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Comparative Evaluation of Chlorophyll Extraction and Bioactive Properties of Selected Medicinal Plant Leaves

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ABSTRACT: The present study investigated chlorophyll extraction and estimation from selected medicinal plant leaves, namely Betel leaf (Piper betle), Madhuca longifolia, a Crassulaceae species, and Coleus amboinicus Lour., with additional assessment of their phytochemical, antioxidant, and microbial characteristics. The study aimed to evaluate these leaves as potential sources of chlorophyll and associated bioactive compounds for further application and development. Fresh leaves were collected locally, washed, drained, dried, crushed, and subjected to solvent extraction. Chlorophyll was extracted using 80% acetone and estimated spectrophotometric ally by measuring absorbance at 645 and 663 nm using Arnon's equations. Qualitative and quantitative phytochemical screening was performed for alkaloids, tannins, flavonoids, saponins, terpenoids, phenols, glycosides, steroids, and anthraquinones. Antioxidant activity was evaluated using a DPPH-based assay, while microbial counts were monitored during incubation and storage. All four plant samples yielded measurable chlorophyll and exhibited a broad range of phytochemical constituents. Phytochemical absorbance values were generally higher at the reported concentration of 4 µg/ml, with Madhuca longifolia and Betel leaf showing comparatively higher values for several phytochemical groups. In the DPPH assessment, the highest reported value was 6.04% for the Crassulaceae sample at 300 nm, followed by Madhuca longifolia (6.03%), Betel leaf (5.97%), and Coleus amboinicus Lour. (5.93%). Microbial counts increased progressively during incubation and after two months of storage. The findings demonstrate that the selected medicinal leaves are potential laboratory sources of chlorophyll and diverse secondary metabolites. Further studies are warranted to optimize pigment extraction and stabilization, characterize individual bioactive compounds, and evaluate their potential applications in food, pharmaceutical, and other value-added products.

1. INTRODUCTION

Chlorophyll Extraction

To improve yield, purity, and stability, chlorophyll extraction has increasingly moved beyond conventional solvent maceration toward optimized, selective, and environmentally friendly approaches. Process design starts with feedstock characterisation and sample approach since pre-treatment, particle size, and raw material selection (leaf vs. microalgae) have a significant impact on extraction efficiency, according to reviews by published reviews.

Solvent selection remains central: traditional organic solvents such as acetone, methanol and ethanol are widely used because they effectively dissolve chlorophylls, but comparative studies emphasize ethanol as a safer, food-grade alternative. Work on spinach, moringa and microalgae demonstrates that methanol and acetone often give higher initial yields, while ethanol and aqueous-ethanol mixtures are preferred when downstream food or nutraceutical applications require non-toxic solvents. (Ferreira et al., 2021; Ngcobo et al., 2024).

Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have been shown to markedly improve chlorophyll recovery and reduce processing time compared with conventional maceration. Reviews and experimental studies report that UAE increases cell disruption and mass transfer—enhancing yields from spinach, carrot or microalgal biomass—while MAE provides rapid heating that liberates pigments efficiently; both methods are frequently optimized by response-surface designs for solvent ratio, temperature, time and power.

Green extraction approaches are expanding, including non-ionic surfactant cloud-point extraction, ionic-liquid-based aqueous systems, and biodegradable solvents such as 2,3-butanediol, which have been investigated to reduce environmental impact and improve selectivity for chlorophyll a and b.(Ferreira et al., 2021; Leite et al., 2018) Process reviews highlight that such solvents can enable food-grade pigment recovery while lowering hazardous solvent use, but they require attention to downstream removal and regulatory acceptance.

Post-extraction stability and formulation are equally important because chlorophylls rapidly degrade (pheophytinization, oxidation). Recent work addresses stabilization via microencapsulation, electrospinning and incorporation into carrier matrices to protect pigments during storage and application; studies demonstrate that encapsulation (e.g., zein nanofibers, liposomes) markedly improves color retention and shelf life for food applications. Scale-up studies (Tamminen et al., 2022) also

show that ultrasound-assisted systems can be translated to pilot continuous operation with careful control of energy input and residence time.

1.1. Madhuca longifolia (Butter Tree)

Figure 1. Madhuca longifolia (Butter Tree)



Madhuca longifolia, commonly known as Mahua or the Indian Butter Tree, is a vital multipurpose tree with significant socioeconomic value, extensively found in the tropical and subtropical regions of the Indian subcontinent. This deciduous tree is resilient, thriving in dry, rocky, and saline conditions. Mahua is prized for its various parts, each offering numerous benefits. The fruit seeds yield mahua butter, rich in linoleic and unsaturated fatty acids beneficial for heart health. The flowers, rich in sugar and containing essential vitamins and minerals, are used for food, liquor production, and animal feed. The leaves and bark have notable antioxidant properties, while the oil extracted from seeds is used in making soap, lubricants,

and candles. Despite its long gestation period, modern grafting techniques have enabled quicker cultivation. The tree's adaptability makes it valuable for arid and semiarid regions. (Devanand et al., 2024).

Madhuca longifolia (Koenig) J.F. Macb., commonly known as Mahua, is a multipurpose tropical tree native to the Indian subcontinent, belonging to the family Sapotaceae. It has been widely recognized for its nutritional, medicinal, and economic importance. Earlier studies have reported that M. longifolia has a long history of ethnomedicinal use among tribal and rural communities in India, where its flowers, seeds, bark, and leaves are utilized for therapeutic and nutritional purposes.(Akshatha et al., 2013) The flowers are rich in sugars, vitamins, and amino acids and are commonly used for fermentation to produce traditional alcoholic beverages, while the seeds yield valuable edible and industrial oils.(Dubey et al., 2023)

Phytochemical analyses revealed that various parts of the plant contain bioactive compounds such as saponins, flavonoids, tannins, steroids, and triterpenes, which contribute to its pharmacological potential. Strong antioxidant and microbial properties in bark and leaf extracts, confirming its traditional use in treating infections, inflammation, and oxidative stress-related disorders. Similarly,

M. longifolia extracts exhibit potent microbial activity against multidrug-resistant bacteria, highlighting its relevance in modern pharmacological research. (Shukla et al., 2024; Dhoubhadel et al., 2023).

1.2. Crassulaceae

Figure 2. Crassulaceae



The Crassulaceae is a mid-sized family of angiosperms comprising roughly 1,400 species in about 30 genera, with a strong representation of herbaceous and succulent perennial plants adapted to arid and semi-arid environments. Crassulaceae species as "mostly herbaceous succulents, usually with 5-merous flowers and one or two whorls of stamens". Many genera (such as Crassula, Sedum, Kalanchoe, Echeveria) are notable for their succulent leaves and adaptive features such as Crassulacean acid metabolism (CAM) photosynthesis, which allow survival in water-limited habitats. (as described in general overview sources) (Liu et al., 2023).

On the phylogenetic and evolutionary front, used plastome (chloroplast genome) data for 52 species representing major clades in Crassulaceae to reconstruct a robust phylogeny, estimate the crown age (≈ 63.9 million years), and trace biogeographic dispersal (origins in southern Africa, spread to the Mediterranean, eastern Asia, Macaronesia, and North America).(Liu et al., 2023)

Research focusing on the large genus Sedum and the subfamily Sempervivoideae has reported substantial non-monophyly in existing taxonomic groupings and emphasized the need for taxonomic revision.(Messerschmid et al., 2020)

1.3. Coleus amboinicus Lour.

Figure 3. Coleus amboinicus Lour.



Coleus amboinicus (synonym: Plectranthus amboinicus (Lour.) Spreng) is a succulent aromatic perennial herb from the family Lamiaceae, widely cultivated in tropical and subtropical regions. The plant is a large, pubescent herb with thick, aromatic leaves traditionally used in Indian medicine for ailments such as asthma, cough, liver and renal affections. In its native or long-cultivated range, the species is also used as a spice, flavoring herb, and ornamental, with the species epithet "amboinicus" referring to Ambon Island in the Maluku archipelago. (Kaliappan and Viswanathan, 2008)

The plant has a rich ethnopharmacological heritage, with traditional uses documented for bronchitis, fevers, digestive ailments, and as a carminative agent, alongside its phytochemical profile and pharmacological investigations. More recently, an experimental study evaluated the anticonvulsant activity of different parts of C. amboinicus.(Bhattacharjee and Majumder, 2013)

C. amboinicus, reporting significant effects and suggesting that its flavonoid, alkaloid and saponin content may underpin this activity. (Rout et al., 2012).

