatomical features and cellular arrangements.

2.4 Physicochemical Standardization

To establish the quality, purity, and identity of the seasonal raw materials, physicochemical parameters were evaluated in strict accordance with the guidelines stipulated by the World Health Organization (WHO) and the Indian Pharmacopoeia^{26, 27}.

Loss on Drying (LOD): Precisely 2.0 g of the powdered bark was placed in a tared, shallow weighing bottle and dried in a hot air oven at 105°C until a constant weight was achieved. The moisture content was calculated and expressed as percentage weight-by-weight (% w/w).

Ash Values: For total ash determination, 2.0g of the sample was incinerated in a tared silica crucible within a muffle furnace at 450°C until a carbon-free white ash was obtained. The acid-insoluble ash was determined by boiling the total ash with 25mL of dilute hydrochloric acid (2M), filtering through ashless filter paper, and incinerating the resulting residue. To determine the water-soluble ash, the total ash was boiled with 25 mL of distilled water, filtered, and the insoluble residue was incinerated. All ash values were calculated and expressed as % w/w.

Extractive Values: Water-soluble and alcohol-soluble extractive values were determined by macerating 5.0 g of the coarsely powdered drug with 100 mL of chloroform-water and 95% ethanol, respectively, in closed flasks for 24 hours. The flasks were agitated frequently during the initial 6 hours. Following filtration, 25 mL of each filtrate was evaporated to dryness in a tared flat-bottomed dish, dried at 105°C , and weighed. The extractive values were expressed as %w/w.

2.5 Quantification of Total Tannin Content (TTC)

The total tannin content of the extracts was determined employing the Folin-Denis spectrophotometric method²⁸. Aliquots of the bark extract were mixed with the Folin-Denis reagent and saturated sodium carbonate (Na_2CO_3) solution. The reaction mixture was allowed to stand, and the absorbance was measured at 700nm using a UV-Visible spectrophotometer. A standard calibration curve was prepared using tannic acid, and the TTC was expressed as milligrams of tannic acid equivalents (TAE) per gram of dry extract.

2.6 Quantification of Total Phenolic Content (TPC)

Total phenolics were quantified utilizing the standard Folin-Ciocalteu assay²⁹. Briefly, an aliquot of the extract was mixed with Folin-Ciocalteu reagent (diluted 1:10 with distilled water) and a 7.5% Na_2CO_3 solution. Following incubation in the dark for 30 minutes at room temperature, the absorbance of the resulting blue complex was recorded at 765nm. Gallic acid was utilized as the reference standard, and the TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract.

2.7 Quantification of Total Flavonoid Content (TFC)

The flavonoid content was estimated via the aluminium chloride colorimetric assay³⁰. The extract was mixed with a 10% aluminium chloride (AlCl_3) solution, 1M potassium acetate, and distilled water. After an incubation period at room temperature, the absorbance of the mixture was measured at 415nm. Quercetin served as the reference standard, and the TFC was expressed as milligrams of quercetin equivalents (QE) per gram of dry extract.

2.8 TLC and HPTLC Fingerprinting and Chemometric Modelling

2.8.1 Thin-Layer Chromatography (TLC): Ethanolic extracts (10 mg/mL) were applied to silica gel 60 F254 plates (Merck). Development utilized a mobile phase of Toluene : Ethyl Acetate : Formic Acid (5:4:1, v/v/v) for optimal phenolic and flavonoid separation. Plates were visualized under UV light (254nm and 366nm) and derivatized with anisaldehyde-sulfuric acid³¹.

2.8.2 High-Performance Thin-Layer Chromatography (HPTLC): Analyses were performed on Silica Gel 60 F254 sheets (Merck) using a CAMAG Linomat V applicator (8 μL per track as 8mm bands). Development occurred in a twin trough chamber saturated with Toluene : Ethyl acetate (7:3, v/v) to an 80 migration distance ($25\pm 2^\circ\text{C}$, RH $50\pm 5\%$). Dried plates were documented (CAMAG TLC Visualizer) and scanned densitometrically in reflectance-absorbance mode (CAMAG TLC Scanner IV, winCATS software).

2.9 In Vitro Antioxidant Assays

2.9.1 DPPH Radical Scavenging Assay: Extracts were evaluated against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical³². Sample dilutions (1mL) were mixed with 0.5mL methanolic DPPH (0.4mM), incubated in the dark for 30 minutes, and absorbance was read at 518nm³³. The IC_{50} was determined via linear regression.

2.9.2 Ferric Reducing Antioxidant Power (FRAP) Assay: Reducing potential was assessed using a modified Oyaizu protocol³⁴. Extracts (300-900 $\mu\text{g/mL}$) were incubated with sodium phosphate buffer (0.2M, pH 6.6) and 1% potassium ferricyanide at 50°C for 20 minutes. Following precipitation with 10% TCA and centrifugation, the supernatant was reacted with 0.1% FeCl_3 . Absorbance of the resulting complex was measured at 700nm^{35,36}, utilizing ascorbic acid as a standard.

2.10 In Vitro Anti-inflammatory Assays

2.10.1 HRBC Membrane Stabilization: Membrane protection against hypotonicity-induced lysis was evaluated using human red blood cells (HRBC)³⁷. A 10% HRBC suspension in isosaline was added to a reaction mixture containing the extract, phosphate buffer (pH 7.4), and hyposaline. Following a 30-minute incubation at 37°C and centrifugation, haemoglobin release was quantified spectrophotometrically at 560nm against a diclofenac sodium standard³⁸.

2.10.2 BSA Protein Denaturation: Inhibition of heat-induced protein denaturation was determined³⁹. Extracts were combined with 1% bovine serum albumin (pH 7.4), incubated at room temperature for 20 minutes, and subsequently heated at 56°C for 20 minutes. Turbidity was measured at 660nm.

3. Results and Discussion

3.1 Macroscopic and Microscopic Validation

Microscopic examination of the powdered bark and transverse section (TS) of *Bauhinia variegata* revealed several characteristic anatomical features that serve as important pharmacognostic markers (Figure 1). The powder microscopy demonstrated abundant parenchymatous cells, appearing as thin-walled, polygonal to irregular cells distributed throughout the powdered material. These cells constituted the major ground tissue and are associated with the storage of metabolites and reserve materials.

