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COMPREHENSIVE PHARMACOGNOSTIC AND PHYTOCHEMICAL PROFILING OF *TABERNAEMONTANA ALTERNIFOLIA* LEAVES: QUANTIFICATION OF PHENOLICS, ALKALOIDS, FLAVONOIDS, AND EVALUATION OF AFLATOXIN AND HEAVY METAL CONTAMINATION

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ABSTRACT

Objectives: The study aimed to establish pharmacognostic standards and quantify major bioactive constituents, phenolics, alkaloids, and flavonoids in the leaves of *Tabernaemontana alternifolia* (Apocynaceae).

Methods: Leaves were collected from the Ratnagiri region of Maharashtra, India, authenticated, and subjected to detailed macroscopic, microscopic, physicochemical, and phytochemical evaluations. Microscopy was used to identify diagnostic anatomical features, whereas physicochemical parameters such as ash values and extractive values were determined using standard procedures. Qualitative and quantitative phytochemical analyses were performed to assess the presence and levels of key metabolites. Heavy metal content was analyzed using atomic absorption spectroscopy, and aflatoxins were assessed using liquid chromatography-tandem mass spectrometry (MS)/MS.

Results: Microscopic examination revealed a dorsiventral leaf with bicollateral vascular bundles and abundant microsphenoidal calcium oxalate crystals. Physicochemical parameters included total ash (5.67±0.76% w/w), acid-insoluble ash (0.54±0.01% w/w), and water-soluble ash (3.21±0.04% w/w), confirming minimal inorganic contamination. Soxhlet extraction produced the highest extractive value (17.93±1.37% w/w). Phytochemical screening confirmed the presence of alkaloids, phenolics, flavonoids, tannins, saponins, proteins, and reducing sugars. Quantitative analysis showed notable levels of total phenolics (11.82 mg gallic acid equivalents/g), flavonoids (7.53 mg rutin equivalents/g), and alkaloids (4.0 mg atropine equivalents/g). Heavy metals (Cd, Pb, As, and Hg) were below detectable limits, and aflatoxins (B1, B2, G1, and G2) were absent.

Conclusion: This study establishes key pharmacognostic and phytochemical benchmarks for *T. alternifolia* leaves. Soxhlet extraction yielded the highest extractive value, whereas quantitative analyses revealed appreciable levels of phenolics, flavonoids, and alkaloids, indicating notable bioactive potential. The absence of heavy metals and aflatoxins confirms material safety, supporting its standardization, quality control, and further pharmacological development.

Keywords: Pharmacognosy, Phenolic content, Flavonoids, Alkaloids, Heavy metal analysis, Aflatoxins, Herbal standardization.

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INTRODUCTION

Plant-derived products have seen a rise in demand in recent years, particularly in industrialized nations, due to their perceived cost-efficiency, safety, and effectiveness. These products are increasingly utilized as medicinal agents, nutraceuticals, and cosmetic ingredients, reflecting the resurgence of interest in herbal therapeutics [1]. To ensure the reliability and reproducibility of herbal formulations, standardization plays a critical role. It encompasses all measures undertaken during manufacturing and quality control processes to achieve consistent composition, quality, and biological activity of herbal products [2].

Pharmacognostic studies form the cornerstone of herbal drug standardization, serving to establish reliable parameters for identification, authentication, and purity determination. Such studies include macroscopic and microscopic evaluations, physicochemical analyses, and phytochemical screening, which collectively ensure product integrity, prevent adulteration, and guarantee therapeutic consistency [3,4]. These investigations are essential for safeguarding public health and providing a scientific foundation for pharmacological and phytochemical research.

Pharmacognostic standardization not only helps in proper identification but also in detecting contamination and adulteration in herbal materials. For example, heavy metal contamination poses a serious risk to the safety of herbal medications. Metals such as cadmium (Cd), copper, lead (Pb), mercury (Hg), and arsenic (As) can accumulate in plants and pose significant toxicological risks when consumed beyond permissible limits [5,6]. Chronic exposure may Pb to neurological disorders, carcinogenic effects, and systemic toxicity. Therefore, it is mandatory to evaluate herbal materials for heavy metal and aflatoxin contamination as part of standard pharmacognostic and safety assessment protocols to ensure food safety and therapeutic efficacy [7,8].

The genus *Tabernaemontana* (Family: Apocynaceae) is named in honor of Jacob Theodor Tabernaemontanus, a 16th-century German physician and botanist. This genus comprises over 100 species of flowering shrubs and small-to-medium-sized trees distributed across tropical and subtropical regions of Asia, Africa, Oceania, and the Americas [9]. Members of this genus are typically characterized by tubular white flowers, follicular fruits containing seeds embedded in colored arils, and the presence of milky latex, earning them the common name "milkweed." These plants are well recognized in traditional medicine for

their wide range of pharmacological activities, including antimicrobial, anti-inflammatory, analgesic, and antitumor effects [10].

Tabernaemontana species are rich in indole alkaloids, particularly monoterpene indole and bisindole alkaloids, which are known for their diverse pharmacological potential [11]. In addition to alkaloids, the genus also contains terpenoids, lactones, steroids, phenolics, and flavonoid compounds that contribute to its biological properties [12,13]. More than 67 species of this genus have been investigated for their alkaloid content, resulting in over 470 isolations representing around 240 unique chemical structures [11]. Such chemical diversity underscores the therapeutic potential of the genus, warranting detailed phytochemical and pharmacognostic exploration of understudied species such as *Tabernaemontana alternifolia*.

