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Comprehensive Phytochemical Profiling and FTIR–GCMS Characterization of Bioactive Metabolites from *Premna tomentosa* Leaves

Bolla Rama Krishna[1,2] and Pandu. Brahmaji Rao*[2]

[1]*Department of Chemistry, Spurthi Institute of Technology, Sathupalli, Telangana, India*
[2]*Department of Environmental Science, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India*

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Corresponding Author: **Pandu. Brahmaji Rao** | E-Mail: **(drbrahmajirao@gmail.com)**

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ABSTRACT

Premna tomentosa is one of the medicinal plants that have high therapeutic versatility and the possibility of being a sustainable source of bioactive phytochemicals for use in pharmaceutical industry and environmental management. This paper evaluates the phytochemical composition of P. tomentosa leaf extracts using solvents of varying polarity (n-hexane, acetone, ethanol, and methanol) based on qualitative and quantitative phytochemical analysis, Fourier Transform Infrared (FTIR) spectroscopy and Gas Chromatography–Mass Spectrometry (GC–MS). Qualitative screening revealed that the methanolic extract had the richest phytochemical composition, having very strong (+++) presence of alkaloids, flavonoids, phenolics, tannins, saponins, glycosides and coumarins. Quantitative analysis indicated that the methanol extract had significantly high levels ($p<0.001$) of total phenolics (151.89±3.02 mg GAE/g), carbohydrates (134.58±2.89 mg GE/g), alkaloids (132.64±2.67 mg AE/g), flavonoids (128.67±2.81 mg QE/g), tannins (82.73±2.46 mg GAE/g), saponins (67.49±2.13 mg DE/g), proteins (61.82±1.86 mg BSAE/g), steroids (58.36±1.89 mg CE/g), glycosides (53.28±1.67 mg DTE/g), and reducing sugars (48.92±1.42 mg GE/g) compared to extracts prepared using other solvents. FTIR spectroscopic analysis confirmed the presence of hydroxyl, carbonyl, aromatic, ether and amine functional groups. GC-MS analysis revealed 25 major bioactive compounds, which included phytol, squalene, campesterol, stigmasterol, β-sitosterol, lupeol and α-tocopherol, compounds known to possess phytoremedationantimicrobial and anti-inflammatory properties. The presence of high amounts of phenolics, flavonoids, terpenoids and phytosterols also implies that P. tomentosa has significant phytoremediation potential since these phytochemicals are responsible for increasing the resistance of plants to oxidative stress due to exposure to heavy metals and organic contaminants. Therefore, methanol was found to be the best solvent for the extraction process, and phytochemical profile shows that Premna tomentosa is a valuable natural resource.

Keywords: *Premna tomentosa; Phytochemical screening; Total phenolic content; FTIR; GC–MS; Bioactive compounds; Methanolic extract; Medicinal plant; Phytoremediation; Environmental remediation.*

Introduction

The vast structural diversity of natural products isolated from medicinal plants continues to be one of the most important sources of novel biologically active lead compounds used in drug discovery, nutraceutical industry, and validation of traditional herbal remedies. From among many studied plant families, those of the order Lamiaceae (previously considered to belong to Verbenaceae) are particularly remarkable due to an extraordinary chemical diversity of their terpenoids, flavonoids, phenylethanoid glycosides, and iridoids [30]. The genera of this order that are especially well-studied in the context of potential biopharmaceuticals include *Premna* L., which comprises more than 200 species widespread in tropical and subtropical areas of Asia, Africa, Australia, and the Pacific islands and frequently used in South and Southeast Asian folk medicine as remedies for inflammation, gastro-intestinal disorders, fever, wounds, and liver diseases.

In particular, *Premna serratifolia*, *Premna integrifolia*, *Premna latifolia*, and *Premna barbata* have increasingly attracted attention of the contemporary researchers in recent years due to their phytochemically rich composition involving phenolic acids, flavonoids, phenylethanoid glycosides, sterols, and volatile terpenoids. For instance, the analysis of ethanolic and aqueous extracts of *Premna serratifolia* using LC-QTOF-MS/MS allowed the detection of phenylethanoid glycosides such as forsythoside A, forsythoside B, and isoacteoside, which corresponded with their pronounced antioxidant properties and enzyme inhibition relevant for treatment of metabolic diseases [22]. Additionally, in vitro cytotoxic testing of the same *Premna serratifolia* leaves against hepatic carcinoma cell lines confirmed the pharmaceutically significant chemical profile of this species [23]. Similarly, the GC-MS analysis of the methanol extract of *Premna latifolia* Roxb. leaves revealed an abundant diversity of fatty acid esters, sterols, and terpenoid derivatives, thus proving the consistent chemotaxonomy of phytoconstitutes within the genus [13]. Pharmacologically meaningful activity of *Premna integrifolia*, which showed increased acceleration of dermal wound healing due to the presence of phenolic- and flavonoid-rich fraction, has been proven by in vivo experiments [2]. The combination of HPLC/TOF-MS and GC-MS analysis of the same *Premna integrifolia* revealed the antiproliferative activity of this species associated with polyphenolic and fatty acid fractions [18].

Despite all of the above evidence about the genus *Premna*, one of the species of this order – *Premna tomentosa* L., which has traditionally been used in folk preparations in peninsular India to treat inflammation and skin diseases – has been insufficiently studied in terms of its systematic, spectroscopic and integrated phytochemical analysis of leaves. Most of the works related to tomentose and closely related species of Lamiaceae or Verbenaceae order have utilized the single-technique approach, which is contrary to the modern trend of the integrated phytochemical research involving the simultaneous utilization of Fourier-transform infrared (FTIR) spectroscopy and gas chromatography–mass spectrometry (GC-MS). The latter allows to confirm functional groups corresponding to certain classes of the secondary metabolites via FTIR and to identify and quantify individual volatile and semivolatile phytoconstituents via GC-MS, thus creating a more complete and mutually correlative chemical profile than that possible with the use of the above-mentioned approaches separately [15].

The above-described approach has been repeatedly applied to various medicinal plant leaves in recent years. In the case of *Cissus assamica*, the combined analysis of GC-MS/MS and FTIR allowed to identify fifteen bioactive phytoconstituents along with the identification of hydroxyl, carbonyl, and aromatic moieties via FTIR and their correlation with antidiarrhoeal, analgesic, and hypoglycemic activities due to in silico docking [26]. Similarly, the FTIR and GC-MS-based analysis of the leaves of *Digera muricata* proved the presence of phenolic, flavonoid, and fatty-acid-derived functional groups underlying the antioxidant activity of this species [20]. Comparable study of Boehmeria caudata leaves revealed the FTIR absorption bands, which corresponded to GC-MS-identified compounds with neuropharmacological activities [1]. Even earlier, FTIR was used to identify aromatic amine, carboxylic acid, ketone, and phenolic functional groups that corresponded with twenty-five GC-MS-identified compounds of *Amomum nilgiricum* leaf and rhizome extracts and were responsible for its antibacterial and antioxidant activity [12]. Moreover, the similar approach can be extended to the stem barks, as proved by the ATR-FTIR and GC-MS analysis of *Magnolia champaca* stem bark that revealed the correlation between the specific spectral bands and thrombolytic and cytotoxic phytoconstitutes through molecular docking [11]. Enzyme-inhibiting constituents, which are relevant for diabetes and hyperpigmentation treatment, have been identified by means of GC-MS and LC-ESI-MS profiling of the leaves of *Strobilanthes glutinosus* [4]. GC-MS-based metabolic profiling of Verbena officinalis, which belongs to the same order as Premna, has revealed the fatty acid- and terpenoid-rich fractions with enzyme-inhibitory and antioxidant activity, proven by in silico docking [16]. Essential oils, rich in volatile phytoconstitutes, of tomentose Lamiaceae-adjacent taxa, such as leaf oil of Citrus grandis 'Tomentosa', can be characterized by means of network pharmacology for identifying the specific molecular targets and pathways [29].

