Chapter IV

4.1: Selection and segregation of appropriate germplasm from different biogeographical zones of Gujarat.

The precise identification of plants is crucial to ethnobotany study because it serves as a crucial connection between scientific understanding and traditional knowledge (Cotton,1996). The medicinal properties of plants are contingent on the usage of appropriate plant material and the concentration of that material. Consequently, accurate identification and classification of each plant is essential and is the first stage in the scientific research of medicinal plants (Metre & Ghorpade, 2013; Bennett & Balick, 2008). Moreover, accurate taxonomy and nomenclature are necessary for repeatability, documentation, and prediction with regard to plant identification (Bennett & Balick, 2014). The data on the tag affixed to the voucher specimen assists in plant identification and offers information on the plant's geographic range, conservation status, possible uses, and regional significance.

Preparing medicinal plants for experimentation is the first stage and a crucial factor in producing high-quality research results. It entails the extraction and evaluation of the quality and amount of bioactive elements prior to the planned biological testing. Despite the fact that extracts, bioactive fractions, and chemicals derived from medicinal plants are employed for a variety of applications, the processes involved in their production are often the same regardless of the desired biological tests. The primary steps involved in gaining a high-quality bioactive molecule include the selection of an acceptable solvent, extraction techniques, phytochemical screening processes, fractionation techniques, and identification procedures. The specifics of these strategies and the precise route used rely exclusively on the study design. Polar solvents (e.g., water, alcohols), intermediate polar solvents (e.g., acetone, dichloromethane), and nonpolar solvents (e.g., acetone, dichloromethane) are often employed to extract medicinal plants (e.g., n-hexane, ether, chloroform). Various chromatographic methods are used to accomplish the fractionation and purification of phytochemical compounds. The resulting compounds are then characterised using a variety of identification methods, including mass spectroscopy, infrared spectroscopy, ultraviolet spectroscopy, and nuclear magnetic resonance spectroscopy.

It is also noted that the existence and type of active components in the plant raw material define the medicinal potential of plants, which in turn relies on environmental and other variables such as time and season of collection, regional variances, and drying and storage techniques.

Due to its direct effect on the concentration of active principles and the effectiveness of botanical products, the standardisation of collection and post-collection methods is of utmost relevance for the quality evaluation of plant raw materials (Prajapati *et al*., 2003; Mukherjee, 2002).

In accordance with this, the following sections detail the taxonomic classification of *Taverniera cuneifolia* as well as the collection conditions, regions of collection, authentication, post-collection processing including drying and post drying variations, pulverising, and storage.

4.1.1 Description and classification

This section provides an explanation of the morphological identifying characteristics of *Taverniera cuneifolia*, focusing on its categorization according to the Bentham and Hooker (1862–1883) method.
Taverniera cuneifolia (Roth) Arn

Botanical name: *Taverniera cuneifolia* (Roth) Arn

Vernacular names

English: East Indian moneywort (ILDIS),
Gujarati: Desi jethimadh (Thaker, 1908), Jethimal, Jethimadh

Classification: (APG IV, 2016; Legume Phylogeny Working Group, 2013)
Clade: Eudicots
Clade: Core eudicots
Clade: Rosids
Clade: Fabids
Order: Fabales
Family: Fabaceae
Sub family: Faboideae/ Papilionoideae
Tribe: Hedysareae
Genus: *Taverniera*
Species: *cuneifolia*

4.1.2 Distribution

Internationally: *T. cuneifolia* has been reported from Africa, Asia and Eurasia along coastal tracts. From Africa - Somalia (Thulin, 1985) while from Asia - Iran (Thulin, 1985); Pakistan (Thulin, 1985; Ali, 1977) and from Eurasia (Middle East): Oman (Thulin, 1985), United Arab Emirates (Thulin, 1985) and Farasan islands (Alfarhan, 2005) (fig. 2.1).

Nationally: *T. cuneifolia* has been reported from (fig. 2.2):

North India: Jammu and Kashmir: Northwest Himalaya (Chauhan *et al*., 2003)**; Punjab** (Bhandari, 1978) - Plains of Punjab (Khare, 2007).

Western and central India: Rajasthan (Bhandari, 1978, Shetty & Singh, 1987) Jodhpur Jadaan; **Gujarat** (Shah, 1978) - Shetrunjaya, Rozimata temple, Narara beyt, Rampara sanctuary, Hingolgadh sanctuary (Nagar, 2008), Kutch (Bhuj) Tapkeshwari Hill Range (Joshi *et al*., 2013), Gir forest, Ghumli, Dwarka (Santapau, 1962); **Maharashtra** (Karthikeyan & Kumar, 1993) - Majalgaon (Khan *et al*., 2012), Osmanabad (Jamdhade *et al*., 2013); **Madhya Pradesh** (Sanjappa, 1992).

Eastern part of India: Orissa (Bairiganjan *et al*., 1985); **West Bengal** (Sanjappa, 1992).

Southern India: Karnataka (Bijapur, Madhbhavi, Raichur and Vijayanagara Sri Krishnadevaraya University, Ballari) (Singh, 1988); **Andhra Pradesh** (Gamble, 1918; Rao *et al*., 2006) - Betam cherala, Kurnool district.

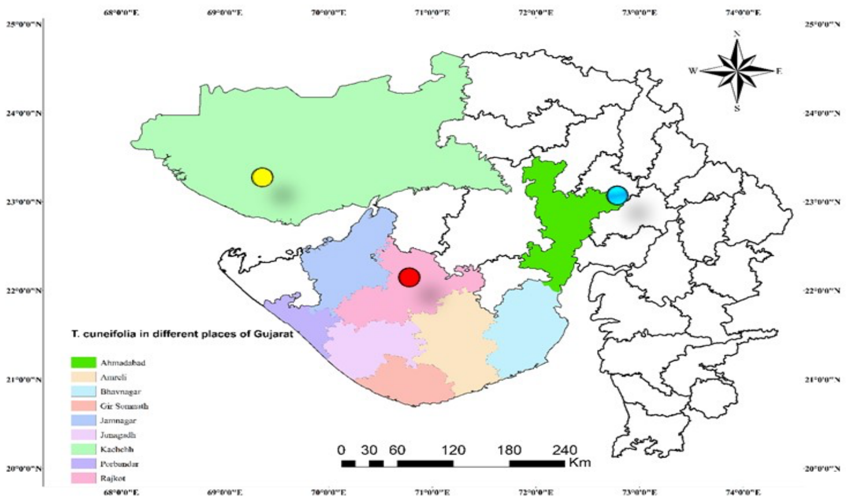


Figure 4.1: Collection of *T. cuneifolia* in different places of Gujarat

The Collection has been done from three different biogeographical zones i.e.,

Table 4.1: Different biogeographical zones with GPS location

Name of the Place	GPS Location
Bagodara	N 22°33'11.5848"; E 72°18'40.9752"
Kutch (Bhuj)	N 23°18’40.1"; E 69°39’53.0"
Rajkot (Munjka village)	N 22°17'22.2389"; E 70°44'34.652"
Jamnagar (Khijadiya)	22° 31' 29.7948" N 70° 10' 0.6744" E

Figure 4.2:*Taverniera cuneifolia* collect from Bagodara

Figure 4.3: *Taverniera cuneifolia* collection from Kutch (Bhuj)

Figure 4.4: *Taverniera cuneifolia* collection from Rajkot (Munjka)

Figure 4.5: *Taverniera cuneifolia* collection from Jamnagar (Khijadiya)

4.1.3 Observations

Plants were collected while in the flowering and fruiting stages, and their identities were double-checked against the Flora of West Pakistan (Ali, 1977), the Flora of the Presidency of Bombay (Cooke, 1910), and the Flora of Gujarat State (Shah, 1978) in the laboratory at the Department of Botany in Vadodara. During the fieldwork, we also made notes on the plants' growth patterns, preferred environments, flower colours, and the communities in which they tend to thrive. The plant was reconfirmed with herbarium sheets of Botanical Survey of India. The plant species was compared with following specimens: BSI, western circle poona, Flora of Eastern Karnataka (Collector, N. P. Singh) Acc. no.-142989, sheet No. 113404, locality (Bagewadi, Muddebihal); Flora of Gujarat (Collector, R. S. Raghvan) Acc. No. 114838, Sheet no. 95696, locality (Lakhpat).

The macromorphology of *Taverniera cuneifolia* is as follows:

The length of the tap root ranges from 30 to 60 cm, while the girth varies between 2 and 4 cm (Fig.2.1). The usual thickness of the stem is 0.5-0.8 cm. It is greenish brown, smooth, and lustrous. Leaflets are 0.6-2.5 cm long, obovate to oblanceolate, entire, mucronate, sparsely pubescent, becoming subglabrous, and have a brownish edge; stipules are connate, amplexicaul, and 3 mm in length. Flowers of violet-pink, Pods have 1-2 seeded joints. The seeds are kidney-shaped and yellowish brown in colour.

4.1.3.1 Morphology

The plant is prostrate to erect, much branched from the ground, 80-100cm height. When it is in full bloom, it is much shrubby.

Shrub, 60-100 cm, branches pubescent. Leaf uni-trifoliolate, leaflets 0.6-2.5 cm long, obovate to oblanceolate, entire, mucronate, pubescent, becoming subglabrous; stipules connate, amplexicaul, ca. 3 mm long. Inflorescence an axillary raceme, up to 10 cm long, Pedicel 1-2.5 mm long, bracts ca. 2.5 mm. Calyx 4-5 mm long, silky, teeth deltoid, ca. 2.5 mm long. Corolla purple, macrescent. Vexillum 10-13 mm long, vexillum and keel larger than the wing. Fruit with 1-3, 1-seeded joints, joints echinate and ovoid, pubescent.

Figure 4.6: Habit of *Taverniera cuneifolia* showing various morphological parts (A) Flowers; (B) Leaves; (C) Roots; (D) Dried flowers; (E) Seeds

Figure 4.7: Roots collected from Bagodara, Kutch and Rajkot (April-June)

Plant collection was done from three pockets (Figure 4.3, 4.4 and 4.5) with good population density and the details of the site are as follows:

Table 4.2: Morphological & Phytochemical observations of different locations

In Native Soil	**Bagodara**	**Kutch**	**Rajkot**
Collection period	Pre monsoon	Pre monsoon	Pre monsoon
Root Girth	2-5.5cm	1-3cm	1-5cm
Root Length	15-40cm	10-20cm	10-35cm
Root Biomass	40-280g	20-180g	20-250g
Soil Type	Sandy loam/clayey	Sandy/gravelleous	Moderate black
Sucrose Content	313.4 µg/mg	34.5 µg/mg	176.7 µg/mg
Seed Viability (Tetrazolium test)	Highest	Low	Medium

The collection from Bagodara has been taken into consideration for future study based on the aforementioned observations.

Table 4.3: Organoleptic characters of powders of *T. cuneifolia* roots

Parameters	***T. cuneifolia***
Color	Yellowish brown/light brown
Odour	Characteristic groundnut like
Taste	Sweet

4.1.3.2 Ecology

The flowers of *Taverniera cuneifolia* are often beautiful to attract pollinators since the plant is predominantly entomophilous. This excess of seed production acts as a survival strategy. They are 2 mm in diameter and brown to golden in colour. The poor success rate of germination contributes to the limited abundance of the species in the wild. The leaves are adapted to terrestrial and salty environments. In coastal regions, the leaves are rather thick (swollen), whereas in terrestrial places they are membranous. Another intriguing adaptation is the winter and summer shedding of leaves, although photosynthesis continues in the green stem. This is likely the reason why even green twigs may be spotted throughout the off-season and sugar storage persists.

The associated species occurring with *T. cuneifolia* in grassland/fallow lands are Aristida sp., Helandia latebrosa, Zizyphus nummularia, Alyscicarpus vaginalis, Bothriochloa pertusa, Indigofera cordifolia, Pulicaria wightiana.

4.1.3.3 Climatic parameters

Based on the available meteorological data from last ten years, the climatic data are as follows:

Rainfall: The average relative humidity is 52%, ranging from 34% in the summer (March) to 78% during monsoon season (July). July is the most humid month, with humidity ranging from 43.0% to 96.7% (Ray *et al*., 2009).

Temperature: On an average temperature is 32-45°C in Saurashtra, with temperature going as high as 43-45°C. Temperature along coastal track is between 32-47°C (Ray *et al*., 2009).

Humidity: Humidity is maximum along coastal track of Jamnagar, Porbandar, Junagadh and Bhavnagar. Humidity goes to a maximum of 80-85% during monsoon with minimum humdity during the months May-July. However, along the coastal track which is most suitable track along which *T. cuneifolia* grows has an average of 43-96% humidity in pre monsoon (Ray *et al*., 2009).

Wind speed: Wind fall along coastal track is 20 km/hr. However, in the terrestrial zones of Rajkot, Girnar the wind fall is 14 km/hr.

Soil type: Geographically, Gujarat is divided into five regions: 1) Kutch Region, 2) North Gujarat, 3) Central Gujarat, 4) South Gujarat (South Gujarat and South Gujarat Hills) and 5) The Saurashtra Peninsula (North and South Saurashtra). The most prevalent soil types in Gujarat are as follows:

Table 4.4: Geographical parameters of Gujarat

Sub Region	Climate	Soil
Kutch	Arid to semi-arid	Sandy, Saline
North Gujarat	Arid to semi-arid	Loamy, alluvium
Central Gujarat	Semi-arid	Medium black
South Gujarat	Semi-arid to dry sub-humid	Deep black, alluvium/ clayey
Saurashtra	Dry sub-humid	Shallow, medium black

(Patel, 2019)

4.1.3.4 Soil Chemical characters

Soil analysis of the visited sites revealed an exceptionally high concentration of Potassium, moderate amount of Zinc, Iron, Manganese, and copper, and a very low concentration of Phosphorus (Table 4.5). The pH level of the soil was tending from neutral to alkaline. The high EC suggested presence of significant concentration of minerals. While, the EC of Bagodara soil varied between 1.66 and 2.70.