The quantification of phenolic, alkaloid, and flavonoid contents is a key step in assessing the therapeutic potency and antioxidant capacity of medicinal plants [1]. These secondary metabolites are known for their pharmacological activities, including anti-inflammatory, antimicrobial, antidiabetic, and anticancer properties [2]. Estimating their levels provides insight into the plant's bioactivity and ensures reproducibility in herbal formulations.

Despite the recognized medicinal potential of *T. alternifolia*, comprehensive pharmacognostic and quantitative phytochemical studies on its leaves remain limited. Previous studies have largely focused on qualitative phytochemical screening, reporting the presence of alkaloids, flavonoids, tannins, phytosterols, cardiac glycosides, terpenoids, reducing sugars, and saponins [7]. Extraction studies have shown that methanol is an effective solvent for isolating phytoconstituents. However, there have been no reports of studies addressing aflatoxin contamination or heavy metal analysis of this species, nor quantitative measurement of total phenolic, alkaloid, and flavonoid contents.

Hence, the present investigation aims to establish comprehensive pharmacognostic standards and generate quantitative phytochemical data for *T. alternifolia* leaves. This study is expected to provide a scientific basis for its quality control, authentication, and potential utilization in herbal formulations and pharmacological applications.

METHODS

Collection and identification of plant material

The plant *T. alternifolia* was collected in December 2022 from the Ratnagiri region of Maharashtra, India. The specimen was authenticated by the College of Forestry, Dapoli (Ratnagiri), and a voucher specimen (MM/COP/3061/2022–23) was deposited for future reference. Fresh leaves were subjected to macroscopic examination, after which the plant materials were thoroughly washed with water and shade-dried at room temperature. The dried samples were then coarsely powdered and stored in a tightly sealed, light-resistant container until further experimental analysis.

Macroscopic assessment

All collected samples were carefully washed and shade-dried for 10–15 days, after which they were evaluated for their macroscopic characteristics, including color, shape, size, odor, taste, fracture, and other surface features. The dried leaves were subsequently pulverized into powder, which was further examined for color, odor, and taste, and subjected to the filter paper test for preliminary assessment [14].

Microscopic evaluation

Mature fresh leaves were collected and thoroughly washed with water for the preparation of transverse sections. To maintain tissue moisture, thin sections were carefully cut from the lamina across the midrib and preserved in water. This technique is employed to identify plant-based drugs at the cellular level by examining their microscopic characteristics, using either whole tissues or specific fragments of coarsely powdered material. Fresh plant parts were used for section cutting, and thin transverse sections (T.S.) of the leaves were prepared by freehand sectioning with a sharp blade, after which the initial microscopic observations were recorded [15].

Ash values

Two to three grams of the accurately weighed powdered drug were placed in a tared silica crucible and incinerated at a temperature not exceeding 450°C for 4 h, until carbon-free ash was obtained [16]. The crucible was then cooled and weighed to determine the total ash content. For acid-insoluble ash determination, the total ash was boiled with 25 mL of 2 M hydrochloric acid for 5 min, filtered through ashless filter paper, washed with hot water, and ignited in a tared crucible at a temperature not exceeding 450°C for 4 h. The residue was cooled in a desiccator and weighed. To determine water-soluble ash, the total ash was boiled with 25 mL of distilled water, filtered through ashless filter paper, washed, and ignited under the same conditions. The weight of the insoluble matter was subtracted from the total ash weight, and the difference represented the water-soluble ash value [17].

Extractive values of plant material

Initially, the *T. alternifolia* plant material was kept in a controlled drying environment. All extractions (Soxhlet, microwave, etc.) were performed on leaves only. The gathered leaves were ground into a fine powder using a mechanical grinder after being shade-dried to retain thermolabile components. Various extraction processes were applied to the powdered material in order to generate crude extracts for additional examination.

Maceration

Maceration was performed using a mechanical shaker. Accurately weighed powdered leaves of *T. alternifolia* (5 g) were placed in a 250 mL conical flask containing 100 mL of methanol [18]. At room temperature, the mixture was constantly stirred for 72 h to guarantee complete extraction. The extract was then filtered through Whatman No. 1 filter paper, and the filtrate was gathered for additional processing [19].

Soxhlet extraction

For Soxhlet extraction, 5 g of dried powdered leaves of *T. alternifolia* were placed in a Soxhlet apparatus fitted with a 250 mL round-bottom flask containing 100 mL of methanol. For 72 h, the extraction process was conducted at a regulated temperature of 65°C. Upon completion, the extract was filtered and concentrated to dryness under reduced pressure [20].

Ultrasonic extraction

An ultrasonic bath was used to perform ultrasound-assisted extraction. 100 mL of methanol was used to extract 5 g of powdered *T. alternifolia* leaves, which were then sonicated for 15 min at room temperature. The resulting mixture was filtered, and the filtrate was evaporated to dryness to obtain the extract [21].

Microwave-assisted extraction

Microwave-assisted extraction was carried out by placing 5 g of dried and powdered leaves of *T. alternifolia* in an Erlenmeyer flask containing 100 mL of methanol. The suspensions were exposed to microwave irradiation (CATA-4R, Mumbai, India) at 490 W for 5 min, ensuring that overheating or superboiling was avoided. The obtained extract was filtered and concentrated to dryness using a tray dryer maintained at 40°C [22].

