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Integrated Phytochemical Characterisation and Toxicological Assessment of *Tabernaemontana divaricata* Leaves using LC-MS/MS-QTOF and Molecular Docking Approaches

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Abstract

Background: *Tabernaemontana divaricata* is a medicinal plant with considerable phytochemical and therapeutic potential; however, comprehensive quality, safety, and metabolite standardisation data are limited. **Aim:** To comprehensively evaluate the pharmacognostic characteristics, phytochemical profile, safety, metabolite composition, and potential bioactivity of *T. divaricata* leaves to support their standardisation and therapeutic relevance. **Methods:** Macroscopic and microscopic characteristics, physicochemical parameters, extractive values, and preliminary phytochemical profiles were evaluated. Total Phenolic Content (TPC), Total Alkaloid Content (TAC) and Total Flavonoid Content (TFC) were determined using validated colorimetric methods. Heavy metals (Pb, Cd, As, and Hg) were assessed by atomic absorption spectroscopy, while aflatoxins were analysed by LC-MS/MS. LC-MS/MS-QTOF was used for metabolite identification, and molecular docking was performed against human cytosolic β-glucosidase (PDB ID: 2ZOX). **Results:** Soxhlet extraction yielded the highest overall extractive value (19.10 ± 0.78% w/w), whereas ultrasound-assisted extraction showed the highest TPC (13.24 ± 0.2343 mg GAE/g) and microwave-assisted extraction the highest TAC (1.751 ± 0.0651 mg AE/g) and Soxhlet extraction yielded the highest TFC (9.1353 ± 0.0251mg RUT/g). Major phytochemical classes, including alkaloids, flavonoids, phenolics, glycosides, terpenoids, steroids, proteins, and tannins, were detected. Heavy metals were below detectable levels, while aflatoxins were within permissible regulatory limits. LC-MS/MS-QTOF identified 25 putative bioactive compounds based on characteristic fragmentation patterns. Molecular docking revealed strong binding affinities for several compounds, with Flavidulol showing the highest docking score (-27.18 kcal/mol), compared with Acarbose (-8.30 kcal/mol). **Conclusion:** The integrated pharmacognostic, phytochemical, toxicological, metabolomic, and *in silico* approach demonstrates the phytochemical richness and safety of *T. divaricata* leaves and provides a comprehensive framework for their standardisation, quality control, and further pharmacological investigation.

Major Findings: The major findings demonstrate that *T. divaricata* leaves possess a rich phytochemical profile with good quality and safety, including undetectable toxic heavy metals and aflatoxin levels within permissible limits. LC-MS/MS-QTOF identified 25 bioactive compounds, while molecular docking revealed strong target interactions, with Flavidulol showing the highest affinity (-27.18 kcal/mol) against human cytosolic β-glucosidase.

Keywords: Aflatoxins, Alkaloids, Flavonoids, Heavy Metal Analysis, Herbal Standardisation, Phenolic Content, *Tabernaemontana divaricate*

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1. Introduction

Herbal medicine has long been a vital component of basic healthcare systems around the world. Eighty percent of people worldwide are thought to utilise herbal treatments for medical purposes[1-3]. The growing awareness of the adverse effects associated with allopathic medicines, coupled with the perceived benefits of herbal therapies, has significantly increased the demand for plant-based medicinal products[4]. However, fraudulent activities have also increased in parallel with this growing demand, such as the use of synthetic adulterants and the replacement of authentic botanical products[5]. As a result, the implementation of rigorous standardisation and quality control measures has become imperative. Furthermore, the intrinsic toxicity of some medicinal plants may also be the cause of side effects linked to herbal remedies, in addition to adulteration, contamination, or incorrect plant species identification. This emphasises the need for thorough toxicological assessment in order to reduce possible safety hazards. Furthermore, the safety and effectiveness of herbal medicines may be further endangered by the inclusion of external pollutants such as pesticide residues, heavy metals, and microbiological agents[6]. The chemical composition and purity of herbal materials can be significantly impacted by variations in cultivation procedures, harvesting techniques, storage conditions, processing processes, and environmental exposure. Therefore, to guarantee the safety, consistency, and therapeutic efficacy of herbal medications, thorough quality assessment and strong standardisation procedures are crucial[7].

J. Thomson Muller, a German physician and botanist, was the first to describe *T. divaricata*, a member of the Apocynaceae family[8]. The genus comprises approximately 100 species distributed across Africa, Asia, Oceania, and the Americas[9]. *T. divaricata* is found throughout the upper Gangetic plains, Garhwal, East Bengal, Kasia Hills, Assam, Burma, and the hills of Visakhapatnam in India[10]. The plant is usually stated to as "milkweed" due to the presence of milky latex. It is also known by several synonyms, including *Ervatamia coronaria*, *Ervatamia microphylla*, *Ervatamia divaricata*, and *Tabernaemontana coronaria*. Common vernacular names include "Crape Jasmine"[11].

Tabernaemontana divaricata is an evergreen shrub with silvery-grey bark and copious amounts of milky latex that grows to a height of around 1.8 to 2.4 m. The leaves have an acuminate tip and slightly undulating margins. They are simple, opposite, elliptic to elliptic-lanceolate in shape, glossy green in colour, and smooth in texture. The plant flowers throughout the year, with peak blooming during spring and summer. The flowers are waxy, pure white, and ruffled at the edges, emitting a strong fragrance, particularly at night. At branch bifurcations, they are grouped in cymes with one to eight flowers; at the bud stage, the corolla lobes overlap to the right. The seeds are irregularly shaped, dark brown, and encased in a pulpy, crimson aril[12]. Ethnomedicinal claims related to *T. divaricata* have been supported by several pharmacological investigations[13]. Its analgesic, anti-inflammatory, antibacterial, antioxidant, antidiabetic, and anticancer properties have been documented in earlier research. The existence of several bioactive secondary metabolites is largely responsible for these biological characteristics. The plant has therapeutic potential due to the presence of indole alkaloids, flavonoids, phenolic compounds, terpenoids, saponins, and glycosides, according to phytochemical studies[14].

Phytochemical profiling and standardisation are essential components of quality control in herbal medicines. Qualitative and quantitative evaluation of bioactive constituents facilitates authentication, detection of adulteration, and maintenance of batch-to-batch consistency in herbal formulations. Furthermore, the development of phytochemical fingerprints and marker-based analytical approaches is fundamental for ensuring the reliability of herbal drugs and promoting their acceptance within modern healthcare systems[15]. Consequently, comprehensive phytochemical characterisation remains a prerequisite for the scientific validation and therapeutic application of medicinal plants.

In addition to phytochemical variability, contamination of herbal materials with toxic substances represents a significant public health concern. Herbal drugs are particularly vulnerable to fungal contamination resulting from inadequate drying, storage, and handling conditions, which may lead to aflatoxin production. Regulators have set acceptable limits for aflatoxins in herbal goods to protect consumer health[16]. Likewise, heavy metal contamination has

emerged as a major safety issue in herbal medicines. Toxic metals like Lead (Pb), Cadmium (Cd), Arsenic (As), and Mercury (Hg) may enter plant materials from contaminated soil, irrigation water, atmospheric deposition, agrochemicals, and industrial activities. These metals can accumulate in plant tissues and pose serious risks to human health. As a result, routine monitoring of heavy metal levels in medicinal plants has become mandatory under pharmacopoeial and regulatory guidelines[17].