Therefore, there are several examples, which prove both pharmacological potential of the genus *Premna* and the applicability of the integrated FTIR-GC-MS profiling for the resolution of phytochemical composition of its leaves. However, the systematic and comprehensive phytochemical profile of *Premna tomentosa* leaves, combining the qualitative phytochemical screening, FTIR functional group analysis, and the identification and relative quantification of volatile and semivolatile phytoconstitutes via GC-MS, has not been reported in contemporary scientific literature yet. Therefore, filling this gap is important not only for the verification of the traditional medical use of this species but also for its placement into the broader chemotaxonomic and pharmacological picture of *Premna*. Hence, the present study was conducted with the aim to perform a comprehensive phytochemical profile of *Premna tomentosa* leaves via preliminary qualitative screening of the major secondary metabolite classes, the FTIR-based functional group analysis, and the identification and relative quantification of volatile and semivolatile phytoconstituents using GC-MS.

Methodology

Collection and preparation of Plant Material

The leaves of *Premna tomentosa* were obtained from Aleru Forest, Nellikudur Mandal, Mahabubabad District, Telangana, India. The obtained leaves were washed with distilled water, shade dried at ambient temperature, and pulverized using a mechanical grinder. About 100 g of the obtained powder was subjected to Soxhlet extraction with the use of n-hexane, acetone, ethanol, and methanol in that particular order. Extraction was carried out at the boiling point of each solvent till exhaustion of the extraction process was obtained. The extracted solution was allowed to cool to ambient temperature and filtered using Whatman No. 1 filter paper. The solutions were then concentrated by rotary vacuum evaporation at reduced pressure. The concentrated extracts were kept in sterile amber bottles and preserved at 4°C for further analysis.

Qualitative Phytochemical Screening of *Premna tomentosa* Leaf Extract

The qualitative phytochemical analysis of *Premna tomentosa* leaves in various solvents such as n-hexane, acetone, ethanol, and methanol involved the use of standard methods for phytochemical testing. The alkaloids present were tested for by using Dragendorff's reagent where an orange yellow precipitate confirmed the presence of these chemicals. The flavonoid content of the leaves was verified through the use of 10% sodium hydroxide solution and dilute hydrochloric acid. In this test, if there is a loss of yellow color, then this means that flavonoids are present. The tannin presence in the leaves was confirmed by using 5% ferric chloride solution resulting in a bluish or greenish black coloration. The steroids were tested for using Liebermann-Burchard test where chloroform, acetic anhydride and sulfuric acid solutions resulted in green coloration. The presence of saponins in the leaves was determined through the froth test where the agitated mixture of the extract with distilled water formed stable foam. Glycosides were tested for using sodium picrate reagent where yellow to orange color developed. The phenolic compound presence in the leaves was determined using the ferric chloride solution where blue, green and violet colors confirmed the presence of phenol.

Quantitative Phytochemical Analysis of *Premna tomentosa* Leaf Extract

Total Alkaloid Content (TAC)

Total amount of alkaloids present in the methanolic leaf extract was determined using bromocresol green (BCG) spectrophotometry method. This involved dissolving 1 mg of the extract in DMSO and adding 1 mL of 2N HCl solution. After filtration, the resulting mixture was added to a separatory funnel along with 5 mL bromocresol green and 5 mL phosphate buffer (pH 4.7).

Chloroform was used to extract the complex, and the chloroform fractions obtained were combined to make up to 10 mL. Atropine (20 – 100 µg mL^{-1}) was used as a calibration standard. The absorbance value was read at 470 nm using a UV-visible spectrophotometer and reported in AE/g.

Total Flavonoid Content (TFC)

The amount of total flavonoids was calculated using the aluminum chloride colorimetric test method. One mL of extract solution was mixed with distilled water, and then 5% sodium nitrate, 10% aluminum chloride, and 1 M sodium hydroxide were added successively following the procedure. Quercetin in the concentration range of 20 – 100 µg mL^{-1} was used as the standard substance. Measurement of the absorbance was done at 510 nm, and flavonoid content was recorded as mg QE/g extract.

Total Phenolic Content (TPC)

Total phenolic compounds were measured using the Folin-Ciocalteau method. One milliliter of the sample extract was mixed with the Folin-Ciocalteau reagent and 7% sodium carbonate solution was added to the mixture. The reaction mixture was incubated for 90 minutes at room temperature and absorbance was read against reagent blank at 765 nm. Calibration curve was prepared using gallic acid concentration ranging from 20 to 100 µg mL^{-1} and results were reported in mg of GAE/g of dry extract.

Total Tannin Content (TTC)

The total amount of tannins was measured using the Folin-Ciocalteau assay, with gallic acid used as the standard. The sample extract was mixed with Folin-Ciocalteau reagent and sodium carbonate solution, after which the absorbance was taken at 725 nm. The amount of tannins was measured in milligrams per gram of dry extract in terms of gallic acid equivalent (GAE).

Total Saponin Content (TSC)

The total saponin content was determined by the vanillin-sulfuric acid method. To 0.25 mL of methanolic extract, 0.25 mL of 8% vanillin solution was added along with 2.5 mL of 72% sulfuric acid. The mixture was heated for 10 min at 60°C and then cooled on ice. The absorbance value was measured at 544 nm. The diosgenin concentration ranging from 20 to 100 µg mL^{-1} was used as the standard. The result was presented as mg DE/g of dry extract.

Total Steroid Content

The total amount of steroids present was determined using the colorimetry technique called the Liebermann-Burchard test. Herein, the extract was mixed with acetic anhydride and sulfuric acid, and the color observed was compared with a standard (cholesterol) and the absorbance read at 640 nm. The results were reported in milligrams of cholesterol equivalents per gram of dried extract.

Total Glycoside Content

Total glycoside content was determined by using the Baljet colorimetry method. Extract was reacted with Baljet's reagent to obtain orange-colored solution and measured with wavelength of 495 nm. Digitoxin was used as a standard and results were obtained in mg of digitoxin equivalent/g of dry extract.

Total Carbohydrate Content

Total carbohydrate was measured by the phenol-sulfuric acid method. The sample solution was mixed with 5% phenol and concentrated sulfuric acid, and then incubated for 30 minutes. The absorbance was taken at 490 nm. Glucose was used as the reference substance and the amount of carbohydrates was measured in mg of glucose equivalent (GE)/g of dry extract.

Total Protein Content

Protein concentration was determined using the Bradford method. The protein extract was mixed with Bradford dye and allowed to stand for 10 minutes at room temperature. The absorbance value was taken at a wavelength of 595 nm, and bovine serum albumin (BSA) was used as the calibration protein. The data was reported in mg BSA/g dry extract.

Total Reducing Sugar Content

The amount of reducing sugars in the samples was determined using the Dinitrosalicylic Acid (DNS) method. The extract was mixed with DNS solution and heated to boiling for 10 minutes. After cooling, absorbance was taken at 540 nm. The standard used was glucose, while the results were presented as mg GE/g dry extract.