Numerous stone cells (sclereids) were observed either singly or in small aggregates. The sclereids were irregular to rounded in shape with heavily lignified, thick secondary walls and narrow lumina. Several sclereids exhibited distinct three-sided wall thickening, representing an important diagnostic characteristic of mature bark tissues. The intense reddish coloration produced after phloroglucinol-HCl staining confirmed their lignified nature.

The transverse section of cork (phellem) exhibited several layers of compact, rectangular to tangentially elongated cells arranged in orderly radial rows. These cork cells lacked intercellular spaces, indicating a well-developed protective periderm. In the surface view, cork cells appeared polygonal, closely packed, and arranged in compact clusters with clearly distinguishable cell boundaries. Such structural organization is characteristic of mature bark and contributes to protection against mechanical injury and environmental stress. Overall, the microscopic profile comprising abundant parenchyma, lignified three-sided sclereids, compact cork tissue in transverse section, and polygonal cork cells in surface view provides reliable anatomical markers

for the authentication and quality evaluation of *Bauhinia variegata* bark.



[Figure 1. Diagnostic microscopic characteristics of *Bauhinia variegata* Linn. bark: (A) abundant parenchymatous cells; (B) lignified three-sided stone cells (sclereids); (C) transverse section of cork showing compact rectangular phellem cells arranged in radial rows; (D) surface view of cork exhibiting compact polygonal cork cells stained with phloroglucinol-HCl]

Microscopic evaluation is one of the most dependable pharmacognostic approaches for the identification and standardization of medicinal plant materials because anatomical characteristics remain relatively unaffected by environmental conditions. The present investigation demonstrated several diagnostic features that are consistent with the reported anatomical characteristics of *Bauhinia variegata* bark.

The abundance of parenchymatous cells observed in the powdered bark indicates the presence of well-developed cortical and secondary phloem tissues. These living cells function primarily in storage and accumulation of bioactive constituents, including polyphenols, tannins, flavonoids, and carbohydrates, which contribute significantly to the medicinal value of the bark. Similar observations have been reported in pharmacognostic studies of *Bauhinia* species, where parenchyma forms a major component of the bark matrix. A characteristic feature of the present study was the occurrence of numerous thick-walled sclereids (stone cells) exhibiting irregular morphology and narrow lumina. Some sclereids displayed three-sided thickening, which represents a valuable taxonomic character for bark identification. The positive phloroglucinol-HCl reaction confirmed the presence of lignified secondary cell walls. Sclereids provide mechanical support and resistance against physical damage while serving as important microscopic diagnostic markers in crude drug authentication.

The cork tissue observed in transverse section consisted of several layers of compact rectangular cells arranged in regular radial rows, whereas the surface preparation revealed tightly packed polygonal cork cells. Such organization is typical of mature periderm and functions as an effective protective barrier against microbial invasion, excessive water loss, and environmental stress. These findings agree with the anatomical descriptions reported in standard pharmacognostic literature and WHO quality control guidelines for herbal materials.

The absence of tissue deterioration and the distinct visualization of parenchyma, sclereids, and cork layers indicate that the bark sample possessed good anatomical integrity and was suitable for pharmacognostic standardization. The combination of abundant parenchymatous tissue, lignified three-sided stone cells, well-developed cork in transverse section, and compact polygonal cork cells in surface view constitutes a characteristic microscopic profile of *Bauhinia variegata* bark. These diagnostic characters can be employed for routine identification, detection of adulteration, and quality control of the crude drug used in herbal formulations.

3.2 Physicochemical Standardization and Seasonal Variation

The evaluation of physicochemical parameters is a critical initial step in establishing the quality, purity, and identity of crude herbal drugs. These parameters provide essential baseline data to detect adulteration, improper handling, or suboptimal storage conditions. The results of the physicochemical analysis for the stem

bark of *Bauhinia variegata* collected across three distinct seasons (Monsoon/Rainy - S1; Winter - S2; Summer - S3) are presented in Table 1. The data clearly indicates that macro-climatic shifts significantly modulate the fundamental physical properties of the plant material.

Table 1: Physicochemical Parameters of *Bauhinia variegata* Bark Across Different Seasons

Parameter	S1 (Rainy Season)	S2 (Winter Season)	S3 (Summer Season)
Loss on Drying (Moisture Content)	12.38%	14.20%	9.19%
Total Ash Value	9.8%	10.2%	9.2%
Water-Soluble Extractive	11.16%	15.9%	7.9%
Alcohol-Soluble Extractive	6.5%	7.7%	6.4%

The physicochemical parameters demonstrated appreciable seasonal variation among the three collection periods. Loss on drying (LOD) was highest in winter (S2; 14.20%), followed by the rainy season (S1; 12.38%), and was lowest during summer (S3; 9.19%). The higher moisture content observed in winter may be associated with lower ambient temperature and reduced evaporative loss, whereas the lower value in summer may reflect enhanced moisture depletion under relatively higher temperature conditions.

The total ash content showed comparatively limited seasonal variation, ranging from 9.2% to 10.2%, with the highest value recorded in winter (S2). This relatively narrow variation suggests that the overall inorganic mineral content of the plant material remained comparatively stable across seasons. In contrast, marked seasonal differences were observed in the extractive values. The water-soluble extractive was maximum in winter (15.9%), followed by the rainy season (11.16%) and summer (7.9%). Similarly, the alcohol-soluble extractive was highest in winter (7.7%), compared with 6.5% in the rainy season and 6.4% in summer. The higher extractive values during winter may indicate enhanced accumulation or retention of polar and moderately polar soluble phytoconstituents.

Overall, winter (S2) exhibited the most favourable physicochemical profile, particularly with respect to water- and alcohol-soluble extractive values, suggesting that seasonal environmental conditions may influence the accumulation of extractable phytoconstituents in the investigated plant material.

3.3 Quantitative Phytochemical Estimations

To empirically validate the seasonal fluctuations observed in the extractive values and qualitative screening, the total tannin, phenolic, and flavonoid contents were quantitatively determined. Polyphenols represent the primary defensive and antioxidant arsenal of the plant, and their quantification is paramount for establishing the optimal harvesting window.

3.3.1 Total Tannin Estimation

Tannins are complex, astringent polyphenolic biomolecules synthesized primarily as defence mechanisms against herbivores and pathogens.