From a phytochemical and bioactivity perspective, C. amboinicus leaves contain phenolic acids (e.g., rosmarinic acid), diterpenoid quinones (such as royleanone), monoterpenoid and sesquiterpenoid essential oils, and strong antioxidant and antimicrobial activities have been reported under contrasting climatic cultivation conditions (Indonesia vs. Poland).(Stevens et al., 1995)

The study further highlighted that C. amboinicus exhibits both constitutive and facultative forms of crassulacean acid metabolism (CAM) photosynthesis — a water-conservation adaptation rare in many members of the mint family — positioning it as an interesting model for evolutionary studies of plant metabolic plasticity..

1.4. Betel Leaf

Figure 4. Betel leaf



Betel leaf (Piper betel L.) is an aromatic creeper, which belongs to Piperaceae family comprising approximately 10 genera comprising more than 2000 species.(Chakraborty and Shah, 2021) This plant is familiar with different vernacular names such as 'Paan' in India, 'Trau' in Vietnam and 'Plue' in Thailand . It is abundantly grown in Southeast Asian countries for commercial purpose. India, Sri Lanka, Bangladesh, Malaysia, Burma, Nepal, Myanmar and Thailand are the major cultivators

of the vine . The leaves of the plant are economically important for being used as the principal component in betel quid (Paan). It is consumed by more than 15-20 million people on a daily basis only in India, whereas the number is over two billion worldwide .

Betel leaf (Piper betel L.) is a commercial crop, which is traditionally consumed in the raw state as a mouth freshener and stimulant in Southeast Asia since antiquity. It possesses various functional and medicinal properties, such as microbial, antioxidant, anti-diabetic, anti- carcinogenic etc. The leaf contains essential oil, which has a unique aroma contributing flavour and fragrance. This essential oil is a mixture of concentrated phytochemicals, among which the major components are estragole, chavicol, chavibetol, B-cubebene, and caryophyllene.

In different scientific researches, betel leaf extracts or essential oil were described for its numerous beneficial functional properties, contributed by these bioactive compounds. However, some health hazards may occur, when the leaf is consumed along with areca nut and tobacco. Varieties of Betel Leaf are based on the morphological characters and essential oil content, betel vine varieties are divided into five main groups viz, Bangla, Desawari, Kapoor, Sanchi, and Meetha..

Betel leaf demand for its products such as herbal drugs, medicines, and natural herbal formulations has increased. The beneficial effects of betel leaves and its products have traditionally exploited for the treatment of several diseases like bad breath, cuts, injuries, inflammations, cold cough, indigestion, etc. The extract of this highly perishable product can be used in organic synthesis, food, and beverage industry, pharmaceuticals, etc., to the environmental issues.

1.5. Rationale for Chlorophyll Extraction

The purpose of chlorophyll extraction lies in utilizing chlorophyll's natural color, antioxidant potential, and health-promoting properties as a functional ingredient in food formulations. Chlorophyll, the green pigment found in plants, is widely recognized for its natural coloring ability, making it a sustainable alternative to synthetic food dyes in gummy production. Studies have highlighted that chlorophyll and its derivatives (such as chlorophyllin) provide a vibrant green hue that remains stable when properly encapsulated or formulated, allowing manufacturers to create visually appealing, plant-based colorants without artificial additives.

Beyond coloration, chlorophyll adds functional health benefits to colorants. Research has shown that it exhibits antioxidant, detoxifying, and potential anti-inflammatory properties, which can support overall wellness when incorporated into food and nutraceutical formulations. Its incorporation helps develop "clean-label" or health-oriented confectionery products that align with the growing consumer demand for natural, plant-based ingredients.

Moreover, extracted chlorophyll can improve the nutritional and marketing value, positioning them as fortified functional foods rather than conventional sweets. Optimized extraction ensures high pigment yield and stability, preserving its bioactivity during preparation. Therefore, the extraction of chlorophyll serves both technological (natural coloring, stability) and functional (health benefit, consumer appeal) purposes, bridging food science and nutrition innovation.

2. MATERIALS AND METHODS

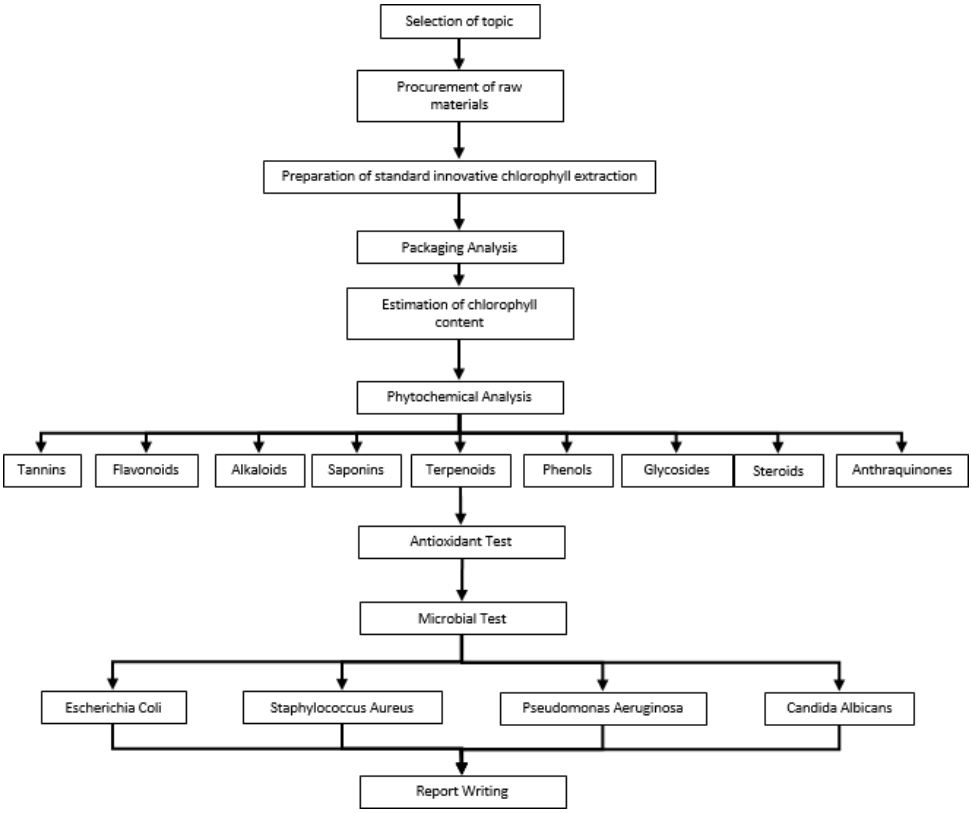
In this chapter, the leaves from selected medicinal plants—particularly Madhuca longifolia, Crassulaceae species, and Coleus—are being investigated mainly for their chlorophyll content, nutritional compounds, and medicinal properties. The work focuses on extracting chlorophyll from these leaves using different extraction methods and then reviewing how the obtained pigments and phytochemicals contribute to health benefits. The leaves are studied scientifically through literature to understand their bioactive components, their role in traditional medicine, antioxidant potential, anti-inflammatory effects, nutritive value, and possible pharmaceutical applications. Overall, researcher used these leaves as natural sources of chlorophyll and phytochemicals and analysing them in terms of extraction, composition, and biological importance. The leaves are also subjected to solvent-based extraction processes in order to isolate chlorophyll efficiently. Different organic solvents such as acetone, ethanol, methanol, petroleum ether and hexane are used because they dissolve chlorophyll and help separate it from the leaf tissues. The extraction mainly involves drying, powdering, and mixing the leaf powders with selected solvents under controlled conditions followed by filtration and evaporation of the solvent to obtain crude chlorophyll. By comparing solvents and conditions, researcher analyse which solvent gives better pigment yield and purity, allowing the researcher to evaluate how extraction efficiency varies among leaves of Madhuca longifolia, Crassulaceae, and Coleus. This

helps in identifying the most effective solvent system that can be applied for laboratory studies as well as possible industrial applications.

2.6.1. Chlorophyll Estimation

2.1. Research Design

Figure 5. Research design



2.2. Procurement of Raw Materials

The basic ingredients required for the process were Betel Leaves, Madhuca Longifolia (Ippa Chettu), Crassulaceae (Ranapala), Coleus amboinicus lour (Karpoorvali). These were produced from local market from Tirupati and few raw materials from Sri Padmavati Mahila Visvavidyalayam premises.