Although several studies have reported the pharmacological and phytochemical properties of *T. divaricata*, systematic investigations integrating phytochemical composition, aflatoxin contamination, and heavy metal content within a single framework remain limited. The absence of such comprehensive data hinders the development of standardised herbal formulations and poses challenges to regulatory compliance. Accordingly, the present study was undertaken to conduct an integrated quality assessment of *T. divaricata* leaves by combining phytochemical profiling with aflatoxin and heavy metal evaluation. The specific objectives of this investigation include qualitative and quantitative analysis of major phytoconstituents, determination of aflatoxin levels using validated analytical methods, and estimation of toxic heavy metals in accordance with international regulatory guidelines.

2. Materials and Methods

2.1 Collection and Identification of Plant Material

In August 2023, leaves of *T. divaricata* L. were gathered from the Karad area of the Satara district in Maharashtra, India. Dr. D. L. Shirodkar, a botanist from the Botanical Survey of India's Western Regional Centre in Pune, verified the plant material's taxonomy. A voucher specimen with the specimen number TDBPV-2 was placed for later use. In compliance with WHO guidelines, freshly collected leaves were subjected to preliminary macroscopic and microscopic examination, thoroughly washed with potable water to remove extraneous matter, and shade-dried at room temperature under controlled conditions. The dried material was then coarsely powdered utilising a suitable grinder and stored in a well-closed, light-resistant container at ambient conditions until further pharmacognostic and phytochemical analysis.

2.2 Macroscopic or Organoleptic Evaluation

Organoleptic evaluation of the freshly collected leaves was carried out to assess fracture, size, shape, taste, odour, and other surface characteristics[18].

2.3 Microscopic Evaluation

Free-hand transverse sections of the leaves were produced and made somewhat translucent for microscopic examination. Anatomical features, including the structure of the lamina and midrib, were examined under an optical microscope. Diagnostic microscopic characters were observed utilising light microscopy at appropriate magnifications. To explore the anatomical characteristics of the leaf tissue, the sections were placed on a glass slide utilising distilled water, covered with a cover slip, and inspected under a microscope[19].

2.4 Ash Value

The entire ash value was calculated by precisely weighing two grams of the powdered drug into a silica China dish that had been previously weighed. The sample was then burnt at 455°C in a muffle furnace (Wise Therm) until all carbon was removed, signifying that all biological matter had been totally oxidised. After cooling in a desiccator, the ash-filled dish was weighed. The usual formula was used to get the total ash content, which was then represented as a percentage of air-dried material.

After adding 25 mL of 2 M hydrochloric acid to the total ash, the mixture was heated in a water bath for five minutes to determine the amount of acid-insoluble ash. To get rid of soluble contaminants, the final solution was filtered utilising Whatman No. 42 ashless filter paper and carefully cleaned with warm water. The filter paper containing the insoluble residue was burnt at 455°C, cooled in a desiccator, and weighed after being put in a silica China dish that had previously been weighed. The proportion of acid-insoluble ash in the air-dried sample was calculated[20].

The amount of water-soluble ash was determined by filtering the insoluble material through ashless filter paper after the full amount of ash was boiled with 25

mL of distilled water for five minutes. The filter paper containing the insoluble residue was incinerated at a temperature below 450°C, then chilled in a desiccator before being weighed. By deducting the weight of the insoluble residue from the total ash, the water-soluble ash content was calculated as a percentage of the air-dried medications[21].

2.5 Extractive Values of Plant Material

2.5.1 Maceration

The powdered leaves of *T. divaricata* were extracted using the maceration technique following the method described by researchers[22], with slight modifications. Methanol was added as the extraction solvent after precisely weighing and transferring 5 g of the leaf powder into conical flasks. To stop sample loss and solvent evaporation during the extraction procedure, the flasks were firmly sealed with aluminium foil. To ensure effective phytoconstituent extraction, the combinations were thereafter put in a shaking water bath and shaken at a speed of 9 for a whole day at room temperature. Following maceration, the extracts were first filtered through Whatman No. 41 filter paper to eliminate coarse particles, and then they were further filtered through Whatman No. 1 filter paper to produce clear filtrates. The filtrates were subsequently collected and stored for further phytochemical and analytical investigations.

2.5.2 Soxhlet Extraction

A 250 mL round-bottom flask with a condenser and methanol as the extraction solvent was linked to a Soxhlet extractor that held a 5 g powdered sample of *T. divaricata* leaves in a thimble. The apparatus was mounted on a heating mantle, allowing the solvent to evaporate, condense, and percolate through the plant material in a continuous cycle. The extraction was carried out at a controlled temperature of 65°C for 72 h. During this process, the condensed solvent accumulated in the extraction chamber and, upon reaching the siphon level, was recycled back into the round-bottom flask, ensuring efficient extraction. Following the extraction process, the extract solution was allowed to reach room temperature before being filtered through Whatman No. One piece of filter paper to get rid of particles. The filtrate was then concentrated under reduced pressure using a rotary evaporator[23] and further dried to obtain a solid residue. For later analysis, the dried extract was kept at 4°C.

2.5.3 Ultrasonic Extraction

Ultrasound-assisted extraction was carried out in an ultrasonic bath. 5 g of powdered *T. divaricata* leaves were extracted utilising 100 mL of methanol and sonicated for fifteen minutes at room temperature. The extract was obtained by filtering the resultant mixture and evaporating the filtrate until it was completely dry[24].

2.5.4 Microwave Assisted Extraction

Microwave-assisted extraction was carried out by placing 5 g of dried and powdered leaves and stems of *T. divaricata* 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 super boiling was avoided. The obtained extract was filtered and concentrated to dryness utilising a tray dryer maintained at 40°C[25].

2.6 Qualitative Phytochemical Tests

The phytochemical evaluation of *T. divaricata* leaf extract was conducted using established qualitative protocols encompassing eleven categories of secondary metabolites. Alkaloids were found utilising Hager's, Wagner's, Mayer's, and Dragendorff's reagents; flavonoids were verified utilising the alkaline reagent, lead acetate, and Shinoda tests. Fehling's and Benedict's tests were used to identify reducing sugars, while Molisch's, iodine, Benedict's, and Fehling's reactions were used to confirm carbohydrates. Glycosides were analysed 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[18].