Table 4.5: Macro and micronutrient analysis of Soil

Soil Name	Macronutrients				Micronutrients				pH	EC
	N/OC	P	K	S	Zn	Fe	Mn	Cu		
Bagodara(Pre-monsoon)	0.575	8.50	255.00	11.55	1.83	15.55	22.3	3.02	7.59	1.66
Bagodara(monsoon)	0.99	8.00	241.00	9.50	4.38	10.98	18.00	5.10	7.58	0.42
Bagodara(post monsoon)	0.7	8.66	175.66	11.53	1.56	9.58	12.30	1.42	8.44	2.70

Very low
High
Very high

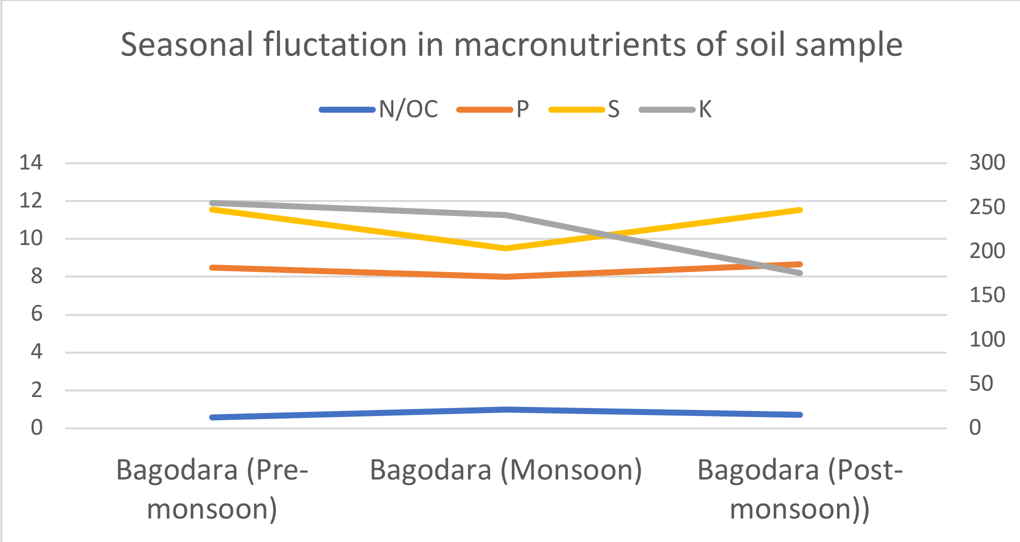


Figure 4.8: Seasonal fluctuations in macronutrients of soil samples

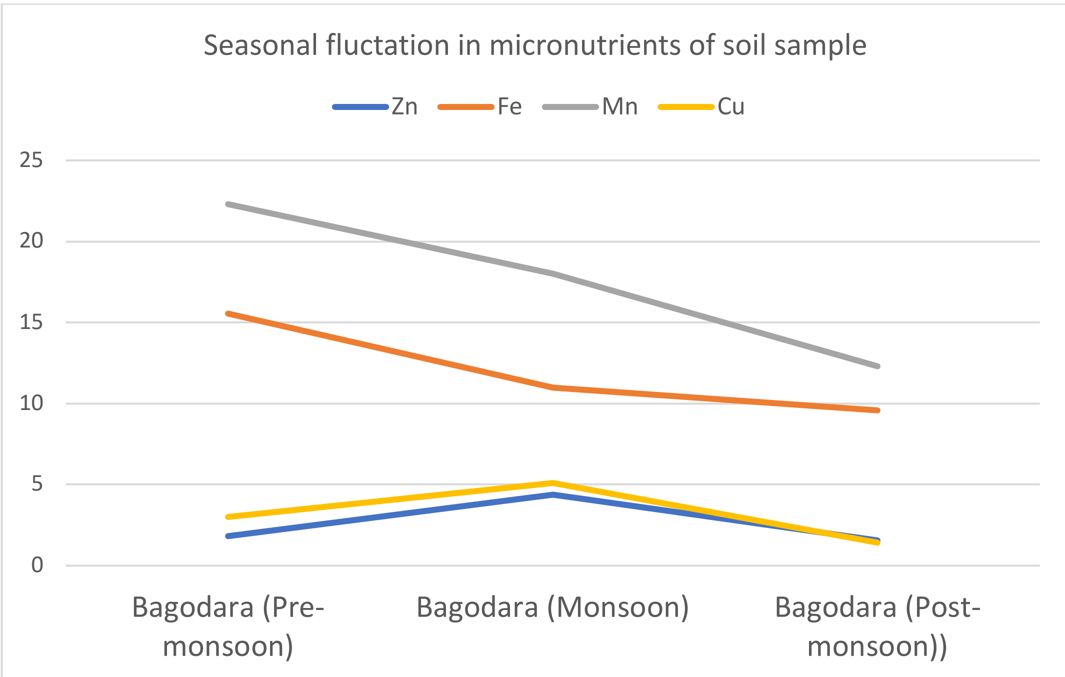


Figure 4.9: Seasonal fluctuations in micronutrients of soil samples

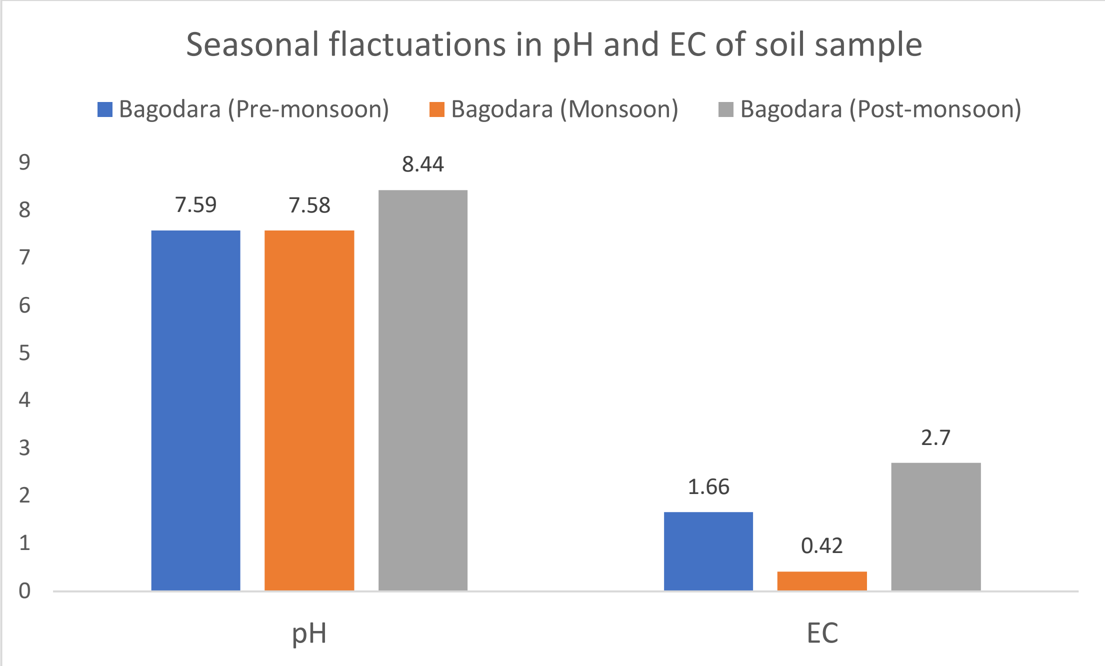


Figure 4.10: Seasonal fluctuations in pH and EC of soil samples

Mission Code :O20220031Z01

National Bureau of Plant Genetic Resources (ARIS Cell)
Pusa Campus, New Delhi-110012

AKMU-7524 IC : 645833 to 645835

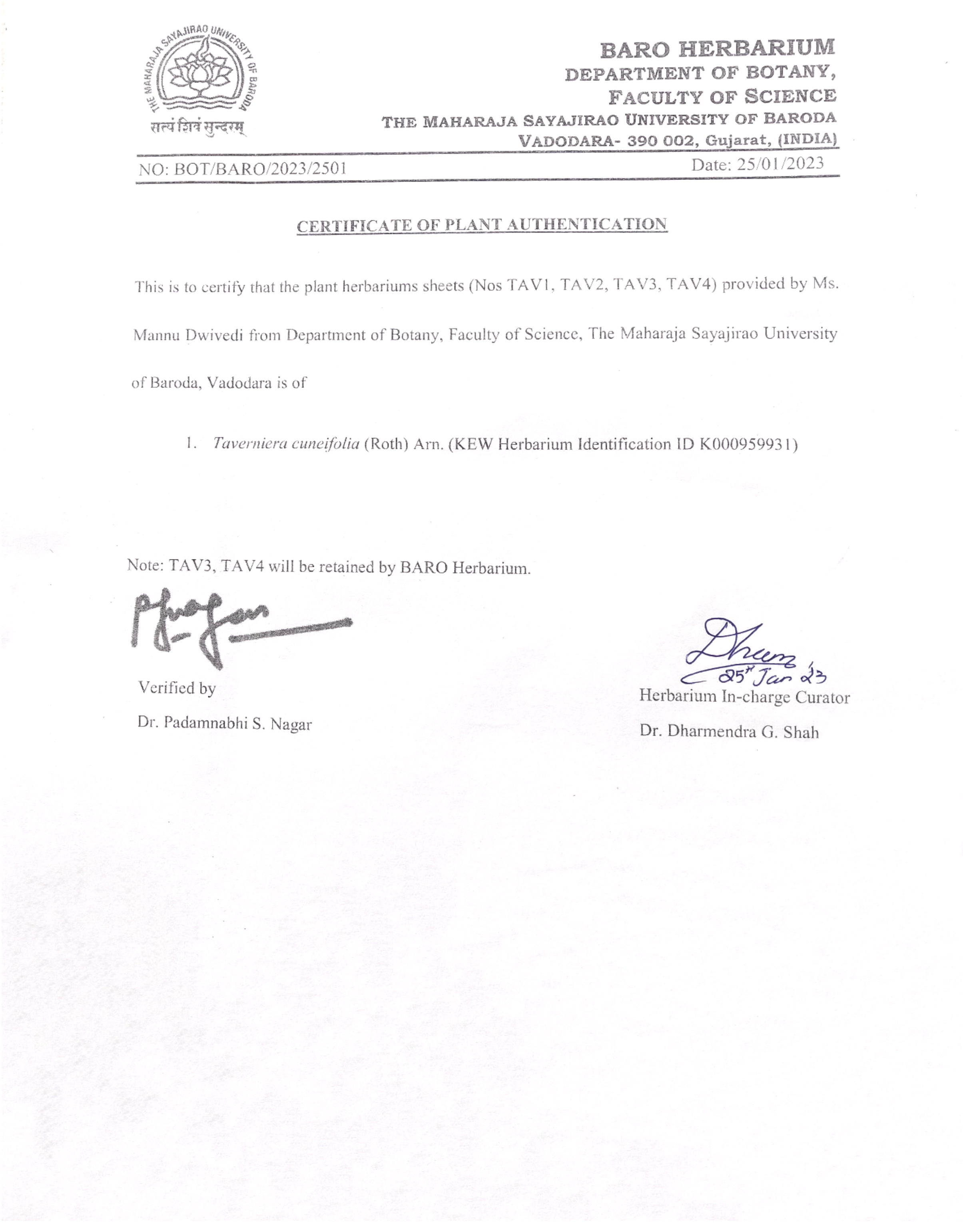
Collaborator(s) :Dr. Padmnabhi S. Nagar Assoc. Prof. Dept of Botaniy, Faculty of Science The Maharaja Sayajirao University of Baroda Vadodara Gujarat, Miss. Mannu Dwivedi Research Scholar Dept of Botaniy, Faculty of Science The Maharaja Sayajirao University of Baroda Vadodara Gujarat

Total 3 Record(s).

S.No.	Accession Material Type	Coll-No Other-Id	Crop Species	Cultivar Name Bio-Status Variety	Sample Method Sample Type	Collection Date Village/District/State	Source Frequency Habitat	Pedigree Donor	Imp Traits Remark
1	IC-0645833 FRUITS	MD.1 -	Indian Licorice *Taverniera cuneifolia*	Indian licorice /Jethimadh WILD -	RANDOM FRUITS	06 Apr 2022 Bhataman /Bagodara /Gujarat	FALLOW FREQUENT DISTURBED	- -	- -
2	IC-0645834 FRUITS	MD.2 -	Indian Licorice *Taverniera cuneifolia*	Indian licorice /Jethimadh WILD -	RANDOM FRUITS	02 May 2022 Bhuj /Kutch /Gujarat	FALLOW FREQUENT CULTIVATED	- -	- -
3	IC-0645835 FRUITS	MD.3 -	Indian Licorice *Taverniera cuneifolia*	Indian licorice /Jethimadh WILD -	RANDOM FRUITS	05 Apr 2022 Rajkot /Gujarat	FALLOW FREQUENT DISTURBED	- -	- -

Figure 4.11: IC number provided by NBPGR, Pusa campus, Delhi

Figure 4.12: Herbarium of *T. cuneifolia* submitted to BARO herbarium



सत्यं शिवं सुन्दरम्

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

BARO HERBARIUM
DEPARTMENT OF BOTANY,
FACULTY OF SCIENCE
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA
VADODARA- 390 002, Gujarat, (INDIA)

NO: BOT/BARO/2023/2501 Date: 25/01/2023

CERTIFICATE OF PLANT AUTHENTICATION

This is to certify that the plant herbariums sheets (Nos TAV1, TAV2, TAV3, TAV4) provided by Ms. Mannu Dwivedi from Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara is of

1. *Taverniera cuneifolia* (Roth) Arn. (KEW Herbarium Identification ID K000959931)

Note: TAV3, TAV4 will be retained by BARO Herbarium.

25th Jan 23

Verified by
Dr. Padamnabhi S. Nagar

Herbarium In-charge Curator
Dr. Dharmendra G. Shah

Figure 4.13: Authentication certificate of *Taverniera cuneifolia* from BARO herbarium, Department of Botany, Vadodara

4.1.4 Discussion

The raw material collected from three different locations and the germplasm submitted to NBPGR, Pusa, Delhi and the herbarium were submitted to BARO herbarium (figure 4.11, 4.12

& 4.13). The raw material collected from Bagodara with gravelly soil was found to have a pH range of 7.5-8.4, an electrical conductivity range of 1.5-2.7, and relatively higher levels of macronutrients such as zinc, manganese, iron, and copper; however, the material with the highest levels of sugars and amino acids as primary metabolites had the highest potassium content (figure 4.5, table 4.10). Thus, it can be concluded that the quality of *T. cuneifolia* was determined based on characteristics such as qualitative and quantitative phytochemical analyses. In order to ensure the quality of the medicine, the fingerprint profiles generated by HPTLC also served as a reference for the phytochemical profile. In the subsequent phase, plant raw materials were used to prepare extracts. The ensuing chapter elaborates on the optimal settings for the manufacture of extract and the parameters examined for their standardisation.

4.2: Standardization and validation of sugars (sweeteners) and amino acids.

Substances with established therapeutic value are what researchers call the "active components" or "active principles" in herbal remedies. For the most part, researchers have turned to natural ingredients to spur the development of novel therapeutics. In the seven decades between the 1940s and the end of 2014, approximately half of the chemical medications authorised by the FDA for the treatment of human illnesses were either directly developed from or inspired by natural products (Newman & Cragg, 2012, 2016). When compared to compounds derived from combinatorial chemistry, natural products provide more drug-like properties in terms of functional groups, chirality, and structural complexity (Atanasov *et al*., 2015; Cragg & Newman, 2013). In most cases, the levels of active components in natural remedies are rather minimal. The use of natural products in medicine development has been limited by the lengthy and laborious process of extracting and isolating these compounds in the laboratory. The initial stage in separating desired natural products from basic materials is extraction. According to the extraction principle, extraction procedures include solvent extraction, the distillation process, pressing, and sublimation. Solvent extraction is the most used technique. The extraction of natural products proceeds as follows: (1) the solvent permeates the solid matrix; (2) the solute dissolves in the solvents; (3) the solute diffuses out of the solid matrix; and (4) the extracted solutes are retrieved. Any component that increases diffusivity and solubility throughout the aforementioned processes will aid extraction. The extraction efficiency is affected by the characteristics of the extraction solvent, the particle size of the raw materials, the solvent-to-solid ratio, the extraction temperature, and the extraction time (Li *et al*., 2008; Li *et al*., 2014; Yi *et al*., 2012; Zhou *et al*., 2012; Du *et al*., 2011).

