Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
Impact Factor (RJIF): 6.35
www.phytojournal.com
JPP 2025; 14(5): 92-101
Received: 06-06-2025
Accepted: 08-07-2025

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Comparative HPTLC fingerprint profiling of metabolites in *Tinospora cordifolia* commercial samples

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DOI: https://www.doi.org/10.22271/phyto.2025.v14.i5b.15557

Abstract
This study employed a comprehensive quality assessment approach to evaluate the phytochemical composition and authenticity of market samples of *Tinospora cordifolia* using standard analytical techniques. Organoleptic and physicochemical parameters were analysed for a standard sample (TCSF) and three commercial samples (TCS1, TCS2, TCS3) of *Tinospora cordifolia*. While most samples met general quality control standards, specific deviations were identified such as sample TCS3 showed significant weight variation, and TCS2 exhibited high loss on drying (8.1%) and elevated acid-insoluble ash content (3.62%), suggesting potential adulteration with inorganic matter. Preliminary phytochemical screening confirmed the presence of bioactive constituents like alkaloids, flavonoids, tannins, and saponins, with the standard (TCSF) and TCS1 containing the highest number of compounds and TCS3 the fewest. All samples were weakly acidic, a property that may enhance bioavailability in the gastrointestinal tract. High-Performance Thin Layer Chromatography (HPTLC) fingerprinting at 254 nm and 366 nm enabled detailed chemical profiling, with TCS1 demonstrating a banding pattern and Rf values most consistent with the reference standard. In contrast, TCS3 showed limited phytochemical diversity and low band intensity. The results underscore the utility of integrated analytical profiling in differentiating authentic *Tinospora cordifolia* products from substandard or adulterated market samples. Among the samples tested, TCS1 was identified as the most authentic, while TCS2 and TCS3 indicate quality concerns requiring further regulatory scrutiny.

Keywords: *Tinospora cordifolia*, Quality control, HPTLC fingerprinting, Phytochemical screening, Physicochemical analysis, Adulteration

1. Introduction
Nutraceuticals represent a vital convergence between nutrition and pharmaceuticals, offering health-promoting bioactive compounds derived from natural sources. These substances, commonly found in dietary supplements and herbal formulations, play a pivotal role in preventing disease and enhancing wellness [1]. Nutritional therapy underscores their potential, using nutraceuticals to correct deficiencies, detoxify the body, and restore metabolic balance. Central to this concept are phytonutrients that are plant-based compounds that interact synergistically with vitamins and minerals to support human health [1, 2].
Today, nearly 80% of pharmaceutical drugs are rooted in botanical sources [3]. The World Health Organization (WHO) estimates that 80% of the global population relies on traditional herbal remedies, with India's Ayurvedic system standing out for its vast pharmacopoeia. Among its most revered medicinal plants is *Tinospora cordifolia* (commonly known as Giloy or Guduchi), celebrated for its immunomodulatory, antioxidant, and adaptogenic properties [4, 5].

1.1 Giloy: A Versatile Medicinal Powerhouse
Tinospora cordifolia is a climbing shrub valued in Ayurveda as a Rasayana (rejuvenator), known for promoting longevity and vitality. Its pharmacological efficacy is attributed to a complex array of bioactive constituents such as alkaloids, flavonoids, glycosides, and saponins, distributed throughout its stems, leaves, and roots [6]. These compounds have demonstrated therapeutic benefits against inflammatory disorders, metabolic syndromes, and liver fibrosis [7]. Traditional Ayurvedic preparations like *Guduchyadi churna* and *Amritashtaka churna* harness these effects to treat fever, digestive complaints, and immune dysfunction [8, 9].
However, the surge in demand for Giloy-based products has led to widespread adulteration and quality variability in commercial markets. Substitutes, contaminants, and substandard processing techniques compromise both safety and efficacy.

Standardization is therefore critical. Advanced analytical tools such as High-Performance Thin Layer Chromatography (HPTLC) enable fingerprint profiling and identification of marker compounds in line with the Ayurvedic Pharmacopoeia [10].

Recent advances in metabolomics and analytical chemistry have significantly enhanced the characterization of *Tinospora cordifolia*'s complex phytochemical profile. Liquid Chromatography-Mass Spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy have identified novel bioactive compounds, including cordioside, tinocordiside, and other metabolites such as berberine, magnoflorine, and S-adenosylmethionine, which exhibit neuroprotective and metabolic regulatory properties [11, 12].

1.2 Bridging Traditional Wisdom with Scientific Rigor

The convergence of traditional Ayurvedic knowledge and modern pharmacological validation is reshaping the role of herbal medicine. For instance, Mechanism of immunomodulatory activity of Giloy have been reviewed highlighting specific pathways including NF-kB pathway and interleukin modulation [13]. Nevertheless, regional differences in preparation methods persist, Ayurveda traditionally emphasizes fresh stem extracts, while most commercial formulations use dried powders of variable potency. Initiatives like the AYUSH-ICMR Advanced Centre for Integrative Health Research are addressing such gaps by developing harmonized protocols that preserve ethnopharmacological integrity while enhancing scientific credibility [14].

1.3 Rationale and Scope of the Study

This study aims to investigate the qualitative consistency of commercially available *T. cordifolia* products using a combination of preliminary phytochemical screening and HPTLC-based fingerprinting. By analysing organoleptic characteristics, extractive values, and metabolite profiles, the study seeks to identify potential adulterants and assess product conformity to authentic reference standards. The findings are expected to contribute to the growing demand for scientifically validated, standardized herbal products that integrate traditional efficacy with modern reliability.

2. Materials and Methods

2.1 Sample Selection and Preparation

2.1.1 Sample Collection

Three commercially available *Tinospora cordifolia* supplements were procured from local markets in Chennai, India as two capsule formulations from different manufacturers and One tablet formulation (Table:1 & Figure 1). Additionally, a certified standard reference material was obtained from M/s All Pure Botanicals, New Delhi, containing 3% dry extract with documented levels of key bioactive compounds.