2.7 Total Phenolic Content

The Total Phenolic Content (TPC) of *T. divaricata* leaf extract was determined using the Folin-Ciocalteu method described by researchers[26], with slight modifications. The extract was first diluted in methanol to produce a stock solution of 1 mg/mL. After adding

20 µL of the stock solution and 100 µL of 10% Folin-Ciocalteu reagent to a 96-well microplate, the mixture was left in the dark for five minutes to allow for the initial reaction. After adding 80 µL of a 7.5% sodium carbonate (Na_2CO_3) solution, the mixture was incubated for one to two hours at room temperature to guarantee full colour development. The absorbance at 760 nm was measured using a microplate reader. Every analysis was done in triplicate to ensure that the results were accurate and repeatable. A standard calibration curve was created using gallic acid values ranging from 12.5 to 800 µg/mL. The calibration curve was utilised to determine the total phenolic content, which was then represented as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

2.8 Total Flavonoids Content

The Total Flavonoid Content (TFC) of *T. divaricata* leaf extract was determined using the aluminium chloride colorimetric method described by researcher[27], with slight modifications. 100 µL of the extract solution (1 mg/mL) and 100 µL of a 2% Aluminium Chloride ($AlCl_3$) solution were mixed, and the mixture was left to form a complex at room temperature for ten minutes. The absorbance of the final solution at 415 nm was measured utilising a UV-visible spectrophotometer. Every measurement was made three times to ensure accuracy and consistency. A standard calibration curve was created using rutin at values ranging from 10 to 400 µg/mL. The total flavonoid content was calculated using the calibration curve and expressed as milligrams of rutin equivalents per gram of extract (mg RE/g extract).

2.9 Total Alkaloids Content

The total alkaloid content of the methanolic extract of *T. divaricata* leaves was quantified by the Bromocresol Green (BCG) colorimetric method with minor modifications. 0.5 g of the crude methanolic extract was accurately dissolved in methanol, and 1 mL of this solution was transferred into individual separatory funnels. To each funnel, 5 mL of bromocresol green reagent along with phosphate buffer (pH 4.7) was added to facilitate complex formation. Chloroform was then added sequentially in volumes of 1, 2, 3, and 4 mL, with vigorous shaking after each addition to ensure efficient extraction of the alkaloid-BCG complex into the organic layer. The chloroform fractions were carefully separated and pooled into 10 mL volumetric flasks, and the final volume was adjusted with chloroform[28]. To create a calibration curve, atropine standard solutions with concentrations of 0.4, 0.6, 0.8, 1.0, and 1.2 µg/mL were made and put through the same extraction process. A UV-visible spectrophotometer was used to measure the absorbance of both standard and sample solutions at 470 nm in comparison to a reagent blank. The standard calibration curve was used to determine the total alkaloid content, which was then represented as milligrams of atropine equivalents per gram of extract (mg AE/g extract).

2.10 Heavy Metal Analysis

To ensure total moisture removal, the obtained samples were finely powdered and dried under controlled circumstances at 55-70°C for 6-8 h. Following drying, 12 mL of Concentrated Nitric Acid (HNO_3) was added to digestion flasks containing 1.0 g of each sample that had been precisely weighed. For a whole day, the combination was left to predigest. The samples were then digested at 120-130°C for 5-6 h until brown fumes stopped and a clear solution formed, signifying full digestion. This was followed by the addition of 5 mL of an acid combination made up of Sulphuric Acid (H_2SO_4) and Nitric Acid (HNO_3) in a 3:1 (v/v) ratio.

After the digested solutions cooled to room temperature, Whatman No. 42 filter paper was used to filter them. The filtrates were quantitatively transferred into 50 mL volumetric flasks and diluted to volume using Milli-Q water. To guarantee analytical accuracy, reagent blanks were made concurrently using the same process. Each sample was evaluated twice, and the procedure was repeated five times to ensure reproducibility.

Elemental analysis was carried out utilising an Atomic Absorption Spectrophotometer (AAS) (Model AA-6300, Shimadzu, Japan) equipped with flame and graphite furnace atomisers, operating within a wavelength range of 185-900 nm and fitted with a photomultiplier detector covering 185-600 nm. Every solvent and reagent utilised was of analytical quality[29]. Standard stock solutions (1000 ppm) of Cd, Pb, As, and Hg were employed to prepare working standard solutions within the concentration ranges specified, and the instrumental operating parameters are presented in Table 1.

2.11 Determination of Aflatoxins

An LCMS-8040 N-series UPLC system (Shimadzu, Japan) was used to develop a highly sensitive and selective LC-MS/MS technique for aflatoxin measurement. Using a Kinetex C18 column (2.1 mm × 100 mm, 1.7 µm particle size) kept at 40°C and a steady flow rate of 0.5 mL/min, chromatographic separation was accomplished. Solvent A (5 mM ammonium acetate in water with 0.1% formic acid) and solvent B (5 mM ammonium acetate in methanol) made up the mobile phase. They were applied in a gradient elution mode as follows: 5% B (0-4 min), 50% B (4-5.5 min), 85% B (6-7.5 min), and re-equilibration to 5% B (8.1-10 min). A fixed injection volume of 5 µL was utilised.

For mass spectrometric detection, two different transitions were tracked for every analyte utilising a heated Electrospray Ionisation (ESI) source operating in positive ionisation mode under Multiple Reaction Monitoring (MRM) conditions. The temperatures at the interface, heat block, and Desolvation Line (DL) were kept at 350°C, 400°C, and 250°C, respectively. The Collision-Induced Dissociation (CID) gas was argon at 350 kPa. Nebulising, drying, and heating gas flow rates were set at 3.0, 10.0, and 10.0 L/min, respectively[30,31].

2.12 LC-MS/MS Characterisation of Extract

A previously reported method was used to identify and map compounds from *T. divaricata* leaf extract obtained by Soxhlet extraction[32]. Analysis was performed using an Agilent 6520 Accurate-Mass QTOF in both positive and negative ion modes. Separation was achieved on a Synergi Hydro-RP Column with a flow rate of 800 µL/min and an injection volume of 15 µL using a gradient mobile phase consisting of (A) 0.1% formic acid in water and (B) 95% acetonitrile with 0.1% formic acid. Mass spectra were recorded in the range of 100-1000 amu under optimised instrument parameters, and phenolic compounds were identified using MassHunter Workstation Software.

Table 1. Atomic absorption spectroscopy instrumentation for heavy metal analysis (Arsenic, Cadmium, Lead, and 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

2.13 Molecular Docking

The structures of the LC-MS/MS-identified compounds in the active fraction and key target proteins were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do), respectively. Molecular docking was performed by analysing the binding positions and binding energies of each ligand. The docking study focused on the crystal structure of the Covalent Intermediate of Human Cytosolic beta-Glucosidase (PDB ID: 2ZOX), obtained from the PDB (https://www.rcsb.org/). Protein preparation was carried out using Molsoft ICM Pro, which involved adding hydrogen atoms, removing water molecules, assigning partial charges, determining protonation states, and applying restraints[33]. After ligand removal, binding sites were identified, and a grid box was generated for docking[34]. Each LC-MS/MS-identified compound in the active fraction was docked into the designated binding site within the grid using Molsoft ICM Pro's Glide module, a rapid and efficient docking program designed for small-molecule docking[35,36]. The program incorporates a scoring function that evaluates shape complementarity, electrostatic interactions, and van der Waals forces between the ligand and receptor[37]. Key interactions at the active site and corresponding docking scores were thoroughly analysed.

3. Results and Discussion

3.1 Macroscopic or Organoleptic Evaluation

The organoleptic and morphological evaluation of *T. divaricata* leaves was systematically carried out, and the results are presented in Figure 1. The leaves exhibited distinct and consistent characteristics, including an acuminate apex, an entire margin, and a symmetrical base, reflecting uniform laminar development. The petiole was short, supporting a well-developed lamina

measuring approximately 10-12 cm in length and 4-6 cm in width. The leaves were green in colour and possessed an aromatic odour with a characteristic bitter taste. The lamina texture was leathery, indicating structural firmness and durability. For the identification and standardisation of *T. divaricata* leaves in pharmacognostic and microscopic investigations, these morphological characteristics function as trustworthy diagnostic criteria.