For solvent extraction, the choice of solvent is critical. In selecting solvents, selectivity, solubility, cost, and safety should be addressed. According to the rule of resemblance and inter-miscibility (like dissolves like), solvents having a polarity value close to the solute's polarity are likely to perform better, and vice versa. Alcohols (EtOH and MeOH) are ubiquitous solvents for phytochemical research solvent extraction. Drying is the first step under many post-collection procedures used for plant collection (Rocha *et al*., 2011). It involves removal of moisture from the crude drug so as to improve its quality and make it resistant towards the growth of microorganisms like bacteria, fungus and mould (Sipahimalani, 2002). The kinetics of drying can define the final properties of the dried material and influence chemical changes in it. Also, the storage of plants for a longer time requires drying (Benbelaid *et al*., 2013). Out of the various techniques of drying, shade drying is the most preferred method for drying the plant sample as it can maintain or minimize loss of colour of leaves or flowers and at low temperatures can minimize the loss of volatile substances.

Storage of the plant material is also of key importance as improper storage facilities can lead to several problems (Rocha *et al*., 2011). The plant powders absorb moisture to the extent of about 25% of their weight during storage and become susceptible to microbial growth. Moisture also increases the bulk of the drug causing impairment in its quality and facilitates enzymatic reaction leading to changes in the physical appearance, decomposition of active constituents or production of metabolites with no effects or toxic effects. Physical factors such as radiation due to direct sunlight, air (oxygen), humidity, and temperature can bring about deterioration directly or indirectly. These factors, alone or in combination, can also lead to the development of organisms such as molds, mites, and bacteria (Kamboj, 2012; Sipahimalani, 2002).

To accurately assess chemical and biological indicators, fingerprint analysis by high performance thin layer chromatography (HPTLC) has emerged as a potent and useful method (Dash *et al*., 2010; Patil *et al*., 2010; Ramya *et al*., 2010). Moreover, it provides a more precise and rapid resolution of the active components.

Improved chromatographic and spectral fingerprinting techniques have contributed significantly in recent years to the quality assurance of sophisticated herbal remedies (Yamunadevi *et al*., 2011). The chemical fingerprinting approach has been used to offer a thorough chemical description of herbal remedies, and it has been given their full endorsement by the World Health Organization (WHO), the FDA of the United States, and the European Medicines Agency (EMA) (Li *et al*., 2020; WHO, 1991; EMA, 2011). Multiple chromatographic and spectroscopic methods may be used to establish the fingerprints, or distinctive profiles and patterns representing the complicated chemical makeup of herbal samples (Bansal *et al*., 2014; Goodarzi *et al*., 2013).

Sugars are the primary source of energy for plants, animals, and humans. Herbs contain three types of sugars: monosaccharides, oligosaccharides, and polysaccharides, with monosaccharides being the primary component of oligosaccharides and polysaccharides. Sugars hold antibacterial qualities, are used to treat wounds and are major source of energy for healthy functioning of human metabolism.

The quantity of amino acids varies from plant to plant based on their metabolic processes (Abed, 2007). These amino acids are readily transmitted by root hairs and plant capillaries. Amino acids have a significant part in a variety of biotic activities, whether they are free or as a component of proteins; hence, their significance and efficacy lie in the development phases of plants. The presence of different amino acids in plant extracts has been determined by a variety of techniques. Using Ninhydrine or 2,4-dinitrofluorobenzene, the total amount of amino acids may be measured colorimetrically (Chen *et al*., 2009). HPLC, capillary electrophoretic, and anion exchange chromatography are the few chromatographic techniques published for the analysis of amino acids (Wang *et al*., 2010; Alcazar *et. al*., 2007; Aucamp *et al*., 2000; Ding, Y., Yu & Mou, 2002; Pongsuwan *et al*., 2008). Due to the absence of a suitable chromophore in their structures, amino acids must be labelled using labelling reagents such as 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl chloride), O-phthaladehyde (OPA), and phenylisothiocyanate. In this situation, derivatization using precolumn or postcolumn reagents and detection with fluorescence, ultraviolet, or diode array detectors are required (DAD) (Wang *et al*., 2010).

HPTLC has been widely used for standardising herbal medications and formulations since it allows for the simultaneous examination of many samples with a little amount of marker component and solvent solution (Patil *et al*., 2015). Herbal medicines are notoriously difficult to standardise and monitor for quality, since they are comprised of a diverse array of phytoconstituents and are therefore prone to substantial variance (Seasotiya *et al*., 2014). Alternative approaches based on high-performance liquid chromatography (HPTLC) are being investigated as potential methods of importance in routine drug analysis and should be taken into account. HPTLC finger printing is beneficial for more than only identifying and checking the quality of a plant species; it also aids in identifying, isolating, purifying, and characterising marker chemical components unique to that species. It's possible to display graphics with the peak data in HPTLC findings (Vijayalaxmi & Ravindhran, 2012).

4.2.1 Solvent Extractive value

Solvent extractive value: Extractive value / yield extractive value is the quantity of phytoconstituents extracted from a given amount of plant material using various solvents (Mukherjee, 2002). The extractive value reveals the type of the chemical ingredients (Patil *et al*., 2015) and indicates the proportion of polar, mid-polar, and non-polar components in the plant material (Singh *et al*., 2011). It also aids in the detection of low-grade, expired medicines (Garg *et al*., 2012). Numerous solvents, such as methanol, ethanol, acetone, and water, have been used to extract bioactive chemicals from plant material. Due to the range of bioactive compounds present in plant materials and their varying solubility in various solvents, the best solvent for extraction is dependent on the plant materials and the chemicals to be extracted (Ajanal *et al*., 2012; Mahdi-Pour *et al*., 2012). As determined by the plant, the content of the extractives followed the order: water-soluble > methanol-soluble > hexane>ethyl acetate-soluble (figure 4.14). This shows that the plant samples have the largest concentration of polar phytoconstituents and the lowest concentration of mid-polar phytoconstituents. The analysis's findings are shown in Figure 4.1.

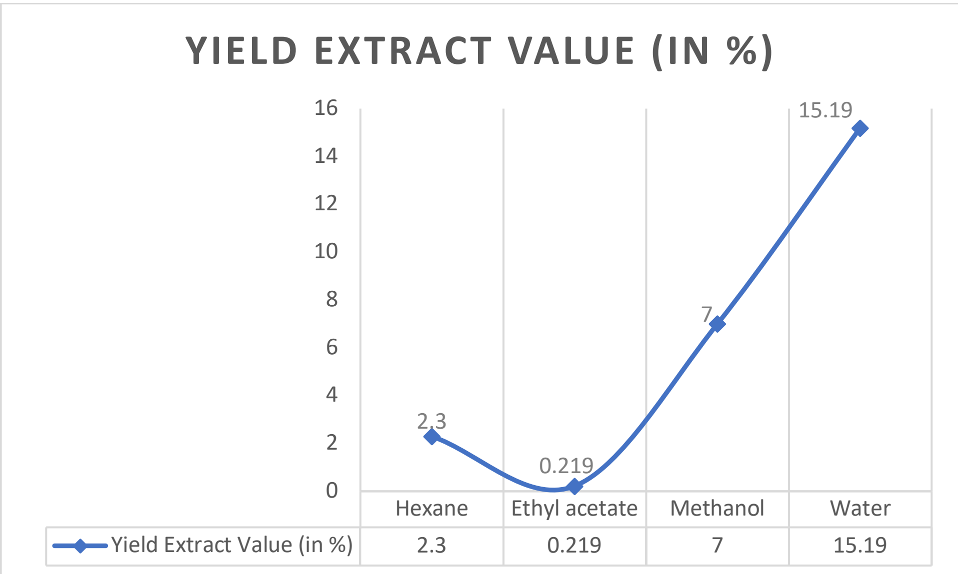


Figure 4.14: Graph showing percentage yield extract value of *T. cuneifolia* plant extract in four different solvents

4.2.2 Fingerprinting analysis

In the current study, an HPTLC fingerprinting profile of secondary metabolites in raw materials has been performed. Extraction and HPTLC analysis were performed on the samples. The extracts produce a distinct pattern of peaks and valleys attributable to one or more recognised or unknown components. Unknown raw materials may be defined by comparing their fingerprints to those of botanically verified materials (Ansari *et al*., 2015).

In the current work, several solvent systems like chloroform, cyclohexane, ethyl acetate, acetone, methanol, formic acid, toluene, glacial acetic acid, etc., in different ratios were tried and tested for development of HPTLC fingerprint. It was observed that the mobile phase of ethyl acetate: Formic acid: Glacial acetic acid: water (15: 1: 1: 2, v/v/v/v) gave good separation of phytoconstituents in *G. glabra, T. abyssinica and T. cuneifolia* methanolic and hydro-alcholic extracts. HPTLC plate photos captured at different wavelengths and the HPTLC chromatograms are given in figure 4.15, 4.17 and 4.19, respectively. Different Rf values along with the response obtained for the phytochemicals using HPTLC technique for *G. glabra, T. abyssinica and T. cuneifolia* are listed in figure 4.16, 4.18 & 4.20. From the results of the fingerprint analysis, it was observed that the water extracts showed more number of bands as compared to the hydro-alcoholic extracts wherein, the *G. glabra* showed 9 bands in each extract before derivatization. Similarly, *T. abyssinica* showed 5 bands in methanolic extract and 4 bands in hydro-alcoholic extract whereas, *the bark extract of T. cuneifolia* showed more number of bands in methanolic extract than the pith portion of extract in hydro-alcoholic extract at 254 nm and 366 nm, respectively.

The derivatization of HPTLC plates with 10% methanolic sulphuric acid permitted the visibility of specific phytoconstituents devoid of inherent quenching and fluorescence intensity. This may be the result of a chemical process that changed the molecular structures of phytoconstituents, hence improving and improving their detection (Kupiec, 2004). At 366 nm and 550 nm, respectively, densitometric scanning revealed a large number of bands in all the samples, with distinct visual distinctions.