FTIR Analysis of Methanolic *Premna tomentosa* Leaf Extract

The Fourier Transform Infrared (FTIR) spectroscopic technique was used for the determination of the important functional groups existing in the methanolic leaf extract of *Premna tomentosa*. The crude methanolic extract was subjected to concentration through reduced pressure using a rotary evaporator at a temperature between 40 and 45 degrees Celsius, and the extract was dried to get rid of the last traces of methanol. A few grams of the dried extract was placed directly onto the crystal surface of the Attenuated Total Reflectance–Fourier Transform Infrared (ATR-FTIR) instrument. An FTIR spectrum was taken in the range of wavenumber 4000-400 cm^{-1} with 4 cm^{-1} resolution. The background spectrum was taken first before the measurement of the sample spectrum. The sample spectrum was subtracted from the background spectrum. A scan of each sample was done between 16 to 32 times to produce a good spectrum. The absorptions were given in terms of percentage transmittance in respect to wavenumbers. Major peaks were picked up and identified by referring to the absorption regions. The functional groups such as hydroxyl, carbonyl, carboxyl, aromatic, alkene, ether, ester, amine, and aliphatic groups were identified using FTIR spectral references. They were then correlated with the possible occurrence of phenolics, flavonoids, tannins, alkaloids, terpenoids, glycosides, fatty acids, and other metabolites in the methanolic extract.

GC–MS Analysis of Methanolic *Premna tomentosa* Leaf Extract

Identification of volatile and semi-volatile metabolites of the methanolic leaf extract of *Premna tomentosa* was performed using Gas Chromatography-Mass Spectrometry (GC–MS) analysis. Extract was dried, reconstituted in HPLC-grade methanol in an appropriate concentration, and filtered through a 0.22 µm filter to remove particulates. The extract was injected into a GC vial for analysis. GC–MS analysis was carried out using a gas chromatograph connected to a mass selective detector. A capillary column of approximately 30 m × 0.25 mm i.d. with a film thickness of 0.25 µm (e.g., DB-5MS or an equivalent non-polar column) was used in this analysis.

Helium gas was used as a carrier gas at a constant flow rate of about 1.0 mL min^{-1}. Approximately 1 µL of the extract was injected into the column in split or splitless injection mode, depending on the concentration of the extract. The temperature of the injector was controlled at approximately 250°C. In oven temperature programming, the initial temperature was set at 60°C and kept at this temperature for 2–3 minutes. Then, the temperature was increased from 5–10°C min^{-1} up to 280°C; at the end, this temperature was held for 5-10 minutes to assure that all the compounds were completely eluted. In the mass spectrometer, electron ionization was performed at 70 eV. The ion source temperature and transfer line temperature were set at approximately 230°C and 280°C, respectively. The spectra were recorded within a proper range of masses, such as 40-600 m/z.

Metabolites were separated based on retention time, and the identity was determined based on their mass fragmentation pattern. Identification of the compounds was done by comparing the recorded spectrum of compounds with reference spectra available in the NIST library or any other standard library. Identification of metabolites was based on the matching score between the recorded spectrum and reference spectrum, along with retention time, molecular formula, molecular weight, and relative peak areas. The percent of each metabolite in the sample was calculated as the ratio of the relative abundance of each compound to the total peak area.

Statistical Analysis

All the quantitative analyses of phytochemicals were done in triplicate (n=3), with values reported as mean ± SD. Any differences between phytochemicals' levels were evaluated using one-way analysis of variance (ANOVA) with post hoc Tukey's test. Significance level was set at $p<0.05$.

Results

Preparation of *Premna tomentosa* Leaf Extracts Using Different Organic Solvents

Fresh leaves of *Premna tomentosa* were sequentially extracted with solvents having different levels of polarity, which included n-hexane, acetone, ethanol, and methanol to obtain crude extracts containing different kinds of phytochemicals. It was evident from the difference in color and texture in the extracts obtained after Soxhlet extraction that there was a difference in solubility of the phytoconstituents in the selected solvents (Figure 1). The n-hexane extract had deep green color, showing that there was effective extraction of non-polar constituents like chlorophylls, waxes, lipids, carotenoids and other hydrophobic metabolites. The acetone extract had light yellow and clear color indicating that moderately polar compounds like pigments, flavonoids and some phenolic compounds had been extracted. The ethanol extract was golden yellow to amber in color indicating that there were polar phytochemicals like flavonoids, tannins, glycosides and phenolic compounds.

On the other hand, the methanol extract had dark brown color indicating effective extraction of highly polar bioactive metabolites like polyphenols, tannins, alkaloids and other oxygenated secondary metabolites**.**

Figure 1. Soxhlet extracts of Premna tomentosa leaves obtained using n-hexane, acetone, ethanol, and methanol

Qualitative Phytochemical Screening of *Premna tomentosa* Leaf Extracts

The qualitative phytochemical analysis of the n-hexane, acetone, ethanol, and methanol leaf extracts of *Premna tomentosa* exhibited significant variations in the profile of biologically active secondary metabolites in relation to the nature of the solvent used for extraction (please refer to Table 1). In terms of their phytochemical profile, the methanolic extract was found to contain all the classes of secondary metabolites tested, which included alkaloids, flavonoids, phenolic compounds, tannins, saponins, glycosides, and coumarins with strong positive responses (+++).

In addition, the methanolic extract was found to contain quinones, cardiac glycosides, anthraquinones, proteins, and carbohydrates in moderate concentrations (++), implying that methanol is an efficient solvent for the extraction of polar and moderately polar phytochemicals. The ethanolic extract of *Premna tomentosa* leaves had a varied phytochemical profile, which included flavonoids and phenolic compounds (+++) while alkaloids, tannins, saponins, glycosides, quinones, coumarins, cardiac glycosides, and carbohydrates had moderate (++), while anthraquinones, steroids, terpenoids, and proteins gave weak to moderate responses (++). On the other hand, the acetone extract contained alkaloids, flavonoids, phenolic compounds, steroids, and terpenoids in moderate amounts (++), while tannins, saponins, glycosides, quinones, coumarins, cardiac glycosides, proteins, and carbohydrates had low levels (+) of occurrence in the acetone leaf extract. In addition, anthraquinones were absent in the acetone extract of *Premna tomentosa*.

Table 1. Qualitative Phytochemical Screening of Different Solvent Extracts of Premna tomentosa Leaves

Phytochemical Constituent	Test Employed	n-Hexane Extract	Acetone Extract	Ethanol Extract	Methanol Extract
Alkaloids	Dragendorff's test	+	++	++	+++
Flavonoids	Alkaline reagent test	–	++	+++	+++
Phenolic compounds	Ferric chloride test	+	++	+++	+++
Tannins	Ferric chloride test	–	+	++	+++
Saponins	Frothing test	–	+	++	+++
Steroids	Liebermann–Burchard test	+++	++	+	+
Glycosides	Sodium picrate test	–	+	++	+++
Terpenoids	Salkowski test	+++	++	+	+
Quinones	Concentrated H_2SO_4 test	–	+	++	++
Coumarins	NaOH test	–	+	++	+++
Cardiac glycosides	Keller–Killiani test	–	+	++	++
Anthraquinones	Borntrager's test	–	–	+	++
Proteins	Biuret test	–	+	+	+
Carbohydrates	Molisch's test	–	+	++	++

Symbols: *– = Absent; + = Weakly present; ++ = Moderately present; +++ = Strongly present.*

The n-hexane extract, on the other hand, contained mostly non-polar phytochemicals. Positive reactions (+++) were noted for steroid and terpenoid content, but alkaloid and phenolic compound contents showed low positive reactions (+). Flavonoid, tannin, saponin, glycoside, quinone, coumarin, cardenolide, anthraquinone, protein, and carbohydrate contents were absent. This is because of the inability of n-hexane to extract polar phytochemicals. The phytochemical analysis performed qualitatively shows that the efficiency of phytochemical extraction increases with the increasing polarity of the solvent used, in the order methanol > ethanol > acetone > n-hexane. Based on the presence of phenolics, flavonoids, tannins, alkaloids, and glycosides in the methanolic extract, the latter would be suitable for further studies involving quantitative phytochemical estimation, FTIR spectrometry, and GC-MS analysis.