Figure 1. A leaf of *T. divaricata*.

3.2 Microscopic Evaluation

The microscopic evaluation of *T. divaricata* leaves revealed characteristic anatomical features that are useful for pharmacognostic identification and quality control (Figure 2). The lamina was protected and supported structurally by the single layer of compactly packed parenchymatous cells that made up the upper epidermis. Cluster-type calcium oxalate crystals were prominently observed in the midrib region, serving as important diagnostic markers. The stomata were predominantly paracytic in nature, with each stomatal aperture accompanied by two subsidiary cells aligned parallel to the guard cells. Quantitative microscopy indicated a stomatal number of 200 ± 25 per mm^2, while the stomatal index was recorded as 10.85 ± 4.09%.

It is dorsiventral in nature, Upper epidermis and lower epidermis are made up of single layered of rectangular cells which are covered with cuticle, Strips of collenchyma present below upper epidermis and

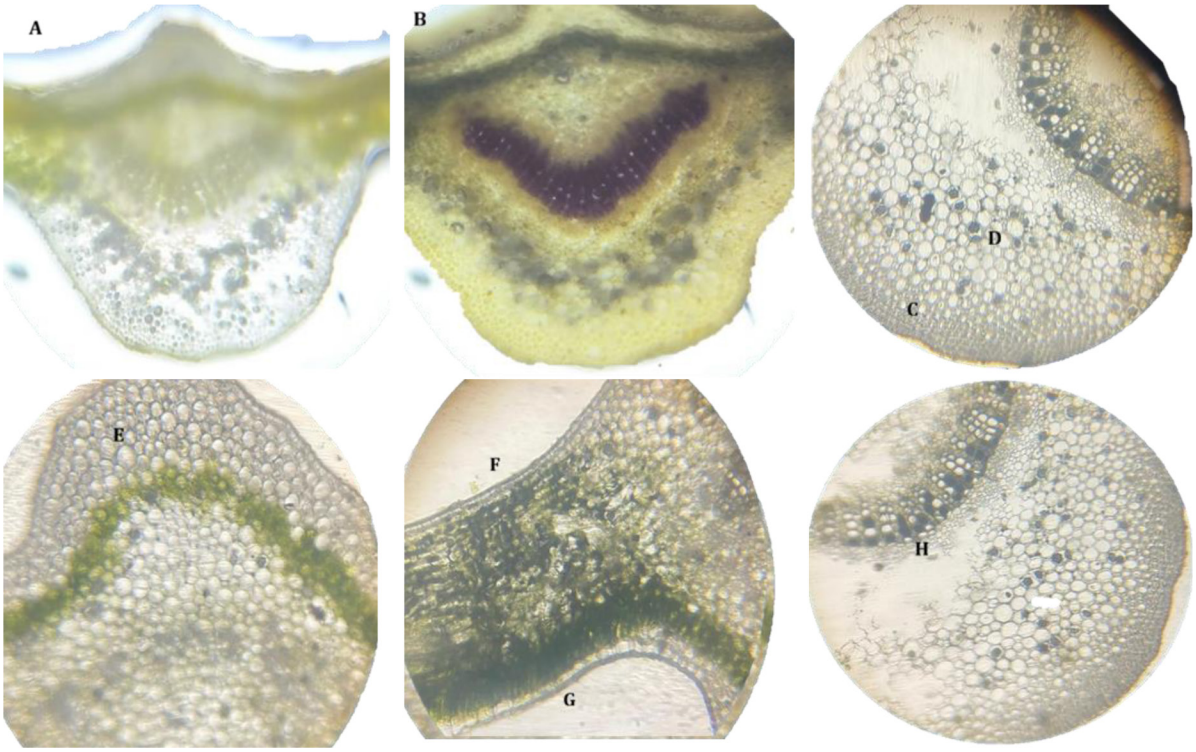


Figure 2. Transverse section of leaves of *T. divaricata,* A. T.S. of Leaf; B. T. S. Stained with Phloroglucinol and Conc. HCL; C. Collenchyma; D. Calcium oxalate crystals; E. Strips of Collenchyma; F. Upper epidermis; G. Lower epidermis; H. Vascular bundle.

above lower epidermis, Mesophyll contain Palisade cell, Bicollateral vascular bundle present in the midrib, compactly arranged spongy parenchyma present below the vascular bundle and contain cluster crystals types of calcium oxalate.

These consistent microscopic features contribute to the authentication and standardisation of *T. divaricata* leaves and support their identification in pharmacognostic and botanical investigations.

3.3 Ash Value

Physicochemical parameters are essential for evaluating the overall quality, purity, and authenticity of crude pharmaceutical materials. Among these, ash value determination provides valuable information regarding the presence of earthy matter, inorganic constituents, and extraneous impurities associated with herbal drugs[38]. In the present study, *T. divaricata* leaf powder exhibited a total ash content of 4.90 ± 0.12% w/w, an acid-insoluble ash value of 0.612 ± 0.01% w/w, and a water-soluble ash value of 1.73 ± 0.24% w/w. These findings indicate acceptable purity levels and contribute to the standardisation and quality control of *T. divaricata* leaf material.

3.4 Extractive Values of Plant Material

Extractive value is a key parameter for evaluating the presence of bioactive constituents and the efficiency of the extraction process. Among the different extraction techniques applied to *T. divaricata* leaves, the Soxhlet method yielded the highest extractive value (19.10 ± 0.78% w/w), indicating superior extraction efficiency. Microwave-assisted extraction also produced a relatively high yield (13.56 ± 1.10% w/w). In comparison, ultrasound-assisted (12.97 ± 0.41% w/w) and maceration (14.13 ± 1.53% w/w) methods resulted in comparatively lower extractive values. Soxhlet extraction is the most effective technique for obtaining a higher concentration of soluble phytoconstituents from *T. divaricata* leaves under the investigated conditions.

3.5 Qualitative Phytochemical Tests

Alkaloids, glycosides, flavonoids, phenolic compounds, terpenoids, steroids, proteins, amino acids, saponins, and tannins were present in *T. divaricata* leaf extracts, according to preliminary phytochemical screening (Table 2).

Table 2. Preliminary phytochemical screening of *T. divaricata* leaf extracts

Phytochemical Test	*T. divaricata* Leaf Extract
Alkaloids	+
Glycoside	+
Flavonoids	+
Phenols	+
Saponin	+
Terpenoids	+
Carbohydrates	-
Steroids	+
Reducing Sugar	-
Protein and amino acid	+
Anthraquinones	-
Tannins	+

(+) Present, (-) Absent

3.6 Total Phenolic Content

Phenolic chemicals are a significant class of antioxidant agents that act as molecules that break chains and scavenge free radicals[39]. A gallic acid standard calibration curve was used to calculate the TPC. The results are shown in milligrams of gallic acid equivalents per gram of extract (mg GAE/g) in Figure 3 and Table 3. The calibration curve, which employed gallic acid as the reference standard, showed a significant correlation coefficient (R^2 = 0.9988) and a linear regression equation of y = 0.0019x + 0.025.