Table 1: Collection of Samples of *Tinospora cordifolia*

Samples of *Tinospora cordifolia*	Short form
Standards	TCSF
TC Sample I - Capsule form	TCS1
TC Sample II - Capsule form	TCS2
TC Sample III - Tablet form	TCS3

2.1.2 Sample Preparation: For analysis, Capsule contents were carefully emptied using clean spatulas. Tablets were crushed to fine powder using a ceramic mortar and pestle. All samples were sieved through #60 mesh (250µm) screens. Prepared samples were stored in amber glass containers with airtight seals at 25±2°C until analysis.

2.2 Physical Characteristic Study and Organoleptic Properties

The Physical characteristic and Organoleptic study of the capsules, tablets and powder such as colour, texture, flavour, taste and weight were studied.

2.2.1 Organoleptic Evaluation

A trained panel of three assessors evaluated the Colour (visual inspection under standardized lighting), Texture (manual assessment of particle size and consistency), Odor (sniff test at consistent distance) and Taste (small quantity placed on tongue, followed by water rinse) Evaluation followed triangle test methodology with three replicates per sample [15].

2.2.2 Weight Variation Analysis

The weight of the product was measured by randomly choosing 10 number of tablets and capsules, then it is compared with the weight mentioned in the label of the product in order to determine the variation of weight of the product. The permissible variations in the weights of individual tablets or capsules, expressed in terms of the allowable deviation from the average weight of a sample were also assessed. The permissible limit of deviation is 2 capsules or tablet (<5% deviation for tablets, <7.5% for capsules).

2.3 Physiochemical Analysis

2.3.1 Determination of Loss on Drying

Pre-weighed porcelain crucibles containing 2gm of each sample were dried at 105±2°C in hot air oven for 5 hours. Then cooled in desiccator containing silica gel. The samples were weighed until constant weight achieved (two consecutive measurements). The following formula was used to calculate Loss on dry.

Loss on drying Percentage = (Loss in weight of the sample*100) / Weight of the sample taken

2.3.2 Determination of Total Ash

2g from samples were weighed in a silica dish. Samples were spread evenly and ignited in a muffle furnace at 600±25°C until it becomes white indicating the absence of carbon. Then the samples were cooled in a desiccator and weighed separately. The samples were duplicated for constant weight. The following formula was used to calculate the total ash.

Percentage of total ash = (Weight of ash*100) / Weight of the sample taken

2.3.3 Determination of Water-Soluble Ash: Total ash of samples was boiled with 45ml of water in three portions of 15ml each time and then boiled gently for 5 minutes and filtered. The insoluble matter is filtered through ash less filter paper (Whatman no: 41) then the filter paper containing the residual matter is ignited at 450±25°C. It is cooled in a desiccator and then weighed. The following formula was used to calculate the percentage of water-soluble ash.

Percentage of water-soluble ash = (Weight of the water-soluble residue *100) / Weight of the sample

2.3.4 Determination of Acid-Insoluble Ash

The method is same as mentioned in determination of water-soluble ash except that the ash samples were boiled with diluted HCL (1:5).

Percentage of acid insoluble ash = (Weight of the acid insoluble residue *100)/ Weight of the sample

2.3.5 Determination of Water-Soluble Extractive

2g of sample were weighed in a stoppered flask. 100ml of distilled water were added and Shaked occasionally for 6 hours. It is allowed to stand for 24 hours. Then it is filtered through Whatman #1 filter paper and 25ml of the filtrate is pipetted out in a pre-weighed 100ml beaker. It is allowed to evaporate on a water bath. Then it is kept on an air oven at 105°Celsius for 6 hours. It is cooled in a desiccator and then weighed. The experiment is repeated twice to take then average value. The following formula was used to measure water soluble extractive.

Percentage of water-soluble extractive = (Weight of the extract*100) / (25*Weight of the sample taken*100)

2.3.6 Determination of Alcohol Soluble Extractive

The method is same as mentioned in determination of water-soluble extractive except that 100 ml of ethanol was used instead of distilled water. The following formula was used to measure acid soluble extractive.

Percentage of alcohol-soluble extractive = (Weight of the extract*100) / (25*Weight of the sample taken*100)

2.4 Phytochemical Screening

Preliminary phytochemical screening was carried out on all the samples. The Phytochemical parameters such as Alkaloids, Steroids, Saponins, Cardiac glycosides, Tannins, Flavonoids, Reducing sugar, Phenol, Protein, Anthraquinone, Quinones, Coumarin, Acid and Glycosides were analysed in triplicate with appropriate positive and negative controls [6, 16, 17].

2.5 HPTLC Analysis of Phytochemicals Present in *Tinospora cordifolia* Samples

2.5.1 Sample Preparation

10g of each sample was extracted with 10 ml of ethanol, filtered using Whatman Ni 1 filter paper and filtrate was made up to 10ml. 10µl of the above extract was applied on a pre-coated silica gel 60 F254 on aluminium plates to a band width of 7 mm using Linom at 5 TLC applicator. The plate was developed in developing chamber (after saturated with solvent vapour) with Toluene: Ethyl acetate: Formic acid (2:9:0.5) mobile phase. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAGREPROSTAR3) and the images at white light, UV254nm and UV366nm were captured. Then the developed plate was sprayed with vanillin sulphuric acid reagent and dried at 100°C in hot air oven. The plate was photo-documented in white light and UV520nm mode using photo-documentation (CAMAGREPROSTAR3) chamber. Rf and colour of the spots and densitometric scan were recorded

3. Results and Discussion

Natural products, particularly *Tinospora cordifolia* (Giloy), are gaining clinical interest due to their medicinal value and minimal side effects compared to conventional drugs. Recently the discovery of active components from the plant and their biological function in disease control has led to active interest in the plant across the globe. This research focuses on analysing the quality and purity of Giloy and its market samples. Utilizing High-Performance Thin-Layer Chromatography (HPTLC), the study aims to establish a fingerprint profile for various forms of processed *Tinospora cordifolia*, including capsules and tablets. This HPTLC method serves as a rapid quality control tool for authenticating herbal samples and ensuring the consistency of their active components, demonstrating its efficacy in verifying the quality of diverse herbal products.

3.1 Certification Analysis of *Tinospora cordifolia* Standard

The *Tinospora cordifolia* standard used in this research was procured from M/s All Pure Botanicals, New Delhi, India. Non-GMO plants were utilized for the extraction of this standard. The stem of *Tinospora cordifolia* was extracted using a hydroalcoholic method, and the resulting extract had a bitter taste.