Table 2: Total Tannin Content of *B. variegata* Bark

Season / Sample	Total Tannin Content (%)
S1 (Rainy Season)	4.23%
S2 (Winter Season)	5.40%
S3 (Summer Season)	2.30%

As shown in Table 3, the total tannin content was heavily influenced by seasonal shifts. The highest accumulation occurred during the winter season (S2: 5.40%), demonstrating a significant increase over the rainy season (S1:4.23%). Conversely, tannin levels plummeted during the summer season (S3: 2.30%). This winter peak aligns with the plant's defensive ecology; as temperature drops and metabolic turnover slows, carbon resources are often re-allocated from primary vegetative growth to the synthesis of stable secondary defence molecules like tannins, effectively fortifying the dormant bark tissue.

3.3.2 Total Phenolic and Flavonoid Content

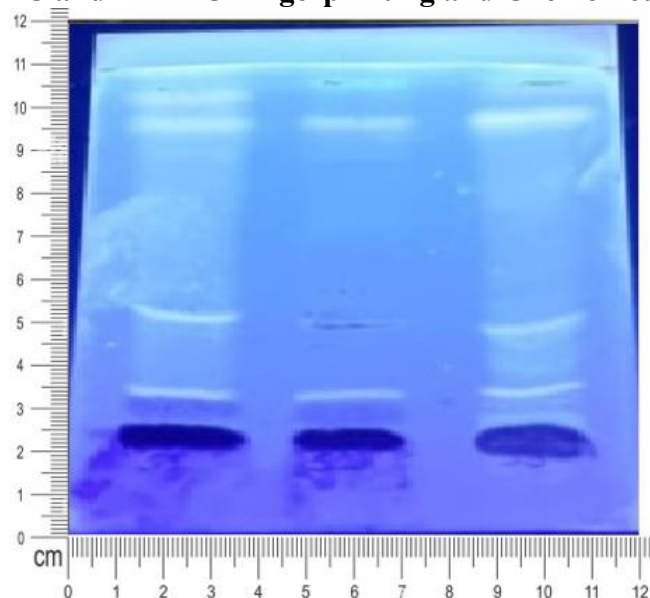
Phenolics and flavonoids are potent natural antioxidants responsible for a vast majority of the biological activities associated with herbal medicines, including anti-inflammatory, hepatoprotective, and anticancer effects. The extracts were evaluated using both aqueous (water) and methanolic solvents at a standard concentration of 50 μ g/mL .

Table 3: Total Phenolic and Flavonoid Contents of *B. variegata* Extracts

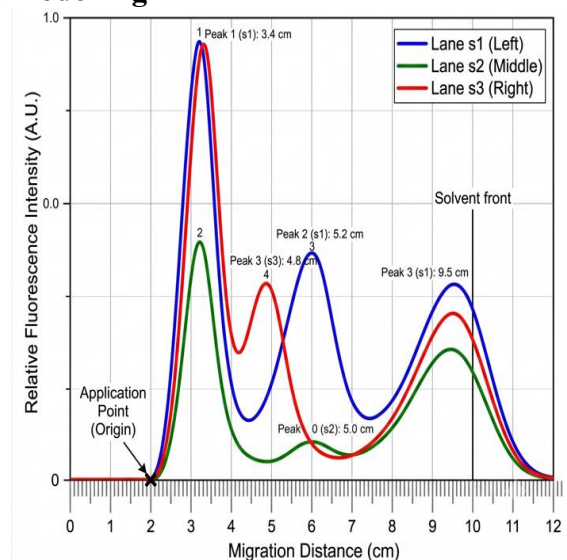
Solvent System	Phytochemical	S1 (Rainy)	S2 (Winter)	S3 (Summer)
Aqueous Extract	Total Phenolics	5.6 μ g/mL	9.27 μ g/mL	6.4 μ g/mL
	Total Flavonoids	15.4 μ g/mL	20.4 μ g/mL	16.2 μ g/mL
Methanolic Extract	Total Phenolics	8.5 μ g/mL	15.6 μ g/mL	7.25 μ g/mL
	Total Flavonoids	23.2 μ g/mL	25.6 μ g/mL	10.5 μ g/mL

All samples tested at 50 μ g/mL concentration.

3.4 TLC and HPTLC Fingerprinting and Chemometric modelling

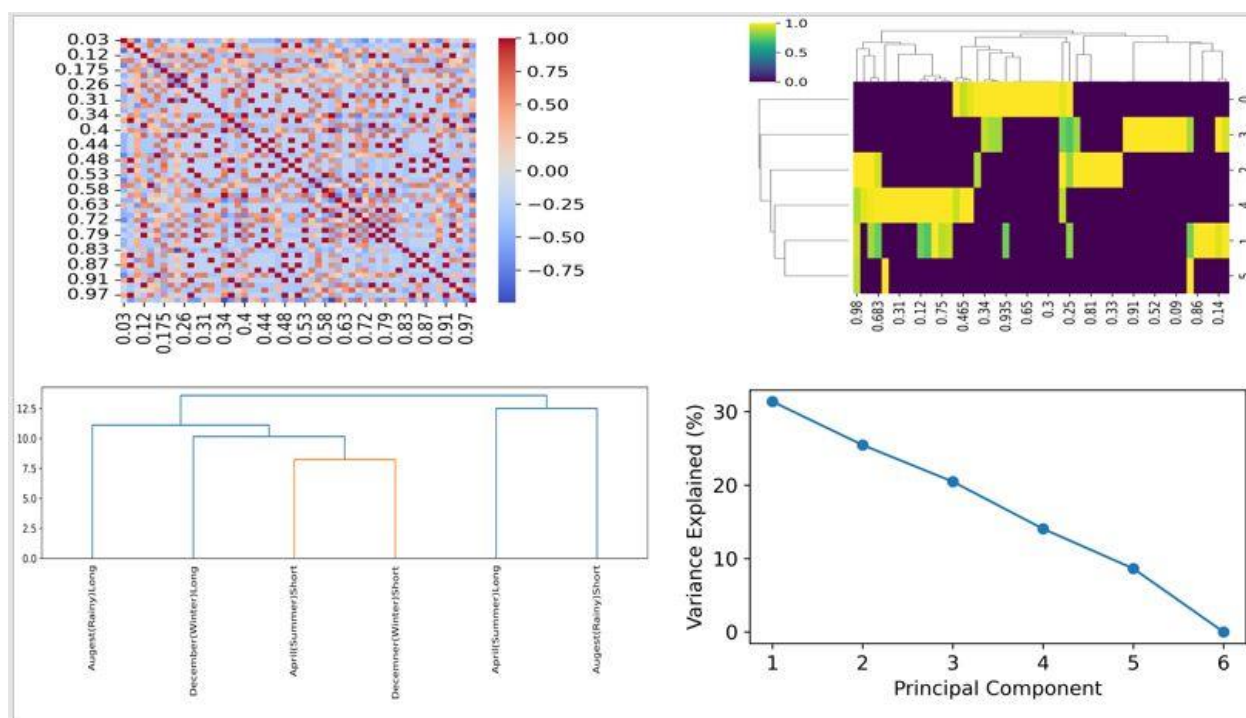


[figure 1:- Thin layer chromatogram]