3.7 Total Flavonoids Content

A significant class of phenolic chemicals, flavonoids are essential for managing and preventing a number of disorders. Their potent antioxidant activity helps to regulate obesity and associated metabolic diseases by reducing oxidative stress and lipid buildup[40]. Bioactive metabolites, including flavonoids, tannins, saponins, and ellagic acid, exert protective effects by reducing oxidative stress in adipocytes, thereby inhibiting obesity development[41]. The total flavonoid content of *T. divaricata* extract is shown in Table 3. The calibration curve was built utilising rutin as the reference standard, as shown in Figure 4. It displayed a linear regression equation of y = 0.0345x − 0.0167 with a significant correlation coefficient (R^2 = 0.9925).

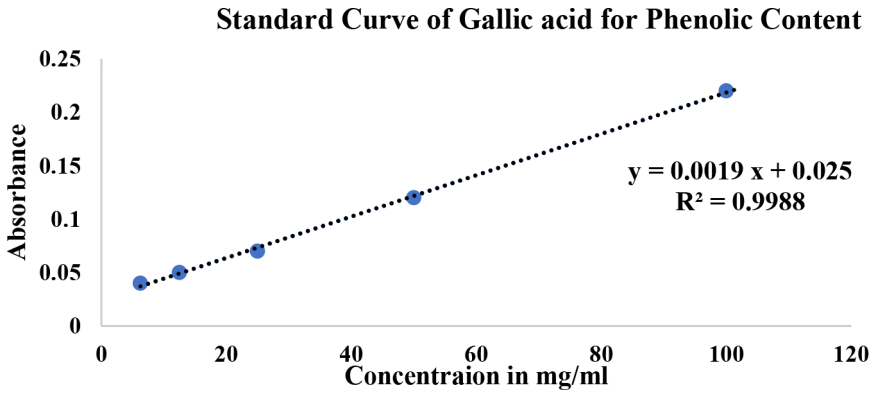


Figure 3. Calibration curve of gallic acid standard for total phenolic content.

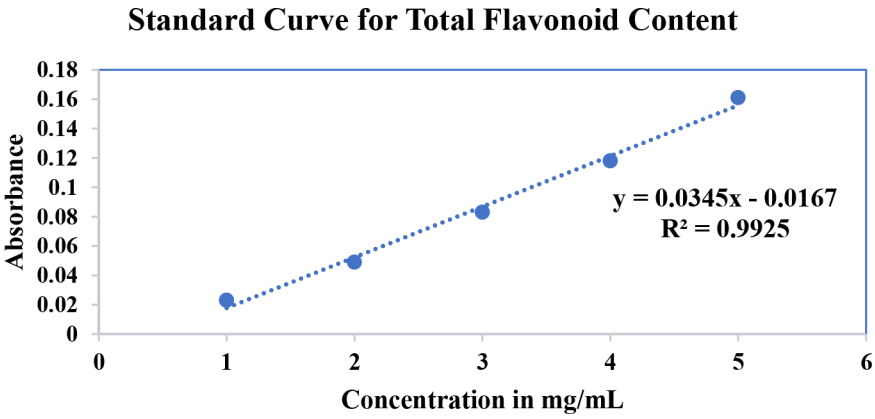


Figure 4. Calibration of rutin standard for total flavonoids content determination.

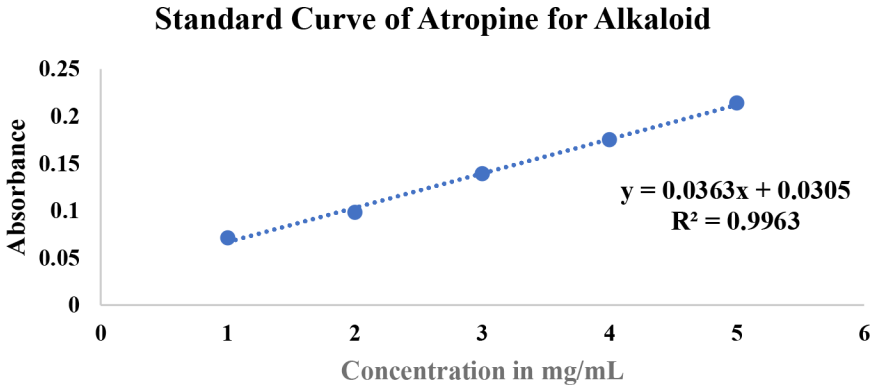


Figure 5. Calibration of Atropine standard for total alkaloids content determination.

3.8 Total Alkaloids Content

The total alkaloid content of the *T. divaricata* extract was quantified and expressed as milligrams of atropine equivalents per gram of extract. The corresponding results are presented in Table 3. A calibration curve was constructed using atropine as the reference standard, yielding a linear regression equation of $y = 0.0363x + 0.0305$ with a high correlation coefficient ($R^2 = 0.9963$), indicating excellent linearity. The calibration graph is depicted in Figure 5.

The extraction methods exhibited distinct efficiencies with respect to overall extractive yield and specific phytochemical constituents. Soxhlet extraction produced the highest extractive yield (19.10 ± 0.78% w/w), suggesting efficient recovery of total soluble constituents. However, the highest TPC was observed

with ultrasound-assisted extraction (13.24 ± 0.2343 mg GAE/g), which was higher than that obtained by Soxhlet extraction (12.77 ± 0.03 mg GAE/g). Similarly, microwave-assisted extraction yielded the highest TAC (1.751 ± 0.0651 mg AE/g), compared with Soxhlet extraction (1.440 ± 0.0264 mg AE/g). Soxhlet extraction yielded the highest TFC (9.1353 ± 0.0251mg RUT/g) compared to microwave-assisted extraction. (These findings indicate that although Soxhlet extraction afforded the highest overall extractive yield, ultrasound-assisted and microwave-assisted extraction were more effective for the recovery of phenolic and alkaloid constituents, respectively.

3.9 Heavy Metal Analysis

Heavy metal analysis was executed utilising Atomic Absorption Spectroscopy (AAS) in positive ionisation mode. The method was optimised using

Table 3. Total phenolic content, total flavonoid content and total alkaloid content of the methanolic extract of *T. divaricata* leaves

Method of extraction	TPC (mg GAE/g TD)		TFC (mg RUT/g TD)		TAC (mg AE/ g TD)	
	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation
SOX	12.77	0.03	9.1353	0.0251	1.440	0.0264
MA	0.893	0.083	7.1567	0.0251	1.036	0.0584
USA	13.24	0.2343	6.4323	0.1004	0.796	0.0104
MAE	5.733	0.1527	7.4682	0.0794	1.751	0.0651