Analysis confirmed that heavy metals were below detectable limits (Arsenic and Cadmium NMT 1 ppm, Lead NMT 3 ppm, Mercury NMT 0.1 ppm, and Total heavy metals NMT 10 ppm). Furthermore, no microbiological contaminations were found, with *E. coli*, *Staphylococcus aureus*, and *Salmonella* all testing negative. Both heavy metal and microbiological contamination levels complied with the protocols of the United States Pharmacopeia. The Giloy (*Tinospora cordifolia*) standard was packed and stored at room temperature in a dry place.

Fig 1: *Tinospora cordifolia* Standard and samples

3.2 Organoleptic and Physical Characteristics of *Tinospora cordifolia* Supplement

3.2.1 Organoleptic Characteristics of *Tinospora cordifolia*

Organoleptic evaluation refers to the assessment of individual drugs and formulations based on characteristics such as colour, odour, taste, and texture [18]. Organoleptic parameters, along with physicochemical and phytochemical analyses, are essential for quality assessment. These evaluations were carried out in accordance with WHO guidelines. The standard sample of *Tinospora cordifolia* (TCSF) was observed to be fine, yellowish-brown in colour, bitter in taste, and possessed a pungent odour. The test samples (TCS1, TCS2, TCS3) were also bitter and astringent in taste, though other characteristics showed slight variations depending on the mode of preparation. Organoleptic characters correspond to perceptions involving the five sense organs, and minor morphological variations may occur due to differences in

sample quality. The bitterness of *Tinospora cordifolia* is primarily attributed to the presence of clerodane glycosides [9]. The detailed organoleptic characteristics are presented in Table 2.

Table 2: Organoleptic Characteristics of *Tinospora cordifolia* market samples

S. No	Parameters	TCSF	TCS1	TCS2	TCS3
1.	Texture	Fine coarse	Fine coarse	Hard Granules	Rough
2.	Colour	Yellowish brown	Dark brown	Dark brown	Creamish brown
3.	Taste	Bitter	Bitter	Astringent	Bitter
4.	Odour	Pungent	Aromatic	Aromatic	Pungent

3.2.2 Analysis of Weight Variance of *Tinospora cordifolia* samples

The weight variation analysis of *Tinospora cordifolia* formulations revealed that TCS1 and TCS2 tablets consistently weighed around 500 mg, while TCS3 tablets were 250 mg as per the label. Ten units from each sample batch were randomly selected and weighed. The weights of TCS1 and TCS2 capsules showed minimal variation, ranging between 500-600 mg. In contrast, TCS3 tablets exhibited a wider range of weight variation, indicating inconsistency in formulation. These variations are visually represented in Figure 2.

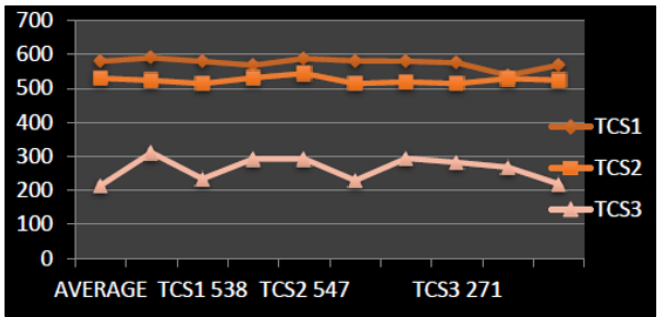

Fig 2: Weight Variance of *Tinospora cordifolia* capsules and Tablet

3.3 Physicochemical Parameters of *Tinospora cordifolia* Samples

Various physicochemical parameters were analysed and recorded for different marketed samples of *Tinospora cordifolia* in capsule and tablet forms as shown in Table 3. These parameters are essential for validating the concentration, purity, and overall quality of herbal drugs. Loss on drying (LoD) was measured as an indicator of moisture content, which directly impacts the stability and shelf life of plant materials. Ash values were evaluated to determine the total mineral content, where water-soluble ash indicates the proportion of inorganic compounds and acid-insoluble ash helps detect contamination from substances such as silica or earthy materials. The results showed higher water-soluble ash values compared to acid-insoluble ash. Extractive values (both water- and alcohol-soluble) were assessed to estimate the concentration of active phytoconstituents.

Table 3: Physico-chemical parameters of *Tinospora cordifolia* standard and market samples

Parameters	TCSF (%)	TCS1 (%)	TCS2 (%)	TCS3 (%)
Loss on drying	2.024	2.123	8.116	4.128
pH	4.83	5.11	5.51	5.57
Total Ash	2.02	2.12	8.11	4.12
Water soluble extract	52.57	68.43	66.116	67.46
Acid soluble extract	52.231	68.43	68.04	65.96
Water soluble ash	1.184	1.532	2.059	2.112
Ash insoluble ash	0.580	0.171	3.628	1.019

3.3.1 Loss on Drying of *Tinospora cordifolia* samples

Loss on drying (LOD) refers to the combined amount of moisture content and volatile matter present in a product. Moisture content in crude drugs is a critical factor, as it directly affects the stability of the product and its susceptibility to microbial growth. Lower moisture content contributes to improved shelf life [19]. According to the Ayurvedic Pharmacopoeia of India [20], the acceptable limit for LOD in *Tinospora cordifolia* is not more than 2% (NMT 2%). In the present study, the LOD values for TCSF, TCS1, TCS2, TCS3 were 2.0%, 2.1%, 8.1%, 4.1% respectively. These results are summarized in Table 4. Among the samples, only the standard sample (TCSF) met the API specification. The LOD values of the other market samples exceeded the acceptable limit, suggesting possible adulteration or improper storage conditions. Notably, the TCS2 sample exhibited the highest moisture content, indicating poor quality. Similar findings were reported by Sharma *et al*. (2013) [8], who observed that the LOD of *Tinospora cordifolia* stem collected in different seasons reached up to 6% when dried at 110 °C (% w/w). These results highlight significant variability and the need for strict quality control in commercial *Tinospora cordifolia* preparations.