Total Alkaloid Content (TAC)

Alkaloids in total content showed significant differences between different solvent extracts of *Premna tomentosa* leaves (one-way ANOVA, $p < 0.001$). The highest alkaloid content was found in methanol extract (132.64 ± 2.67 mg AE/g dry extract), while ethanol and acetone extracts had lower contents (98.76 ± 2.45 and 67.82 ± 2.11 mg AE/g dry extract, respectively). The lowest alkaloid content was noted in n-hexane extract (31.45 ± 1.23 mg AE/g dry extract). The increased yield of alkaloids in the methanol extract is consistent with results of qualitative phytochemical analysis, in which the presence of alkaloids was strong (+++). Thus, methanol can be considered the best solvent for extraction of alkaloids from leaves of P. tomentosa. Values are means ± SD (n = 3) (Fig. 2).

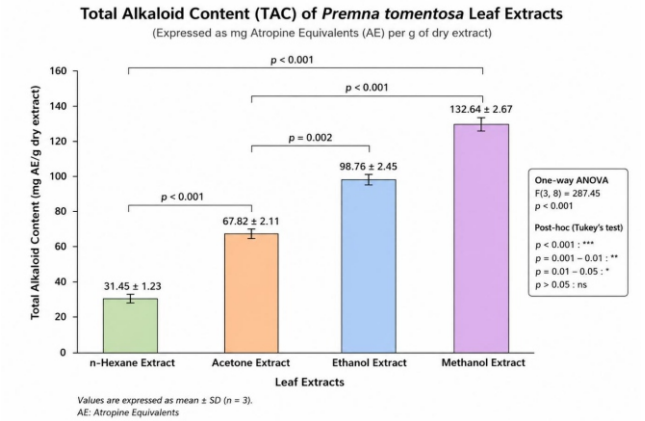

Figure 2. Total alkaloid content (TAC) of different solvent extracts of Premna tomentosa leaves expressed as mg atropine equivalents (AE) per g dry extract.

Total Flavonoid Content

Total flavonoid contents differed greatly among the solvent extracts of *Premna tomentosa* leaf extracts ($F(3, 8) = 245.67$, $p < 0.001$). Methanol extracts had the highest flavonoid content (128.67 ± 2.81 mg QE g^{-1} dry extract) compared to ethanol extracts (96.42 ± 2.58 mg QE g^{-1} dry extract) and acetone extracts (56.34 ± 2.03 mg QE g^{-1} dry extract). n-Hexane extracts had the least flavonoid content (14.78 ± 1.12 mg QE g^{-1} dry extract). Post hoc analysis using Tukey's test confirmed significant differences among solvent extracts. This indicated that polar solvents, such as methanol, were better in extraction of flavonoids than non-polar solvents. Data are expressed as mean ± SD (n = 3) (Fig. 2).

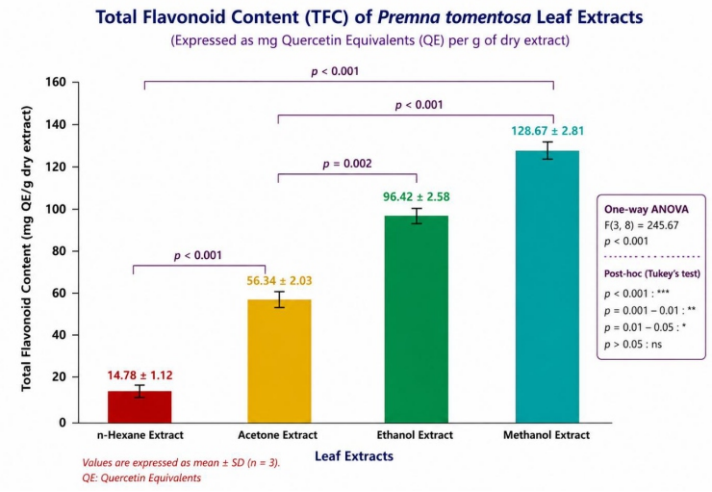

Figure 2. Total flavonoid content of different solvent extracts of Premna tomentosa leaves (mg QE/g).

Total Phenolic Content

There was a highly significant difference in the total phenolic content between the different solvent extracts of *P. tomentosa* leaves ($F(3, 8) = 312.46$, $p < 0.001$). Methanol extract had the highest total phenol content (151.89 ± 3.02 mg GAE/g dry extract) compared to other extracts. Ethanol extract had the second highest total phenolic content (109.64 ± 2.73 mg GAE/g dry extract), followed by acetone extract (62.78 ± 2.15 mg GAE/g dry extract), while n-hexane extract had the least amount of total phenolics (28.36 ± 1.41 mg GAE/g dry extract). Post hoc test using Tukey's HSD showed highly significant difference among the solvent extracts ($p < 0.001$). Hence, methanol is the best solvent for phenolic compound isolation.

Results of this quantification were consistent with those of phytochemical screening where phenolic compounds were identified as +++ in methanol and ethanol extracts. All data are presented as mean ± SD (n = 3) (Fig 3).

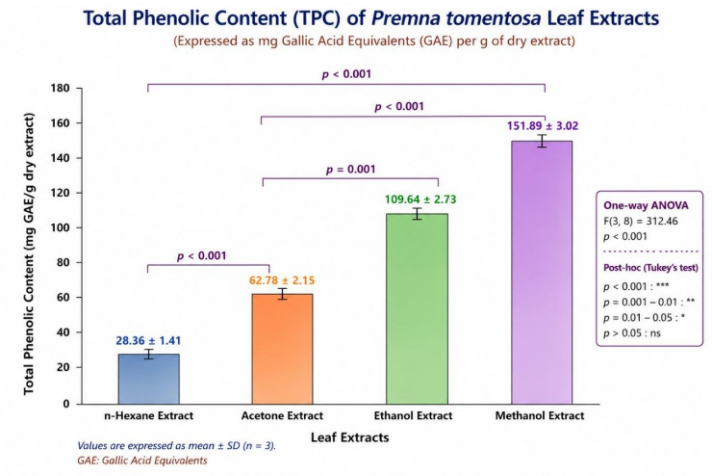

Figure 3. Total phenolic content (TPC) of different solvent extracts of Premna tomentosa leaves (mg GAE/g).

Total Tannin Content

There was a considerable variation in the tannin content of the *Premna tomentosa* leaf extracts in relation to the extraction solvents used ($F(3, 8) = 256.89$, $p < 0.001$). The methanol extract had the highest tannin content (82.73 ± 2.46 mg GAE/g dry extract), while the ethanol extract was second (60.21 ± 1.89 mg GAE/g dry extract) and the acetone extract third (33.48 ± 1.27 mg GAE/g dry extract). The n-hexane extract contained the least amount of tannins (15.62 ± 0.98 mg GAE/g dry extract). According to the Tukey's post hoc test, there were significant variations in the solvent extracts ($p < 0.001$), which suggested that methanol was the most efficient solvent in extracting tannins. These quantitative analyses corresponded to the results from the qualitative phytochemical screening test, where tannins were identified as being abundant (+++) in the methanol extract and moderate (++) in the ethanol extract (Fig. 4).

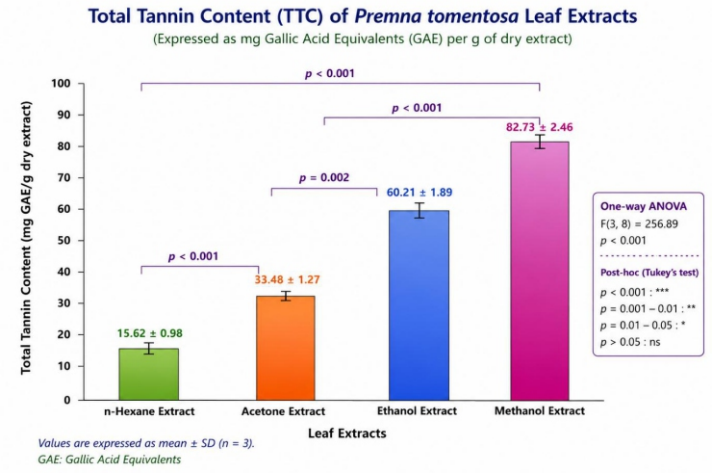

Figure 4. Total tannin content (TTC) of different solvent extracts of Premna tomentosa leaves (mg GAE/g).