[figure 2:- Densitometric analysis of TLC plate lanes]

High-Performance Thin-Layer Chromatography (HPTLC) Data derived from samples collected across different seasons (August/Rainy, December/Winter, April/Summer) and storage durations (Long, Short) were subjected to multi-variate statistical analysis to identify patterns and correlations. The analysis included a correlation matrix, a clustered heatmap, a dendrogram (Hierarchical Clustering Analysis), and Principal Component Analysis (PCA).



[Figure 3 :- Correlation matrix, A clustered heatmap, Dendrogram (Hierarchical Clustering Analysis), and Principal Component Analysis (PCA).]

Correlation Matrix (Top Left):

A correlation matrix was generated to assess the linear relationships between various variables (labelled with numerical values ranging from 0.03 to 0.97, likely representing R_f values or specific spectral features). The heatmap displays correlation coefficients ranging from -1.0 (strong negative correlation, blue) to +1.0 (strong positive correlation, red). The diagonal shows perfect self-correlation (dark red). The matrix reveals a complex pattern of positive and negative correlations across the measured variables, with several distinct clusters of highly positively correlated features and regions showing strong negative correlations.

Clustered Heatmap (Top Right):

A clustered heatmap was constructed to visualize the intensity of features (columns, corresponding to the variables from the correlation matrix) across six distinct sample groups (rows, labelled 0-5). The color scale ranges from 0.0 (dark purple/low intensity) to 1.0 (yellow/high intensity). Hierarchical clustering was applied to both the rows (samples) and columns (features).

- **Feature Clustering:** The dendrogram above the heatmap groups features with similar intensity patterns across the samples.
- **Sample Grouping:** The row clustering groups the six samples based on their feature profiles. For example, samples 2 and 4 show similar high-intensity patterns for a specific subset of features (left side of the map), while sample 0 has a distinct high-intensity profile for a different feature subset (middle).

Hierarchical Clustering Dendrogram (Bottom Left):

Hierarchical Clustering Analysis (HCA) was performed on the specific sample classifications (Season/Duration) to visualize their similarities based on the HPTLC data. The y-axis represents the distance (dissimilarity) between clusters. The resulting dendrogram reveals three main groupings:

1. **August (Rainy) Short** is the most distinct sample, clustering separately from all others at a high distance (~12.5).
2. **August (Rainy) Long** forms its own distinct branch, though more closely related to the remaining group than the "Short" sample.
3. The remaining samples form a central cluster consisting of two sub-groups: **December (Winter) Long** and a sub-cluster containing **April (Summer) Short** and **December (Winter) Short**.

Principal Component Analysis (PCA) Scree Plot (Bottom Right):

PCA was utilized to reduce the dimensionality of the dataset and determine the principal factors contributing to the variance. The scree plot displays the percentage of variance explained by each of the first six Principal Components (PCs).

- PC1 accounts for the highest variance, exceeding 30%.
- PC2 explains approximately 25% of the variance.
- The cumulative variance explained by the first two components (PC1 + PC2) is roughly 55%.
- The variance explained decreases steadily with each subsequent component, with PC6 explaining nearly 0%.

The multivariate statistical analysis of the HPTLC data provides significant insights into the chemical variability of the samples based on seasonal collection and storage duration.

Feature Relationships and Profiling:

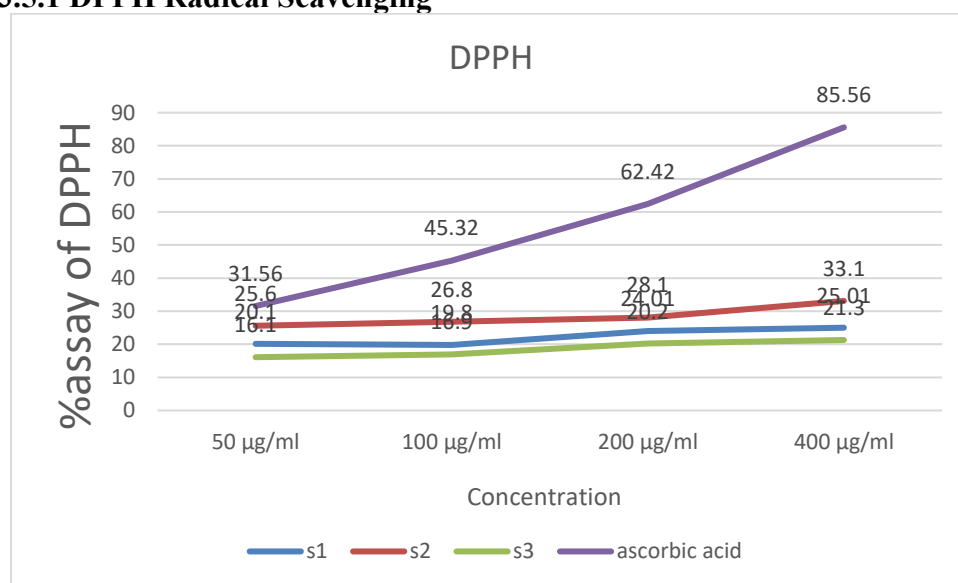
The correlation matrix indicates that the chemical profile consists of numerous inter-dependent features, suggesting complex metabolic relationships or co-eluting compounds. The clustered heatmap further refines this by demonstrating that specific sets of features (R_f values/compounds) are characteristic of different sample groups (0-5). The clear presence of "blocks" of high intensity (yellow) indicates that certain sample groups can be defined by the elevated concentration of specific biomarker clusters. This confirms that the HPTLC method successfully captured distinct chemical signatures for the different sample sets.