4.3.2 Ash Analysis of *Tinospora cordifolia* samples

Ash value analysis was conducted to assess the presence of inorganic impurities such as sand, siliceous matter, and low-grade materials in the powdered samples of *Tinospora cordifolia*. Total ash typically includes both physiological ash and non-physiological ash, containing inorganic components such as phosphates, silicates, and silica. Acid-insoluble ash specifically measures the siliceous content, such as sand and earthy contaminants, while water-soluble ash reflects the portion of inorganic matter that dissolves in water. These parameters are essential for evaluating the purity and overall quality of herbal drugs [21].

According to the Ayurvedic Pharmacopoeia of India [20], the standard limit for total ash in *Tinospora cordifolia* is not more than 16%. In the present study, the total ash values were found to be 2.02% for TCSF (standard), 2.12% for TCS1, 8.11% for TCS2, and 4.12% for TCS3. All market samples were within the permissible limit, with TCS1 showing better quality based on its lower ash value in comparison to others.

The acid-insoluble ash values were also evaluated, with a standard limit of not more than 3%. The recorded values were 0.58% for TCSF, 0.17% for TCS1, 3.62% for TCS2, and 1.01% for TCS3. While TCSF, TCS1, and TCS3 met the standard, TCS2 exceeded the acceptable limit, indicating a higher level of siliceous contamination, possibly due to the presence of adulterants like sand or rice husk.

Water-soluble ash, which represents the water-soluble portion of the total ash, was found to be 1.18% for TCSF, 1.53% for TCS1, 2.05% for TCS2, and 2.11% for TCS3. All values were within acceptable limits. However, TCS2 and TCS3 had

slightly higher levels of water-soluble inorganic compounds compared to TCS1 and the standard.

All the samples complied with the total ash limits, while the acid-insoluble ash value for TCS2 exceeded the standard, suggesting possible adulteration and inferior quality. TCS1 and TCS3 had relatively fewer siliceous impurities. Since total ash may also include physiological components naturally present in the plant, acid-insoluble ash is considered a more reliable indicator of purity in herbal formulations. The results indicate that ash value analysis is a useful tool to detect contamination and assess the overall quality of herbal nutraceutical supplements available in the market.

3.3.3 Extractive Analysis of *Tinospora cordifolia* Samples

The determination of water- and alcohol-soluble extractive values is an important method for assessing the quality and purity of chemical constituents in crude herbal drugs. These values help evaluate the presence of active phytochemicals and detect adulteration or the use of exhausted material. Water extraction isolates primarily water-soluble compounds, although some insoluble components may also be retained due to straining. In contrast, alcohol extraction yields alcohol-soluble constituents, and higher values may also indicate the presence of alcohol-soluble starches. Extraction can be performed using maceration with cold water or continuous extraction through a Soxhlet apparatus [21].

According to the Ayurvedic Pharmacopoeia of India [20], the standard extractive values for *Tinospora cordifolia* are not more than 11% for water-soluble extractives and not less than 3% for alcohol-soluble extractives. In the present study, these values were measured to evaluate and compare the selected market samples.

The water-soluble extractive values for the samples were as follows: TCSF (52.57%), TCS1 (68.43%), TCS2 (66.11%), and TCS3 (67.4%). All values were significantly higher than the minimum limit, indicating the presence of abundant water-soluble phytochemicals. These may include glycosides, saponins, tannins, flavonoids, and other polar compounds. Higher water extractive values reflect good quality, whereas lower values could suggest the addition of exhausted material or improper processing during drying, storage, or formulation.

The alcohol-soluble extractive values were also found to be within the acceptable range: TCSF (52.23%), TCS1 (68.43%), TCS2 (68.43%), and TCS3 (65.96%). These values indicate the presence of alcohol-soluble constituents such as polyphenols, alkaloids, steroids, glycosides, terpenoids, and other secondary metabolites [22]. As with water-soluble extractives, a lower alcohol extractive value may suggest adulteration or poor-quality material.

Comparing both extractive values, it was observed that water-soluble extractive percentages were consistently higher than alcohol-soluble ones across all samples. This indicates a greater proportion of water-soluble phytoconstituents in *Tinospora cordifolia*, which are likely responsible for its therapeutic effects. These findings emphasize the importance of extractive value analysis in evaluating the quality and standardization of herbal products. The extractive values for all samples are presented in Table 6.

3.3.4 pH Analysis of *Tinospora cordifolia* Samples

The pH value conventionally represents the acidity or alkalinity of a substance and is a critical parameter in evaluating the quality of herbal formulations. Monitoring the pH of samples is important for a wide range of applications, including drug absorption, stability, and taste. Weakly acidic drugs are generally better absorbed in acidic environments such as the stomach, whereas weakly basic drugs may require more alkaline conditions for optimal absorption. The pH of a formulation also influences the availability of nutrients and affects the behaviour of physical and chemical reactions, which in turn can impact the consistency and palatability of the product [23]. In the present study, the pH values of *Tinospora cordifolia* market samples were found to be: TCSF - 4.83, TCS1 - 5.11, TCS2 - 5.51, and TCS3 - 5.57. All samples were weakly acidic in nature and remained within acceptable standard limits. A slightly higher pH can enhance the digestibility and nutritional value of herbal products [24]. Therefore, the observed pH range suggests good formulation characteristics and supports the potential effectiveness of the marketed *Tinospora cordifolia* samples.

3.4 Phytochemical Screening of *Tinospora cordifolia* Samples

In the preliminary phytochemical screening, different samples of *Tinospora cordifolia* (TCSF, TCS1, TCS2, and TCS3) were extracted with ethanol and analysed to determine the presence of bioactive phytoconstituents. The results revealed a wide range of compounds, including alkaloids, flavonoids, tannins, proteins, phenols, acids, glycosides, and others. Phytochemical analysis plays a crucial role in medicinal plant research and forms the basis for the discovery and development of novel therapeutic agents. Earlier studies have demonstrated the presence of such bioactive compounds in *Tinospora cordifolia* extracts [21, 24, 9]. In the current study, variations were observed in the presence of these compounds across the market samples, reflecting differences in formulation or plant material quality.

Phytochemical analysis is an essential step in medicinal plant research and plays a critical role in identifying compounds that may serve as leads for drug discovery and development. The presence of specific compounds varies across different market samples, which reflects differences in raw material quality, processing, or formulation. In this study, glycosides and quinones were present in all samples, while alkaloids, saponins, and steroids were found in most. Phenols and organic acids were less prominent but are recognized for their antiseptic and anti-inflammatory properties.