Total Saponin Content

The average amount of saponins in the leaf extracts of *Premna tomentosa* varied significantly depending on the type of extraction solvent ($F(3, 8) = 228.34$, $p < 0.001$). The extract using methanol had the highest level of saponin concentration (67.49 ± 2.13 mg DE/g dry extract) than ethanol extract (45.63 ± 1.56 mg DE/g dry extract) and acetone extract (24.87 ± 1.12 mg DE/g dry extract). The extract using n-hexane was observed to contain the least quantity of saponin (10.26 ± 0.78 mg DE/g dry extract). Post hoc analysis using Tukey's test showed a significant difference among the solvents ($p < 0.001$), thus showing that methanol is the best extraction solvent for saponins. This quantification concurs with the phytochemical qualitative screening where saponins were strongly present (+++) in methanol extract, moderately present (++) in ethanol extract, weakly present (+) in acetone extract, and not present (–) in n-hexane extract (Fig. 5).

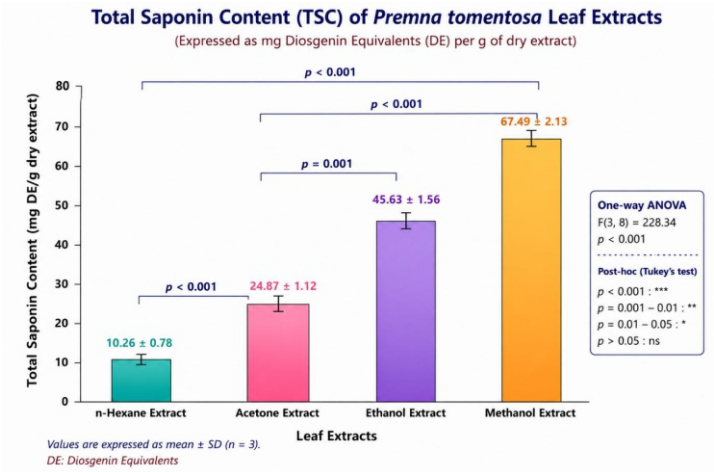

Figure 5. Total saponin content (TSC) of different solvent extracts of Premna tomentosa leaves (mg DE/g).

Total Steroid Content

There was a statistically significant difference in the total amount of steroids in the leaves of *Premna tomentosa* using different extraction solvents ($F(3, 8) = 286.71$, $p < 0.001$). Methanolic extract had the highest content of steroidal compounds (58.36 ± 1.89 mg CE/g dry extract), followed by ethanol (37.83 ± 1.42 mg CE/g dry extract) and acetone (22.18 ± 1.03 mg CE/g dry extract). N-hexane extract showed the lowest amount of steroidal compounds (11.42 ± 0.71 mg CE/g dry extract). Using Tukey's post hoc test, there were significant differences among solvent extracts ($p < 0.001$), suggesting that methanol gave the highest amount of steroidal compounds recovered. This result was confirmed with the results from phytochemical screening showing the presence of steroids in all solvent extracts, but with different extraction efficiencies (Fig. 6).

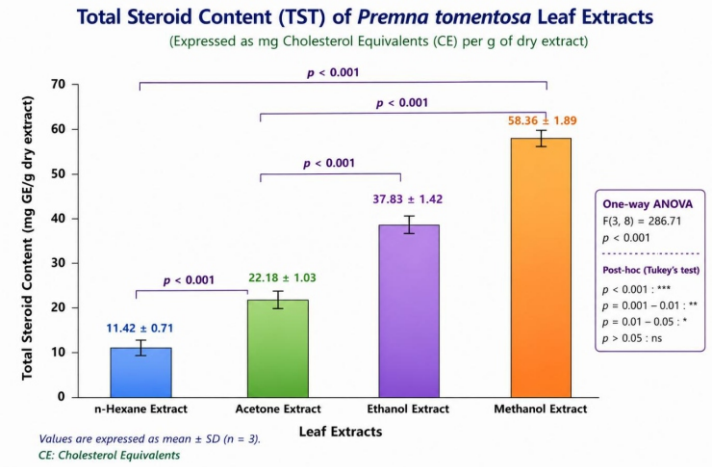

Figure 6. Total steroid content (TST) of different solvent extracts of Premna tomentosa leaves (mg CE/g).

Total Glycoside Content

Glycoside content in leaves of P. tomentosa differed significantly among various solvents used in the extraction process ($F(3, 8) = 274.36$, $p < 0.001$). In terms of solvent efficiency in extraction of glycosides, methanol had the highest glycoside content (53.28 ± 1.67 mg DTE/g dry extract), followed by ethanol (36.52 ± 1.25 mg DTE/g dry extract), acetone (22.96 ± 0.98 mg DTE/g dry extract), and n-hexane (11.27 ± 0.64 mg DTE/g dry extract). Post hoc Tukey test showed a highly significant difference between solvent extracts ($p < 0.001$) which suggests that methanol is the most efficient solvent in extracting glycosides from the leaves. The above quantified data is consistent with the qualitative phytochemical screening results where glycosides were identified as strong (+++) in methanol extracts, moderate (++) in ethanol extracts, weak (+) in acetone extracts, and not found (-) in n-hexane extracts (Fig. 7).

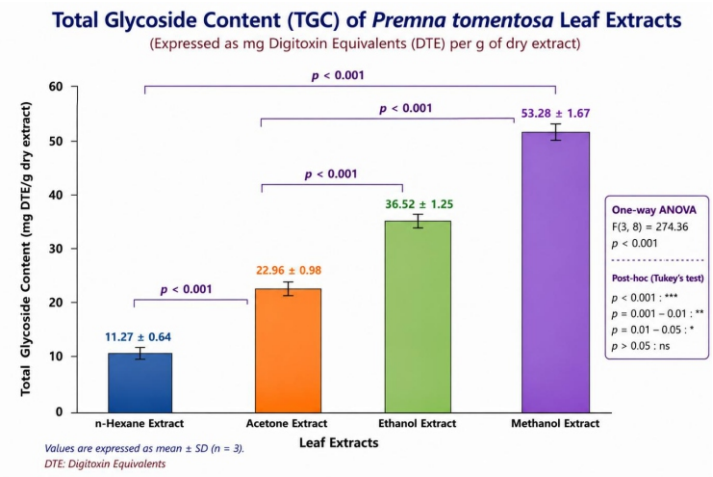

Figure 7. Total glycoside content (TGC) of different solvent extracts of Premna tomentosa leaves (mg DTE/g).

Total Carbohydrate, Protein, and Reducing Sugar Content

Quantitative analysis exhibited substantial differences in terms of carbohydrate, protein and reducing sugar contents of the different solvent extracts of *Premna tomentosa* leaves.

With regard to total carbohydrate content, the highest value was recorded for the methanol extract (134.58 ± 2.89 mg GE/g dry extract) while the other solvent extracts had ethanol extract (92.76 ± 2.13 mg GE/g dry extract), acetone extract (48.67 ± 1.45 mg GE/g dry extract) and n-hexane extract (21.34 ± 0.92 mg GE/g dry extract) respectively.