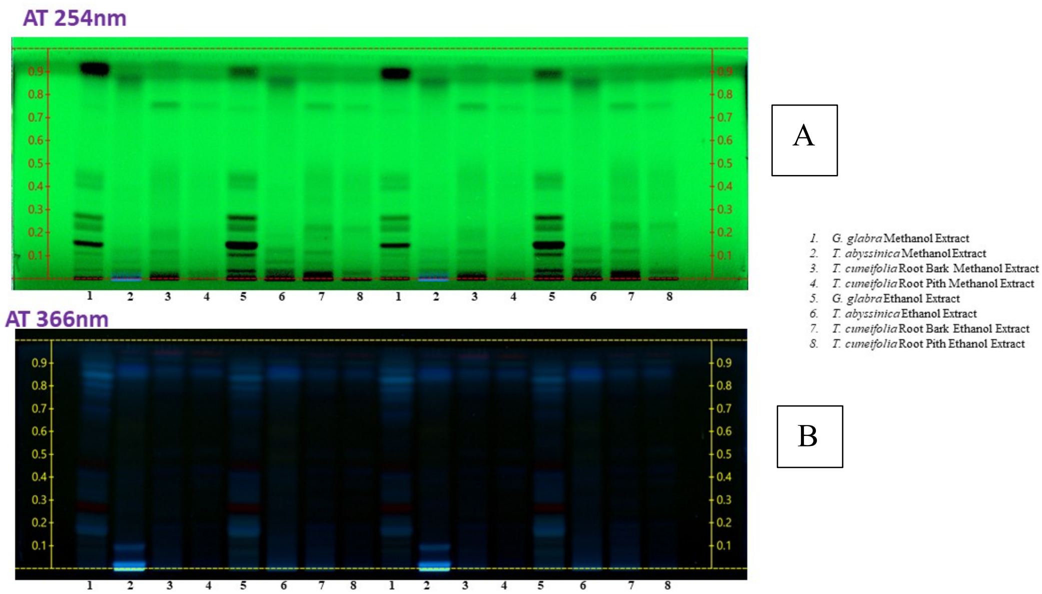


Figure 4.15: After development before derivatization at (A) 254nm and (B) 366nm

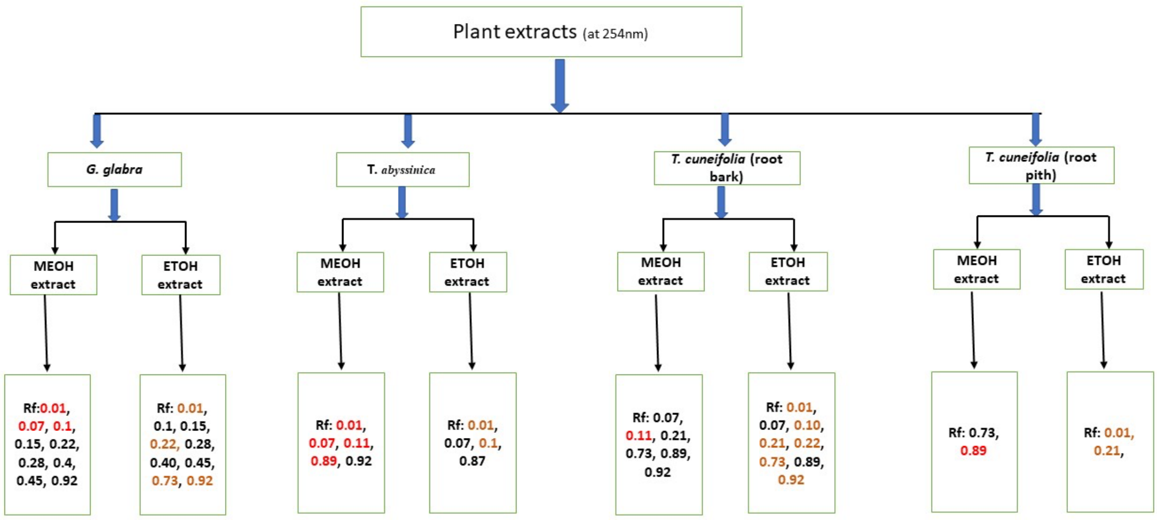


Figure 4.16: Schematic diagram of chromatographic fingerprint Rf

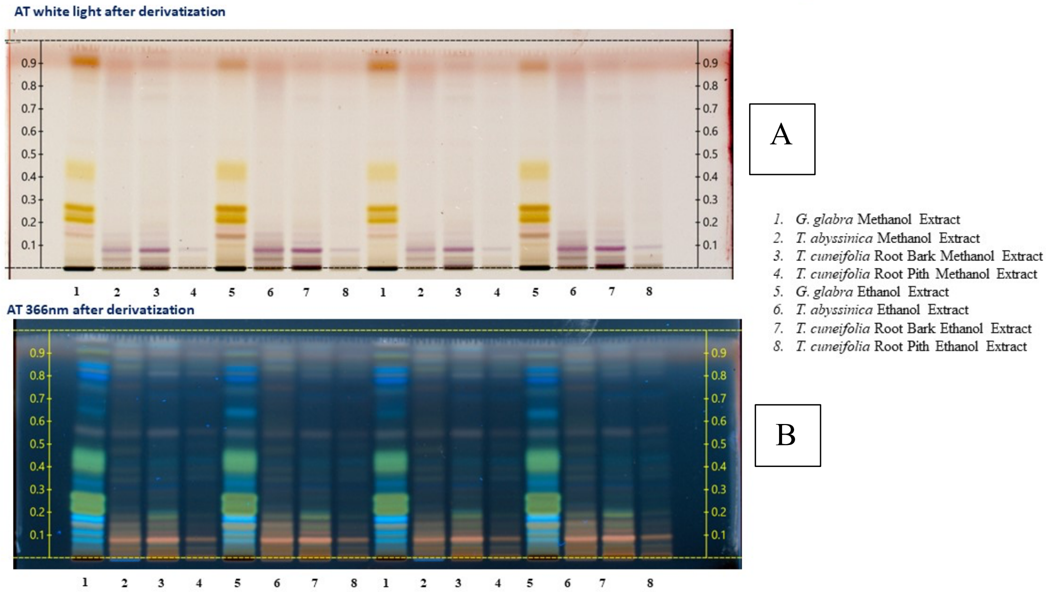
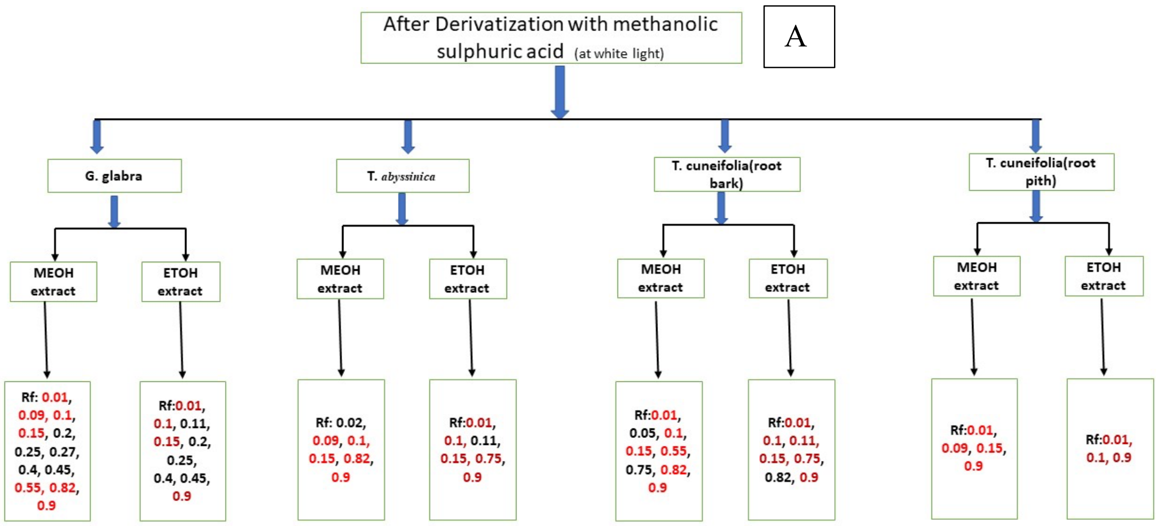


Figure 4.17: After derivatization with 10% methanolic sulphuric acid at (A) 254nm and (B) 366nm



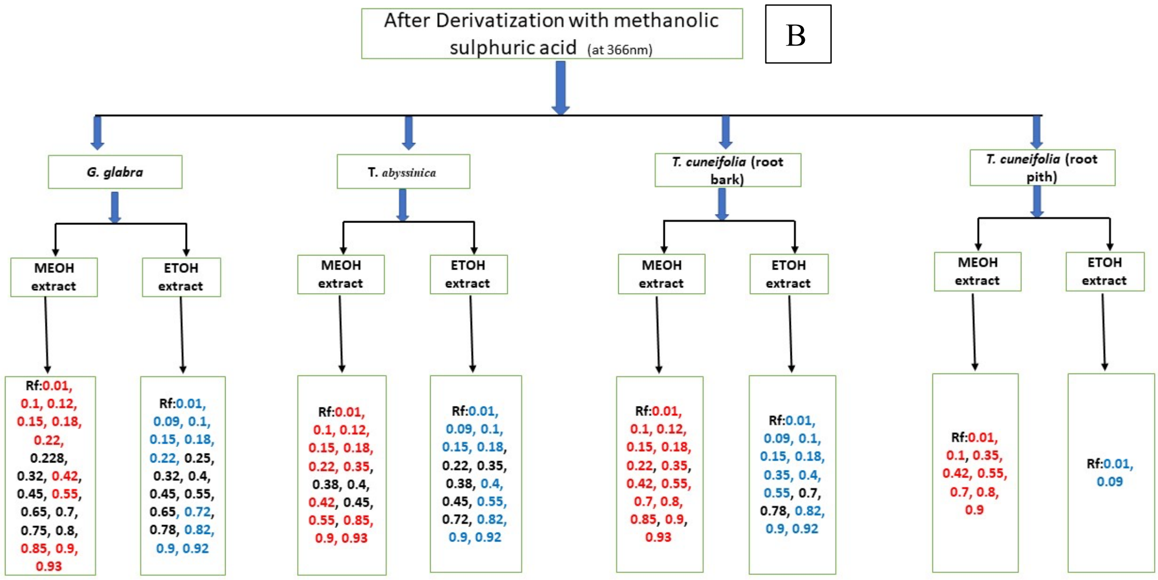


Figure 4.18: Schematic diagram of chromatographic fingerprint Rf after derivatization (A) at Visible light (B) at 366nm

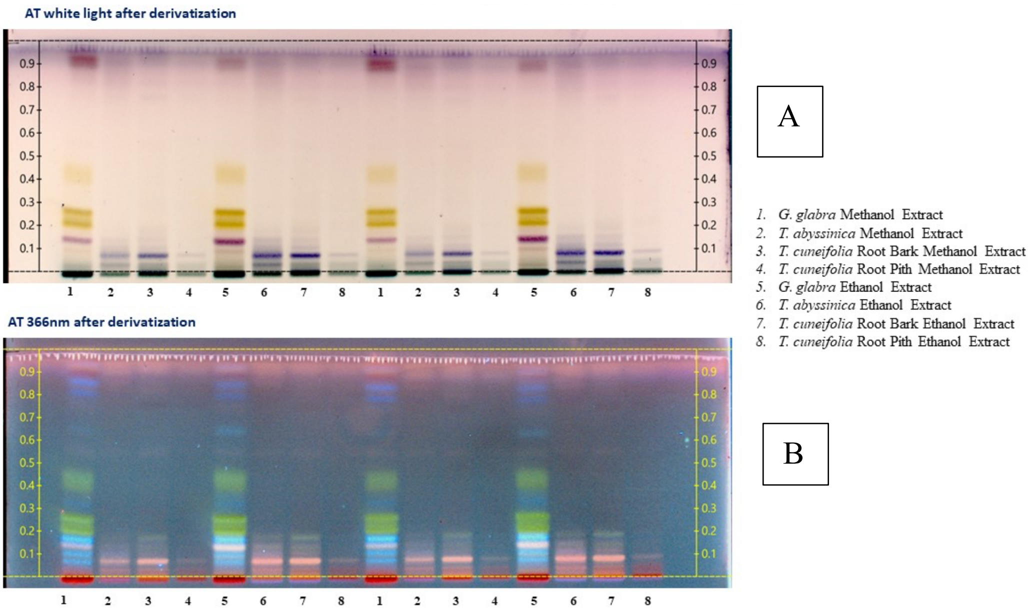


Figure 4.19: After derivatization with anisaldehyde sulphuric acid reagent at (A) 254nm and (B) 366nm

Figure 4.20: Schematic diagram of chromatographic fingerprint Rf after derivatization (A) at Visible light (B) at 366nm

The mentioned data indicate that samples of *Glycyrrhiza glabra* and *Taverniera cuneifolia* have a small number of similarities as well as differences. The apparent resemblance is attributable to the existence of common phytotoconstituents (as validated by Rf and colour), and variances may be the result of changes in the concentrations and therefore the intensities of these phytoconstituents. Thus, the created fingerprints might indicate the similarities and differences that occur across various plant samples. In the absence of a pure marker compound, several of the reported common, well-resolved phytoconstituents may serve as marker peaks that give insight on the influence of geographical variation, collecting circumstances, climate, temperature, and other similar variables on the components of plants (Mukherjee, 2007).

4.2.3 Standardisation and validation of Sugars

During the present research the quantitative TLC profiling of *T. cuneifolia* roots has been done along with fourteen sugar standards (arabinose, glucose, fructose, maltose, manitol, galactose, inositol, lactose, mannose, rhamnose, ribose, sorbitol, sucrose and xylose) (Table 4.6). These have been preliminary tried on TLC plates along with plant extracts. Out of these only glucose, fructose and sucrose are detected in the roots of plant extracts. These standards were selected for final results on HPTLC. Being highly polar compounds, the analysis of sugars (in this study glucose, fructose and sucrose) on HPTLC silica gel 60 F254 plates requires strongly polar solvent system. To sharpen obtained bands, the addition of a small volume of a suitable acid (e.g., formic acid, acetic acid) was also assumed to be beneficial. On the basis of these considerations, a number of solvent systems in different ratios were tested during the method development phase of this study. The sample information are: S1 (Rajkot-June), S2 (Kutch-June), S3 (*G. glabra*), S4 (Bagodara, August), S5 (Bagodara, June), S6 (Bagodara, April), S7 (Bagodara, February), S8 (Bagodara, September), R1 (Rajkot, September), R2 (Rajkot, February), K1 (Kutch, February), K2 (Kutch, September).

Table 4.6: Identification of various sugar markers in the sample *T. cuneifolia* and *G. glabra*

Plant	**Sugar markers tested**													
	Arabinose	Glucose	fructose	Maltose	Manitol	Galactose	Inositol	Lactose	Mannose	Rhamnose	Ribose	Sorbitol	Sucrose	Xylose
***T. cuneifolia* roots**	-	+	+	-	-	-	-	-	-	-	-	-	+	-
***G. glabra* roots**	-	+	+	-	-	-	-	-	-	-	-	-	+	-

Three sets of images (R white, T white and RT white) were taken for analysis. Each set of images was analysed by vision CATS software, and T white was found to give the most consistent results over the entire concentration range of the calibration curve (data not presented) with an adequate correlation coefficient (r^2) for the respective calibration curves. For quantitative analysis, both absorbance peak areas and absorbance peak heights versus concentration of the respective sugar standard curves were plotted. Peak areas versus concentration produced more reliable and accurate analysis results compared to peak heights versus concentration over the entire calibration range.

4.2.4 Chromatographic results

Glucose, fructose and sucrose were separated and quantified by HPTLC. After derivatisation with aniline–diphenylamine–phosphoric acid reagent, the chromatographic plate image background was white in colour and the individual sugars presented in different bright colours, glucose dark ash coloured, fructose pink-red and sucrose dark brown (figure 4.21, 4.22, 4.23 & 4.24). Their respective RF values were found to be 0.33 (glucose), 0.27 (sucrose) and 0.15 (fructose).

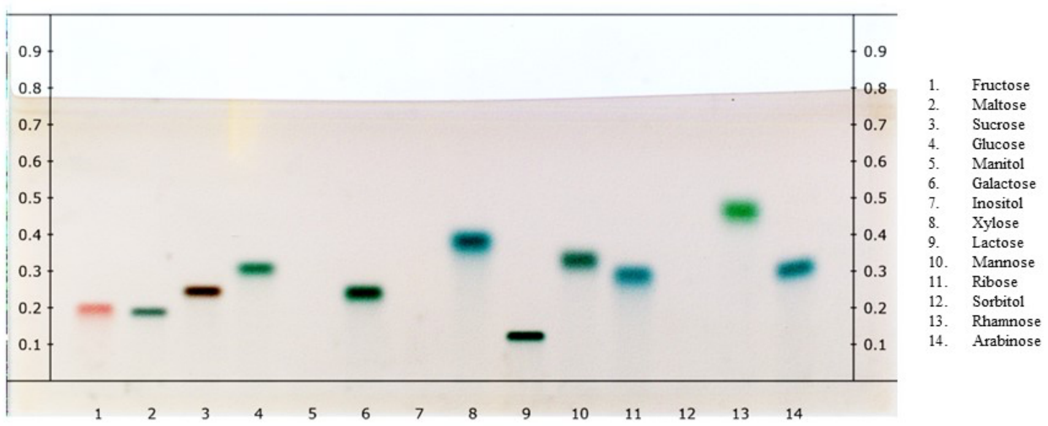
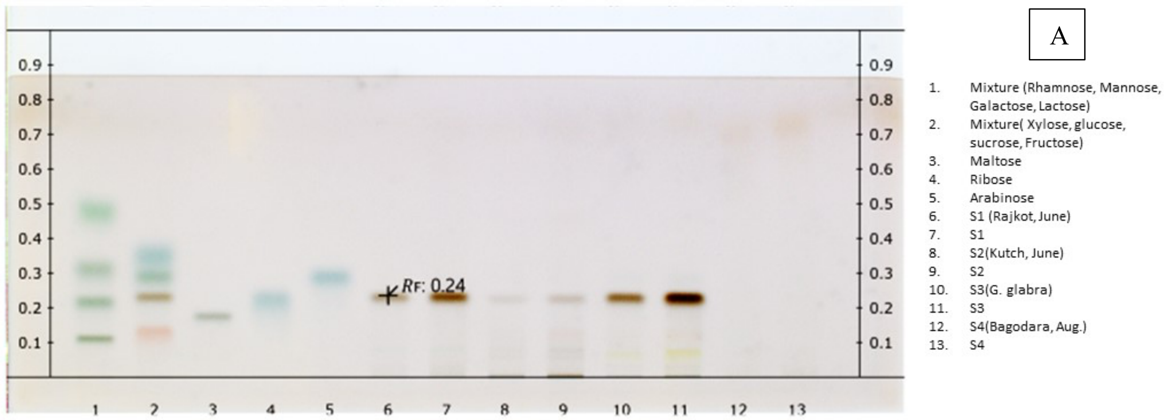


Figure 4.21: HPTLC chromatogram for the separation of standards of sugars(after derivatization)



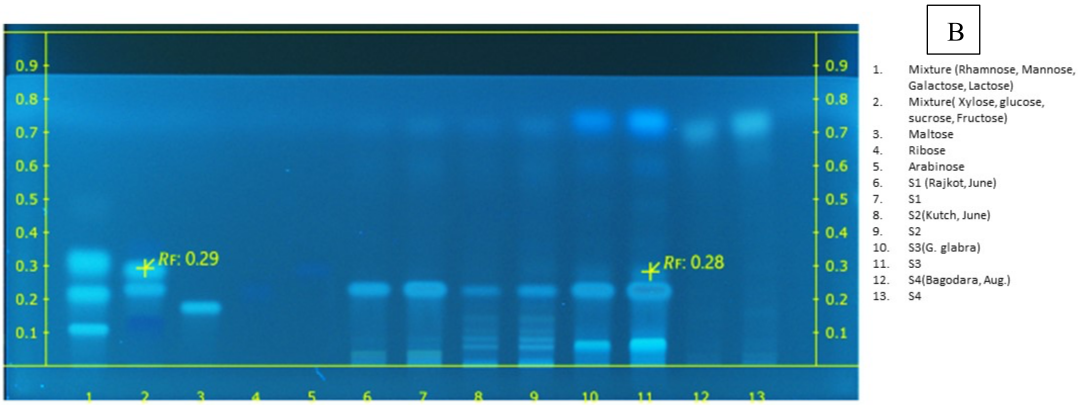


Figure 4.22: HPTLC chromatogram for the for the identification of sugars in the samples (A) Visible (B) at 366nm