The variability in phytochemical content among the samples suggests that *Tinospora cordifolia* is a valuable medicinal plant, rich in antioxidant compounds. These phytochemicals serve as nutraceutical agents that help alleviate oxidative stress and may contribute to the prevention or mitigation of degenerative diseases, offering significant health benefits [26]. In this study ethanolic extracts were effectively used to identify the presence of these phytoconstituents. The results affirm the therapeutic potential and rich phytochemical profile of *Tinospora cordifolia* samples available in the market.

Comparative analysis showed that the reference standard sample (TCSF) contained 11 phytochemical constituents, including flavonoids, saponins, tannins, acids, coumarins, alkaloids, proteins, quinones, reducing sugars, and glycosides. Notably, anthraquinones, cardiac glycosides, and steroids were absent in the standard. Among the market samples, TCS1 exhibited 10 phytochemical compounds, showing close similarity to the standard. TCS2 contained 8 compounds, while TCS3 had only 4, indicating a lower phytochemical profile and potentially reduced therapeutic value. These findings are summarized in Table 8, highlighting that the market samples TCS1 and TCS2 closely matched the reference standard, while TCS3 showed the least similarity.

The variation in phytochemical content suggests differences in the quality of raw material or processing techniques used in market formulations.

Overall, the results emphasize the rich phytochemical composition of *Tinospora cordifolia*, especially in standard and higher-quality market samples. The observed variation also highlights the importance of proper standardization and quality control in the manufacturing of herbal products.

Table 4: Qualitative analysis of secondary metabolites of *Tinospora cordifolia* standard and market samples

S. No.	Phytochemical Analysis	TCSF	TCS1	TCS2	TCS3
1	Phenol	Absent	Absent	Absent	Absent
2	Tanin	Present	Absent	Absent	Present
3	Flavonoids	Present	Absent	Absent	Absent
4	Triterpenoids	Absent	Present	Absent	Absent
5	Proteins	Present	Present	Present	Absent
6	Glycosides	Present	Present	Present	Present
7	Reducing sugar	Present	Absent	Present	Absent
8	Anthraquinones	Absent	Present	Present	Absent
9	Quinones	Present	Present	Present	Present
10	Alkaloids	Present	Present	Present	Absent
11	Saponins	Present	Present	Present	Present
12	Cardiac glycoside	Present	Present	Absent	Absent
13	Steroids	Present	Present	Present	Absent
14	Coumarin	Present	Present	Absent	Absent
15	Acids	Absent	Absent	Absent	Absent

3.5 HPTLC Screening of Phytochemical Present in *Tinospora cordifolia* Samples

One of the most significant challenges in the formulation of herbal nutraceuticals is the lack of comprehensive evaluation methods. Such evaluation is essential to ensure the quality, safety, and purity of herbal products [27, 28, 29]. In this study, High-Performance Thin Layer Chromatography (HPTLC) was employed to fingerprint the *Tinospora cordifolia* market samples and compare them with a reference standard to assess product quality. HPTLC is an advanced form of thin-layer chromatography offering improved resolution, reproducibility, and efficiency. It is well-suited for qualitative, quantitative, and micro-preparative analyses. The system was standardized using parameters such as the plant extraction solvent system, mobile phase composition, scanning wavelength, and detection conditions [30, 31].

The HPTLC method employed a silica gel 60 F254 plate as the stationary phase, and the mobile phase used was a mixture of Toluene: Ethyl acetate: Formic acid (5:5:0.5, v/v/v). The inclusion of a small proportion of polar solvent, such as ethyl acetate, enhanced the eluting power of the mobile phase. By analysing the Rf values, various phytoconstituents including phenols, saponins, tannins, alkaloids, glycosides, acids, and quinones were identified in the TCSF, TCS1, TCS2, and TCS3 samples. These Rf values also provided insight into the polarity of the compounds present. Polar compounds (Rf 0.1-0.4) are generally water-soluble, distribute in the bloodstream, and are eliminated primarily via the kidneys. Mid-polar compounds (Rf 0.4-0.6) show intermediate properties, while non-polar compounds (Rf 0.6-0.9) are lipid-soluble, distribute into tissues and fat, and are generally excreted through bile and feces.

After method development, the mobile phase comprising Toluene: Ethyl acetate: Formic acid (2:9:0.5) was identified as optimal for separating phytochemicals in *Tinospora cordifolia*. This combination yielded well-resolved bands and was consistent with previous findings [32]. Horizontal developing chambers were selected for their efficiency, flexibility, and reproducibility. To enhance reagent application, a Derivatizer (a closed, automated spraying device) was employed. This ensured uniform reagent distribution, reduced solvent consumption (4 mL for 20 × 20 cm plates; 2 mL for 20 × 10 cm plates), and provided safe and reproducible results. Chromatograms were evaluated under white light and ultraviolet light at different wavelengths (200-400 nm). *Tinospora cordifolia*, a widely used medicinal plant in traditional formulations, was effectively fingerprinted using HPTLC. The stationary phase used was silica gel 60 F254, and the optimized mobile phase successfully facilitated the separation and analysis of bioactive constituents in the root extract and its herbal formulations.

3.5.1 HPTLC Screening of Phytochemical Present in *Tinospora cordifolia* Samples at 254 nm

Phytochemical visualization was carried out under UV light at 254 nm. The F254 indicator used in silica gel plates functions based on phosphorescence quenching. In this context, phosphorescence refers to the fluorescence that persists briefly after the excitation source is removed, typically lasting longer than 10 seconds [33]. The F254 fluorescent indicator, when excited at a UV wavelength of 254 nm, emits a green fluorescence. Compounds that absorb radiation at this wavelength quench the fluorescence, resulting in dark violet spots against a green fluorescent background, corresponding to the locations of the compound zones [34].

This quenching effect is typically caused by compounds containing conjugated double bonds. Classes of phytochemicals detectable at 254 nm include anthraglycosides, coumarins, flavonoids, propylphenols (commonly found in essential oils), and various alkaloids such as indole, isoquinoline, and quinoline types [35].