Qualitative phytochemical tests

The phytochemical evaluation of *T. alternifolia* leaf extract was conducted using established qualitative protocols encompassing 11 categories of secondary metabolites. Hager's, Wagner's, Mayer's, and Dragendorff's reagents were used to detect alkaloids, whereas the alkaline reagent, Pb acetate, and Shinoda tests were used to confirm flavonoids. Fehling's and Benedict's tests were used to identify reducing sugars, whereas Molisch's, iodine, Benedict's, and Fehling's reactions

were used to confirm carbohydrates. Glycosides were analyzed through Liebermann's and Keller–Kiliani tests. The presence of saponins was assessed by the foam test, proteins and amino acids by Biuret and ninhydrin assays, phytosterols by Salkowski and Liebermann–Burchard's reactions, and anthraquinones by Borntrager's and ammonium hydroxide tests [14].

Total phenolic content

Total phenolic content was quantified using the Folin–Ciocalteu reagent, following a slightly modified protocol [23]. Gallic acid was used as the standard for calibration, and a standard curve was prepared using gallic acid solutions ranging from 5 to 125 mg/L. A test sample was prepared by dissolving 10 mg of the extract in 10 mL of methanol. To each standard and sample solution, 0.5 mL was mixed with 2.5 mL of 50% Folin–Ciocalteu reagent and 2.5 mL of distilled water. After a 5-min incubation, 2 mL of 7.5% w/v aqueous sodium carbonate solution was added. The mixture was shaken thoroughly and kept at room temperature in the dark for 15 min. The absorbance was then measured at 765 nm using a Cecil CE7410 ultraviolet (UV)-vis spectrophotometer, and the results were expressed as milligrams of gallic acid equivalents (GAE)/100 mg of dry leaf weight [23]. The total phenolic content was performed in triplicate (n=3), and results are expressed as mean±standard deviation (SD).

Total flavonoid content

The total flavonoid content was determined using the aluminum chloride colorimetric method, following the procedure described by [24] with slight modifications. Briefly, 1 g of the methanolic extract was dissolved in 100 mL of 80% methanol. From this solution, 0.5 mL was mixed with 1.5 mL of 95% methanol, 0.1 mL of 10% aluminum chloride solution, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The reaction mixture was incubated at room temperature (25°C) for 30 min. The absorbance of both test and blank solutions was measured at 415 nm using a UV-visible spectrophotometer. Quantification was carried out using a calibration curve prepared with rutin (1–5 µg/mL), and all measurements were performed in triplicate. The total flavonoid content was expressed as milligrams of rutin equivalents per gram of dry weight of the extract [25]. The total flavonoid content was performed in triplicate (n=3), and results are expressed as mean±SD.

Total alkaloid content

A 0.5 g portion of the crude methanolic extract of *T. alternifolia* leaves was accurately dissolved in methanol. From this solution, 1 mL was transferred into separate separatory funnels. To each funnel, 5 mL of bromocresol green solution and phosphate buffer (pH 4.7) were added, followed by the addition of 1, 2, 3, and 4 mL of chloroform with vigorous shaking. The chloroform layers were collected in 10 mL volumetric flasks and made up to the mark with chloroform. Similarly, a series of atropine standard solutions (0.4, 0.6, 0.8, 1.0, and 1.2 µg/mL) was prepared using the same procedure. The absorbance of the test and standard solutions was measured at 470 nm using a UV-visible spectrophotometer against a reagent blank. The total alkaloid content was calculated and expressed as milligrams of atropine equivalents (AE) per gram of extract [26]. The total alkaloid content was performed in triplicate (n=3), and results are expressed as mean±SD.

Heavy metal analysis

The collected samples were finely ground and dried in a controlled environment at 55–70°C for 6–8 h to ensure complete removal of moisture. After drying, an accurately weighed 1.0 g portion of each sample was transferred into a digestion flask and treated with 12 mL of concentrated nitric acid (HNO_3) for 24 h. Subsequently, 5 mL of an acid mixture composed of sulfuric acid (H_2SO_4) and nitric acid (HNO_3) in a 3:1 ratio was added to each flask. The mixture was then digested at 120–130°C for 5–6 h until the evolution of brown fumes ceased and a clear solution was obtained, indicating complete digestion. After cooling to room temperature, the digested solution was filtered through Whatman No. 42 filter paper, and the filtrate was quantitatively transferred into a 50 mL volumetric flask. The final volume was made up to the mark with Milli-Q water. A reagent blank was prepared alongside each batch of samples following the same procedure to ensure analytical accuracy. Each sample was analyzed in duplicate, and the procedure was repeated 5 times to ensure reproducibility. Elemental analysis was performed using an atomic absorption spectroscopy (AAS) (Model AA-6300, Shimadzu, Japan) equipped with both flame and graphite furnace atomizers. The instrument operated over a wavelength range of 185–900 nm, with a photomultiplier detector covering 185–600 nm. All reagents and solvents used were of analytical grade. Standard stock solutions (1,000 ppm) of Cd, Pb, As, and Hg were used to prepare working standard solutions within the concentration ranges specified in Table 1. The instrumental operating parameters for trace and heavy metal determination are summarized in Table 2 [27]. Table 3 presents the standard permissible limits for heavy metals as recommended by the World Health Organization (WHO) and the U.S. Food and Drug Administration (FDA).

Permissible limits for heavy metals as per WHO (2011) and US FDA (2018) guidelines for medicinal plants and herbal products.