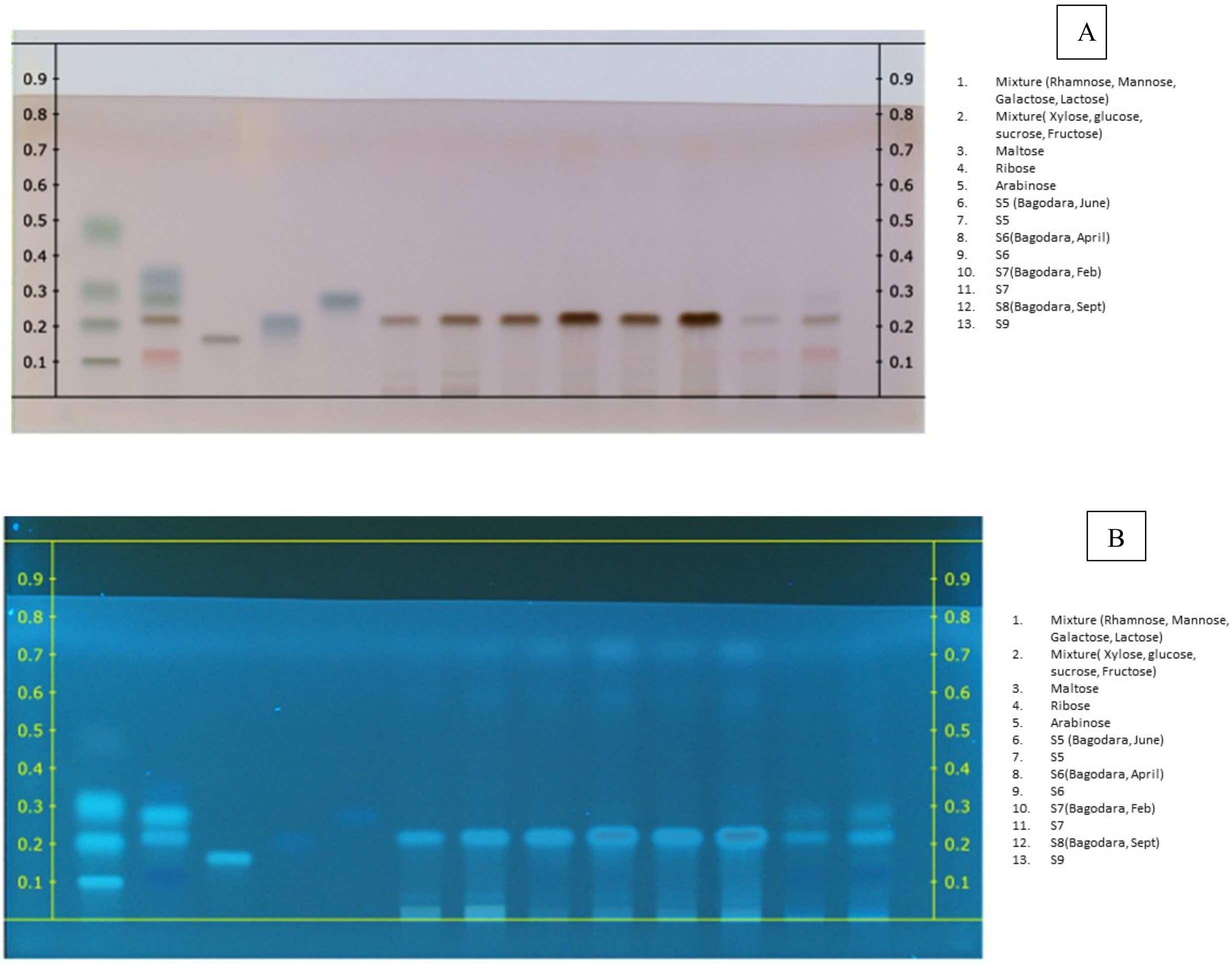


Figure 4.23: HPTLC chromatogram for the for the identification of sugars in the samples (A) Visible (B) at 366nm

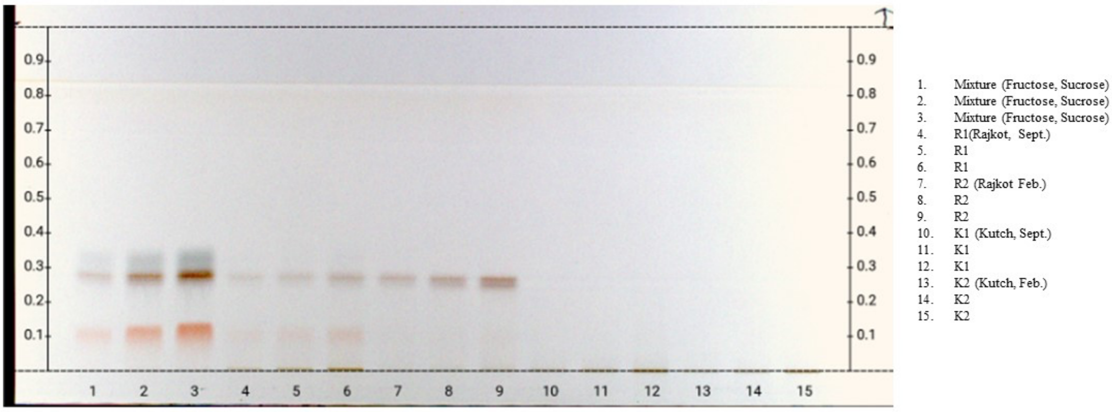


Figure 4.24: HPTLC chromatogram for the for the identification of glucose in the samples in visible light

4.2.4.1 Method validation

The absorbance intensity at white light after derivatisation of the individual bands was recorded. The specificity of the method was determined by measuring individual bands at their specific RF values. For quantitative analysis, the peak intensity was plotted against concentration. The linearity was tested for range of 250–1250 ng/band. Using polynomial analysis, the linear regression and correlation coefficient (r^2) of the standard curves validated the linearity of the analytical method.

By using the trend line equation of the three different calibration curves, the sensitivity of the method in terms of LOD and LOQ was calculated. For the three different sugars, the LOD and LOQ were 95.512 ng and 289.430 ng for fructose; 49.439 ng and 149.816 ng for glucose; and 118.822 ng and 360.068 ng for sucrose, respectively (Table 4.7). In comparison to the findings of previous similar studies (Morlock *et al.,* 2014; Puscas *et al*., 2013; Pukl & Prošek, 1990) the sensitivity of the method proposed in this paper has been greatly improved.

The accuracy of the method in terms of sample recovery was determined by calculating % mean recovery of each of the three sugars using the standard addition method. The accuracy of % mean recoveries were found to be in acceptable range as per the ICH guidelines (Table 4.9, 4.10, 4.11, 4.12 & 4.13).

The precision of the method was carried out as intra-day and inter-day precision, which determined the repeatability and intermediate precision of the optimised method. The precision studies were performed for the three sugars three times (n=3) at three different concentrations, indicating that the method can be considered precise with high level of confidence (Table 4.7, 4.8 & 4.9). Repeatability is an indicator of system precision, which was expressed as SD and

%RSD. The obtained %RSD values were within the acceptable limit indicating that the method can be considered repeatable with high level of confidence.

The parameters of the optimised methods were intentionally varied to investigate the robustness of the developed method. Specifically, robustness was examined by making small changes in the saturation time, mobile phase volume and composition. Each time the experimental conditions were changed slightly, the changes in the responses for all the sugars were noted. It was found that variation in the studied parameters had no noticeable influence on the separation and quantification of fructose, glucose and sucrose using the proposed method. In these experiments, the sugars were effectively separated with almost unchanged Rf values. The values of % recoveries with unaffected Rf values indicate the robustness of the developed method according to the ICH guidelines.

Table 4.7: The chromatographic and calibration parameters, LOD and LOQ

Compound	**Rf**	**Regression equation**	**Correlation coefficient (r^2)**	**Coefficient of variation (CV)**	**LOD (ng/µL)**	**LOQ (ng/µL)**
Glucose	0.338	$Y=4.662x10^{-9} X+3.204x10^{-4}$	0.9964	2.69	49.439	149.816
Fructose	0.15	$Y=1.462x10^{-9}X$	0.99	1.33	95.512	289.430
Sucrose	0.27	$Y=3.504x10^{-9} X+5.366x10^{-4}$	0.999	1.69	118.822	360.068

Table 4.8: Precision of the method (intra-day)

Theoretical concentration band (ng)	**Precision of the method (intra-day)**					
	Plate 1	**Plate 2**	**Plate 3**	**Mean**	**SD**	**%RSD**
Glucose	2.60	2.65	2.59	2.61	0.032	1.22
Fructose	2.46	2.49	2.40	2.45	0.045	1.83
Sucrose	2.98	2.91	2.98	2.95	0.040	1.35

Table 4.9: Precision of the method (inter-day)

Theoretical concentration band (ng)	Precision of the method (inter-day)					
	Day 1	Day 1	Day 1	Mean	SD	%RSD
Glucose	2.60	2.85	2.64	2.69	0.134	4.98
Fructose	2.46	3.15	2.48	2.69	0.39	14.49
Sucrose	2.98	3.34	3.16	3.16	0.18	5.69

Table 4.10: Recovery of glucose S8

Compounds	Amount of Compounds in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recovery (% recovery)
Glucose						
S8	187.39 187.39 187.39	200 250 300	387.39 437.39 487.39	365.182 408.750 447.729	94.26 93.45 91.86	93.19

Table 4.11: Recovery of fructose S8

Compounds	Amount of Compounds in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recovery (% recovery)
Fructose						
S8	114.66 114.66 114.66	100 125 150	214.66 239.66 264.66	195.57 215.69 236.85	91.10 89.99 89.49	90.19

Table 4.12: Recovery of sucrose S8

Compounds	Amount of Compounds in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recovery (% recovery)
Sucrose						
S3(GG)	114.66 114.66 114.66	100 125 150	214.66 239.66 264.66	192.04 216.90 241.33	89.46 90.50 91.18	90.38
S5	295.49 295.49 295.49	100 125 150	395.49 420.49 445.49	388.22 392.23 421.31	98.16 93.27 94.57	95.33
S6	277.90 277.90 277.90	100 125 150	377.9 402.9 427.9	379.99 401.27 401.48	100.55 99.59 93.82	97.98
S7	317.17 317.17 317.17	100 125 150	417.17 442.17 467.17	405.24 431.03 474.08	97.14 97.48 101.47	98.54
S8	189.06 189.06 189.06	100 125 150	289.06 314.06 339.06	260.51 273.46 278.72	90.12 87.07 82.20	86.76

Table 4.13: Recovery of sucrose in Rajkot sample

Compounds	Amount of Compounds in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recovery (% recovery)
Sucrose						
S1(Rajkot)	376.03 376.03 376.03	100 125 150	476.03 501.03 526.03	415.91 428.29 427.96	87.37 85.48 81.62	89.82
R1(Rajkot)	323.51 323.51 323.51	200 250 300	523.51 573.51 623.51	511.62 520.19 521.50	97.72 90.72 83.63	90.69
R2(Rajkot)	385.69 385.69 385.69	200 250 300	585.69 635.69 685.69	602.72 606.96 596.73	102.92 95.48 87.02	95.14

Table 4.14: Recovery of fructose in Rajkot sample

Compounds	Amount of Compounds in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recovery (% recovery)
Fructose						
R1(Rajkot)	303.94 303.94 303.94	200 250 300	503.94 553.94 603.94	505.81 526.48 524.54	100.37 95.04 86.85	94.08

Table 4.15: Recovery of sucrose in Kutch sample

Compounds	Amount of Compounds in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recovery (% recovery)
Sucrose						
S2(Kutch)	189.80 189.80 189.80	100 125 150	289.8 314.8 339.8	274.27 285.26 287.24	94.64 90.61 84.53	89.92

Table 4.16: Results from the quantitative analysis of sugars detected in *T. cuneifolia* (Bagodara)

Seasonal plant extract (Bagodara samples)	Amount of sugars of various seasons in *T. cuneifolia* (µg/mg of dry weight)		
	Glucose	**Fructose**	**Sucrose**
S3(GG)	ND	ND	264.3
S4(Aug)	ND	ND	ND
S5(June)	ND	ND	120.5
S6(April)	ND	ND	313.4
S7(Feb)	ND	ND	379.5
S8(Sept)	5.334	115.2	47.89

*ND=not detected

Table 4.17: Results from the quantitative analysis of sugars detected in *T. cuneifolia* (Rajkot)

Seasonal plant extract	**Amount of sugars of various seasons in *T. cuneifolia* (µg/mg of dry weight)**		
	Glucose	**Fructose**	**Sucrose**
R2	ND	ND	152.0
S1	ND	ND	176.7
R 1	ND	43.39ug	53.67

*ND=not detected

Table 4.18: Results from the quantitative analysis of sugars detected in *T. cuneifolia* (Kutch)

Seasonal plant extract	**Amount of sugars of various seasons in *T. cuneifolia* (µg/mg of dry weight)**		
	Glucose	**Fructose**	**Sucrose**
K2	ND	ND	ND
S2	ND	ND	34.35
K1	ND	ND	ND

*ND=not detected

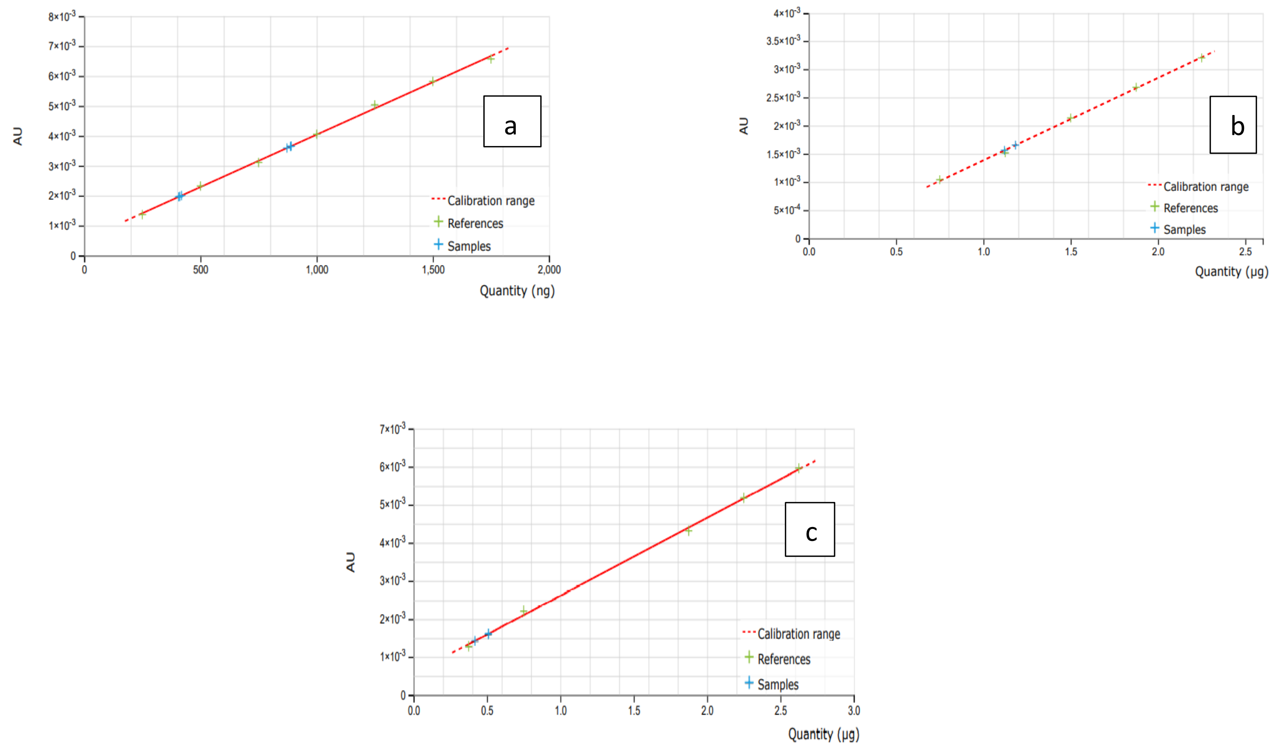


Figure 4.25: Calibration curve of (a) Glucose (b) Fructose (c) Sucrose

4.2.5 Discussion

In the current study, an HPTLC technique was devised to compare the sugar content of *T. cuneifolia* root samples from three distinct sites to that of *G. glabra* root samples. The procedure has been standardised and validated in accordance with ICH recommendations.