Dimensionality and Variance:

The PCA scree plot confirms that the complexity of the HPTLC data can be effectively reduced. While a substantial portion of the variance (~55%) is captured by the first two principal components, indicating primary underlying trends (likely corresponding to the seasonal or storage differences observed in HCA), a significant amount of variance is distributed across PC3-PC5. This suggests that the chemical differences between the samples are multifactorial and cannot be reduced to a single simple variable.

3.5 In Vitro Antioxidant Assays

3.5.1 DPPH Radical Scavenging



[figure 4 :- DPPH radical scavenging]

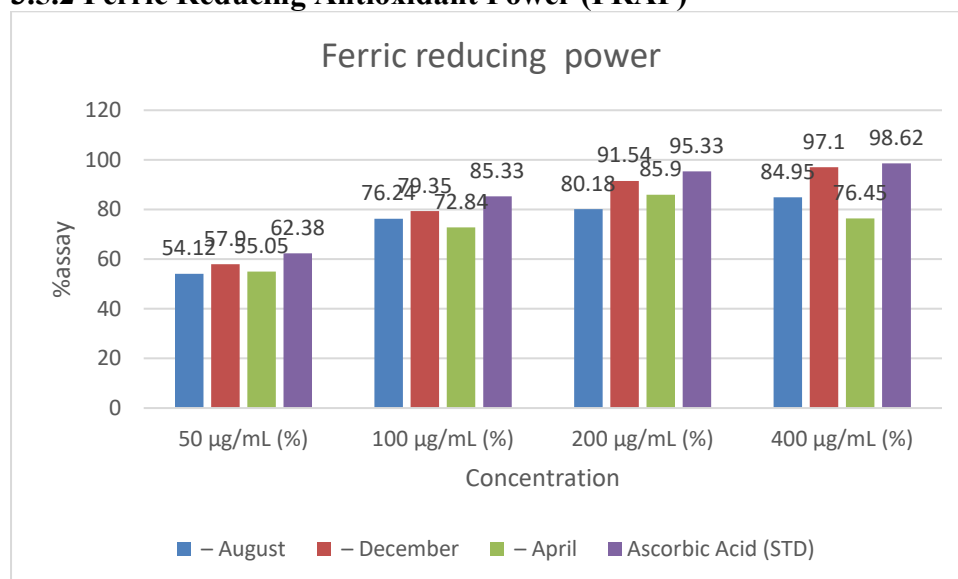
The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen or electron-donating abilities. DPPH is a stable free radical; to become a stable diamagnetic molecule, it accepts an electron or hydrogen radical⁴⁰. Figure 4 illustrates a concentration-dependent increase in the DPPH scavenging ability of the tested *Bauhinia variegata* methanolic extracts (S1, S2, S3) and the ascorbic acid standard across concentrations of 50, 100, 200, and 400 µg/mL.

As shown in the graph, ascorbic acid (standard) demonstrated robust scavenging activity, ranging from 31.56% at 50 µg/mL to a maximum of 85.56% at 400 µg/mL. The different methanolic extracts exhibited lower scavenging activities, which ranged from 20.1% to 25.01% for S1, 25.6% to 33.1% for S2, and 16.1% to 21.3% for S3.

The results clearly indicate that while the methanolic extract of S2 exhibits the highest DPPH scavenging activity among the tested plant samples, its efficacy is still relatively weak when compared directly with the standard ascorbic acid. In its radical form, DPPH disappears upon reduction by an antioxidant compound or

a radical species to become a stable diamagnetic molecule, resulting in a color change from violet to yellow, which is taken as an indication of the hydrogen-donating ability of the tested sample.

3.5.2 Ferric Reducing Antioxidant Power (FRAP)



[Figure 5 :- Ferric Reducing Antioxidant Power (FRAP) assay]

The Ferric Reducing Antioxidant Power (FRAP) assay was conducted to evaluate the electron-donating ability of the plant extracts collected across different seasons—S1 (August), S2 (December), and S3 (April)—compared to the standard antioxidant, Ascorbic Acid. The reducing power was measured as the percentage of assay activity at varying concentrations (50, 100, 200, and 400 µg/mL). The results, including calculated IC_{50} and EC_{50} values, are presented in the accompanying table and corresponding bar chart.

Overall, the extracts and the standard demonstrated a concentration-dependent increase in reducing power, with higher concentrations generally yielding greater antioxidant activity.

Ascorbic acid (standard) exhibited the highest reducing capacity across all tested concentrations, starting at 62.38% at 50 µg/mL and reaching 98.62% at 400 µg/mL. Among the tested plant samples, the S2 (December) extract showed the most potent reducing power, closely trailing the standard. S2's activity ranged from 57.9% at 50 µg/mL to an impressive 97.1% at 400 µg/mL.

The S1 (August) extract displayed moderate reducing power, steadily increasing from 54.12% at 50 µg/mL to 84.95% at 400 µg/mL. The S3 (April) extract showed a similar initial trajectory (55.05% at 50 µg/mL to 85.9% at 200 µg/mL), but notably, its reducing activity unexpectedly declined to 76.45% at the highest concentration of 400 µg/mL.

The potency of the samples was further confirmed by their IC_{50} and EC_{50} values, where lower values indicate higher antioxidant effectiveness. Ascorbic acid recorded the lowest values (IC_{50} = 36.96 µg/mL, EC_{50} = 37.81 µg/mL). True to the percentage activity trends, the S2 (December) extract was the most effective among the plant samples (IC_{50} = 38.1 µg/mL, EC_{50} = 38.95 µg/mL), followed by S1 (August) (IC_{50} = 40.85 µg/mL, EC_{50} = 41.72 µg/mL), and finally S3 (April) (IC_{50} = 42.2 µg/mL, EC_{50} = 43.15 µg/mL).

The FRAP assay measures the antioxidant potential of a substance based on its ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) through electron donation. The significant reducing power exhibited by the extracts indicates that they contain compounds capable of acting as electron donors, thereby terminating free radical chain reactions and acting as effective antioxidants.