Determination of aflatoxins

A highly sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was established for the quantification of aflatoxins using an LCMS-8040 N-series UPLC system (Shimadzu, Japan). Chromatographic separation was achieved on a Kinetex C18 column (2.1 mm×100 mm, 1.7 µm particle size) maintained at 40°C, with a constant flow rate of 0.5 mL/min. The mobile phase consisted of solvent A (5 mM ammonium acetate in water containing 0.1% formic acid) and solvent B (5 mM ammonium acetate in methanol), employing the following gradient program: 5% B (0–4 min), 50% B (4–5.5 min), 85% B (6–7.5 min), followed by re-equilibration to 5% B (8.1–10 min). The injection volume was 5 µL. Mass spectrometric detection was performed using a heated electrospray ionization source operating in positive ion mode under multiple reaction monitoring conditions, monitoring two characteristic transitions for each analyte. The interface, block, and desolvation line temperatures were maintained at 350°C, 400°C, and 250°C, respectively. Argon served as

Table 1: Heavy metal content in *Tabernaemontana alternifolia* leaves

Standard/ sample	Weight (g)	Volume (mL)	Dilution factor	Absorbance	Conc. (ppm)
Standard	-	-	-	0.295	3
Standard	-	-	-	0.466	5
Standard	-	-	-	0.653	7
TA1				0	0
TA2	1	1	1	0	0
TA3	1	1	1	0	0
Standard	-	-	-	0.058	3
Standard	-	-	-	0.071	5
Standard	-	-	-	0.093	7
TA1	1	1	1	0.009	0
TA2	1	1	1	0.008	0
TA3	1	1	1	0.009	0
Standard	-	-	-	0.331	3
Standard	-	-	-	0.552	5
Standard	-	-	-	0.745	7
TA1	1	1	1	0.000	0
TA2	1	1	1	0.000	0
TA3	1	1	1	0.000	0
Standard	-	-	-	0.278	3
Standard	-	-	-	0.475	5
Standard	-	-	-	0.651	7
TA1	1	1	1	0.000	0
TA2	1	1	1	0.000	0
TA3	1	1	1	0.000	0

N.D. (W/B): Not detected (Within Blank). The limit of detection (LOD) for each metal was: Arsenic- 0.5 ppm, Cadmium - 0.5 ppm, Mercury - 0.5 ppm, Lead - 0.5 ppm. Values reported as 0 indicate concentrations below the respective LOD

Table 2: Instrumental conditions for heavy metal analysis by atomic absorption spectroscopy (Arsenic, Cadmium, Lead, Mercury)

AAS specification	Elements			
	Arsenic	Cadmium	Lead	Mercury
Wavelength	193.70	228.30	283.70	253.7
Current (mA)	5.0	5.0	5.0	5.0
Burner horizontal	0.20	0.30	0.20	0.20
Burner height (mm)	3.3	4.5	3.3	2.1
Fuel (L/min)	2.27	2.02	2.08	3.08
Pmt (V)	267.4	432.5	267.4	246.4

Table 3: Standard permissible limits for heavy metals as per WHO and FDA guidelines

Heavy metals	Permissible limit (PPM)
Arsenic	Not more than 3
Cadmium	Not more than 1
Mercury	Not more than 5
Lead	Not more than 1

WHO: World Health Organization, FDA: Food and Drug Administration

the collision-induced dissociation gas at a pressure of 350 kPa. The nebulizing, drying, and heating gas flow rates were set at 3.0, 10.0, and 10.0 L/min, respectively [28,29].

RESULTS AND DISCUSSION

Macroscopic assessment

The findings of the macroscopic and morphological analyses are shown in Table 4 and Fig. 1. Morphological assessment serves as a fundamental step in the authentication of crude drugs, as it involves the examination of external characteristics, including color, size, shape, taste, odor, and texture, which collectively aid in the identification and quality assurance of the plant material.

Microscopic studies

The transverse section of the *T. alternifolia* leaf (Fig. 2) exhibits a distinct dorsiventral structure with well-differentiated tissue organization. The upper epidermis is composed of a double layer of compactly arranged parenchymatous cells, covered externally by a thin cuticle that provides protection against desiccation and mechanical injury. A single layer of palisade parenchyma, which is composed of long cells that are closely packed with chloroplasts, lies beneath the epidermis and plays a major role in photosynthetic activity. The lower part of the mesophyll is made up of spongy parenchyma, which is made up of loosely packed, irregularly shaped cells with lots of intercellular gaps that allow gaseous exchange.

The midrib region is prominently developed and shows a centrally located bicollateral vascular bundle surrounded by compact parenchymatous ground tissue. The xylem is directed toward the upper epidermis, while the phloem is oriented toward the lower epidermis. The vascular bundle is encased by a well-defined bundle sheath composed of small, thin-walled parenchymatous cells.

Calcium oxalate crystals of micro-sphenoidal type are abundantly distributed throughout the mesophyll and midrib regions, often localized within parenchymatous cells, serving as a characteristic diagnostic feature of the species.

The polygonal epidermal cells have anticlinal walls that range from straight to somewhat wavy. Each of the paracytic-type stomata has two subsidiary cells that are positioned parallel to the guard cells. Quantitative microscopic parameters revealed an average stomatal number of 185±36.05/mm^2 (×15) and a stomatal index of 9±2.03%, indicating a moderate density of stomata on the leaf surface.