The comparative research found seasonal differences between glucose, sucrose, and fructose in contrast to *G. glabra* (figure 4.21, 4.22, 4.23 & 4.24). Nonetheless, the analyses also showed the presence of Arabinose and Rhamnose, although in negligible quantities. On a similar note, Magalorkar reported in 2014 the presence of Mannose, Fructose, Arabinose, Ribose, Glucose, Lactose Mohohydrate, Maltose Monohydrate, Xylose, Galactose, and Sucrose sugars in HPTLC instrument, and GC MS analysis of roots revealed the presence of 5-Hydroxymethylfurfural, Furfuryl alcohol, Maltose, However, comparison studies with conventional sugars revealed the existence of large quantities of Sucrose, Fructose, and Glucose. In contrast *G. glabra* showed 5–15% as glucose, sucrose, and mannitol and starch (approximately 20% of the dried root) (Blumenthal 2000). Additionally, the roots of *G. glabra* contains 1.6% of water-soluble polysaccharides comprised of rhamnose, arabinose, mannose, glucose, and galactose, as well as 9.7% of total polysaccharides (Dzhumamuratova *et al*. 1978). Rhamnose, arabinose and galactose were located in *T. cuneifolia*; however, their concentration was not significant.

The composition of monosaccharide (glucose, fructose, arabinose and rhamnose) and disaccharides (sucrose) in *T. cuneifolia* were identified whereas Denisova *et al*. (2003) reported that more than 50% of the ethanol extract of licorice root was composed of monosaccharides and disaccharides (7 to 8 mass % of the dry raw material) in *G. glabra*. The major constituent was saccharose (46.78%).

Among the detected sugars, significant amounts of D -mannopyranose (9.06 %), β- D -glucopyranose (7.06 %) and 2- O -hydroxyethylglucose (12.84 %) and smaller quantities of sorbose (4.12 %), α- D - fructose (2.01 %), β- D -fructose (2.56 %) and β- D - galactofuranose (1.88 %) and Insignificant levels of the sugar alcohols mannopyranosyl- D -glucitol (3.49 %), ribitol (0.95 %), mannitol (1.33%), and myo-inositol (0.33%) were detected. Though mannitol, myo-inositol were recorded by Mangalorkar (2012) in LCMS and GC studies the present studies couldn't contribute significantly to this aspect.

In the present investigations, the pre-monsoon period was characterised by a high sucrose concentration, which likely led to an increase in secondary metabolites. From the investigations, it is evident that *T. cuneifolia* has a greater sucrose content than *G. glabra*. However, no glucose nor fructose were detected in *G. glabra* in this investigation.

In earlier study done by Mangalorkar in 2014, the HPTLC examination of Sugars in the roots of *T. cuneifolia* revealed the presence of Mannose, Fructose, Arabinose, Ribose, Glucose, Lactose Mohohydrate, Maltose Monohydrate, Xylose, Galactose, and Sucrose, however it was not validated. Regional investigations reveal that, among the three samples from Kutch, Bagodara, and Rajkot, Bagodara samples had the highest sugar content. And when seasonal analysis was performed on samples from Bagodara, the February sample exhibited the highest level of sugar (sucrose) and was greater than *G. glabra*. Thus, these procedures provide a dependable method for detecting, separating, and quantifying sugars in the methanolic extracts of *T. cuneifolia* and *G. glabra*. Complete validation of the procedures yielded good results for the parameters that were studied. The study's findings suggest that the procedures were rapid, simple, reliable, accurate, linear, selective, sensitive and cost-effective as well as having enough recovery and acceptable accuracy. Thus, these validation experiments proved the methodologies' capacity to deliver accurate laboratory measurement. These techniques may be advised for the determination of sugar concentrations in plant extracts.

4.2.6 Standardisation and validation of Amino acids

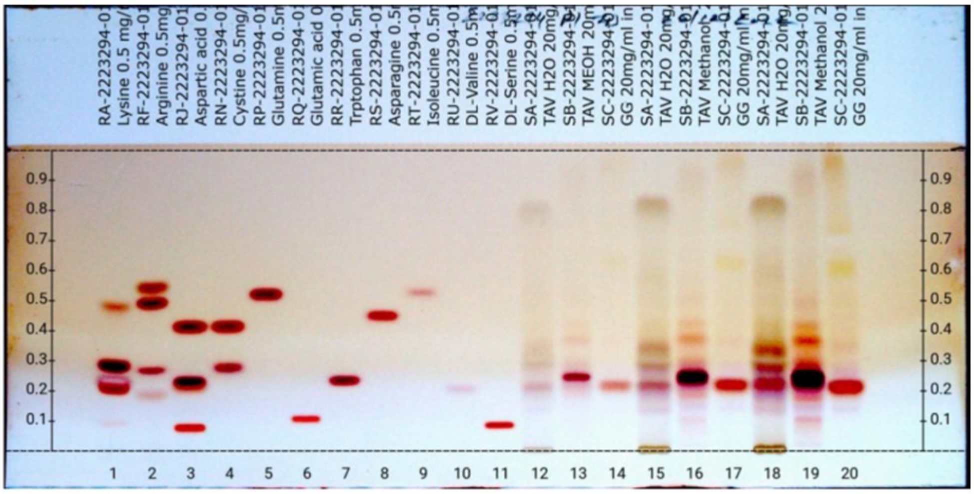
The primary metabolites are crucial to the organism's survival. Glycolysis, the Krebs cycle, and the Calvin cycle all use them as inputs or outputs, respectively. In addition to their primary role, they also serve as a final product and a precursor for other compounds known as secondary metabolites. Primary metabolites are used as building blocks in the production of certain antibiotics, such as actinomycin, which is derived from the amino acid tryptophan (Raghuveer *et al.,* 2015).

Amino acids are the building blocks of proteins and other metabolites, and they also exist in plants in their free form as primary metabolites. There are over 20 distinct amino acids that can be extracted from proteins, with others found naturally in plants and bacteria. While plants and bacteria can produce all 20 essential amino acids, humans can produce just approximately half of them. Those amino acids that animals can produce on their own are called "non-essential" and aren't required in the diet, whereas the rest of the amino acids are called "essential" and must be received from the diet (Wu *et al.*, 2013). Biosynthesis of many secondary metabolites begins with a single amino acid (primary metabolites). Secondary metabolites, such as alkaloids, are produced from amino acids that serve as precursors. Precursors for secondary metabolites (biomolecules), amino acids are used for a wide variety of cellular repair and growth processes in plants and animals (Raghuveer *et al.,* 2015).

The amino acids in *T. cuneifolia* (both water and methanol extracts) and *G. glabra* (methanol extract) have been identified and validated using an HPTLC instrument in the current investigation. The solvent system of n-Butanol: acetic acid: water (3: 1: 1 V/V/V) was successful in detecting four amino acids i.e., Arginine, Proline, Valine and Lysine after several trials with other solvent systems.

4.2.7 Chromatographic results

Bands of a comparable Rf value were seen for arginine, proline, valine, and lysine on the identification plate. The arginine concentration in the TAV methanol extract and the proline concentration in the GG sample were both determined. Proline and arginine were validated using a TAV methanol extract and a GG methanol extract. Their respective Rf values were found to be 0.11 (arginine), 0.21 (proline) and 0.08 (lysine) and 0.41 (valine) (fig. 4.26, table 4.19).



A

1. Lysine
2. Arginine
3. Aspartic acid
4. Cystine
5. Glutamine
6. Glutamic acid
7. Tryptophan
8. Asparagine
9. Isoleucine
10. Dl-Valine
11. DL-Serine
12. TAV (H2O extract)
13. TAV (MEOH extract)
14. GG (MEOH extract)
15. Repeat 11,12,13

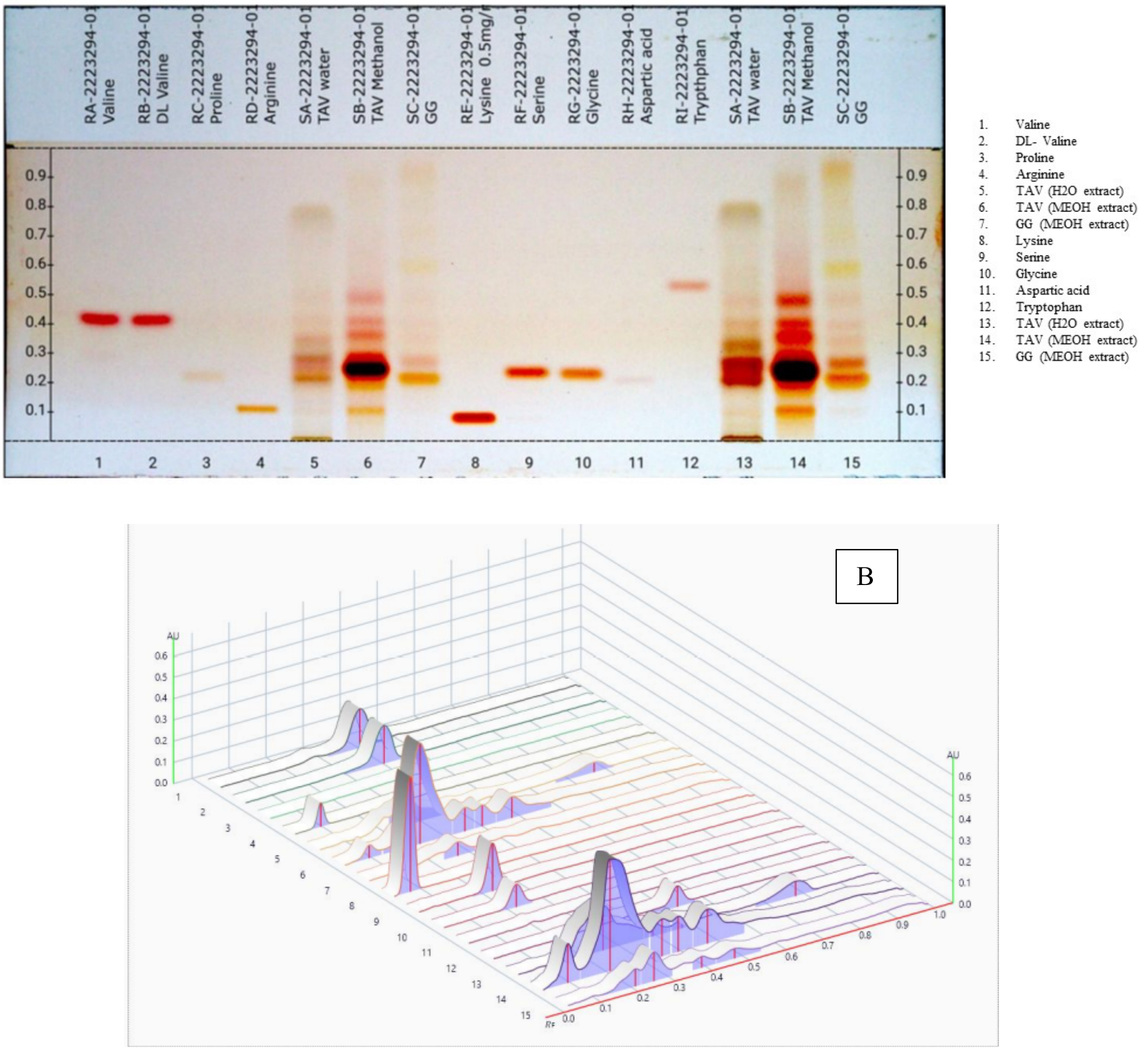


Figure 4.26: Identification plate of amino acid detected in *T. cuneifolia* and *G. glabra*. (TLC plate) (B) 3D overlay

Table 4.19: Identification of amino acid in *T. cuneifolia* in comparison with *G. glabra*

Sr. No.	Standards of Amino acids	*T. cuneifolia* water extract	*T. cuneifolia* methanol extract	*G. glabra* extract
1	Alanine	ND	ND	ND
2	Asparagine	ND	ND	ND
3	Arginine	Present	Present	ND
4	Aspartic acid	ND	ND	ND
5	Cystine	ND	ND	ND
6	Glycine	present	present	present
7	Glutamic acid	ND	ND	ND
8	Glutamine	present	present	present
9	Histidine	ND	ND	ND
10	Isoleucine	ND	ND	ND
11	Leucine	ND	ND	present
12	Lysin	ND	ND	ND
13	Methionine	ND	ND	ND
14	Phenylalanine	ND	ND	ND
15	Proline	present	Present	present
16	L- Serine	ND	ND	ND
17	DL- serine	ND	ND	ND
18	Tyrosine	ND	ND	ND
19	Threonine	ND	ND	ND
20	Tryptophan	ND	ND	present
21	L- Valine	ND	ND	ND
22	DL- Valine	Present	Present	present

*ND=Not detected

4.2.8 Method validation

The absorbance intensity at white light after derivatisation of the individual bands was recorded. The specificity of the method was determined by measuring individual bands at their specific RF values. For quantitative analysis, the peak intensity was plotted against concentration. The linearity was tested for range of 250-1500 ng/band (figure 4.27). Using polynomial analysis, the linear regression and correlation coefficient (r^2) of the standard curves validated the linearity of the analytical method.