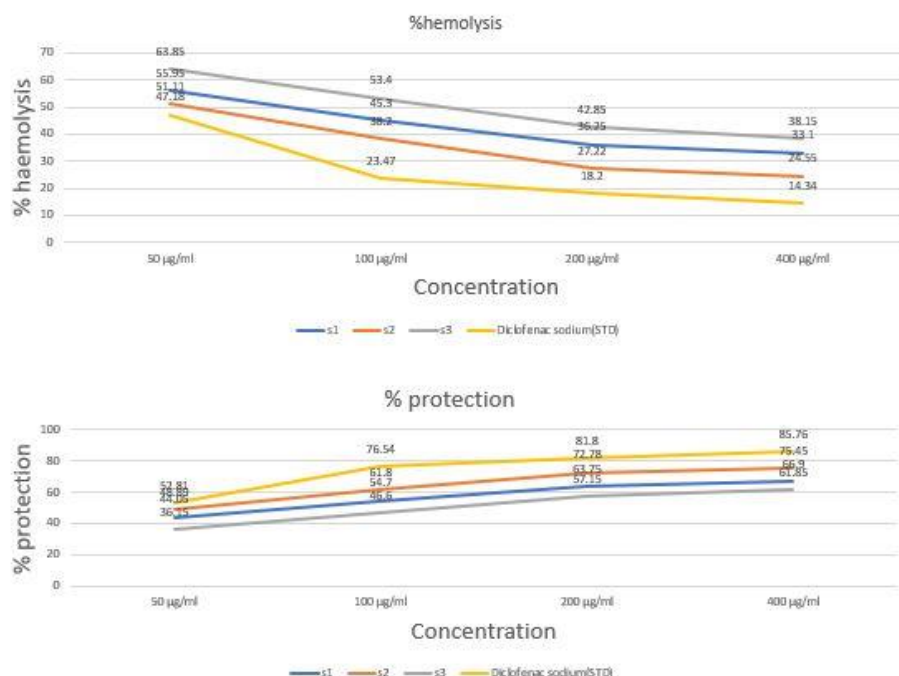
The data reveals a distinct seasonal influence on the antioxidant capacity of the plant material. The extract collected in December (S2) possesses a notably superior reducing power compared to those collected in August (S1) and April (S3). Furthermore, the IC_{50} and EC_{50} values of the S2 extract (38.1 and 38.95 µg/mL, respectively) are highly comparable to those of the pure synthetic standard, Ascorbic acid (36.96 and 37.81 µg/mL). This suggests that the environmental conditions or the plant's growth stage during the winter month of December optimize the synthesis and accumulation of potent reducing phytoconstituents. All tested extracts possess notable ferric reducing abilities, confirming their antioxidant potential. However, harvesting

the plant material in December (S2) yields the most robust antioxidant profile, demonstrating an efficacy that rivals standard Ascorbic acid.

3.6 In-Vitro Anti-inflammatory Assays

3.6.1 In-vitro activity HRBC Membrane Stabilization

HRBC



[figure 6:- Hymolysis & protection for HRBC anti-inflammatory activity]

The *in vitro* anti-inflammatory activity of the plant extracts collected across different seasons (S1 - August, S2 - December, S3 - April) was evaluated using the Human Red Blood Cell (HRBC) membrane stabilization assay. The assay measures the ability of the extracts to prevent hypotonicity-induced lysis of erythrocyte membranes, comparing their efficacy to the standard non-steroidal anti-inflammatory drug (NSAID), Diclofenac sodium. The results are expressed as percentage haemolysis and percentage protection at concentrations ranging from 50 to 400 µg/mL.

The data, presented in the accompanying figure 6, demonstrates a clear, concentration-dependent relationship for all tested samples. As the concentration of the extracts and the standard increased, the percentage of haemolysis decreased, corresponding to an increase in the percentage of membrane protection.

Percentage Haemolysis: At the lowest concentration tested (50 µg/mL), Diclofenac sodium exhibited the lowest haemolysis at 47.18%. Among the extracts, the S2 (December) sample showed the least haemolysis (51.11%), followed by S1 (August) at 55.95%, and S3 (April) at 63.85%. This trend continued at the highest concentration (400 µg/mL), where Diclofenac sodium minimized haemolysis to 14.34%. The S2 extract remained the most effective plant sample, reducing haemolysis to 24.55%, while S1 and S3 reduced haemolysis to 33.10% and 38.15%, respectively.

Percentage Protection: Conversely, the percentage protection represents the stabilizing effect on the HRBC membrane. Diclofenac sodium provided the highest protection across all concentrations, increasing from 52.81% at 50 µg/mL to a peak of 85.76% at 400 µg/mL.

The bark extracts also exhibited significant membrane-stabilizing properties. The S2 (December) extract demonstrated the most potent protective effect among the seasonal samples. Its protective activity started at 48.89% at 50 µg/mL and climbed steadily to 75.45% at 400 µg/mL. The S1 (August) extract provided moderate protection, ranging from 44.05% to 66.9%. The S3 (April) extract showed the weakest protective effect, starting at 36.15% and reaching 61.85% at the highest concentration.

The HRBC membrane stabilization assay is a widely accepted *in vitro* model for evaluating anti-inflammatory activity. The erythrocyte membrane is structurally analogous to the lysosomal membrane. During inflammation, the release of lysosomal enzymes into the cytosol exacerbates tissue damage and the inflammatory response. Therefore, compounds that stabilize the HRBC membrane against hypotonic lysis are

inferred to have the potential to stabilize lysosomal membranes, thereby inhibiting the release of these inflammatory mediators.

The results clearly indicate that all three seasonal extracts possess notable membrane-stabilizing properties, suggesting anti-inflammatory potential. The concentration-dependent increase in protection implies that the active phytoconstituents responsible for this effect are present in functional quantities within the extracts.