One-way ANOVA indicated a highly significant difference between the extracts ($F(3, 8) = 512.74$, $p < 0.001$). There was also significant variation in terms of total protein content ($F(3, 8) = 468.21$, $p < 0.001$) with the methanol extract having the highest total protein content (61.82 ± 1.86 mg BSAE/g dry extract) followed by ethanol extract (44.73 ± 1.27 mg BSAE/g dry extract), acetone extract (25.36 ± 0.89 mg BSAE/g dry extract) and n-hexane extract (12.48 ± 0.58 mg BSAE/g dry extract) respectively. There were also significant variations in total reducing sugar content of the different solvent extracts ($F(3, 8) = 389.65$, $p < 0.001$). For total reducing sugar content, the methanol extract had the highest total reducing sugar content (48.92 ± 1.42 mg GE/g dry extract) followed by ethanol extract (31.68 ± 1.04 mg GE/g dry extract), acetone extract (18.35 ± 0.73 mg GE/g dry extract) and n-hexane extract (8.76 ± 0.41 mg GE/g dry extract) respectively. Tukey's test also indicated highly significant difference between all solvent extracts ($p < 0.001$). It may be concluded that high contents of carbohydrate, protein and reducing sugar in methanol extract coincide with the results of phytochemical qualitative analysis (Fig. 8).

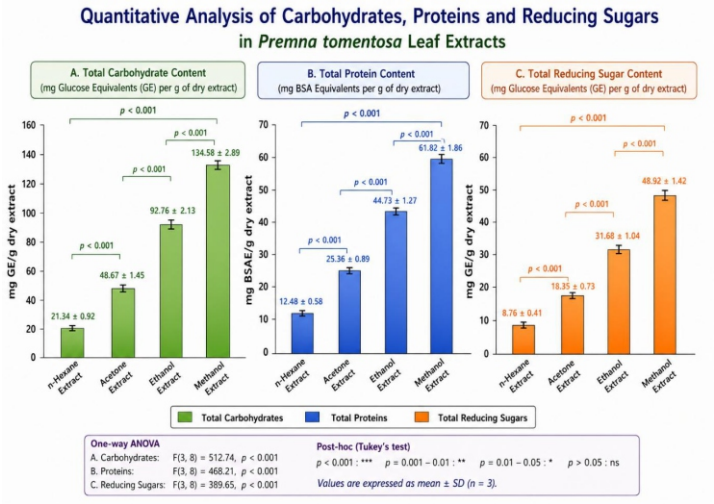

Figure 8. Quantitative analysis of total carbohydrate, protein, and reducing sugar contents in different solvent extracts of Premna tomentosa leaves. (A) Total carbohydrate (mg GE/g); (B) Total protein (mg BSAE/g); and (C) Total reducing sugar (mg GE/g).

Fourier Transform Infrared (FTIR) Analysis of *Premna tomentosa* Methanolic Leaf Extract

The FTIR spectrum of the methanol extract of the leaves of *P. tomentosa* showed several characteristic absorption peaks for different functional groups (Figure 9; Table 2). A prominent peak found between 3200 and 3450 cm^{-1} (recorded at 3450, 3383, 3341, 3277, and 3226 cm^{-1}) was assigned to stretching vibrations of O-H bonds of phenols and alcohols together with N-H vibrations of amine groups.

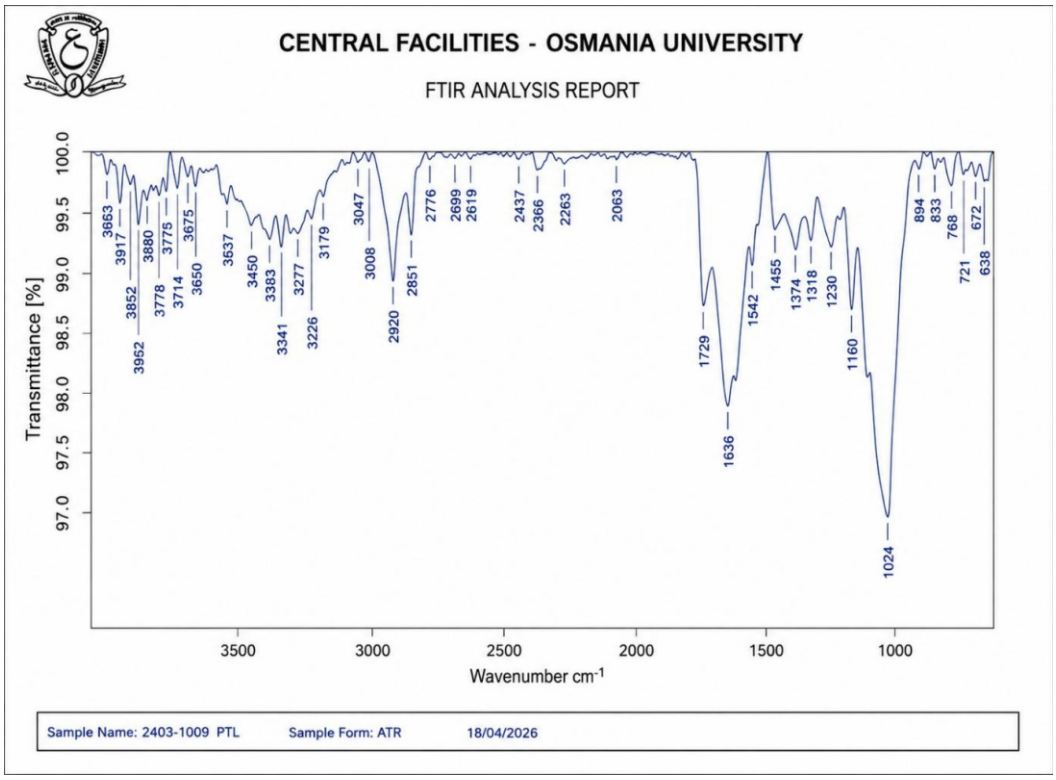

Table 2. Major FTIR absorption bands and functional group assignments of the methanolic leaf extract of Premna tomentosa

Peak (cm^{-1})	Functional group	Vibrational assignment	Possible phytochemical constituents
3450–3226	O–H, N–H	O–H/N–H stretching	Phenolics, flavonoids, tannins, proteins
3047	=C–H	Aromatic C–H stretching	Aromatic compounds
3008	C–H	Unsaturated C–H stretching	Terpenoids, aromatic hydrocarbons
2920	$–CH_2$	Asymmetric C–H stretching	Lipids, terpenoids
2851	$–CH_3$	Symmetric C–H stretching	Aliphatic compounds
1729	C=O	Carbonyl stretching	Esters, aldehydes, carboxylic acids
1636	C=C / Amide I	Aromatic C=C stretching	Polyphenols, proteins
1542	N–H/C=C	Amide II or aromatic vibration	Alkaloids, proteins
1455	C–H	CH_2 bending	Terpenoids, lipids
1374	C–H	Methyl bending	Aliphatic compounds
1318	C–N	C–N stretching	Alkaloids, amines
1230	C–O	Phenolic C–O stretching	Phenolics, flavonoids
1160	C–O–C	Ether stretching	Glycosides, polysaccharides
1024	C–O	Alcohol/C–O stretching	Carbohydrates, glycosides
894–638	C–H	Aromatic out-of-plane bending	Aromatic phytochemicals

The FTIR spectrum shows important absorptions in the regions of 3047, 3008, 2920, and 2851 cm^{-1} due to the stretching vibrations of the C–H group of aromatic and aliphatic hydrocarbons. The absorption peak seen at 1729 cm^{-1} suggests the carbonyl (C=O) groups typical for esters, aldehydes, or carboxylic acids. The peak appearing at 1636 cm^{-1} could be due to C=C stretching in aromatic rings or to amide I vibrations. Absorptions in the regions of 1542 and 1455 cm^{-1} could represent aromatic skeletal vibrations or amide II, respectively. The absorptions in the regions of 1374, 1318, 1230, 1160, and 1024 cm^{-1} were explained by the stretching vibrations of the C–O, C–O–C, and C–N groups, confirming the existence of alcohols, ethers, glycosides, and phenolic derivatives. Absorptions in the region from 894 to 638 cm^{-1} represent aromatic C–H bends and out-of-plane vibrations.