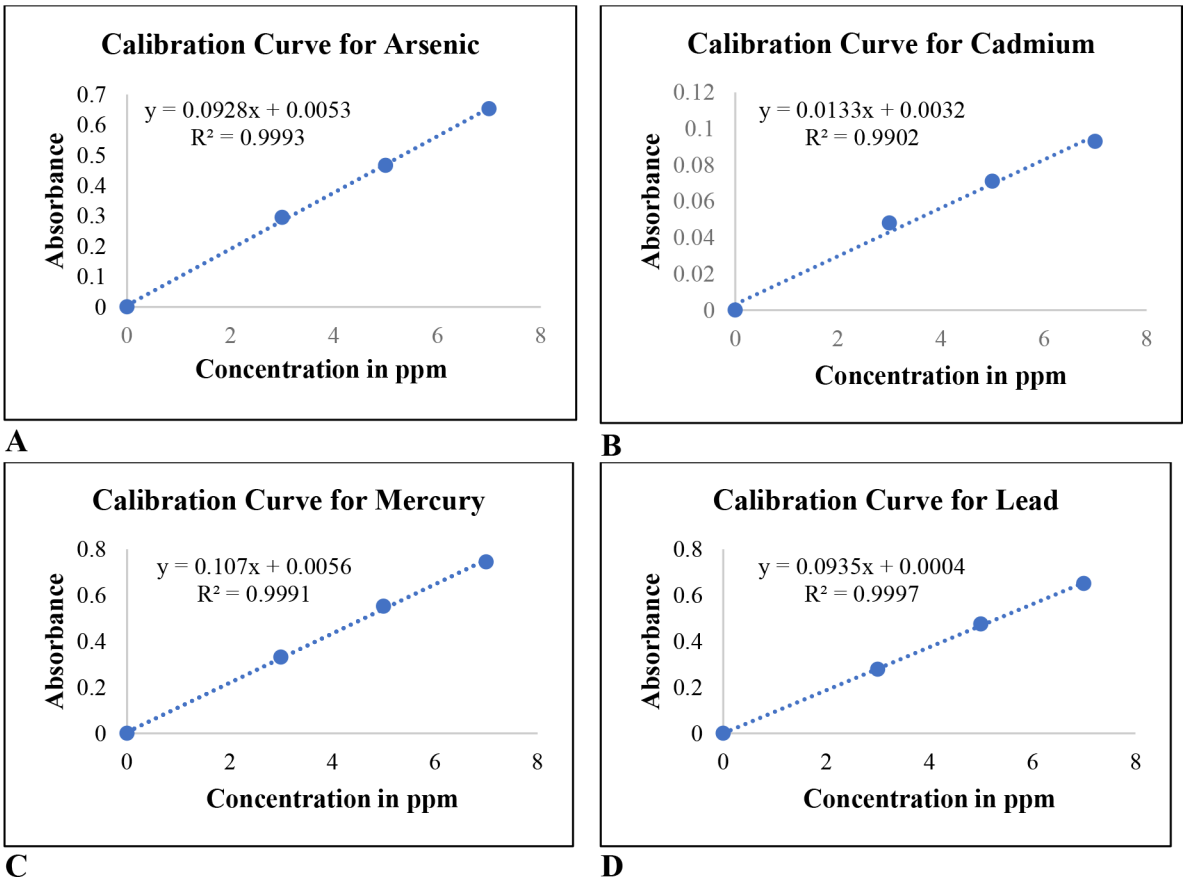


Figure 6. Calibration curve for **(A).** Arsenic, **(B).** Cadmium, **(C).** Mercury, **(D).** Lead. The obtained results (Figures 6-8, Table 4) were assessed in accordance with the maximum permissible limits established by WHO. The examined plant material may be deemed safe for ingestion and therapeutic uses since none of the targeted heavy metals were found.

standard calibration curves prepared from known concentrations of each metal. Calibration curves were generated by plotting instrumental response against concentration, yielding linear relationships for all analytes. Cd, Pb, As, and Hg were analysed at their respective characteristic wavelengths, and the most intense ion signal was selected for quantitative evaluation. According to the research, *T. divaricata* leaves did not exhibit any discernible spectral peaks for Cd, Pb, As, or Hg.

3.10 Aflatoxins

The quantitative analysis of aflatoxins (B1, B2, G1, and G2) in *T. divaricata* leaves is presented in Table 5. The standard solutions of all four aflatoxins exhibited consistent chromatographic responses with a concentration of 20.0 pg/mL and 100% accuracy, confirming the reliability and precision of the analytical technique. In the analysed plant samples, aflatoxin B1, G1, and G2 were detected at trace levels, with concentrations of 0.006, 0.005, and

Table 4. Heavy metal content in *T. divaricata* leaves

Std/ Sample	Weight (gm)	Volume (ml)	Dilution Factor	Absorbance	Conc. (ppm)
Arsenic					
Standard -		-	-	0.295	3
Standard	-	-	-	0.466	5
Standard	-	-	-	0.787	7
TD1	1	1	1	0	0
TD2	1	1	1	0	0
TD3	1	1	1	0	0
Cadmium					
Standard	-	-	-	0.058	3
Standard	-	-	-	0.071	5
Standard	-	-	-	0.157	7.54
TD1	1	1	1	0.009	0
TD2	1	1	1	0.009	0
TD3	1	1	1	0.009	0
Mercury					
Standard	-	-	-	0.331	3
Standard	-	-	-	0.552	5
Standard	-	-	-	0.745	7
TD1	1	1	1	0.000	0
TD2	1	1	1	0.000	0
TD3	1	1	1	0.000	0
Lead					
Standard	-	-	-	0.278	3
Standard	-	-	-	0.376	5
Standard	-	-	-	0.677	7
TD1	1	1	1	0.000	0
TD2	1	1	1	0.000	0
TD3	1	1	1	0.000	0

0.014 pg/mL, respectively, whereas aflatoxin B2 was not detected. The corresponding peak areas were markedly lower than those of the standards, indicating minimal contamination. The detected levels of aflatoxins in *T. divaricata* leaves were substantially below the permissible limits recommended by international regulatory agencies, demonstrating that the plant material is microbiologically safe with respect to mycotoxin contamination. The negligible presence of these toxic metabolites suggests appropriate post-harvest handling and storage conditions and supports the suitability of *T. divaricata* leaves for medicinal and pharmaceutical applications.

3.11 LC-MS/MS Characterisation of Extract

The compounds present in *T. divaricata* leaf extract were identified using LC-MS/MS-QTOF analysis (Table 6). A total of 25 compounds were characterised based on the fragmentation patterns obtained from the MS/MS-QTOF mass spectra.