In the Indian market, numerous products containing *Tinospora cordifolia* are available in various formulations, including traditional Ayurvedic preparations and modern herbal capsules, tablets, and powders. Given its widespread therapeutic use, it is essential to develop a reliable HPTLC method for the qualitative estimation of *Tinospora cordifolia* in these commercial products. The results from the HPTLC analysis of different *Tinospora cordifolia* samples revealed a range of bioactive components. The TLC plates developed showed multiple bands with similar Rf values, indicating the presence of comparable phytoconstituents across the samples. These findings are summarized in Table 9.

Table 5: R*f* values of *Tinospora cordifolia* standard and market samples at 254 nm

TCSF		TCS1		TCS2		TCS3	
R_f	Colour	R_f	Colour	R_f	Colour	R_f	Colour
0.43	Dark	0.43	Dark	0.43	Dark	0.58	Dark
0.51	Dark	0.50	Dark	0.51	Dark		
0.56	Dark	0.55	Dark	0.56	Dark		
				0.71	Dark		

Qualitative estimation and photo-documentation of the ethanol extract (Figure 10) of the standard sample (TCSF) showed characteristic Rf values indicating the presence of phytochemicals. In the TCSF sample, three prominent dark spots were observed at Rf values of 0.43, 0.51, and 0.56. Sample TCS1 showed three dark spots at Rf 0.43, 0.50, and 0.55, while TCS2 revealed four spots at Rf 0.43, 0.51, 0.56, and 0.71. TCS3 exhibited a single dark spot at Rf 0.58 under short-wave UV light (254 nm). The short-wave UV (254 nm) detection allowed for visualization of high, mid, and non-

polar compounds, suggesting the presence of key phytochemicals such as phenols, saponins, tannins, alkaloids, glycosides, acids, and quinones. HPTLC fingerprinting at 254 nm confirmed that the extracts were derived from genuine *Tinospora cordifolia* plant parts. The similarity in banding patterns between standard and market samples (capsules, tablets, and powders) indicates consistency in the source material. However, Principal Component Analysis (PCA) revealed significant variation in phytochemical content and extractive values across different market products, indicating discrepancies in quality among commercial formulations.

3.4.2 HPTLC Screening of Phytochemical Present in *Tinospora cordifolia* Samples at 366 nm

The HPTLC method was found to be specific, accurate, and suitable for the qualitative estimation of crude extracts of *Tinospora cordifolia*. This method is particularly effective for fingerprinting and high-throughput analysis of market samples. The standard extract of *Tinospora cordifolia* and various market samples were visualized under UV light at 366 nm. Detection at 366 nm is based on fluorescence quenching, where fluorescence does not persist once the excitation source is removed. This quenching phenomenon is typically exhibited by compounds such as anthraglycosides, coumarins, flavonoids, phenol carboxylic acids, and certain alkaloids, including those found in *Rauwolfia* and *Ipecacuanha* [34, 36].

Table 6: R*f* values of *Tinospora cordifolia* standard and market samples at 366 nm

TCSF		TCS1		TCS2		TCS3	
R_f	Colour	R_f	Colour	R_f	Colour	R_f	Colour
0.15	Blue	0.15	Blue	0.27	Blue	0.04	Blue
0.21	Blue	0.21	Blue	0.34	Red	0.46	Blue
0.28	Blue	0.28	Blue	0.37	Red	0.53	Blue
0.43	Blue	0.43	Blue	0.44	Red	0.62	Blue
0.45	Red	0.45	Red	0.47	Red	0.94	Blue
0.50	F Blue	0.51	F Blue	0.53	Red		
0.54	Red	0.53	Red	0.79	Red		
0.91	Blue	0.91	Blue	0.85	R White		
				0.91	Red		

Under long-wave UV light at 366 nm, distinct banding patterns were observed in the standard and market samples of *Tinospora cordifolia*, as illustrated in Figure 11. These bands are indicative of various phytochemical constituents present in the samples. Sample 1 (TCSF) displayed eight bands at Rf values of 0.15 (blue), 0.21 (blue), 0.28 (blue), 0.43 (blue), 0.45 (red), 0.50 (fluorescent blue), 0.54 (red), and 0.91 (blue). Sample 2 (TCS1) showed a similar profile with eight bands at Rf values 0.15 (blue), 0.21 (blue), 0.28 (blue), 0.43 (blue), 0.45 (red), 0.51 (fluorescent blue), 0.53 (red), and 0.91 (red).
Sample 3 (TCS2) exhibited nine bands with Rf values at 0.27 (blue), 0.34 (red), 0.37 (red), 0.44 (red), 0.47 (red), 0.53 (red), 0.79 (red), 0.85 (reddish white), and 0.91 (red), indicating a broader range of phytochemicals. Sample 4 (TCS3) showed a simpler profile with five bands at Rf values 0.04 (blue), 0.46 (blue), 0.53 (blue), 0.62 (blue), and 0.94 (blue). These banding patterns reflect the presence of important bioactive groups such as saponins, acids, alkaloids, quinones, and glycosides.
The Rf values obtained in these samples fall largely within the range for non-polar compounds, indicating a predominance of such constituents. Specifically, the Rf values observed in TCSF (0.15-0.91) and TCS1 (0.15-0.91) confirm the presence of several non-polar phytochemicals. Similarly, the broad spread in TCS2 and more limited pattern in TCS3 indicate differences in chemical complexity and possibly the quality or authenticity of these market samples.
In TLC, compound polarity significantly influences mobility on the silica gel plate. Non-polar compounds, having low affinity for the polar stationary phase, move faster and thus show higher Rf values. In contrast, polar compounds are retained longer and exhibit lower Rf values. The classification based on Rf values includes high-polar peaks (Rf 0.1-0.4), mid-polar peaks (Rf 0.4-0.6), and non-polar peaks (Rf 0.6-0.9). The dominance of bands in the non-polar region across the samples further supports the presence of non-polar compounds such as phenols, tannins, saponins, quinones, acids, and glycosides.
The HPTLC fingerprinting profile observed at 366 nm (Figure 11) validates the identity and authenticity of the *Tinospora cordifolia* plant material used in the commercial formulations (capsules, tablets, and powders). The similarity of banding patterns between the standard extract and market samples suggests that the products were derived from genuine plant sources. Moreover, these consistent chemical fingerprints confirm the suitability of HPTLC as a reliable technique for quality control, fingerprinting, and standardization of herbal products.