Overall, these FTIR absorptions confirm the existence of hydroxyl, carbonyl, aromatic, ether, and aliphatic functional groups, representing various phytochemicals.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis of *Premna tomentosa* Methanolic Leaf Extract

The GC-MS analysis of the methanol extract of leaves of *Premna tomentosa* resulted in identification of a wide variety of plant chemicals with different retention times (Figure 10 and Table 3). Overall, 25 different major chemicals have been identified using their retention times and compared with the NIST mass spectra library. Some significant peaks have been observed at 3.980, 5.912, 20.718, 23.018, 25.789, 28.556, 31.303, and 33.348 minutes.

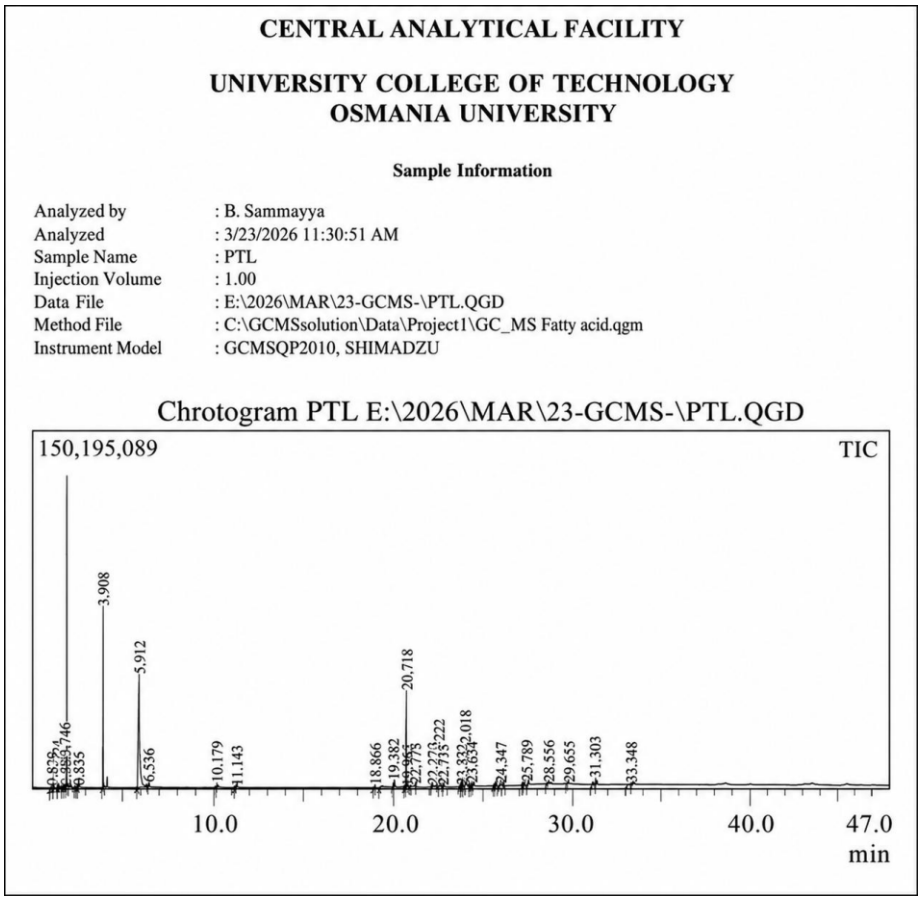

Table 3. Major phytochemicals identified in the methanolic leaf extract of Premna tomentosa by GC–MS analysis

Peak No.	Retention Time (min)	Tentative Compound	Molecular Formula	Molecular Weight (g/mol)	Compound Class	Reported Biological Activity
1	3.980	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.11	Furan derivative	Phytoremediation, antimicrobial
2	5.912	Phenol, 2-methoxy-4-vinyl-	$C_9H_{10}O_2$	150.17	Phenolic compound	Phytoremediation, antimicrobial
3	10.179	Phytol	$C_{20}H_{40}O$	296.53	Diterpene alcohol	Anti-inflammatory, antioxidant
4	11.143	Neophytadiene	$C_{20}H_{38}$	278.52	Diterpenoid	Antimicrobial
5	18.866	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	270.45	Fatty acid ester	Antioxidant
6	19.382	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	Fatty acid	Antimicrobial
7	20.718	9,12-Octadecadienoic acid (Linoleic acid)	$C_{18}H_{32}O_2$	280.45	Unsaturated fatty acid	Anti-inflammatory
8	22.075	Oleic acid	$C_{18}H_{34}O_2$	282.47	Fatty acid	Cardioprotective
9	22.273	Stearic acid	$C_{18}H_{36}O_2$	284.48	Saturated fatty acid	Nutritional
10	23.222	Methyl linolenate	$C_{19}H_{32}O_2$	292.46	Fatty acid ester	Phytoremediation, Antioxidant
11	23.018	Ethyl linoleate	$C_{20}H_{36}O_2$	308.50	Fatty acid ester	Anti-inflammatory
12	23.634	Octadecanoic acid methyl ester	$C_{19}H_{38}O_2$	298.51	Fatty acid ester	Antioxidant
13	24.347	Squalene	$C_{30}H_{50}$	410.72	Triterpene	Anticancer, antioxidant
14	25.789	Campesterol	$C_{28}H_{48}O$	400.68	Phytosterol	Hypocholesterolemic
15	28.556	Stigmasterol	$C_{29}H_{48}O$	412.69	Phytosterol	Anti-inflammatory
16	29.655	β-Sitosterol	$C_{29}H_{50}O$	414.71	Phytosterol	Antidiabetic, antioxidant
17	31.303	Lupeol	$C_{30}H_{50}O$	426.72	Triterpenoid	Anti-inflammatory
18	33.348	α-Tocopherol	$C_{29}H_{50}O_2$	430.71	Vitamin E derivative	Potent antioxidant
19	6.536	Benzyl alcohol	C_7H_8O	108.14	Alcohol	Antimicrobial
20	18.920	9-Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	296.49	Fatty acid ester	Antioxidant, Phytoremediation
21	22.832	Palmitoleic acid	$C_{16}H_{30}O_2$	254.41	Fatty acid	Antimicrobial
22	23.018	Ethyl palmitate	$C_{18}H_{36}O_2$	284.48	Fatty acid ester	Phytoremediation, Antioxidant
23	24.950	Farnesol	$C_{15}H_{26}O$	222.37	Sesquiterpene alcohol	Antimicrobial, Phytoremediation
24	30.870	Cycloartenol	$C_{30}H_{50}O$	426.72	Triterpenoid	Anti-inflammatory
25	32.990	γ-Sitosterol	$C_{29}H_{50}O$	414.71	Phytosterol	Phytoremediation, Antioxidant

The compound categories largely involved fatty acids, fatty acid esters, terpenoids, phenolic compounds, phytosterols, hydrocarbons, and long chain alcohols that have been reported for phytoremediation, antimicrobial, anti-inflammatory and medicinal properties. The variety of compounds determined through GC-MS confirms the presence of phytochemical diversity in *P. tomentosa*, further supporting the phytochemical studies conducted qualitatively and quantitatively.