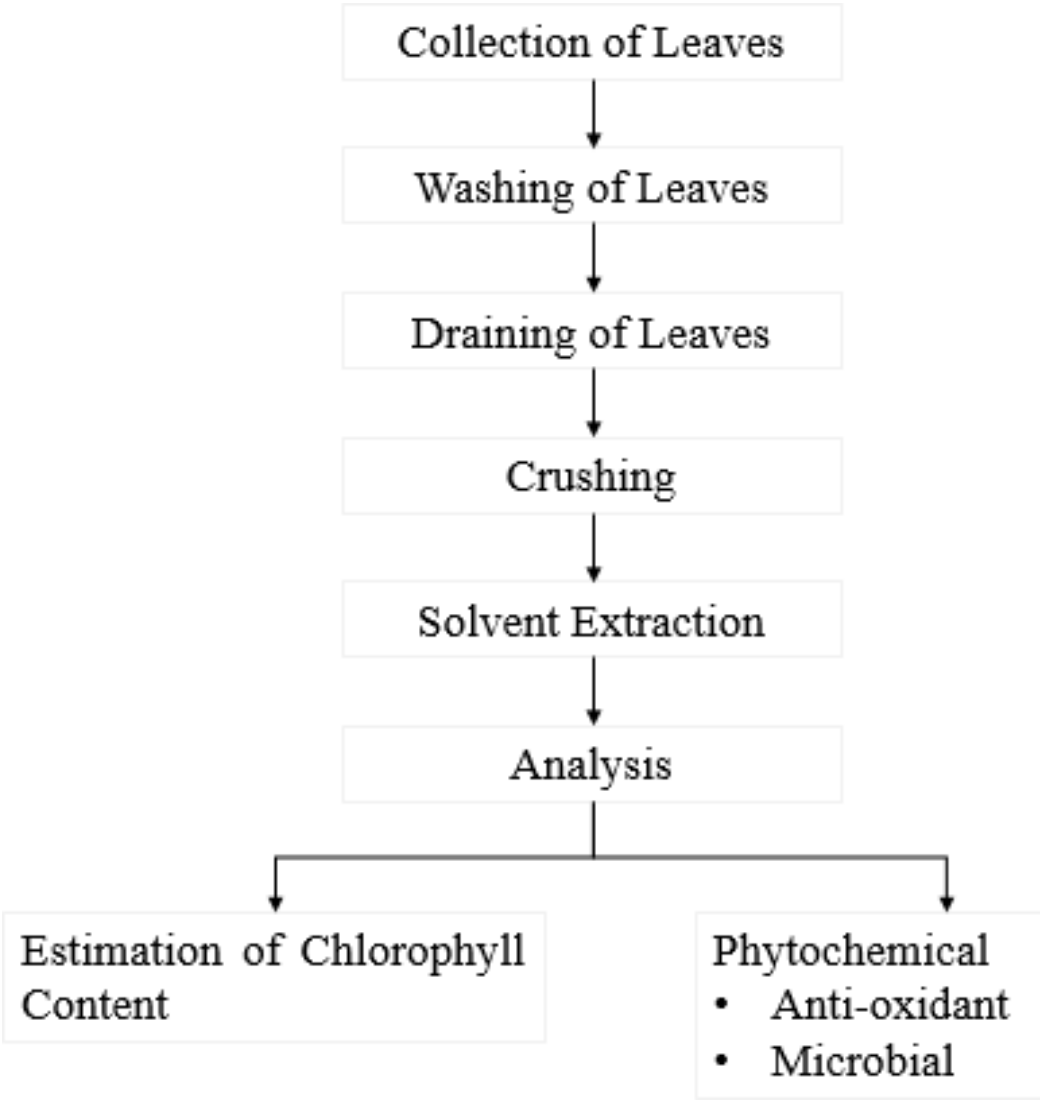
2.3. Plant Materials

Four medicinal plant leaves were selected for the study: betel leaf (Piper betle L.), Madhuca longifolia, Crassulaceae (Ranapala/Kalanchoe pinnata), and Coleus amboinicus Lour. Fresh, mature and apparently healthy leaves free from visible fungal growth or physical damage were used for extraction and subsequent analysis.

2.4. Processing of Ingredients

This procedure explains how fresh medicinal leaves are collected and processed to extract important plant compounds. The extracted solution is later used to study its natural properties such as chlorophyll content and phytochemical behavior like antioxidant and antimicrobial effects. This helps us understand how useful the leaves are in medicinal or biochemical studies.

Figure 10. Processing and analytical flowchart



Collection of Leaves

Fresh and healthy leaves are collected from the plant source in the premises of Sri Padmavati Mahila Visvavidyalayam, Tirupati. Usually, mature leaves without any visible fungal growth or damage are selected so that the extract quality remains high and free from contamination.

Washing of Leaves

The collected leaves are washed thoroughly using clean running water to remove dust, soil particles, or insects. Sometimes distilled or purified water is used to make sure no external impurities affect the extraction results.

Draining of Leaves

After washing, leaves are allowed to drain so that excess surface water is removed. This step prevents dilution of extract and avoids interference in the solvent extraction process. Sometimes leaves may be gently dried using filter paper.

Crushing

The drained leaves are crushed, ground, or blended into a paste or powder form. Crushing helps break the cell structure so that the internal plant chemicals are easily released when solvent extraction takes place.

Solvent Extraction

In this step, crushed leaf material is mixed with a suitable solvent such as ethanol, methanol, acetone, or water. The solvent dissolves useful phytochemicals present in the plant. Afterwards, the liquid extract is separated for testing. This step basically pulls out the active compounds from the leaves.

Analysis

The obtained extract is analysed using laboratory methods. This may include checking colour, absorption, chemical reactions, or using instruments depending on the study purpose which includes estimation of chlorophyll content and phytochemical studies. The analysis will show what kind of useful compounds are present in the plant.

2.5. Solvent Extraction

Solvent extraction is the process where useful plant chemicals are separated from leaf material using a suitable liquid solvent. First, the crushed or powdered leaves are transferred into a clean container, and a solvent such as ethanol, methanol, acetone, or even water is added in a measured amount. The solvent must be chosen depending on the type of compounds we want to extract—polar solvents like water dissolve hydrophilic substances, whereas organic solvents like acetone help extract pigments and phytochemicals that are not easily soluble in water. Once the solvent is added, it starts dissolving the chemical components present in the plant cells.

The mixture is then normally placed in a water bath or maintained at a controlled temperature. Heating the mixture in a water bath gently increases the movement of molecules, allowing the solvent to penetrate deeper into the crushed leaf tissues. This mild heating helps release compounds more effectively, but without boiling the solvent or damaging heat-sensitive plant molecules. During this period, pigments, phenols, flavonoids, chlorophylls and other phytochemicals slowly move into the solvent solution.

After a suitable extraction time, the mixture is removed from the water bath and allowed to cool. The liquid extract is separated from solid particles using filtration or decantation. What remains is a solvent-rich liquid that holds dissolved plant compounds. In many cases, the solvent is then evaporated using gentle heat so that only the concentrated extract remains at the bottom. This concentrated sample contains the active phytochemicals that will be used for chemical analysis, antioxidant studies, chlorophyll estimation or microbial testing. Through this method, most of the important bioactive compounds are successfully recovered from the leaves in an efficient and controlled way.

2.6. Analytical Methods

After extracting the solvent-based leaf extract, the solution is subjected to different analytical tests to understand its chemical composition and biological properties. These tests help us identify the presence of pigments like chlorophyll and also measure phytochemicals responsible for antioxidant and antimicrobial activities. By analysing the extract, we can evaluate how useful the leaves are medicinally and compare the effectiveness of different plant samples.

2.6.1. Estimation of Chlorophyll Content

After the solvent extraction, the chlorophyll present in the liquid extract is measured to determine how much green pigment has been dissolved from the leaves. This is usually done by checking the absorbance of the extract using a spectrophotometer at specific wavelengths that correspond to chlorophyll A and chlorophyll B. The values obtained help calculate the total chlorophyll content present in the plant sample. This estimation allows comparison between different leaves and also shows how efficiently the extraction process worked..

2.6.2. Phytochemical Analysis

Phytochemical analysis is performed to detect and estimate the presence of bioactive compounds in plant extracts. These compounds, known as secondary metabolites, are responsible for the therapeutic and medicinal properties of plants. In leaf extracts, common phytochemicals analysed include tannins, flavonoids, alkaloids, saponins, terpenoids, phenols, glycosides, steroids, and anthraquinones.

Tannins are detected using ferric chloride, producing a blue-black or greenish-black colour.

Flavonoids are identified by reaction with magnesium and hydrochloric acid, giving pink or red coloration.

Alkaloids are detected using Mayer's or Dragendorff's reagent, forming cream or orange precipitate.