By using the trend line equation of the three different calibration curves, the sensitivity of the method in terms of LOD and LOQ was calculated. For the two different amino acid, the LOD and LOQ were 79.29ng/µL and 240.30ng/µL for proline; 167.24ng/µL and 506.79ng/µL for arginine respectively (Table 4.20).

The accuracy of the method in terms of sample recovery was determined by calculating % mean recovery of proline and arginine using the standard addition method. The accuracy of % mean recoveries were found to be in a range of 85.59% for proline, 85.22% for arginine (Table 4.23), which were all within the acceptable range of the ICH guideline.

The precision of the method was carried out as intra-day and inter-day precision, which determined the repeatability and intermediate precision of the optimised method. The precision studies were performed for proline and arginine three times (n=3) at three different concentrations. The obtained %RSD values were within the acceptable limit (Tables 4.21, 4.22), indicating that the method can be considered precise with high level of confidence.

Repeatability is an indicator of system precision, which was expressed as SD and %RSD. The obtained %RSD values were within the acceptable limit (Table 4.21 & 4.22), indicating that the method can be considered repeatable with high level of confidence.

The parameters of the optimised methods were intentionally varied to investigate the robustness of the developed method. Specifically, robustness was examined by making small changes in the saturation time, mobile phase volume and composition. Each time the experimental conditions were changed slightly, the changes in the responses for all the sugars were noted. It was found that variation in the studied parameters had no noticeable influence on the separation and quantification of proline and arginine using the proposed method. In these experiments, the amino acids were effectively separated with almost unchanged Rf values. The values of % recoveries with unaffected Rf values indicate the robustness of the developed method according to the ICH guidelines.

Table 4.20: The chromatographic and calibration parameters, LOD and LOQ

Compound	Rf	Regression equation	Correlation coefficient (r^2)	Coefficient of variation (CV)	LOD (ng/µL)	LOQ (ng/µL)
Proline	0.21	$Y=6.17x10^{-9} X+2.707x10^{-4}$	0.99	0.94%	79.29	240.30
Arginine	0.11	$Y=-1.023x10^{-16} X^2 + 1.117x10^{-8} X-2.349x10^{-3}$	0.99	2.19	167.24	506.79

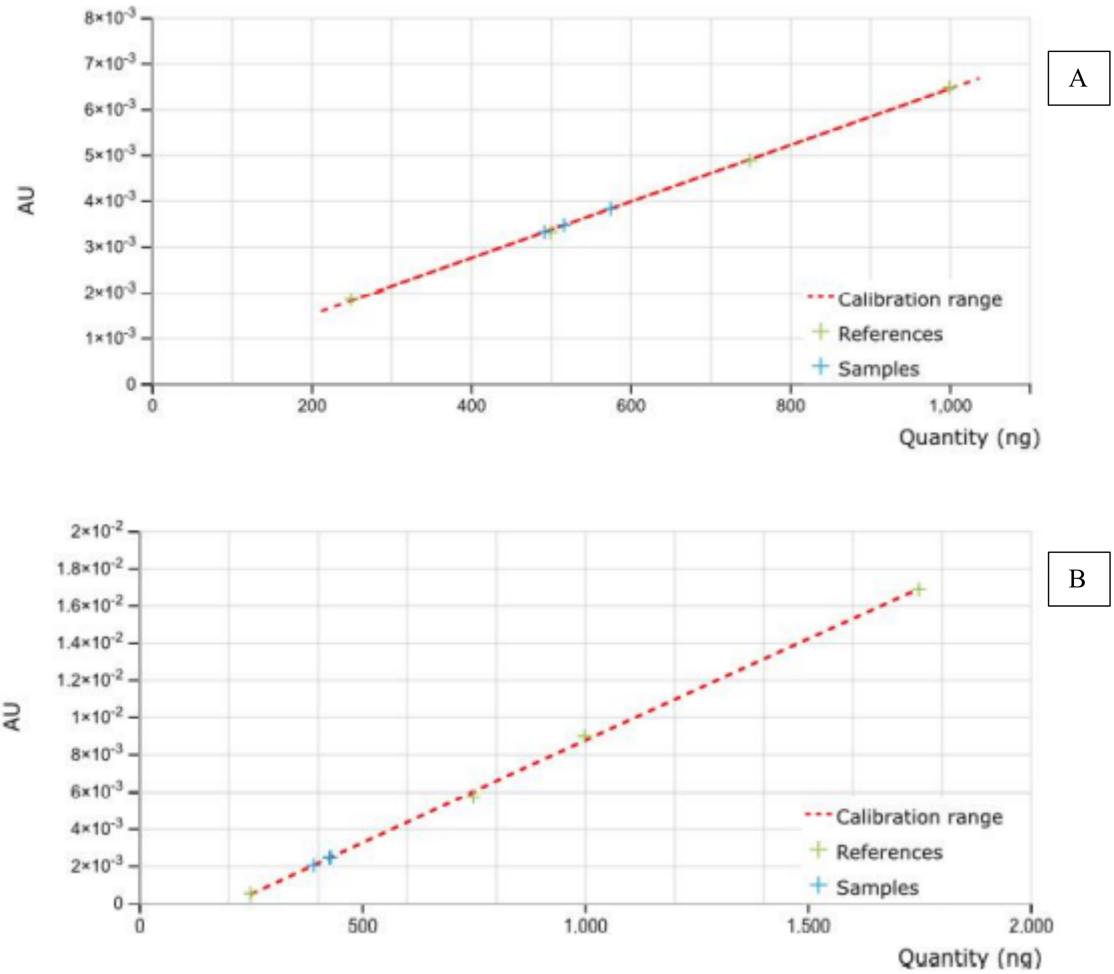


Figure 4.27: Linearity graph of (A) Proline and (B) Arginine

Table 4.21: Precision of the method (intra-day)

Theoretical concentration band (ng)	Precession of the method (intra-day) Intermediate presicion					
	Day 1 plate 1	Day 2plate 2	Day 3plate 3	Mean	SD	%RSD
Proline	2.12	2.09	2.81	2.34	0.407	17.39
Arginine	2.04	2.86	2.29	2.39	0.420	17.57

Table 4.22: Precision of the method (inter-day)

Theoretical concentration band (ng)	Precession of the method (inter-day)					
	Day 1	Day 2	Day 3	Mean	SD	%RSD
Proline	2.12	2.12	2.91	2.38	0.456	19.15
Arginine	2.04	2.35	1.92	2.10	0.221	10.52

Table 4.23: Recovery of Proline & Arginine

Compounds	Amount of Compounds in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recovery (% recovery)
Arginine						
In MEOHTAV	331.56 331.56 331.56	400 500 600	731.50 831.56 931.56	631.25 707.73 785.18	86.28 85.10 84.28	85.22
Proline						
In GGMEOH	520.35 520.35 520.35	400 500 600	920.35 1020.35 1120.35	782.32 888.00 949.60	85.00 87.02 84.75	85.59

Table 4.24: Results from the quantitative analysis of amino acid detected in *T. cuneifolia* and *G. glabra*

Seasonal plant extract	Amount of amino acid in *T. cuneifolia* and *G. glabra* (µg/20mg of dry weight)	
	Proline	Arginine
In MEOHTAV	-	52.06
In GGMEOH	264.2	-

4.2.9 Discussion on amino acids

A high level of proline in a plant could be an indicator that it is under stress or that it has a genetic propensity to produce more proline in response to adversity. The plant's ability to adapt to its changing environment may be facilitated by its high proline content. The ability of a plant to withstand stress may be measured by its proline concentration. Proline buildup is a recognised phenomenon under conditions of water deficit (Hare *et al.,* 1998*),* salinity (Munns, 2005; Rhodes *et al.,* 2002),10 low temperature (Naidu *et al*., 1991), heavy metal exposure and UV radiation (Bassi & Sharma, 1993; 1993; Schat *et al*., 1997; Sharma & Dietz, 2006) etc. In addition to its role as an anosmolyte in osmotic adjustment, proline also helps stabilise sub-cellular structures (such as membranes and proteins), clean up free radicals, and buffer cellular redox potential in times of stress (Ashraf *et al.,* 2007). Its accumulation normally occurs in cytoplasm where it works as molecular chaperons stabilizing the structure of proteins and its accumulation buffers cytosolic pH and maintains cell redox state. There is speculation that its buildup is an adaptive response-altering stress signal (Hayat *et al*., 2012).

There have been reports of the accumulation of various other free amino acids under stress circumstances, including aspartic acid, glutamic acid, and glutamine in cotton (Hanower & Brzozowska, 1975); asparagine, aspartic acid, serine, and glycine in maize (Slukhai & Shvedova, 1972; Thakur & Rai, 1982) and ornithine, arginine, and glutamate in detached rice leaves (Yang *et al*., 2000).

Several crucial metabolic processes in plants require the amino acid arginine. It's a building block for making polyamines, which are vital to plant development and survival in times of stress. Nitric oxide (NO) is a signalling molecule involved in many different physiological processes, including defence response, cell division, and cell development, and arginine is a precursor for its creation. Presence of a high concentration of arginine in a plant may be indicative of a genetic propensity for the plant to generate large amounts of this amino acid. Perhaps this high arginine content is linked to a specific plant function, such stress resistance or growth stimulation. Plants that are able to withstand abiotic stresses like drought or excessive salinity often have higher than average arginine levels because this amino acid acts as an antioxidant and protects cells from damage caused by shifts in osmotic pressure. A plant's high arginine content may also be an indicator that its defences are robust. Because arginine is a necessary building block in the manufacture of several defense-related secondary metabolites (e.g., alkaloids, flavonoids, and others), it is often considered to be a signal molecule. In the present study, we have identified 5 amino acids (arginine, proline, DL-valine, Glycine, Glutamine) in *T. cuneifolia* and 7 amino acids (arginine, proline, DL-valine, Glycine, Glutamine, Leucine and Tryptophan) in the roots of *G. glabra* (Table 4.19, Figure 4.26). After the identification, only those amino acid was validated which were showing very high intensity or absorption in UV lamp. Arginine was quantified in *T. cuneifolia* whereas the proline was quantified in *G. glabra* (Table 4.24).

In 2014, Mangalorkar reported 15 amino acids in the seeds of *T. cuneifolia*, 13 amino acids in the roots, 13 amino acids in the leaves, and 14 amino acids in the fruit cover of the seeds. The Essential amino acids were Arginine, Methionine, Phenylalanine, Tryptophan, lysine, Histidine, Isoleucine, Leucine, Valine and Threonine and non essential amino acids: Alanine, Asparagine, Cystein, Glutamine, Glutamic acid, Glycine, Proline, Serine, and Tyrosine. Various amino acids were recognised by Vora &Testa (2004) as being present in licorice extract. Licorice extract contains around 18 distinct amino acids with a total concentration of roughly 5.3%. In addition, Sherif *et al*. (2013) noted that *G. glabra* lacked Tyrosine and phenylalanine out of a total of 22 amino acids analysed, with 15 of them being essential.

Further study is needed to interpret high arginine levels in plants and understand the exact processes and context in which they arise, as well as the physiological role of arginine in plant's development and stress response.

4.3: Standardization and validation of active phyto-constituents of *T. cuneifolia.*

In the field of modern analytical chemistry, chromatography stands out as the most flexible and widely used method. It is crucial to the process of standardising phyto-therapeutics (Srivastava, 2011; Kamboj, 2000, Marston, 2007). The chemical patterns of herbs may be seen in a chromatogram owing to chromatographic procedures, which are advantageous since they break down a complex system into more smaller portions (Feng & Runyi, 2006). Additionally, the development of these methods has allowed for the identification, assay, and quantification of chemical components contained in the complex plant matrix (Jayaprakasam *et al*., 2014).

This makes chromatographic procedures the most accessible and cost-effective means of identifying phytoconstituents and presenting the phytochemical profile of plant extracts (Marston, 2007).

High-performance liquid chromatography (HPLC) is a chemical analytical method with excellent selectivity, making it well-suited for the study of various botanical extracts and plant materials. In addition to quantitative data on the concentrations of various components in the sample, this one-of-a-kind, flexible, ubiquitous, and well acknowledged instrument also gives qualitative information about the sample's composition (Maji *et al*., 2014; Saroya, 2011). Several characteristics have contributed to its rise to prominence as a useful chromatographic method, including its excellent accuracy and precision, simplicity of use and sample preparation, chemical specificity, high sensitivity, etc (Moresco *et al*., 2014; Marston, 2007).

Because of their many advantages, HPTLC and HPLC/LC are the most used methods for performing chromatographic analysis on plant extracts. On top of that, owing to the complexity of plant extracts, a holistic strategy incorporating a mix of various methodologies is often proposed (Mukherjee, 2015; Gad *et al*., 2013; Hall, 2006). The identification and validation of glycyrrhizin was of the highest importance since our key objective was to determine if *T. cuneifolia* might serve as a potential alternative for *G. glabra*. Later, the remaining markers were standardised. The findings are addressed in depth in the following sections.

4.3.1 Standardisation and validation of Glycyrrhizin using LC-MS/MS

Glycyrrhizin is a glycoside triterpene produced from licorice root (*Glycyrrhiza glabra*). At the C-3 position, it comprises glycyrrhetic acid and two molecules of glucuronic acid. Glycyrrhizin has been shown to be useful in the treatment of several forms of liver inflammation (Manns *et al*., 2012; Abe *et al*., 1982; Yasui *et al*., 2011; Arase *et al*., 1997; Ikade, 2007; Wang *et al*., 2004; Cao *et al*., 2002; Ni *et al*., 2009; Lin *et al*., 2008; Gupta *et al*., 2013 & Montoro *et al*., 2011), lung (Journal *et al*., 2015), kidney, intestine, and spinal cord (Genovese *et al*., 2009). Currently, it has been shown to be effective in significantly reducing steatosis and necrosis of liver cells (Korenaga *et al*., 2011), inhibition of lung cancer and fibro sarcomas (Journal *et al*., 2015), treatment of Hepatitis C (Parvaiz *et al*., 2014), and potent inhibitor of bile acid-induced apoptosis and necrosis (Gumpricht *et al*., 2005). Glycyrrhizin also possesses proapoptotic properties in a hepatocyte model of cholestatic liver injury (Gumpricht *et al*., 2005), antiviral activity and chemo-preventive activity (Rahman & Sultana 2006; Wang *et al*., 2013). It inhibits SARS-CoV multiplication, as well as virus adsorption and penetration, at an early stage of the replicative cycle (Hoever *et al*., 2005; Cinatl *et al*., 2005).

Glycyrrhizin is now one of the most promising phytomolecules. As the demand for glycyrrhizin continues to rise, the quest for alternate sources has become necessary to close the supply-and-demand imbalance. *Taverniera cuneifolia* contains glycyrrhizin and other comparable phytoconstituents (vanillic, syringic, ferulic, o-coumaric, melilotic, and p-Hydroxy benzoic acids and sugars Amit & Daniel, 2014) to those of liquorice (Zore *et al*., 2008; Mangalorkar *et* *al*., 2014a, b). However, the species' glycyrrhizin content has yet to be confirmed and standardised.