Discussion

In the present study, the impact of increasing polarity of solvent was investigated to explore the patterns of phytoconstituent extractions from leaves of *Premna tomentosa* and relating them with qualitative phytochemical screening, quantitative estimation, FTIR functional group profiling, and GC–MS phytoconstituent profiling. As expected, the general tendency where the highest content of phytoconstituents was revealed in the methanolic extract, followed by ethanol, acetone, and n-hexane extracts, is consistent with the known facts of the selectivity of plant phytochemicals based on the polarity of the solvent [28]. The polar solvent like methanol and ethanol disturbs the hydrogen bonding and dipole interaction more efficiently than the non-polar solvents, and therefore facilitates the extraction of polyphenols, flavonoids, tannins, alkaloids, saponins, and glycosides usually associated with the plant cell wall [7]. The deep green color of the n-hexane extract containing chlorophylls, waxes, and other lipophilic substances, along with the golden and dark brown colors of the ethanol and methanolic extracts correspondingly, proves this difference.

Qualitative phytochemical screening shows the gradation of secondary metabolites content in all the four extracts, where the methanolic extract demonstrates the strongest reactions for alkaloids, flavonoids, phenolics, tannins, saponins, glycosides, and coumarins. This gradation corresponds with previous studies on the effectiveness of methanol as the most efficient solvent for extraction of phytoconstituents in different medicinal plants [8,25]. At the same time, the low content of flavonoids, tannins, saponins, glycosides, and anthraquinones in the n-hexane extract in combination with the high content of steroids and terpenoids is caused by the affinity of the non-polar solvent to the lipid skeleton of steroidal and triterpenoid compounds [6]. The moderate profile of the acetone extract with the high content of alkaloids, flavonoids, and phenolics, but poor in tannins, saponins, and glycosides reflects the status of this solvent as the moderate one that extracts both categories of phytoconstituents [17].

The quantitative estimates of total alkaloids, flavonoids, and phenolics are the confirmation of the qualitative findings. The significant advantage of the alkaloid content in the methanolic extract compared with the other three extracts corresponds with the known information about preferential extraction of alkaloids, mainly the salts of organic acids, in the polar protic solvent [14]. At the same time, the significantly greater flavonoid and phenolic contents in the methanolic and ethanol extracts are also consistent with the correlation between the solvent polarity and the extraction efficiency of hydroxylated aromatic compounds, because of numerous hydroxyl groups in the structure of flavonoids and phenolics [9]. The method for phenolic content estimation, Folin–Ciocalteu assay, follows the standard procedure described by Singleton et al. [24]. The value of phenolic content for the methanolic extract is typical of the other therapeutically valuable plants from the Lamiaceae family using comparable extraction and estimation procedures [3].

Similar solvent-dependent pattern was found for the tannin and saponin contents, where methanol showed the highest content and n-hexane – the lowest one. Since tannins are the high-molecular weight polyphenolic polymers and contain numerous hydroxyl groups, and saponins are amphiphilic glycosides, it is natural that the polarity of solvent plays the key role in their extraction, which is proved by the numerous comparative solvent-extraction studies of the medicinal plants [5]. Low tannin and saponin yield in the acetone and n-hexane extracts proves that the extraction of these compounds in non-polar and semi-polar solvents is inefficient, which is typical for other ethnomedicinal plant screenings [25].

Contrary to the flavonoids, phenolics, tannins, and saponins, the steroid content in the methanolic extract was the highest, but the content in the non-polar and semi-polar extracts was significantly lower. It is due to the amphiphilic structure of plant steroidal compounds, which makes them soluble in the broad polarity range, as is typical for related terpenoid-rich species [21]. At the same time, the glycoside content in the four extracts demonstrated the similar solvent dependence as phenolics and flavonoids, because many of the glycosides in the plant tissue are the sugar conjugates of flavonoid or phenolic aglycones [7].

The carbohydrate, protein, and reducing sugar contents in the methanolic extract were also significantly higher compared with the other three extracts. Since the primary metabolites such as soluble sugars and low-molecular weight proteins are inherently hydrophilic, the preference of their extraction in methanol rather than in hexane and acetone is not surprising and was already proved in other phytochemical investigations of leafy material [8]. The parallel increase in carbohydrate, protein, and reducing sugar contents with the increasing solvent polarity confirms the reliability of the extraction protocol used in the present study.

The FTIR analysis of the methanolic extract confirmed the biochemistry of the extract through the functional groups of the molecules contained in it. Thus, the broad O–H/N–H stretching vibration in the 3200–3450 cm^{-1} region indicates phenolic hydroxyl and amine functionalities and is typical of the FTIR analysis of polyphenol-rich plant extracts [21]. The carbonyl stretching vibration near 1729 cm^{-1} and aromatic C=C/amide I stretching vibration near 1636 cm^{-1} indicate the coexistence of ester or carboxylic acid functionalities together with aromatic polyphenolic and proteinaceous structures, and C–N stretching vibration around 1318 cm^{-1} indicates alkaloidal nitrogen-containing moieties. C–O and C–O–C stretching vibrations in the 1230–1024 cm^{-1} region are indicative of glycosidic and carbohydrate linkage, which is consistent with functional groups reported for the other methanolic plant extracts analyzed by FTIR [19]. Thus, the FTIR analysis confirms, from the level of functional groups, the presence of phenolics, flavonoids, tannins, glycosides, alkaloids, and terpenoids in the methanolic extract.

The GC-MS profiling of the methanolic extract showed twenty-five phytoconstituents, mainly fatty acids, fatty acid esters, phytosterols, and terpenoids, some of which are associated with phytoremediation, antimicrobial, anti-inflammatory, and cardioprotective properties. The presence of the diterpenoid derivatives phytol and neophytadiene, with known anti-inflammatory and antimicrobial effects, along with the squalene, campesterol, stigmasterol, β-sitosterol, and lupeol is consistent with the studies describing similar terpenoid and phytosterol profile of the methanolic leaf extract in other important medicinal plants [6].

The presence of the natural antioxidant α-tocopherol and multiple unsaturated fatty acids, like linoleic and oleic acid, is consistent with the high antioxidative effect, which can be attributed to the high phenolic and flavonoid content of the extract. These results suggest the contribution of the phytosterols, terpenoids, and fatty acids to the medicinal value of the plant leaves.

Thus, the combination of the qualitative screening, quantitative estimation, FTIR, and GC-MS analyses provide consistent and mutually supportive picture of the solvent polarity-dependent extraction efficiency and composition of *Premna tomentosa* leaf extracts, where methanol turned out to be the most suitable solvent for the extraction of pharmaceutically important phytoconstituents. This coherence between the results of independent analytical approaches confirms the reliability of the findings and is consistent with the known phytochemical literature proving the priority of methanol for comprehensive metabolite profiling of medicinal plants [27]. However, this study is limited to a single extraction method and single plant part; future research including the alternative extraction methods, additional plant parts, and bioactivity-guided fractionation will help to identify the compounds responsible for biological activities of this species.

Conclusion

The present research study shows that the choice of the extraction solvent has a significant impact on the efficiency of phytochemical extraction from P. tomentosa leaves with the methanol being the most efficient extraction solvent. The methanolic extract has shown significantly higher amounts of alkaloids, flavonoids, phenolics, tannins, saponins, steroids, glycosides, carbohydrates, proteins, and reducing sugars than extracts from ethanol, acetone, and n-hexane ($p < 0.001$). FTIR spectroscopic analysis has revealed the presence of hydroxyl, carbonyl, aromatic, ether, and amine functional groups. GC-MS analysis has determined the presence of twenty-five bioactive compounds, which include phytosterols, terpenoids, fatty acids, and phenolic compounds known for their high capacity for phytoremediation, antimicrobial activity, and anti-inflammatory properties. It can be concluded that the high phytochemical diversity found in the methanolic extract implies a high potential of this plant species not only as a medicinal but also as a phytoremediating plant. High amounts of phenolic compounds, flavonoids, terpenoids, and phytosterols might increase tolerance to the oxidative stress and improve metal binding and detoxification capabilities. The results obtained will be useful for further researches devoted to the evaluation of the phytoremediation potential of *P. tomentosa*.

Conflict of interest: The authors were declaring no conflict of interest to report regarding this research work

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