Saponins give persistent froth upon shaking the extract.

Terpenoids are detected by the Salkowski test, where a reddish-brown interface indicates presence.

Phenols react with ferric chloride to produce blue or green colour.

Glycosides are detected by the Keller-Kiliani test, giving a reddish-brown colour.

Steroids are identified using the Liebermann-Burchard test, producing blue or green coloration.

Anthraquinones are detected with Borntrager's test, where the ammoniacal layer turns pink to red.

2.6.3. Antioxidant Analysis

The antioxidant activity of leaf extracts is analysed to determine their ability to neutralize free radicals and prevent oxidative damage. Typically, the crude extract obtained through solvent extraction (ethanol, methanol, or water) is tested using assays such as DPPH, ABTS, or FRAP, which measure radical scavenging capacity or reducing power. A decrease in the absorbance of the radical solution indicates higher antioxidant activity. Sometimes, results are compared with standard antioxidants like ascorbic acid or Trolox to quantify the extract's scavenging potential. This analysis provides an estimate of the extract's capacity to act as a natural antioxidant.

2.6.4. Antimicrobial Analysis

The antimicrobial activity of leaf extracts is evaluated using solvent-extracted crude samples against bacterial and fungal strains. Common methods include the agar well diffusion, disc diffusion, or broth dilution assays, where inhibition of microbial growth indicates antimicrobial potential. The results are often compared with standard antibiotics like streptomycin or ampicillin to assess the extract's effectiveness.

3. RESULTS

3.1. Chlorophyll extraction and estimation

Measurable chlorophyll extracts were obtained from all four selected leaf materials using the applied 80% acetone extraction procedure. The extraction response varied among the plant samples and tested concentrations, with relatively stronger responses observed for Madhuca longifolia and Coleus amboinicus under the study conditions.

Across the tested concentration series, the strongest chlorophyll assay responses were recorded at 4 µg/g, followed by lower responses at higher concentrations. Madhuca longifolia and Coleus amboinicus showed comparatively higher responses for chlorophyll a and chlorophyll b.

Chlorophyll a (mg/g) = 12.7 (A663) – 2.69 (A645) x V/1000 x W

Chlorophyll b (mg/g) = 22.9 (A645) – 4.689 (A663) x V/1000 x W

Total chlorophyll (mg/g) = 20.2 (A645) – 8.02 (A663) x V/1000 x W

Table 1. Estimation of chlorophyll content

S No.	Particulars	Observation Values
1	Weight of the fresh tissue	1gm
2	Final volume of extraction made with 80% acetone	10 ml
3	Absorbance reading at 645	20.2 mg/g
4	Absorbance reading at 663	8.02 mg/g

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Figure 11. Graphical representation of chlorophyll content

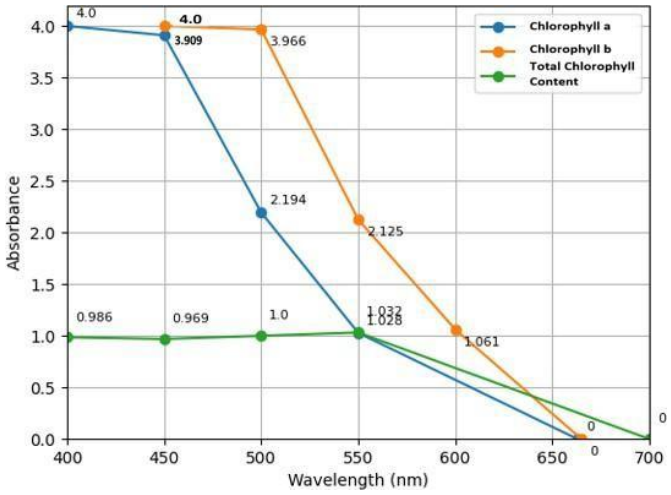
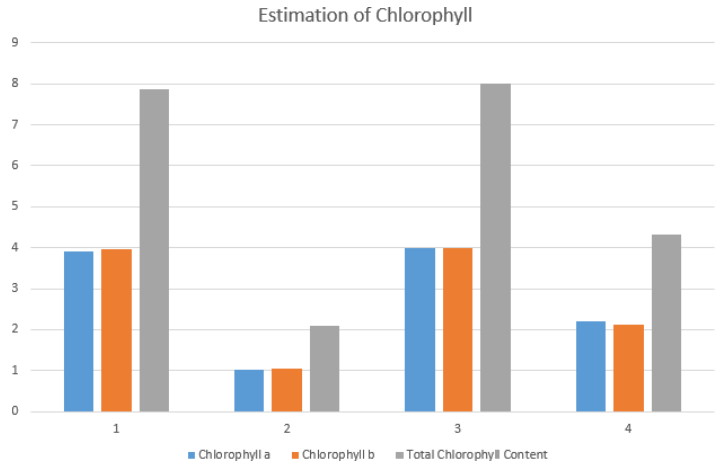


Figure 12. Estimation of chlorophyll



3.2. Phytochemical profile

3.2.1. Alkaloids

Alkaloid-associated responses were detected in all four chlorophyll extracts. The highest assay response occurred at 4 µg/g, with Madhuca longifolia and Coleus amboinicus showing comparatively higher responses.

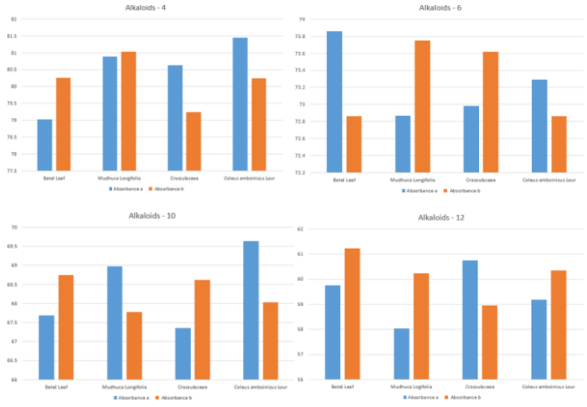
The assay therefore demonstrated a relative alkaloid-associated response across the extracts, with variation among the plant materials.

Table 2. Absorbance values of alkaloids

Leaves	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels
Leaves	4 (µg/g)	4 (µg/g)	6 (µg/g)	6 (µg/g)	10 (µg/g)	10 (µg/g)	12 (µg/g)	12 (µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)

Betel Leaves	80.24%	79.53%	73.86%	72.86%	67.69%	68.75%	59.76%	61.23%
Mudhuca Longifolia	81.74%	80.65%	72.87%	73.75%	68.97%	67.78%	58.03%	60.23%
Crassulaceae	80.59%	79.28%	72.98%	73.62%	67.35%	63.62%	60.75%	58.96%
Coleus amboinicus Lour	81.68%	80.43%	73.29%	72.86%	69.64%	68.03%	59.18%	60.34%

Figure 13. Graphical representation of alkaloids content



3.2.2. Tannins

Tannin-associated responses were detected in all four extracts. The highest assay responses occurred at 4 µg/g, with Madhuca longifolia and betel leaf showing comparatively strong responses.

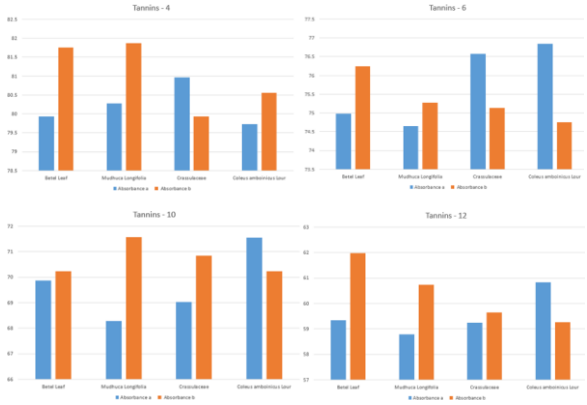
The present data showed clear differences among the four extracts in the magnitude of the tannin-associated response.