Table 4: Morphological evaluation of *Tabernaemontana alternifolia* leaves

Characteristics	*Tabernaemontana alternifolia* leaves
Apex	Acuminate
Margin	Entire
Base	Symmetrical
Petiole	Short
Length	15–16 cm
Width	5–6 cm
Color	Faint green
Odor	Aromatic
Taste	Bitter
Texture	Leathery

Fig. 1: Fresh leaf of *Tabernaemontana alternifolia*

Ash values

When evaluating crude pharmaceuticals' overall quality and purity, physicochemical criteria are essential. Among these, ash value determination provides insight into the presence of earthy materials, inorganic constituents, and extraneous impurities that may be associated with the herbal material [30]. The present investigation determined that the leaves of *T. alternifolia* had total ash, acid-insoluble ash, and water-soluble ash values of 5.67±0.76% w/w, 0.543±0.01% w/w, and 3.21±0.04% w/w, respectively.

Extractive values

The extractive value serves as an important parameter to assess the presence of active constituents and the efficiency of the extraction method employed. All extraction methods were performed on leaves exclusively, ensuring consistency in comparing their efficiencies. Among the various extraction techniques used for *T. alternifolia* leaves, the Soxhlet extraction method yielded the highest extractive value (17.93±1.37% w/w), indicating its superior efficiency in extracting phytoconstituents due to continuous solvent circulation and controlled heating [31]. The maceration method also produced a comparatively high yield (16.70±1.23% w/w), suggesting effective solvent penetration during prolonged contact at ambient conditions. In contrast, the

ultrasound-assisted (13.12±0.69% w/w) and microwave-assisted (13.62±0.37% w/w) extraction methods showed relatively lower yields, which may be attributed to shorter extraction durations and limited solvent-solute interaction. These results collectively suggest that Soxhlet extraction is the most efficient technique for obtaining a higher concentration of soluble phytoconstituents from *T. alternifolia* leaves under the studied conditions.

Qualitative phytochemical tests

The qualitative phytochemical analysis of *T. alternifolia* leaf extracts demonstrated the presence of alkaloids, flavonoids, phenols, tannins, saponins, reducing sugars, and proteins (Table 5). These bioactive compounds are recognized for their wide-ranging therapeutic properties, highlighting the medicinal potential of *T. alternifolia* leaves and supporting their prospective use in herbal formulations.

Total phenolic content

Phenolic compounds are a class of antioxidant agents that act as free radical terminators [23]. A standard gallic acid curve was used to determine the total phenolic content. In addition, Fig. 3 illustrates the results using the milligrams equivalent of gallic acid (mg GAE/g) ratio. Table 6 displays the outcomes of the total phenolic compounds. The line equation for the calibration curve with gallic acid as a standard is y=0.0019×+0.025 (R^2=0.9988). SD was not calculated for calibration standards as values represent instrument responses of prepared standard concentrations.

Table 5: Preliminary phytochemical screening of the *Tabernaemontana alternifolia* leaves extracts

Sr. No.	Phytochemical test	*Tabernaemontana alternifolia* leaf extract
1	Alkaloids	+
2	Cardiac glycoside	+
3	Flavonoids	+
4	Phenols	+
5	Saponin	+
6	Terpenoids	+
7	Carbohydrates	−
8	Steroids	−
9	Reducing sugar	+
10	Protein and amino acid	+
11	Anthraquinones	−
12	Tannins	+

(+) Present, (−) Absent; the detected phytochemicals indicate potential bioactive properties relevant to therapeutic applications

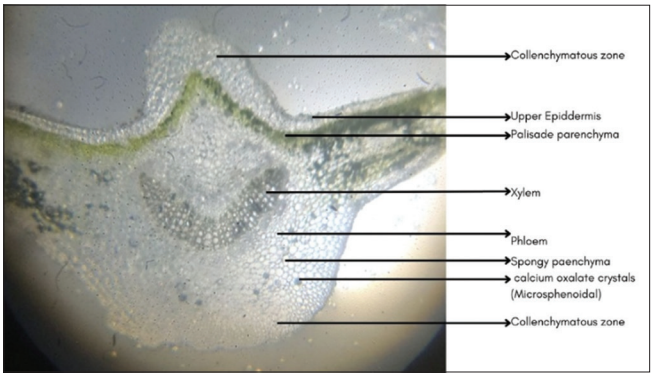

Fig. 2: Microscopic view of *Tabernaemontana alternifolia* leaf tissue without staining (15×)

Total flavonoid content

Flavonoids are a major class of phenolic compounds known for their significant role in the prevention and management of various diseases. Their strong antioxidant potential contributes to the reduction of oxidative stress and fat accumulation, thereby aiding in the treatment of obesity and associated metabolic disorders [32]. Bioactive metabolites such as flavonoids, tannins, saponins, and ellagic acid exert antioxidant effects by lowering oxidative stress levels in adipocytes, thus preventing obesity [33]. Several studies have reported that plants of the *Tabernaemontana* genus contain diverse flavonoid constituents,