3.5.3 HPTLC Screening of Phytochemical Present in *Tinospora cordifolia* Samples at 520 nm

Although chromatogram development is one of the most crucial steps in the HPTLC procedure, several important parameters are often overlooked [37]. The use of twin-trough chambers fitted with filter paper provides the best reproducibility for HPTLC plate development. These chambers help avoid solvent vapor preloading and minimize the effect of humidity, thereby improving chromatographic performance. Detection of the separated compounds on the sorbent layers is typically enhanced through fluorescence quenching when exposed to UV light in the range of 200-400 nm [36, 38].
Following post-derivatization, the developed HPTLC plates revealed distinct banding patterns under white light. For Sample 1 (TCSF), nine bands were observed at Rf values of 0.27 (pinkish violet), 0.32 (pinkish violet), 0.37 (pinkish violet), 0.47 (blue), 0.52 (pinkish violet), 0.56 (pinkish violet), 0.67 (pinkish violet), 0.71 (pinkish violet), and 0.82 (pinkish violet).
Sample 2 (TCS1) also showed nine bands with Rf values at 0.27, 0.32, 0.37, 0.47, 0.52, 0.55, 0.66, 0.70, and 0.80, all of which were pinkish violet in colour. Sample 3 (TCS2) demonstrated nine bands with Rf values at 0.12, 0.36, 0.47, 0.51, 0.60, 0.64, 0.76, 0.81, and 0.86, all appearing as pinkish violet spots under white light.
Sample 4 (TCS3), however, showed a comparatively simpler profile with only six bands observed at Rf values of 0.39, 0.40, 0.54, 0.61, 0.68, and 0.85, all exhibiting pinkish violet coloration. These post-derivatization profiles indicate the presence of various phytochemical compounds and offer a clear means of differentiating the quality and composition of different market samples in comparison to the standard extract.

Table 7: R*f* Values of *Tinospora cordifolia* standard and market samples after derivatization

TCSF		TCS1		TCS2		TCS3	
R_f	**Colour**	**R_f**	**Colour**	**R_f**	**Colour**	**R_f**	**Colour**
0.27	P Violet	0.27	P Violet	0.12	P Violet	0.39	P Violet
0.32	P Violet	0.32	P Violet	0.36	P Violet	0.48	P Violet
0.47	P Violet	0.37	P Violet	0.47	P Violet	0.54	P Violet
0.52	P Violet	0.47	P Violet	0.51	P Violet	0.61	P Violet
0.56	P Violet	0.52	P Violet	0.60	P Violet	0.68	P Violet
0.67	P Violet	0.55	P Violet	0.64	P Violet	0.85	
0.71	P Violet	0.66	P Violet	0.76	P Violet		
0.82	P Violet	0.70	P Violet	0.81	P Violet		
		0.80		0.86	P Violet		

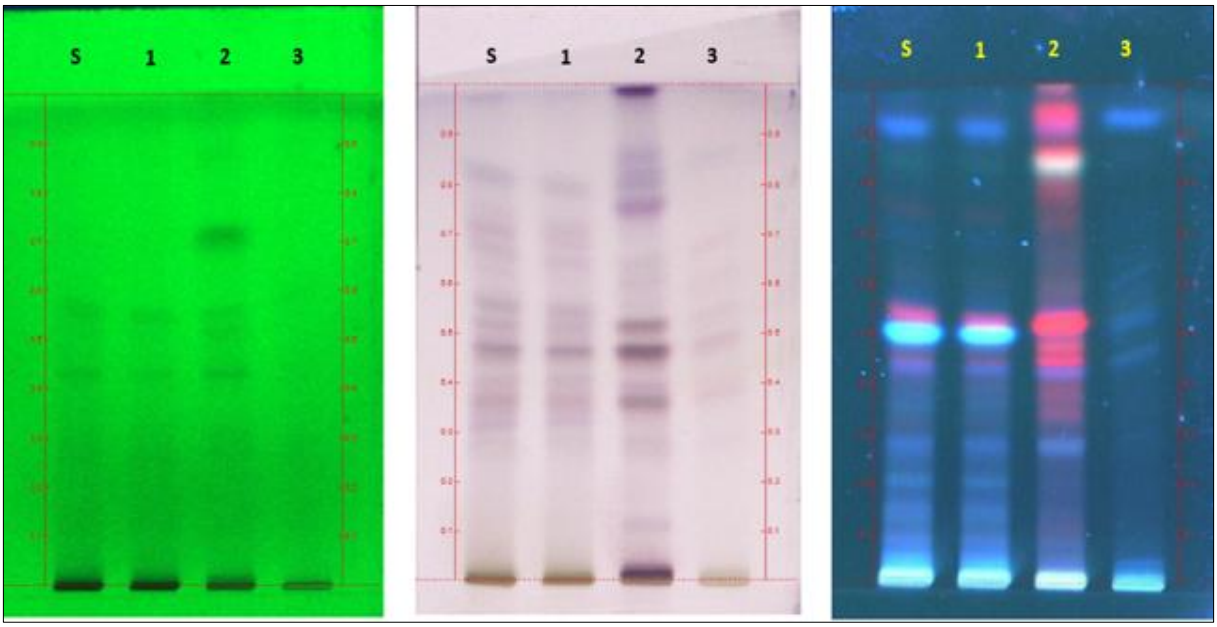

Fig 3: Developed HPTLC Plate photograph of Ethanolic extract of samples. A Plate at UV 254 nm; B: Visible mode after derivatization, C: Plate at UV 366 nm; S = TCSF; 1 = TCS1; 2 = TCS2; 3 = TCS

HPTLC fingerprint analysis was performed on different samples of *Tinospora cordifolia* using a densitometric scanning system. The ethanol extract of the standard sample (TCSF) revealed nine peaks under UV scanning [10, 39]. Prominent peaks were observed at Rf 0.43 (area 23.08%), 0.55 (21.59%), 0.70 (10.79%), and 0.89 (11.66%). The highest peak appeared at Rf 0.43, corresponding to 23.08% of active compounds, which is indicative of bitter compounds, specifically clerodane glycosides. The minimum peak was detected at Rf 0.26, accounting for 4.04% of the compounds. These findings confirm the presence of multiple phytochemicals in the standard extract with varying concentrations [34].