Table 3. Absorbance values of tannins

Leaves	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	79.93%	81.75%	74.98%	76.24%	69.87%	70.23%	59.34%	61.98%
Mudhuca Longifolia	80.27%	81.87%	74.65%	75.27%	68.29%	71.57%	58.78%	60.74%
Crassulaceae	80.96%	79.93%	76.57%	75.13%	60.02%	70.84%	59.24%	59.64%

Coleus amboinicus Lour	79.73%	80.56%	76.84%	74.75%	71.56%	70.24%	60.83%	59.26%

Figure 14. Graphical representation of tannins content



3.2.3. Flavonoids

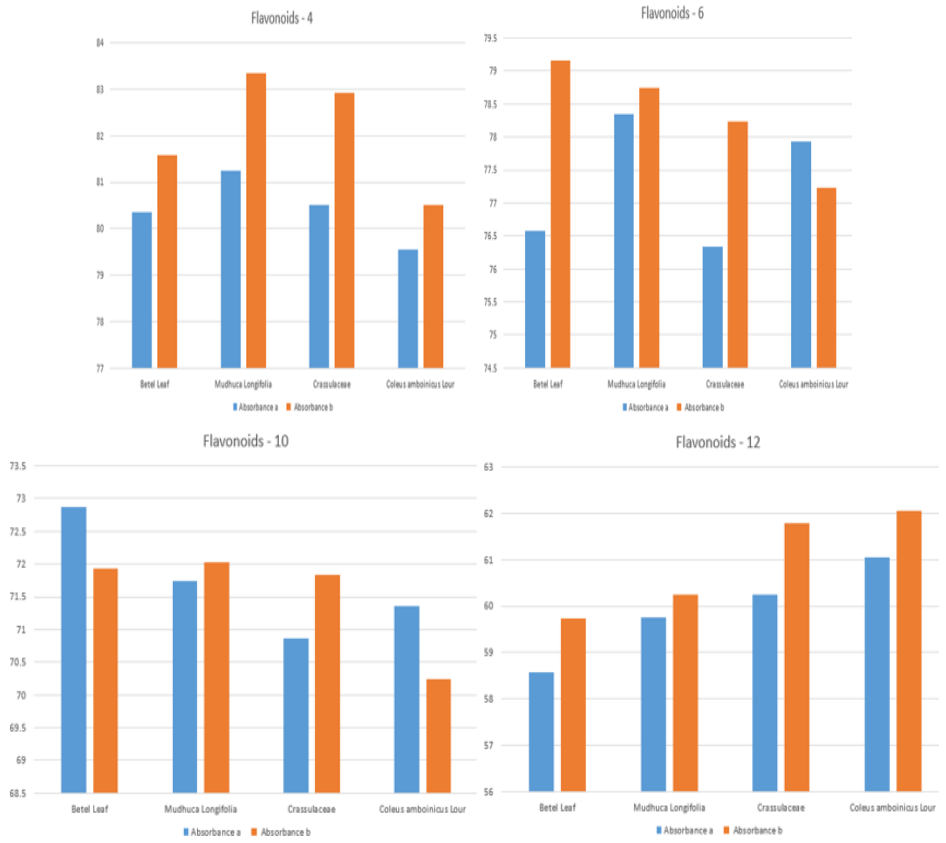
Flavonoid-associated responses were detected in all four extracts. The highest assay responses occurred at 4 µg/g, with Madhuca longifolia and Crassulaceae showing comparatively strong responses.

The flavonoid response was present across all samples and varied among the four plant extracts.

Table 4. Absorbance values of flavonoids

Leaves	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	80.35%	81.57%	76.57%	79.16%	72.87%	71.93%	58.57%	59.74%
Mudhuca Longifolia	81.25%	83.35%	78.34%	78.74%	71.74%	72.03%	59.75%	60.24%
Crassulaceae	80.50%	82.91%	76.34%	78.24%	70.87%	71.84%	60.25%	61.78%
Coleus amboinicus Lour	79.56%	80.52%	77.93%	77.23%	71.35%	70.24%	61.04%	62.06%

Figure 15. Graphical representation of flavonoids content



3.2.4. Saponins

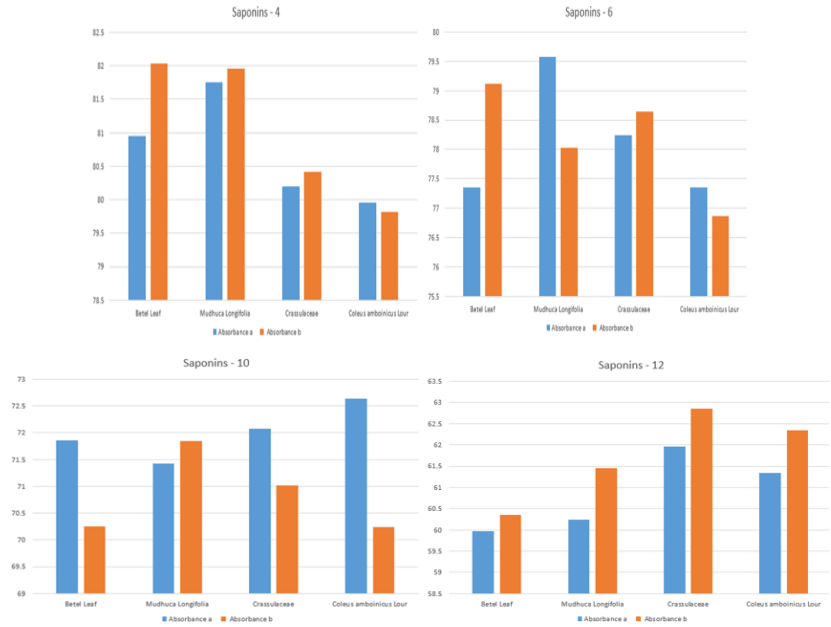
Saponin-associated responses were detected in all four extracts. The strongest responses occurred at 4 µg/g, with relatively small differences among the samples.

The results indicate variation in the relative saponin-associated response among the plant extracts.

Table 5. Absorbance values of saponins

Leaves	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	80.95%	82.04%	77.35%	79.12%	71.86%	70.25%	59.97%	60.35%
Mudhuca Longifolia	81.75%	81.96%	79.57%	78.03%	71.43%	71.85%	60.24%	61.46%
Crassulaceae	80.20%	80.41%	78.24%	78.64%	72.07%	71.02%	61.96%	62.86%
Coleus amboinicus Lour	79.96%	79.82%	77.36%	76.86%	72.64%	70.24%	61.34%	62.35%

Figure 16. Graphical representation of saponins content



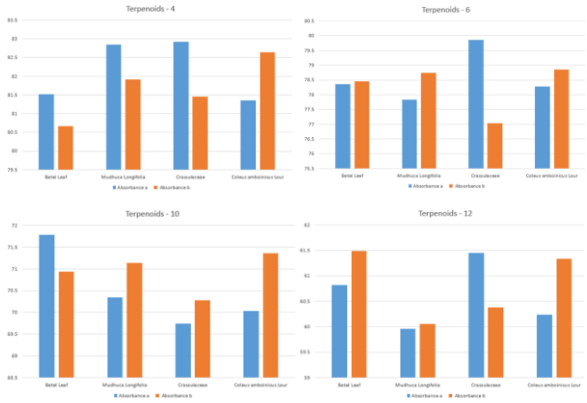
3.2.5. Terpenoids

Terpenoid-associated responses were detected in all four extracts, with the strongest responses at 4 µg/g. Crassulaceae and Madhuca longifolia showed comparatively high responses, while betel leaf and Coleus amboinicus also showed clear responses. The four extracts showed distinct but consistent terpenoid-associated responses across the tested concentration range.

Table 6. Absorbance values of terpenoids

Leaves	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**	**Concentration Levels**
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	81.52%	80.66%	78.35%	78.46%	71.78%	70.93%	60.82%	61.49%
Mudhuca Longifolia	82.85%	81.91%	77.83%	78.74%	70.34%	71.14%	59.96%	60.05%
Crassulaceae	82.92%	81.46%	79.86%	77.03%	69.74%	70.28%	61.45%	60.38%
Coleus amboinicus Lour	81.35%	82.64%	78.27%	78.85%	70.03%	71.36%	60.24%	61.34%

Figure 17. Graphical representation of terpenoids content



3.2.6. Phenols

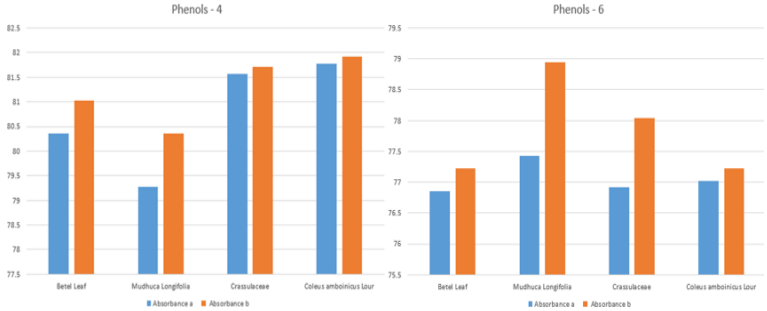
Phenolic-associated responses were observed in all four extracts, with the strongest responses at 4 µg/g. Coleus amboinicus and Crassulaceae showed comparatively strong responses, while betel leaf and Madhuca longifolia also showed substantial responses.