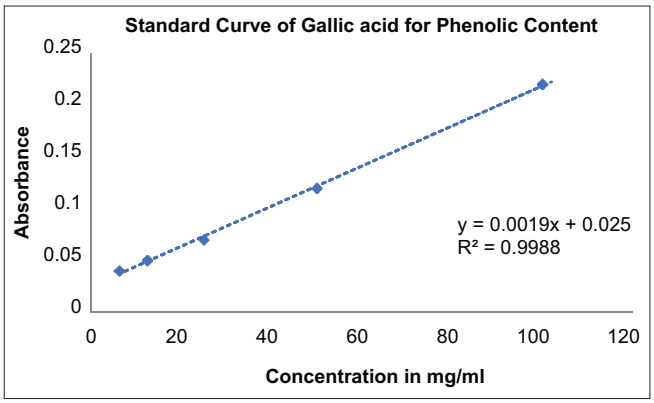

Fig. 3: Calibration curve of gallic acid for phenolic content: y=×0.0019+0.025, R^2=0.9988

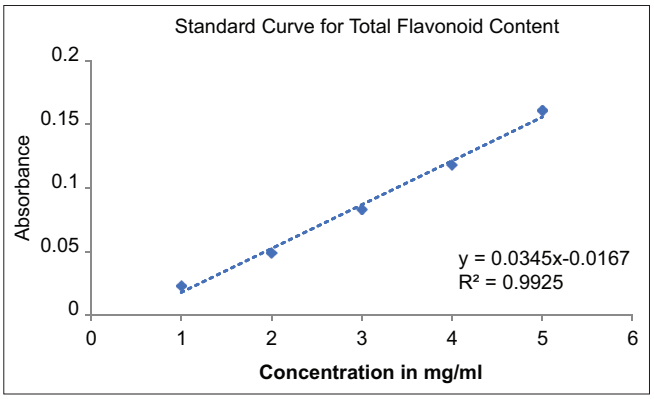

Fig. 4: Calibration curve of Rutin for flavonoid: y=×0.0345–0.0167, R^2=0.9925

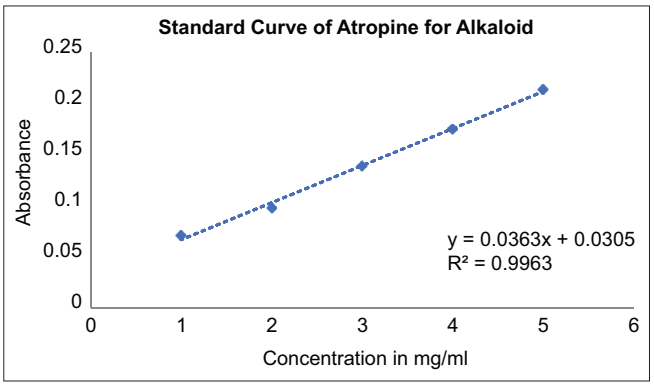

Fig. 5: Calibration curve of atropine for alkaloid: y=×0.0363+0.0305, R^2=0.9963

including rutin, robinin, and quercetin [34], Pinocembrin [10], and Kaempferol [35].

The total flavonoid content of the *T. alternifolia* extract is presented in Table 6. As shown in Fig. 4, which displayed the linear regression equation y=0.0345×–0.0167 with a correlation coefficient (R^2=0.9925), rutin was used as the reference standard for building the calibration curve.

Total alkaloid content

AE milligrams per gram of *T. alternifolia* extract were used to represent the overall alkaloid content. Table 6 presents the findings. As shown in Fig. 5, the calibration curve was prepared using Atropine as the standard. This resulted in the regression equation y=0.0363×+0.0305 with a correlation coefficient (R^2=0.9963).

In comparison with previously reported phytochemical profiles of *Tabernaemontana* species, the total phenolic and flavonoid contents determined in *T. alternifolia* were comparable to, and in certain cases exceeded, those reported for other members of the genus. For instance, *T. divaricata* leaf extracts have been reported to contain total phenolics of up to 48.8 mg GAE/g and flavonoids of up to 18.46 mg QE/g [36], while quantitative analyses of its root extracts by Khatoon *et al.* (2022) also demonstrated appreciable levels of these constituents [7]. Similarly, *T. catharinensis* has been shown to possess notable phenolic (23.34 mg GAE/g) and flavonoid contents in crude extracts, in line with its reported antioxidant potential [10]. Other species, such as *T. ventricosa*, exhibit variable yet significant levels of phenolics and flavonoids across different plant parts and solvent fractions [9]. These comparative data underscore the phytochemical richness of *Tabernaemontana* species and support the relevance of the quantitative values obtained in the present study.

Heavy metal analysis

Analysis using atomic absorption spectroscopy (AAS) was carried out in the positive ionization mode. The method was optimized using standard calibration curves prepared from various known concentrations. Calibration curves were constructed by plotting the instrumental response against the concentration, yielding a linear relationship for each metal. The heavy metals Cd, Pb, As, and Hg were analyzed at their specific wavelengths, and the ion exhibiting the highest intensity was selected as the primary analytical signal. The analysis revealed that no detectable spectral peaks corresponding to Cd, Pb, As, or Hg were observed in *T. alternifolia* leaves. The results obtained (Fig. 6a-d and Table 1) were evaluated against the maximum permissible limits for these metals as established by the WHO. As none of the targeted heavy metals were detected, the analyzed plant material can be considered safe for consumption and medicinal use. Concentrations reported as 0 ppm indicate that the levels of Cd, Pb, As, and Hg were below

Table 6: Total phenolic compound (TPC), total flavonoid content (TFC), and total alkaloid content (TAC) of *Tabernaemontana alternifolia*