Similarly, the densitometric scan of the ethanol extract of sample TCS1 showed eleven peaks, with prominent bands at Rf 0.43 (15.96%), 0.56 (21.12%), 0.64 (10.08%), and 0.69 (10.29%). The maximum peak was recorded at Rf 0.56, suggesting the presence of bitter compounds such as clerodane glycosides. The least concentration was seen at Rf 0.67 (4.37%). These results, including peaks at Rf 0.43 and 0.64, further reflect the presence of a wide range of active phytochemicals.

The ethanol extract of sample TCS2 also displayed eleven peaks, with notable areas at Rf 0.40 (20.14%), 0.54 (24.07%), 0.64 (13.50%), and 0.89 (13.27%). However, the maximum peak in this case was observed at Rf 0.54 (24.07%), differing from the standard profile. Although some Rf values align with those in the standard, others do not, indicating possible variations in the phytochemical composition.

For sample TCS3, nine peaks were identified. The most prominent peaks were recorded at Rf 0.42 (28.32%), 0.58 (29.08%), and 0.69 (11.27%). After derivatization and white light scanning, the highest peak appeared at Rf 0.58 (29.08%), which correlated well with the standard, suggesting the presence of clerodane glycosides. The lowest peak was seen at Rf 0.02, with a concentration of 1.40%. Other significant peaks were found at Rf 0.42 (28.32%) and Rf 0.64 (10.29%).

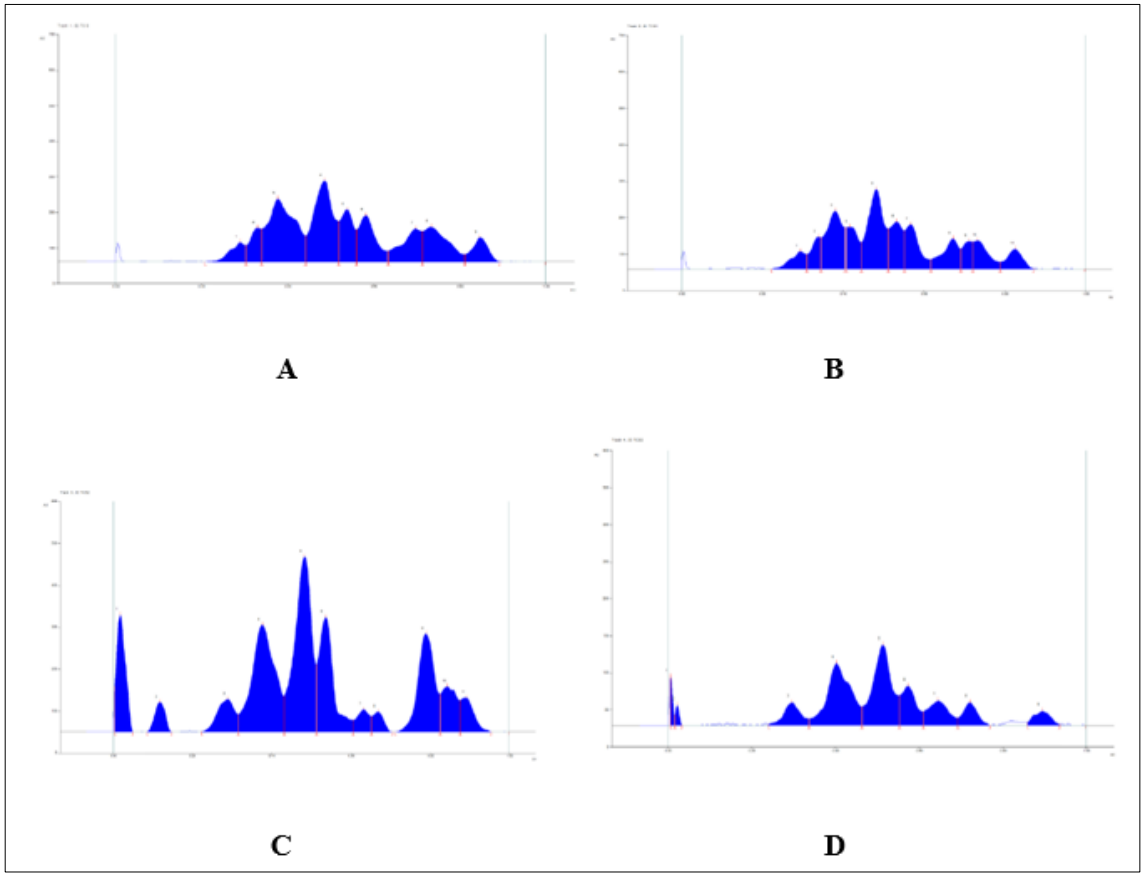

Fig 4: Comparison of Active Ingredients of Standard with Market Samples by HPTLC densitograms at 520 nm A = TCSF; B = TCS1; C = TCS2; D = TCS

Overall, the comparison of market samples (TCS1, TCS2, TCS3, and TCSF) with the standard (TC004) demonstrates the degree of similarity and variance in phytochemical profiles. As illustrated in Figure 17, the high peak observed in the standard is matched by peaks in sample TCS1, which also shows a broader peak similar to that in TCSF, indicating the presence of bitter clerodane glycosides. This is further supported by the Certificate of Analysis for the standard sample.

The phytochemical composition of TCS3 also aligns with the standard to a significant extent, suggesting that it contains similar active constituents. However, TCS2 does not show matching peaks with the standard, suggesting a possible absence of bitter active contents, particularly glycosidic acids. Additionally, TCSF displays a sharp peak, which may indicate the absence of other phytochemicals, pointing to a difference in extract quality or composition.

4. Conclusion

It can be concluded from the present study to analyse physico-chemical and phytochemical properties present in marketed samples (TCS1, TCS2, TCS3) in different forms of *Tinospora cordifolia* with standard TCSF. TCS1 is closely matched with standard limit. HPTLC is one of the most effective, reliable, precise and accurate method for compound identification and authentication. Thus, retention factor (Rf values) obtained from ethanolic extracts at different concentration of each sample can be used to identify each component due to their specificity, uniqueness and specifications of the quality profile of the drug *Tinospora cordifolia* stem.

5. Acknowledgements: None.

6. Declarations: We have nothing to declare. No funding was received for conducting this study. The authors have no relevant financial or non-financial interests to disclose.

7. Conflict of Interests: None.

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