Phenolic-associated responses varied among the extracts, with all four plant materials showing measurable signals.

Table 7. Absorbance values of phenols

Leaves	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	80.35%	81.03%	76.85%	77.23%	69.86%	68.92%	60.24%	58.91%
Mudhuca Longifolia	79.27%	80.35%	77.43%	78.95%	70.26%	69.23%	59.57%	59.98%
Crassulaceae	81.57%	81.71%	76.92%	78.04%	68.93%	69.36%	61.83%	60.82%
Coleus amboinicus Lour	81.78%	81.92%	77.02%	77.23%	69.02%	70.24%	60.74%	61.63%

Figure 18. Graphical representation of phenols content



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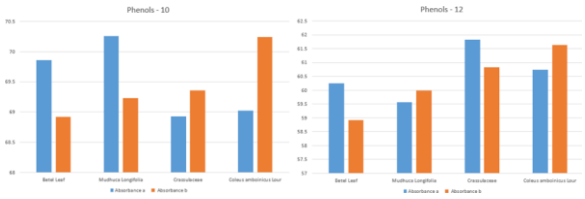


3.2.7. Glycosides

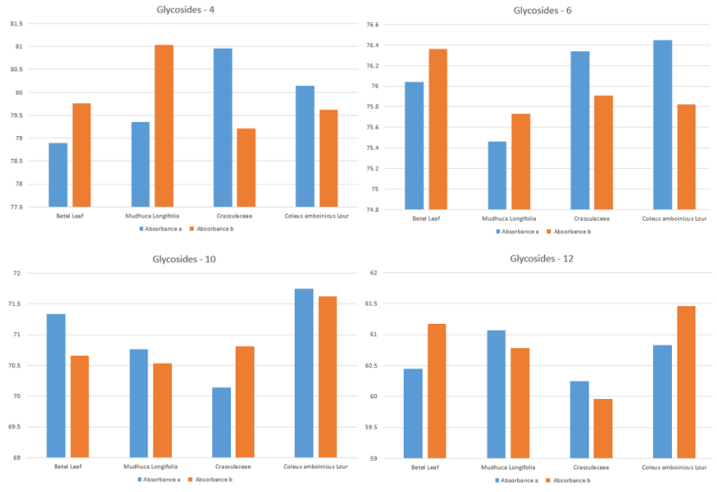
Glycoside-associated responses were detected in all four chlorophyll extracts. The strongest responses occurred at 4 µg/g, with Madhuca longifolia and Crassulaceae showing comparatively high responses.

The extracts showed measurable glycoside-associated responses across the tested concentration range.

Table 8. Absorbance values of glycosides

Leaves	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	78.89%	79.76%	76.04%	76.36%	71.34%	70.66%	60.45%	61.17%
Mudhuca Longifolia	79.35%	81.03%	75.46%	75.73%	70.76%	70.53%	61.07%	60.78%
Crassulaceae	80.96%	79.21%	76.34%	75.91%	70.14%	70.81%	60.25%	59.96%
Coleus amboinicus Lour	80.14%	79.62%	76.45%	75.82%	71.75%	71.62%	60.83%	61.46%

Figure 19. Graphical representation of glycosides content



3.2.8. Steroids

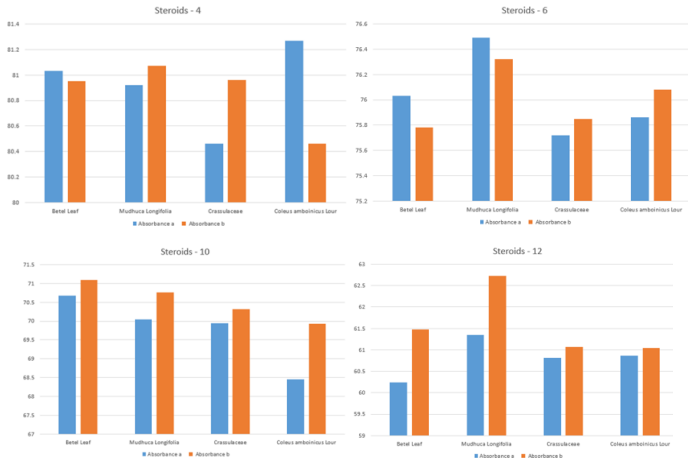
Steroidal responses were detected in all four extracts, with the strongest responses at 4 µg/g. Coleus amboinicus and betel leaf showed comparatively strong responses, while Madhuca longifolia and Crassulaceae also showed measurable responses.

The steroid-associated response was detected across all four plant materials, with modest differences among samples.

Table 9. Absorbance values of steroids

Leaves	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	81.03%	80.95%	76.03%	75.78%	70.67%	71.09%	60.24%	61.48%
Mudhuca Longifolia	80.92%	81.07%	76.49%	76.32%	70.05%	70.76%	61.35%	62.72%
Crassulaceae	80.46%	80.96%	75.72%	75.85%	69.94%	70.31%	60.81%	61.07%
Coleus amboinicus Lour	81.27%	80.46%	75.86%	76.08%	68.46%	69.93%	60.87%	61.04%

Figure 20. Graphical representation of steroids content



3.2.9. Anthraquinones

Anthraquinone-associated responses were detected in all four extracts, with the strongest responses at 4 µg/g. Madhuca longifolia and Coleus amboinicus showed comparatively strong responses.

Anthraquinone-associated responses varied among the samples but were measurable in all four extracts.

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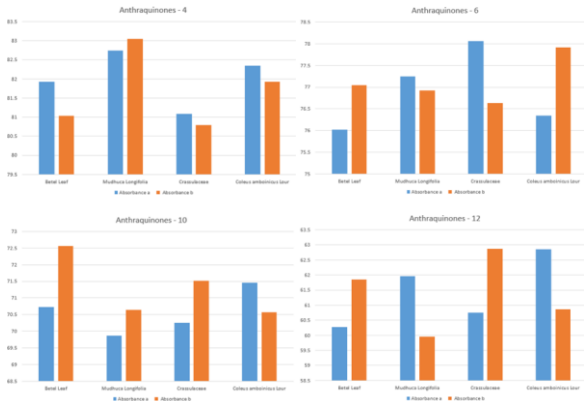
Table 10. Absorbance values of anthraquinones

Leaves	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels	Concentration Levels
Leaves	4(µg/g)	4(µg/g)	6(µg/g)	6(µg/g)	10(µg/g)	10(µg/g)	12(µg/g)	12(µg/g)
Chlorophyll (a)/(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Betel Leaves	81.93%	81.04%	76.02%	77.04%	70.73%	72.56%	60.28%	61.85%
Mudhuca Longifolia	82.74%	83.05%	77.24%	76.92%	69.87%	70.64%	61.96%	59.96%
Crassulaceae	81.08%	80.79%	78.06%	76.63%	70.25%	71.52%	60.75%	62.87%
Coleus amboinicus Lour	82.35%	81.92%	76.34%	77.92%	71.46%	70.57%	62.86%	60.86%

Figure 21. Graphical representation of anthraquinones content



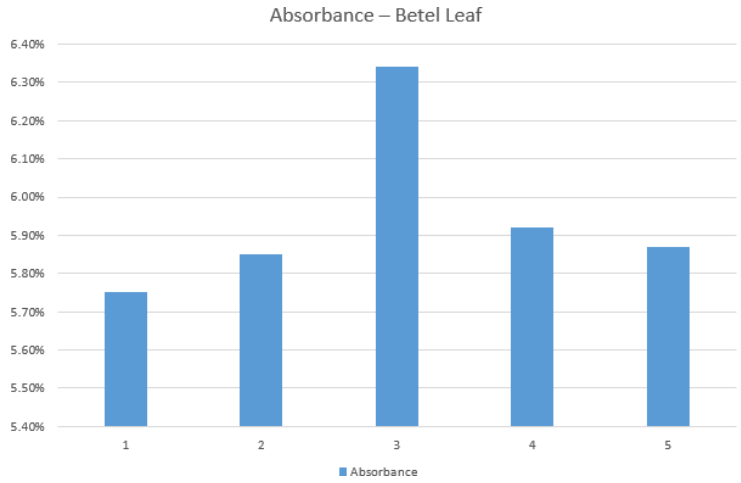
3.3. Antioxidant analysis

All four extracts produced measurable responses in the DPPH assay. The highest reported response was 6.04% for Crassulaceae at 300 nm, followed by 6.03% for Madhuca longifolia, 5.97% for betel leaf, and 5.93% for Coleus amboinicus Lour.