Method of extraction	TPC (mg GAE/g TA)		TFC (mg RUT/g TA)		TAC (mg AE/g TA)	
	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation
SOX	10.37	0.15	7.53	0.30	2.7	0.20
MA	3.47	0.1664	7.03	0.15	1.9	0.15
USA	11.82	0.280	6.71	0.21	2.3	0.15
MAE	10.31	0.1258	7.32	0.23	4.0	0.05

Values are expressed as mean±SD (n=3). TPC: Total phenolic compound, TFC: Total flavonoid content, TAC: Total alkaloid content, SOX: Soxhlet, MA: Maceration, USA: Ultrasonic, MAE: Microwave assisted extraction, GAE: Gallic acid, RUT: Rutin, AE: Atropine, SD: Standard deviation

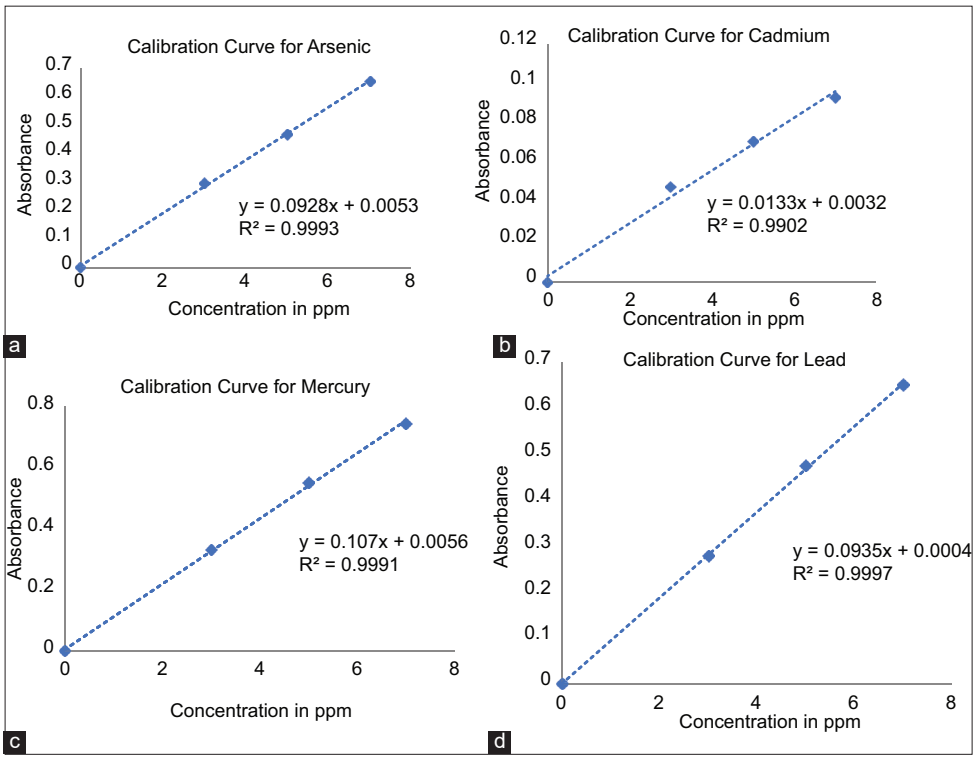

Fig. 6: Calibration curves for heavy metals in *Tabernaemontana alternifolia* leaf extracts: (a) arsenic; (b) cadmium; (c) mercury; (d) lead

Table 7: Quantitative analysis of aflatoxins (B1, B2, G1, and G2) in *Tabernaemontana alternifolia* leaves

Sample name	Sample type	Dilution factor	Area	Conc.pg/mL	Accuracy (%)
Aflatoxin B1	Standard	1.000	938737	20.000	100
Aflatoxin B2	Standard	1.000	686409	20.000	100
Aflatoxin G1	Standard	1.000	759774	20.000	100
Aflatoxin G2	Standard	1.000	257207	20.000	100
TA analysis for (Aflatoxin B1)	Sample	1.000	0.500	N.D.(W/B)	-
TA analysis for Aflatoxin B2	Sample	1.000	311	0.009	-
TA analysis for Aflatoxin G1	Sample	1.000	249	0.007	-
TA analysis for Aflatoxin G2	Sample	1.000	303	0.024	-

N.D. (W/B): Not detected (Within Blank)

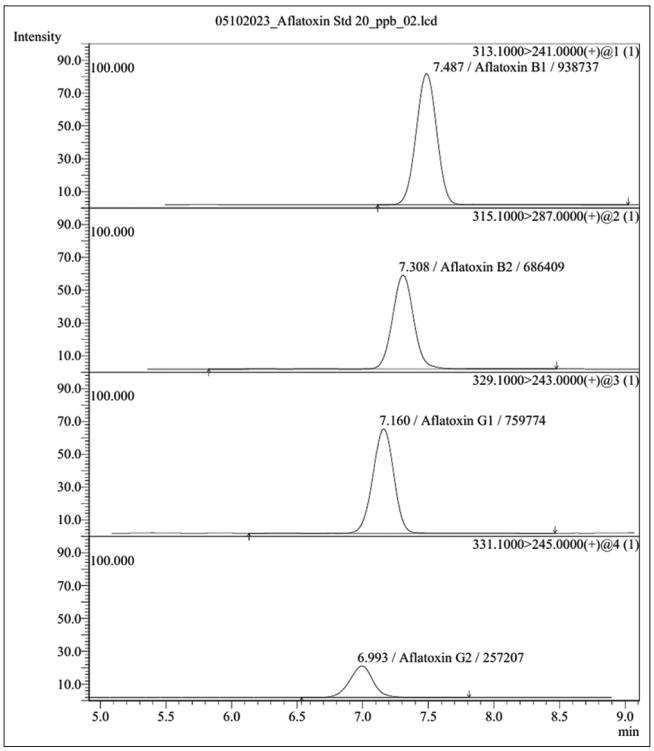

Fig. 7: Chromatogram of standard Aflatoxins B1, Aflatoxins B2, Aflatoxins G1, Aflatoxins G2obtained using liquid chromatography-tandem mass spectrometry (MS)/MS in multiple reaction monitoring mode