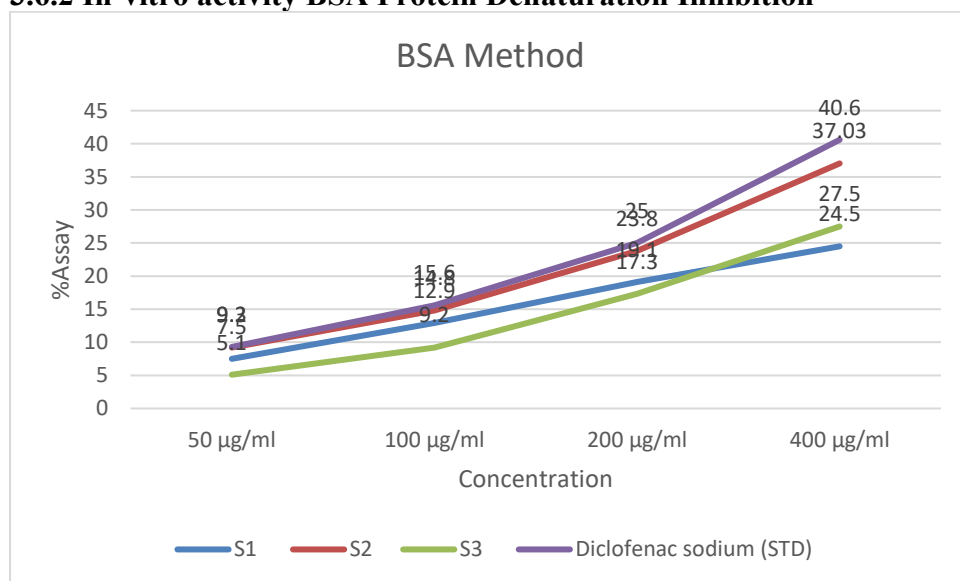
Crucially, the data reveals a significant seasonal variation in the efficacy of the extracts. The sample collected in December (S2) consistently outperformed those collected in August (S1) and April (S3) across all tested concentrations. While it did not quite match the potency of the pure standard Diclofenac sodium, the S2 extract achieved a substantial 75.45% protection at 400 $\mu\text{g/mL}$, demonstrating strong anti-inflammatory potential.

This variation suggests that the environmental conditions during the winter month of December (e.g., temperature, photoperiod, water stress) may trigger an upregulation in the biosynthesis of secondary metabolites responsible for membrane stabilization. These could include flavonoids, tannins, or triterpenoids, which are known to interact with membrane lipids and proteins, enhancing membrane integrity.

Conversely, the S3 (April) sample exhibited the lowest anti-inflammatory activity. This could be due to a depletion of these active constituents during the summer months or a shift in the plant's metabolic priorities toward growth rather than defence mechanism synthesis.

In conclusion, the plant extracts demonstrate significant *in vitro* anti-inflammatory activity by stabilizing HRBC membranes. The optimal harvesting time for maximizing this specific pharmacological effect appears to be December (S2), which yields an extract with efficacy approaching that of a standard NSAID. Further *in vivo* studies and phytochemical profiling are necessary to isolate the active compounds and elucidate their exact mechanisms of action.

3.6.2 In-vitro activity BSA Protein Denaturation Inhibition



[Figur 7 :- anti-inflammatory activity BSA]

The *in vitro* anti-inflammatory activity of the bark extracts collected across different seasons—S1 (August), S2 (December), and S3 (April)—was further evaluated using the Bovine Serum Albumin (BSA) protein denaturation assay. The assay measures the ability of the extracts to prevent heat-induced protein denaturation, a well-documented cause of inflammation. The percentage inhibition of denaturation was recorded at concentrations ranging from 50 to 400 $\mu\text{g/mL}$ and compared against the standard non-steroidal anti-inflammatory drug (NSAID), Diclofenac sodium. The results are summarized in the accompanying table and represented graphically in the dose-response curve.

The data demonstrates a clear, dose-dependent relationship for all tested samples; as the concentration of the extracts increased, the percentage of protection against protein denaturation also increased.

Percentage Inhibition of Denaturation: Diclofenac sodium (standard) exhibited the highest inhibitory activity across the entire concentration range, starting at $9.3 \pm 0.02\%$ at 50 $\mu\text{g/mL}$ and reaching a maximum of $40.6 \pm 0.02\%$ at 400 $\mu\text{g/mL}$.

Among the plant extracts, the S2 (December) sample demonstrated the most potent anti-inflammatory activity, closely mirroring the efficacy of the standard drug. The S2 extract effectively inhibited denaturation from $9.2 \pm 0.05\%$ at the lowest concentration to $37.03 \pm 0.01\%$ at the highest concentration.

The S1 (August) and S3 (April) extracts displayed moderate to lower anti-inflammatory potential. At lower concentrations (50-200 $\mu\text{g/mL}$), S1 exhibited stronger inhibition than S3 (e.g., 19.1% vs. 17.3% at 200 $\mu\text{g/mL}$). However, an interesting shift occurred at the maximum concentration of 400 $\mu\text{g/mL}$, where the trajectory of the S3 extract sharply increased to $27.5 \pm 0.02\%$, surpassing the S1 extract, which recorded $24.5 \pm 0.01\%$.

Protein denaturation is a complex process that leads to the alteration of electrostatic, hydrogen, hydrophobic, and disulfide bonds, ultimately resulting in the loss of biological function. In living tissues, the denaturation of tissue proteins is a primary marker for inflammatory diseases, leading to the production of auto-antigens. Therefore, agents that can prevent protein denaturation are considered to possess significant anti-inflammatory potential.

The results of the BSA assay strongly corroborate the findings from the HRBC membrane stabilization assay, confirming that the tested bark extracts possess notable *in vitro* anti-inflammatory properties. The ability of the extracts to stabilize the BSA protein against heat-induced degradation suggests the presence of active secondary metabolites—such as flavonoids, polyphenols, or triterpenoids—that can bind to the protein matrix and prevent structural alterations.

Crucially, the data further substantiates the significant impact of seasonal variation on the pharmacological efficacy of the bark. The extract derived from the material harvested in December (S2) consistently demonstrated superior anti-inflammatory activity, nearly matching the potency of the standard NSAID, Diclofenac sodium. This strongly indicates that the environmental conditions during the winter season are optimal for the biosynthesis and accumulation of the specific phytoconstituents responsible for inhibiting protein denaturation.

The bark extracts demonstrate substantial *in vitro* anti-inflammatory activity by inhibiting protein denaturation. The December harvest (S2) yields the most highly active extract, reinforcing the conclusion that winter is the optimal harvesting season for maximizing this plant's anti-inflammatory therapeutic potential.