Table 5. Quantitative analysis of Aflatoxins (B1, B2, G1, and G2) in *T. divaricata* 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
TD Analysis for (Aflatoxin B1)	Sample	1.000	265	0.006	-
TD Analysis for Aflatoxin B2	Sample	1.000	6	0.000	-
TD Analysis for Aflatoxin G1	Sample	1.000	175	0.005	-
TD Analysis for Aflatoxin G2	Sample	1.000	186	0.014	-

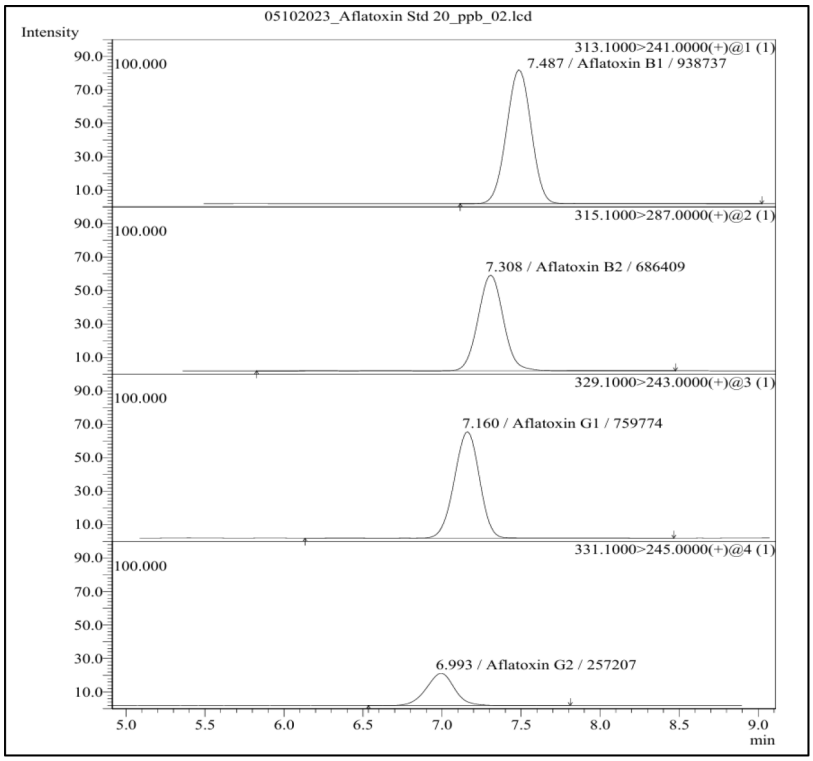


Figure 7. Chromatogram of Standard Aflatoxins B1, B2, G1 and G2.

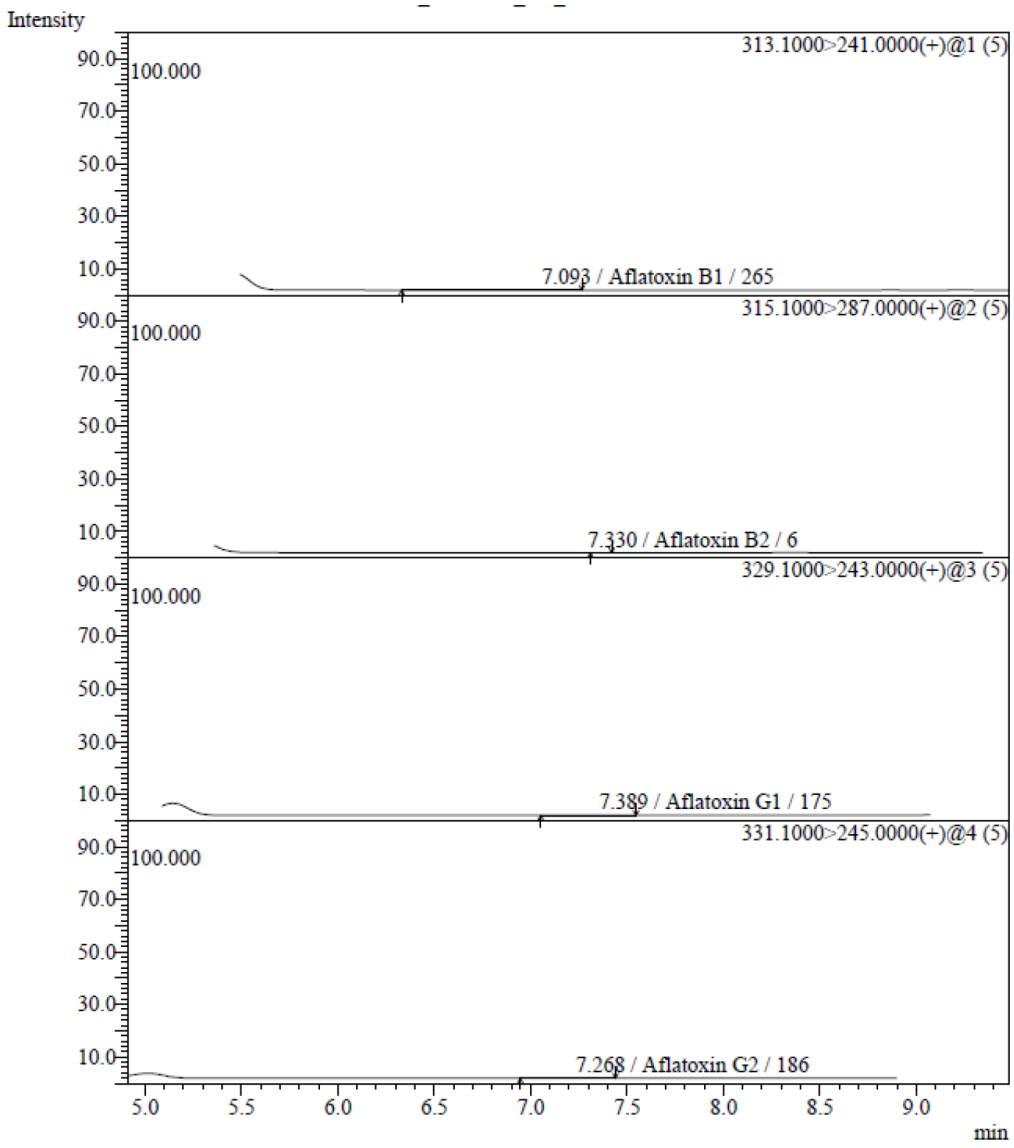


Figure 8. Chromatogram of *T. divaricata* leaves for Aflatoxins B1, B2, G1 and G2.

3.12 Molecular Docking

A molecular docking study was performed using 25 compounds identified from the Soxhlet-derived leaf extract of *T. divaricata*, with human cytosolic β-glucosidase selected as the target macromolecule. The compounds and reference inhibitor Acarbose were docked using Molsoft ICM-Pro to evaluate their binding affinities and interactions within the active site of the enzyme. Among the tested compounds, Flavidulol exhibited the highest binding affinity (-27.18 kcal/mol) against the covalent intermediate of human cytosolic β-glucosidase (PDB ID: 2ZOX), compared with Acarbose (-8.30 kcal/mol). Other compounds, including PGP (16:0/18:1(9Z)), Carolinianine, and 1-Hexanol arabinosylglucoside, also demonstrated notable binding interactions with the target. Overall, 23 of the 25 identified compounds showed favourable binding affinities toward the target protein. The 2D and 3D interactions of Flavidulol and Acarbose within the active binding pocket are presented in Figure 9, while the docking scores and key molecular interactions are summarised in Table 7.