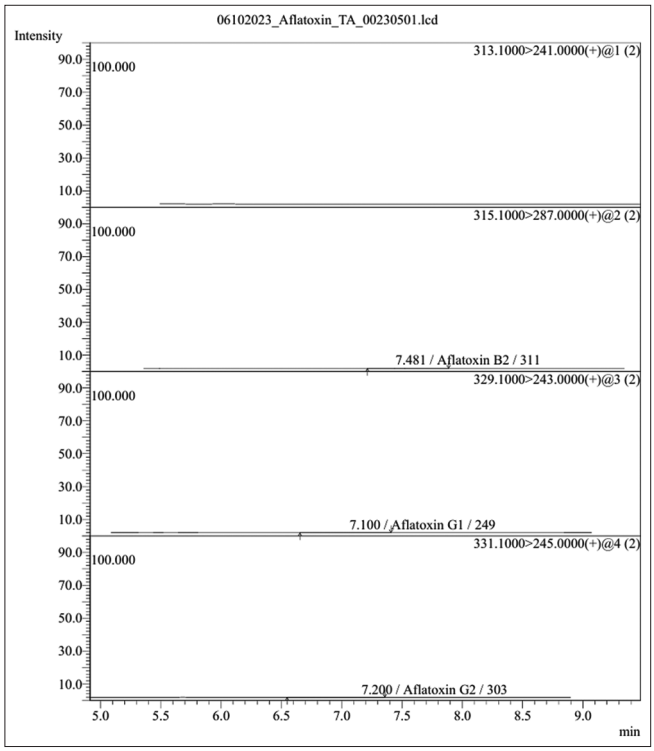

Fig. 8: Chromatogram of *Tabernaemontana alternifolia* leaves for Aflatoxins B1, Aflatoxins B2, Aflatoxins G1, Aflatoxins G2 obtained using liquid chromatography-tandem mass spectrometry (MS)/MS in multiple reaction monitoring mode

the analytical sensitivity of the AAS method. The limits of detection (LOD) and limits of quantification were determined using signal-to-noise ratios of 3:1 and 10:1, respectively; signals below the LOD were therefore considered not detected and reported as 0 ppm rather than implying absolute absence.

Determination of aflatoxins

The aflatoxin analysis of *T. alternifolia* leaf samples was performed using a validated standard calibration approach. The standard solutions of aflatoxins B1, B2, G1, and G2 exhibited expected concentrations of 20 pg/mL with 100% accuracy, confirming the reliability of the method. In the analyzed plant samples, aflatoxin B1 was not detected, whereas trace amounts of aflatoxins B2, G1, and G2 were observed at concentrations of 0.009, 0.007, and 0.024 pg/mL, respectively (Table 7). These values are substantially lower than the permissible limits established for medicinal plant materials, indicating negligible contamination. In addition, the aflatoxin B1 result has been clarified by explicitly stating that the observed trace area signal was below the validated LOD of the LC-MS/MS method and therefore does not represent a quantifiable presence of aflatoxin B1. Overall, the results demonstrate that the leaf samples are essentially free from aflatoxin contamination, confirming their safety for medicinal use (Figs. 7 and 8).

CONCLUSION

The present investigation provides the first comprehensive pharmacognostic and quantitative phytochemical characterization of *T. alternifolia* leaves. Distinct macroscopic and microscopic diagnostic features, together with established physicochemical parameters, offer reliable benchmarks for authentication and quality control. Quantitative analysis revealed appreciable levels of phenolics, flavonoids, and alkaloids, underscoring the phytochemical richness and therapeutic relevance of the plant. Importantly, heavy metals were not detected, and aflatoxins were either absent or present only at trace levels well below the maximum permissible limits recommended by the WHO, thereby confirming the safety and regulatory compliance of the studied plant material for medicinal applications. While these findings establish essential baseline data for standardization and quality evaluation, future investigations should focus on the isolation and structural characterization of individual bioactive alkaloids and other secondary metabolites. In addition, systematic *in vivo* pharmacological

and toxicological studies are warranted to substantiate the biological efficacy and safety of *T. alternifolia*, facilitating its rational development into phytopharmaceutical and nutraceutical formulations.

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AUTHOR CONTRIBUTION

Ms. Pallavi Bhokare and Dr. Prasad Kadam conceptualized and designed the study, with Pallavi Bhokarehandling data collection. Dr. Prasad Kadamconducted data analysis and prepared the initial draft of the article. Dr. Manohar Patil supervised the study, contributed to data analysis and interpretation, and provided essential revisions. All authors have reviewed and approved the final version of the manuscript. The authors confirm that no paper mill or artificial intelligence was used.

COMPETING INTERESTS

The authors declare no conflict of interest.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CLINICAL TRIAL NUMBER

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

All data are available upon request.

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