Table 11. Absorbance values of Betel leaf antioxidant assay

Absorbance	Wavelength
5.97%	100
5.74%	200
5.69%	300
5.86%	400
5.82%	500

Figure 22. DPPH antioxidant activity of Betel leaf



The antioxidant assay showed measurable activity in all four extracts, with only modest differences among the reported responses.

Table 12. Absorbance values of Madhuca longifolia antioxidant assay

Absorbance	Wavelength
5.94%	100
5.75%	200
6.03%	300
5.82%	400
5.76%	500

Figure 23. DPPH antioxidant activity of Madhuca longifolia

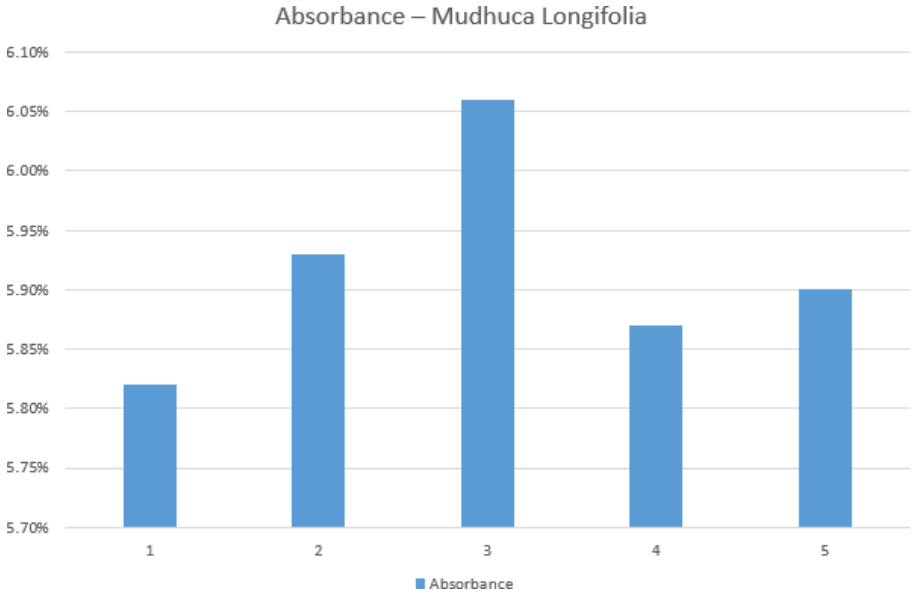


Table 13. Absorbance values of Coleus amboinicus Lour. antioxidant assay

Absorbance	Wavelength
5.83%	100
5.76%	200
5.96%	300
5.69%	400
5.72%	500

Figure 24. DPPH antioxidant activity of Coleus amboinicus Lour.

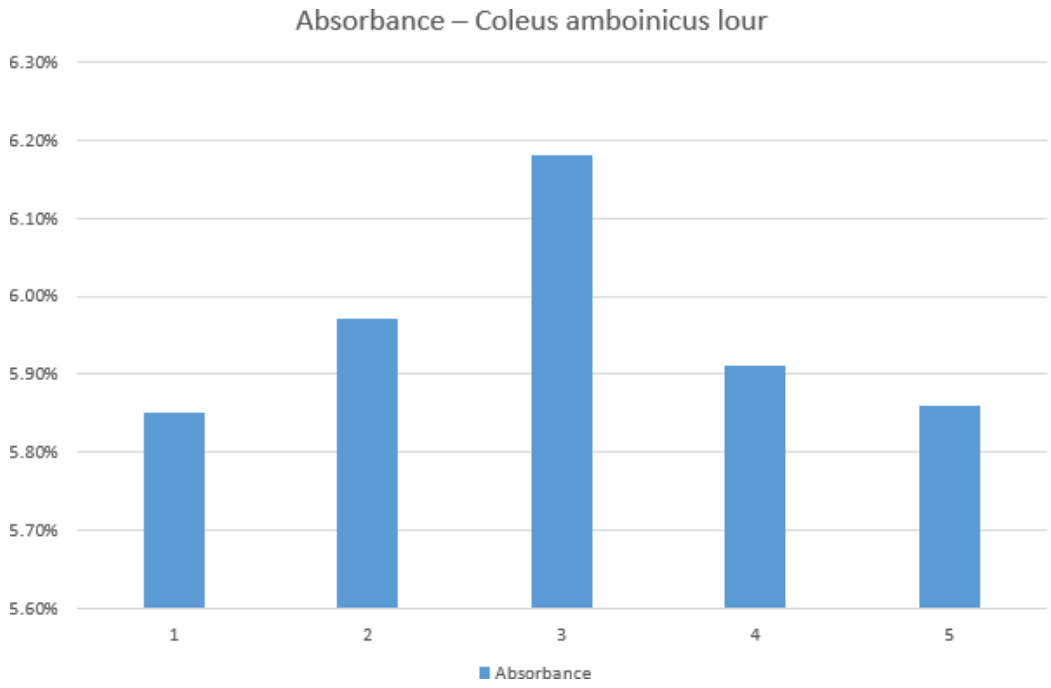
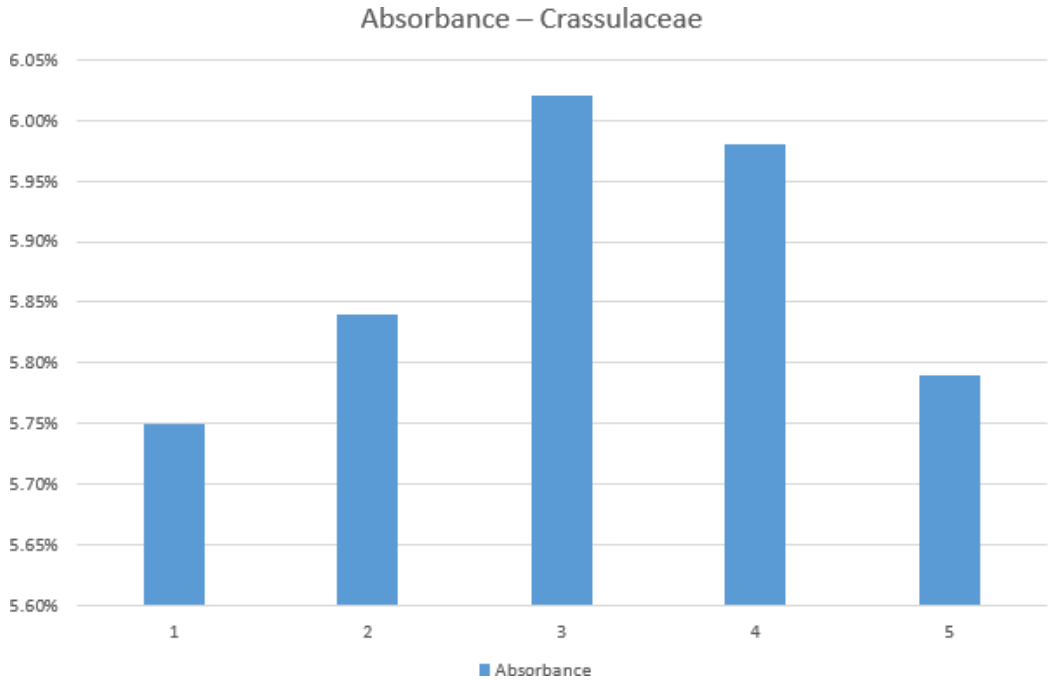


Table 14. Absorbance values of Crassulaceae antioxidant assay

Absorbance	Wavelength
5.93%	100
5.62%	200
6.04%	300
5.73%	400
5.88%	500

Figure 25. DPPH antioxidant activity of Crassulaceae



3.4. Microbial analysis

Microbial load increased progressively during storage in all four extracts. The direction of change was similar among samples, indicating increasing microbial proliferation during the observation period under the tested storage conditions.