Table 6. Characterisation of compounds from *T. divaricata* leaf extract by LC-MS/MS-QTOF

Name	Formula	RT	Mass
Flavidulol C	$C_{34}H_{42}O_4$	24.174	514.3078
1-Hexanol arabinosylglucoside	$C_{17}H_{32}O_{10}$	18.457	396.2007
1-Octen-3-ol-3-o-beta-Dxylopyranosyl (1->6)-beta-D-glucopyranoside	$C_{19}H_{34}O_{10}$	15.186	422.2159
13-(2-Methylcrotonoyl) oxylupanine	$C_{20}H_{30}N_2O_3$	15.045	346.2248
alpha-Ionol O-[arabinosyl-(1->6)-glucoside]	$C_{24}H_{40}O_{10}$	16.537	488.2620
beta-Obscurine	$C_{17}H_{24}N_2O$	11.588	272.1888
Carolinianine	$C_{16}H_{24}N_2O_2$	11.112	276.1833
Camptothecin	$C_{20}H_{16}N_2O_4$	21.2	348.36
Cinnamodial	$C_{17}H_{24}O_5$	15.472	308.1624
Ethopropazine	$C_{19}H_{24}N_2S$	3.635	312.1660
Flabellidine	$C_{18}H_{28}N_2O$	14.717	288.2206
gamma-L-Glutamylgamma-L-glutamyl-L-methionine	$C_{15}H_{25}N_3O_8S$	10.880	407.1363
Glaucarubin	$C_{25}H_{36}O_{10}$	19.508	496.2309
Gallic acid	$C_7H_6O_5$	1.56	170.12
Histidinyl-Lysine	$C_{12}H_{21}N_5O_3$	3.093	283.1635
Monoolein	$C_{21}H_{40}O_4$	28.623	356.2910
Neryl rhamnosyl-glucoside	$C_{22}H_{38}O_{10}$	19.607	462.2464
Rutin	$C_{27}H_{30}O_{16}$	9.68	610.517
Oxyphencyclimine	$C_{20}H_{28}N_2O_3$	12.662	344.2097
PGP (16:0/18:1(9Z))	$C_{40}H_{78}O_{13}P_2$	28.051	828.4913
PI (16:0/18:1(11Z))	$C_{43}H_{81}O_{13}P$	27.146	836.5399
Rhazidigenine Nb-oxide	$C_{19}H_{26}N_2O_2$	14.011	314.1987
Ropinirole	$C_{16}H_{24}N_2O$	11.870	260.1894
Sauroxine	$C_{17}H_{26}N_2O$	13.226	274.2048
Septentriodine	$C_{37}H_{52}N_2O_{11}$	23.477	700.3568

Table 7. Molsoft ICM-Pro docking scores (kcal/mol) of Acarbose (Standard) and *T. divaricata* compounds against the crystal structure of the covalent intermediate of human cytosolic β-glucosidase (PDB ID: 2ZOX)

Name of the Compound	Dock Score	H Bond Score	Lipophilic Score
Flavidulol C	-27.18	0	-12.34
PGP (16:0/18:1(9Z))	-23.94	-9.5	-13.92
Carolinianine	-21.22	-17.79	-6.5
1-Hexanol arabinosylglucoside	-20.8	-17.79	-6.5
Acarbose (Standard)	-8.30	-13.03	-8.112

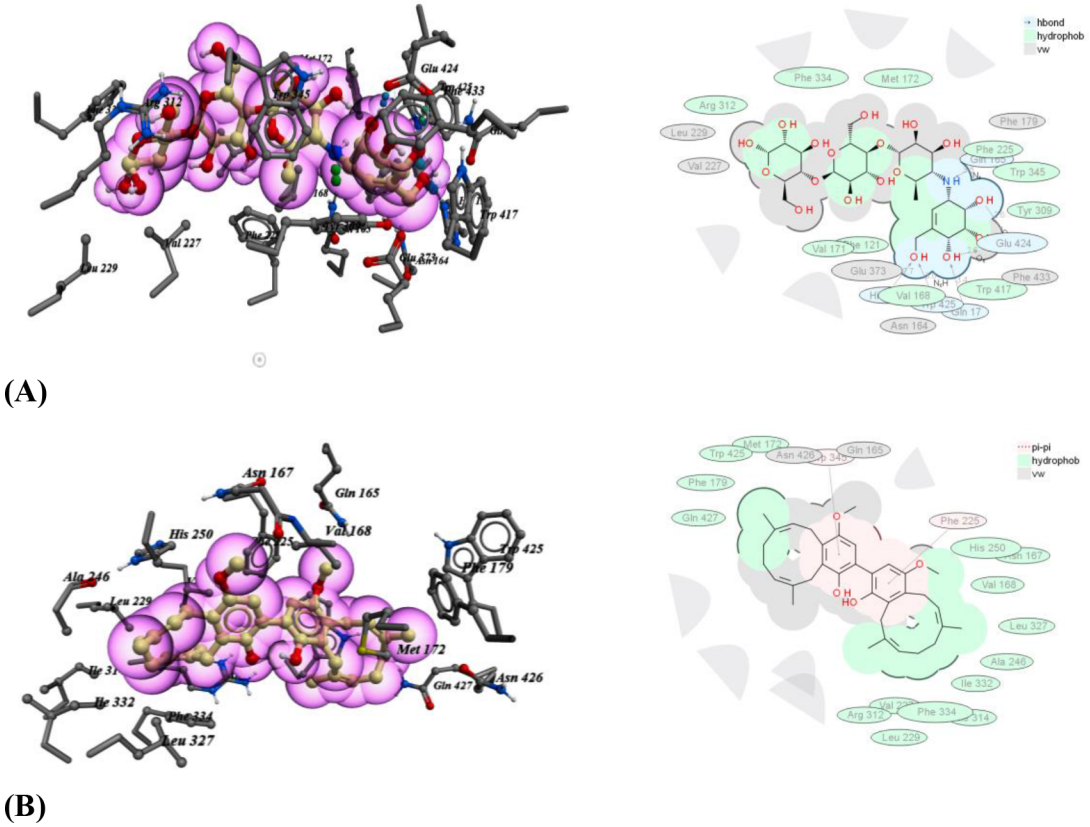


Figure 9. 3D and 2D visualisation of **(A).** Standard (Acarbose) and **(B).** Flavidulol.

4. Conclusion

The present study establishes a comprehensive and multidimensional quality evaluation of *T. divaricata* leaves by integrating pharmacognostic, phytochemical, toxicological, and computational approaches. Distinct macroscopic and microscopic characteristics confirmed the authenticity of the plant material, while physicochemical parameters and extractive values validated its purity and consistency. Phytochemical investigations revealed a rich composition of bioactive secondary metabolites, with significant levels of phenolics, flavonoids, and alkaloids contributing to its therapeutic potential. Safety assessment demonstrated the absence of toxic heavy metals (Pb, Cd, As, Hg), and LC-MS/MS-based aflatoxin analysis confirmed only trace contamination well within acceptable limits, ensuring the suitability of the plant material for medicinal use. Advanced LC-MS/MS-QTOF profiling enabled the identification of 25 compounds, providing a detailed chemical fingerprint essential for standardisation. Importantly, molecular docking studies revealed strong interactions between several identified phytoconstituents and Human Cytosolic β-Glucosidase, with Flavidulol showing the highest binding affinity, significantly exceeding that of the standard drug Acarbose. The integrated methodological framework presented herein can serve as a reliable model for the standardisation, quality control, and drug discovery exploration of medicinal plants.

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Author Contribution

Ms. Pallavi Bhokare and Dr. Prasad Kadam conceptualised and designed the study, with Pallavi Bhokare handling data collection. Dr. Prasad Kadam conducted 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 read and agreed to the published version of the manuscript.