Conclusion:

In conclusion, the statistical analysis demonstrates that the chemical composition of the studied material is significantly influenced by both the season of collection and the duration of storage. The winter season (December) produces a highly distinct chemical profile compared to summer and rainy. These findings highlight the importance of standardizing harvest timing to ensure the consistency of the chemical profile for this material.

References

1. Holopainen JK, Gershenzon J. Multiple stress factors and the emission of plant VOCs. *Trends Plant Sci.* 2010;15(3):176-184.
2. Ramakrishna A, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal Behav.* 2011;6(11):1720-1731.
3. Wink M. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry.* 2003;64(1):3-19.
4. Dixon RA. Natural products and plant disease resistance. *Nature.* 2001;411(6839):843-847.
5. Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep.* 2009;26(8):1001-1043.
6. Dai J, Mumper RJ. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules.* 2010;15(10):7313-7352.
7. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod.* 2016;79(3):629-661.
8. Atanasov AG, Zotchev SB, Dirsch VM, Orhan IE, Banach M, Rollinger JM, et al. Natural products in drug discovery: advances and opportunities. *Nat Rev Drug Discov.* 2021;20(3):200-216.
9. Bourgaud F, Gravot A, Milesi S, Gontier E. Production of plant secondary metabolites: a historical perspective. *Plant Sci.* 2001;161(5):839-851.
10. Harvey AL. Natural products in drug discovery. *Drug Discov Today.* 2008;13(19-20):894-901.

11. Ncube B, Finnie JF, Van Staden J. Quality from the field: the impact of environmental factors as quality determinants in medicinal plants. *S Afr J Bot.* 2012;82:11-20.
12. Sampaio BL, Edrada-Ebel R, Da Costa FB. Effect of the environment on the secondary metabolic profile of *Tithonia diversifolia*: a model for environmental metabolomics of plants. *Sci Rep.* 2016;6:29265.
13. Borges CV, Minatel IO, Gomez-Gomez HA, Lima GPP. Medicinal plants: influence of environmental factors on the content of secondary metabolites. In: Ghorbanpour M, Varma A, editors. *Medicinal Plants and Environmental Challenges*. Springer, Cham; 2017. p. 259-277.
14. Briskin DP. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant Physiol.* 2000;124(2):507-514.
15. Kumar D, Kumar S. *Bauhinia variegata* Linn.: a review on its pharmacognosy, phytochemistry, and pharmacology. *J Pharm Res.* 2011;4:2039-2041.
16. Sharma V, Rajani GP. Evaluation of *Bauhinia variegata* bark extract for its anti-ulcer activity. *Indian J Pharmacol.* 2011;43(2):167.
17. Sharma PV, editor. *Charaka Samhita* (Text with English translation). Varanasi: Chaukhambha Orientalia; 1981.
18. Rajkapoor B, Jayakar B, Muruges N. Antitumour activity of *Bauhinia variegata* on Dalton's ascitic lymphoma. *J Ethnopharmacol.* 2003;89(1):107-109.
19. Bodakhe SH, Ram A. Hepatoprotective properties of *Bauhinia variegata* bark extract. *Yakugaku Zasshi.* 2007;127(9):1503-1507.
20. Mali RG, Mahajan SG, Mehta AA. Rakta Kanchanar (*Bauhinia variegata*): a review. *Pharmacogn Rev.* 2007;1(2):314-319.
21. Kunle OF, Egharevba HO, Ahmadu PO. Standardization of herbal medicines - A review. *Int J Biodivers Conserv.* 2012;4(3):101-112.
22. Azwanida NN. A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Med Aromat Plants.* 2015;4(3):196.
23. Harborne JB. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*. 3rd ed. London: Chapman and Hall; 1998.
24. Khandelwal KR. *Practical Pharmacognosy: Techniques and Experiments*. 19th ed. Pune: Nirali Prakashan; 2008.
25. Evans WC. *Trease and Evans' Pharmacognosy*. 16th ed. Edinburgh: Saunders Elsevier; 2009.
26. World Health Organization. *Quality control methods for herbal materials*. Geneva: World Health Organization; 1998.
27. Indian Pharmacopoeia Commission. *Indian Pharmacopoeia*. 8th ed. Ghaziabad: Ministry of Health & Family Welfare, Government of India; 2018.
28. Latimer GW, editor. *Official Methods of Analysis of AOAC International*. 18th ed. Gaithersburg, MD: AOAC International; 2005.
29. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic.* 1965;16(3):144-158.
30. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal.* 2002;10(3):178-182.
31. Wagner H, Bladt S. *Plant Drug Analysis: A Thin Layer Chromatography Atlas*. 2nd ed. Berlin: Springer; 1996.
32. Shajisvelin CD, Muthu AK. Antioxidant activity of various extracts from whole plant of *Flacourtia sepiaria* Roxb. *Int J Curr Pharm Res.* 2011;3(3):36-37.
33. Thambiraj J, Paulsamy S. Evaluation of in vitro antioxidant activity of methanolic extract of *Acacia caesia* (L.) Willd. (Mimosaceae). *Asian Pac J Trop Med.* 2012;5(5):S173-S177.
34. Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn J Nutr Diet.* 1986;44:307-315.
35. Hemalatha S, Lalitha P, Arulpriya P. Antioxidant activities of the extracts of the aerial roots of *Pothos aurea* (Linden ex Andre). *Der Pharma Chem.* 2010;2:84-89.
36. Aparadh VT, Naik VV, Karadge BA. Antioxidant activity of echinoid crotalaria (*Crotalaria echinoides* Benth.). *J Pharm Res.* 2012;5:2244-2246.

37. Gandhisan R, Thamaraichelvan A, Baburaj S. Anti-inflammatory action of *Lannea coromandelica* by HRBC membrane stabilization. *Fitoterapia*. 1991;62:81-83.
38. Anosike CA, Obidoa O, Ezeanyika LU. Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (*Solanum aethiopicum*). *DARU J Pharm Sci*. 2012;20(1):76.
39. Sakat S, Juvekar AR, Gambhire MN. In vitro antioxidant and anti-inflammatory activity of *Oxalis corniculata* Linn. *Int J Pharm Pharm Sci*. 2010;2(1):146-155.
40. Ho YL, Huang SS, Deng JS, Lin YH, Chang YS, Huang GJ. In vitro antioxidant properties and total phenolic contents of wetland medicinal plants in Taiwan. *Bot Stud*. 2012;53(1):55-66.