Table 15. Microbial load during initial storage assessment

Leaves	**Day**	**Day**	**Day**
Betel Leaves	0.5x108	1.3x108	2.3x108
Mudhuca Longifolia	0.6x108	1.5x108	2.4x108
Crassulaceae	0.6x108	1.6x108	2.4x108
Coleus amboinicus Lour	0.6x108	1.5x108	2.3x108

Table 16. Microbial load after two months of storage

Leaves	**Day**	**Day**	**Day**
Betel Leaves	1.4x108	1.8x108	2.7x108
Mudhuca Longifolia	1.6x108	1.7x108	2.7x108
Crassulaceae	1.5x108	1.7x108	2.6x108
Coleus amboinicus Lour	1.4x108	1.8x108	2.8x108

4. DISCUSSION

4.1. Chlorophyll extraction and estimation

The recovery of measurable chlorophyll from all four plant materials supports the feasibility of the applied acetone-based extraction procedure. This observation agrees with studies showing that solvent composition and extraction conditions strongly influence pigment recovery and selectivity (Ferreira et al., 2021; Leite et al., 2018). Recent work has also emphasized the importance of optimizing extraction conditions for plant matrices, particularly when extraction is intended to retain pigment quality and improve process efficiency (Ngcobo et al., 2024; Tamminen et al., 2022).

The stronger responses observed for Madhuca longifolia and Coleus amboinicus indicate plant-to-plant variation in pigment recovery under identical analytical conditions. Such differences may reflect variation in pigment abundance, leaf structure, moisture content, and solvent accessibility. Because the present assay responses are concentration- and absorbance-dependent, they should be interpreted as comparative responses within the experimental system rather than as direct measurements of absolute pigment yield unless the calculation inputs are fully reported.

4.2. Phytochemical profile

The detection of alkaloid-associated constituents in all four extracts is consistent with earlier phytochemical investigations of Madhuca longifolia, Coleus amboinicus, and Crassulaceae. Alkaloids and related secondary metabolites have been documented in these plant groups, supporting the present screening pattern (Devanand et al., 2024; Bhattacharjee and Majumder, 2013; Stevens et al., 1995). The present assay should, however, be interpreted as qualitative or semi-quantitative screening and not as identification of individual alkaloids.

The tannin-associated responses, particularly in Madhuca longifolia and betel leaf, are compatible with published phytochemical reports for these plants and related species (Bom et al., 2023; Shubha and Bhatt, 2015; Stevens et al., 1995). Differences between the present responses and values reported elsewhere may arise from differences in plant part, extraction solvent, assay chemistry, and reporting units; therefore, direct numerical comparisons should be avoided.

The broad flavonoid response across the extracts agrees with previous reports for Piper betle, Madhuca longifolia, Crassulaceae, and Coleus amboinicus (Shubha and Bhatt, 2015; Deviani and Putri, 2024; Khare and Khare, 2023; Oyedeji et al., 2019). The simultaneous occurrence of flavonoid-associated responses and measurable antioxidant activity is biologically plausible because flavonoids can contribute to radical-scavenging capacity. Nevertheless, the present study does not establish a causal relationship between a particular flavonoid and the antioxidant response.

The detection of saponin-associated constituents in all four extracts is supported by published phytochemical studies on Piper betle, Madhuca longifolia, and Coleus amboinicus, as well as related plant materials (Poudel et al., 2025; Yasmin et al., 2025; Ramadan, 2015; Adeyemi et al., 2024). The relatively similar responses among samples suggest a broad distribution of saponin-associated compounds, but further chromatographic or spectrometric analysis would be required to identify individual saponins.

Terpenoid-associated responses were observed across all four plant materials, which is consistent with reports describing terpenoid constituents in betel leaf, Madhuca longifolia, Crassulaceae, and Coleus amboinicus (Singh et al., 2025; Sharma et al., 2024; Li and Zhang, 2023; Adebayo et al., 2024). The variation among samples may reflect species-specific differences in terpenoid composition and extraction behavior. The present screening therefore provides evidence of occurrence rather than structural identification.

The phenolic responses observed in all four extracts are in agreement with published reports of phenolic constituents in the selected plant groups (Prakash et al., 2024; Verma et al., 2023; Alam et al., 2022; Ncube et al., 2023). Phenolic compounds may contribute to antioxidant behavior, and their concurrent detection with flavonoid-associated responses suggests that multiple secondary metabolites may contribute to the measured biological activity. However, compound-specific contributions cannot be established from the present screening alone.

The glycoside-associated responses add to the chemical diversity observed in the extracts and are consistent with earlier reports for Piper betle, Madhuca longifolia, Crassulaceae, and Coleus amboinicus (Mishra et al., 2024; Kulkarni et al., 2023;

Kumar et al., 2021; Oladeji et al., 2022). Because the screening approach does not distinguish among different classes of glycosides, the findings should be considered an indication of glycosidic constituents requiring further characterization.

Steroidal responses across the four extracts are consistent with reports of phytosterols and steroidal constituents in betel leaf, Madhuca longifolia, Crassulaceae, and Coleus amboinicus (Kumar et al., 2024; Nair et al., 2023; Muthukumar et al., 2021; Okeke et al., 2022). The modest inter-sample differences indicate that steroid-associated compounds were retained during extraction, but their individual identities and concentrations remain to be established.

Anthraquinone-associated responses were also detected in all four extracts, in agreement with published phytochemical reports for the selected plant groups (Verma et al., 2024; Patil et al., 2023; Patel et al., 2021; Adekunle et al., 2022). Considered together with the other phytochemical classes, this pattern indicates a chemically diverse extract. The screening result should not, however, be taken as evidence for the presence of specific anthraquinone structures or their pharmacological effects without confirmatory analysis.

4.3. Antioxidant activity

All four extracts showed measurable responses in the DPPH assay, supporting the presence of radical-scavenging capacity under the reported test conditions. Antioxidant activity has previously been described for Piper betle, Madhuca longifolia, Coleus amboinicus, and Crassulaceae species (Chattopadhyay et al., 2006; Singh et al., 2021; Matkowski et al., 2021; Heredia-Mercado et al., 2022). The present findings are therefore consistent with the broader phytochemical profile of the extracts, particularly the detection of phenolic and flavonoid-associated constituents.

The numerical antioxidant responses should be interpreted cautiously because the present dataset reports absorbance or percentage responses at different wavelengths for the different samples. Direct ranking of antioxidant strength between species may therefore be affected by assay conditions. A future comparison using a common extract concentration, identical wavelength, validated calibration, and a defined antioxidant endpoint would provide a stronger basis for inter-species comparison.

4.4. Microbial stability during storage

The progressive increase in microbial load during storage indicates that the extracted materials were not microbiologically stable under the tested conditions. This result is important for product development because the presence of chlorophyll and secondary metabolites does not by itself ensure preservation during storage. The findings highlight the need for hygienic processing, suitable preservation strategies, moisture control, packaging, and storage-condition optimization before practical application.

4.5. Overall implications

Taken together, the findings support the working hypothesis that the selected medicinal leaves can provide chlorophyll-containing extracts with a broad secondary-metabolite profile and measurable antioxidant responses. The results suggest potential value as plant-derived pigment and bioactive sources, but the present study should be considered a comparative laboratory investigation rather than evidence of therapeutic efficacy. Future work should focus on pigment stability, quantitative identification of individual phytochemicals, standardized antioxidant measurements, and microbial-control strategies. These steps are necessary to translate the observed laboratory responses into reproducible and safe product applications.

CONCLUSION

The study demonstrates that Betel leaf, Madhuca longifolia, Crassulaceae, and Coleus amboinicus can yield measurable chlorophyll extracts under the applied acetone-based procedure and that the extracts contain a broad range of phytochemical responses. The strongest responses for several assays occurred at the lowest tested concentration, while antioxidant activity was measurable across all four samples. These findings support the use of the selected leaves as laboratory sources of chlorophyll-associated bioactive constituents, but they also show that the extracts require further chemical characterization and microbial-stability optimization before application in food, nutraceutical, or other product systems.

Future studies should prioritize pigment stability during storage, confirmation and quantification of individual bioactive compounds, standardized antioxidant assays, and preservation or packaging strategies that limit the microbial increase

observed during storage. The present work therefore provides a foundation for process optimization and for evaluating the potential of these plant-derived chlorophyll extracts in value-added applications.

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AUTHOR CONTRIBUTIONS

V. Nikitha: Investigation, experimental work, data collection, laboratory analysis, and preparation of the initial manuscript.

Dr. M. Niharika: Conceptualization, methodology, Guidance, supervision, validation, interpretation of results, and manuscript preparation and editing.

Dr. D. Madhavi: Review of the manuscript

Dr. G. Sireesha: Scientific guidance, validation, interpretation of findings, and critical revision of the manuscript.

All authors have read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS STATEMENT

This study did not involve human participants, human biological samples, or experimental animals. The research was conducted using plant materials collected locally. Therefore, ethical approval was not required for this study.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request. All relevant experimental observations and results obtained during the study are included in the manuscript..

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