4.3.2 TLC of *T. cuneifolia* and *G. glabra*

TLC for glycyrrhizin in *T. cuneifolia* roots was carried out in conjunction with *G. glabra* during the present study. Several solvent systems, including butanol, glacial acetic acid, and water (Zore *et al*., 2008; Marjan, 2019) were tried during the method development phase of this study. The best result was obtained using an optimised combination of butanol, glacial acetic acid, and water (6:1:3 v/v). The brown colour band was clearly apparent in the extracts at Rf 0.5 (Fig .4.28).

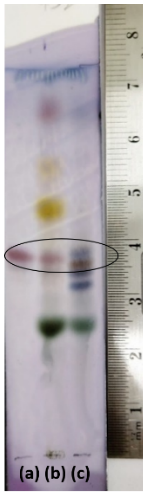


Figure 4.28: TLC of glycyrrhizin in plant samples: (a) Glycyrrhizin standard (b) *Glycyrrhiza glabra* root extract and (c) *Taverniera cuneifolia* root extract.

4.3.3 LC-MS/MS summary

Satisfactory separation was obtained for ionizing the glycyrrhizin in the mass spectrometer using the APCI (+) ionization mode with corona discharge voltage of 4V. The desolvation temperature was kept low at 200°C to prevent glycyrrhizin from thermal decomposition. The MRM transition m/z 823<453 was optimized for quantitative estimation of glycyrrhizin (Glycyrrhizic acid ammonia salt) depicting protonated molecular ion at m/z-823, $[M+H]^+$ and ammonia adduct ion $[M+NH3]^+$ at m/z-840 in APCI positive scan (Fig. 4.29) while APCI negative depicted at m/z-821. Precursor mass m/z-823 was selected for MRM optimization owing to higher intensity.

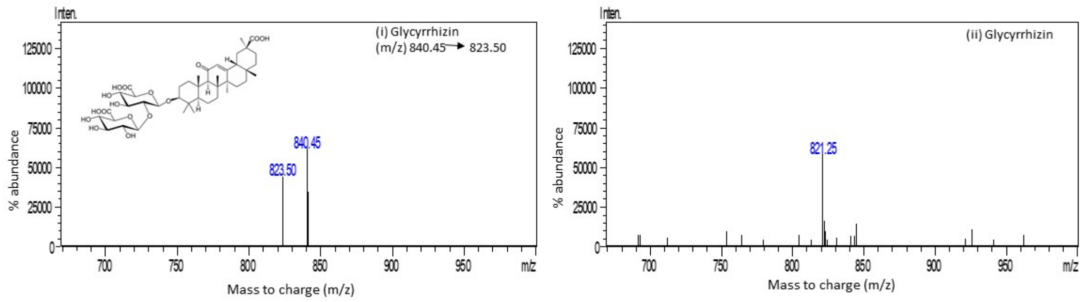


Figure 4.29: Structure and product ion mass of glycyrrhizin in positive and negative mode

4.3.4 Method validation summary

4.3.4.1 Linearity

A good linearity was achieved in the concentration ranges of 5 ng mL^{-1} – 500 ng mL^{-1} Glycyrrhizin (Fig. 4.30). The correlation of coefficient was $R^2 = 0.9997$.

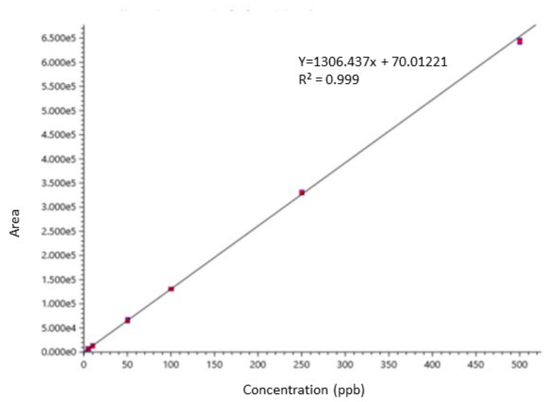


Figure 4.30: Calibration curve of glycyrrhizin

4.3.4.2 Specificity

The retention times for the root extracts of *Taverniera cuneifolia* and *Glycyrrhiza glabra* were 2.75 and 2.73, respectively (Fig. 4.31 & 4.32).

The mean assay value of TC at 150 ppm with a % RSD of 0.83 was higher than the mean assay value of GG at 850 ppm with a % RSD of 0.77. As a consequence, the technique was found to be more selective and specific.

4.3.4.3 Accuracy as Recovery

Three replicates of glycyrrhizin were collected in the concentration ranges of 50 ng/mL, 100 ng/mL, and 200 ng/mL in order to determine the recovery, and the mean recovery was 89% (table 4.25).

4.3.4.4 Precision

The intra-day and inter-day % RSD values for Glycyrrhizin were 0.81 and 0.48 percent, respectively (table 4.25).

4.3.4.5 Limit of Detection (LOD) and Limits of Quantitation (LOQ)

The LOD and LOQ of Glycyrrhizin were discovered to be 2 ng mL^{-1} and 5.0 ng mL^{-1} respectively (table 4.25).

4.3.5 Glycyrrhizin quantification in plant extracts using LC-MS/MS

The proposed method was utilised to assess the concentration of glycyrrhizin in plant root extracts from TC and GG. It was found that the content of glycyrrhizin was 8681997.68 ng mL^{-1} in GG root extract and 153072.85 ng mL^{-1} in TC root extract respectively (table 4.26). This will be the first report of a validated technique for rapid detection and quantification of glycyrrhizin in TC root extract versus GG.

Table 4.25: Parameters of Glycyrrhizin

Parameters	Glycyrrhizin
Linearity range[ng mL^{-1}]	5-500
Slope [m][1]	1306.437
Intercept [c][1]	70.01221
Correlation Coefficient [R^2]	0.9997
LOD [ng /mL][2]	2
LOQ [ng /mL][2]	5
Intraday precision(n=5 COV)	0.81
Interday precision (n=5 COV)	0.48

[1] of the equation $y = mx + c$, where y is peak area, m is the slope, x is the concentration, and c is the intercept.
[2] LOD (Level of Detection) and LOQ (Level of Quantitation) were calculated based on S/N ratio using LABSolutions software, Shimadzu.

Table 4.26: Applicability of the developed method for the determination of Glycyrrhizin in *Glycyrrhiza glabra* (GG) and *Taverniera cuneifolia* (TC) sample

Sample	Retention time	Concentration (ng mL^{-1})
***Glycyrrhiza glabra* root (GG)**	2.734	8681997.68
	2.732	8578341.68
	2.736	8520091.63
***Taverniera cuneifolia* root (TC)**	2.753	153072.85
	2.750	149842.69
	2.757	152024.19

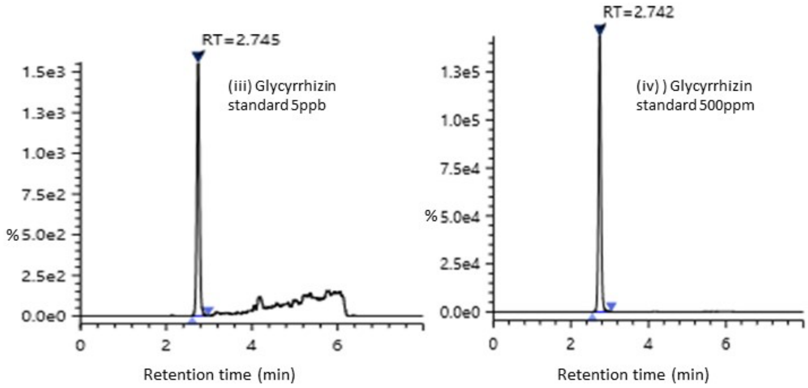


Figure 4.31: Representative graph for Glycyrrhizin standard in 5ppb and 500ppb

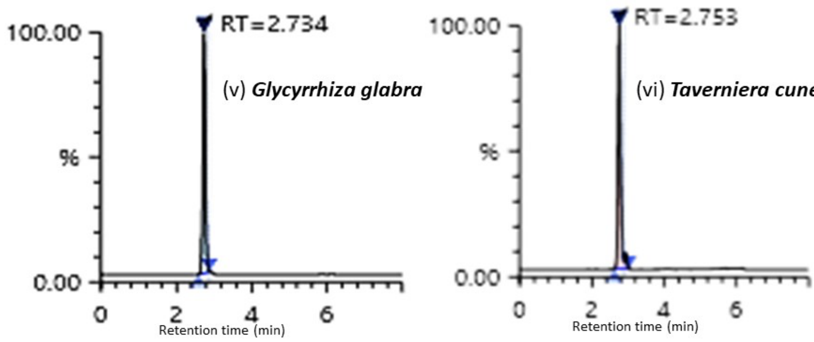


Figure 4.32: Representative graph for *Glycyrrhizin sample* in *G. glabra* & *T. cuneifolia*

4.3.6 Simultaneous method development of phytoconstituents using HPLC

Secondary metabolites are chemicals produced as by-products of metabolic processes in plants (Shrikumar & Ravi, 2007). Bioactives are chemicals or groupings of substances that contribute to therapeutic activity (Kushwaha *et al*., 2010). The essential processes involved in marker-based standardisation of plant extracts are the identification of important and distinctive bioactives in plants as markers and the development of analytical methods for their monitoring (Subramanian *et al*., 2014).

Flavonoids are one of the most widespread classes of naturally occurring polyphenolic chemicals; they are formed from flavans and are extensively dispersed within the plant kingdom. According on their substituent, they are often classified as flavonols, flavanols, anthocyanins, flavones, flavonones, and isoflavones (Sanghavi *et al*., 2014; Subramanian *et al*., 2014; Saroya, 2011; Moutsatsu, 2007). As a major active constituent, flavonoids play a significant role in a variety of pharmacological activities, including anti-allergic, anti-inflammatory, estrogenic, and anti-oxidant (Desire *et al*., 2015; Subramanian *et al*., 2014; Alam *et al*., 2012; Kulkarni *et al*., 2012; Vijayanarayana *et al*., 2007). Sterols also play vital functions in a number of biological processes. In addition to their crucial role in cell membrane support and fluidity, they have a significant role as hormone precursors and in biotic and abiotic stress responses (London, 2002; Arnqvist *et al*., 2007; Sewelam *et al*., 2014; Valitova *et. al*., 2016; Aboobucker & Suza, 2019). Sterols belong to the vast group of isoprenoids generated by the lanosterol (animals and fungi) or cycloartenol (plants) route, and they share a fundamental structure with a four-cyclic hydrocarbon ring termed gonane and a hydroxyl group at position C-3. Cholesterol, the most well-studied sterol, is produced mostly in mammals. Plants, on the other hand, often have a variety of C-24 sterols, including ***β***- sitosterol, campesterol, and stigmasterol (collectively known as phytosterols). Sterols are essential substances in plants and other eukaryotes because they are part of the structure of membranes, which they control in terms of permeability and fluidity (Schaller, 2003). Mammals and fungi typically have one dominant sterol (cholesterol or ergosterol, respectively) in their cell membranes, while plants have a more complicated sterol mixture (Hartmann, 1998). The sterol C22-desaturase enzyme, which is a member of the cytochrome P450 710 family, catalyses the solitary desaturase reaction at position C22 on the sterol side chain, converting -sitosterol into stigmasterol (Morikawa *et al*., 2006; Nelson, 2006).

Liquiritigenin, quercetin, naringenin, genistein, kaempferol, apigenin and glabridin are all well-known flavonoids which are responsible for many biochemical properties. The terpenoidal glycosides such as glycyrrhetinic acid are the major bioactive component in licorice with diverse pharmacological activities (Kim *et al*., 2006; Cho *et al*., 2010; Yu *et al*., 2015; Tang *et al*., 2015). The following table provides a description of several previously used techniques for the separation of the aforementioned substances (table 4.26, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32 & 4.33).

Table 4.27: Summary of some reported phytochemical studies on Apigenin estimation using HPLC

Sample (Apigenin)	Mobile phase	Wavelength (nm)	Rf of Apigenin	References	Flow rate (ml/min)
Apigenin	55% methanolin 0.1mammonium acetate buffer (pH 5.1) containing0.27 mm EDTA as isocratic mobile phase	335 nm	5.2 min	Cai, *et al.*, 2005	1
Apigenin in bulk powder & in nanoliposomes	Acetonitrile and 0.1% formic acid, 55:45 (v/v).	269 nm	4.21 min.	Shetti & Jalalpure, 2021.	1
From commercial formulation	methanol and 0.5 % trifluroacetic acid (80:20 %, v/v).	269		Gomathy et.al., 2020.	1
Clerodendrum serratum	methanol-acetonitrile-acetic acid-orthophosphoric acid-water (40:20:0.05:0.05:40).	352 nm	14.8 min	Rajagopal *et al.*, 2017.	0.6
Scutellaria L	Gradient: A was H2O, the mobile phase B was ACN both containing 0.02% AA.	340	33.82 min	Bardakci *et al.*, 2019.	0.8

Table 4.28: Summary of some reported phytochemical studies on Glabridin estimation using HPLC

Sample (Glabridin)	Mobile phase	Wavelength (nm)	Rt of Glabridin	References	Flow rate (ml/min)
Glycyrrhiza glabra	mixture of acetonitrile and water containing 0.2% acetic acid	280 nm	27 min	Viswanathan & Mukne, 2016.	1
Glycyrrhiza plant	gradient elution of mobile phase A (KH2 PO4 buffer) & mobile phase B (pure acetonitrile).	280 nm	51 min	Kulkarni *et al.*, 2021.	1
Licorice roots	methanol/water (30:70, v/v, containing 1% acetic acid)	252 nm		Tian *et al.*, 2008.	0.5 ml/min
Glycyrrhiza glabra	Mixture of acetonitrile-water containing 2% AcOH (70:30 v/v)	280	7.34 min	Shanker *et al.*, 2007.	1 ml/min
Synthetic drug	Etha-nol-ethyl acetate-dichloromethane-chloroform (1.2:2:5:3 v/v/v/v).	254		Jadhav *et al.*, 2016.	0.15 UL/Sec.

Table 4.29: Summary of some reported phytochemical studies on Genistein estimation using HPLC

Sample (Genistein)	Mobile phase	Wavelength (nm)	Rt of Genistein	References	Flow rate (ml/min)
Trifolium pretense	ammonium acetate: methanol (40:60)	254 nm		Kumar, *et al.*, 2016.	1
Glycine max	methanol and 0.1% acetic acid (53:47)	254 nm	10.08 min	Sulistyowati *et al.*, 2019.	1 ml/min

mangifera fruit	Methanol buffer (10: 50: 40)	260 nm	3.4 min	He & A, 2008.	1
Genista species	methanol:water (70:30, v/v)	248 nm		Orhan *et al.*, 2011.	0.7 ml/min
Genistein	Methanol: water, gradient	260 nm	7.726 min	Riswanto *et al.*, 2020	0.81 ml/min

Table 4.30: Summary of some reported phytochemical studies on Glycyrrhizin estimation using HPLC

Sample (Glycyrrhizin)	Mobile phase	Wavelength (nm)	Rt	References	Flow rate (ml/min)
Glycyrrhiza glabra	Binary gradient solvent system (30–100% B in A over30 min) where solvent A consisted of 0.1% v/v TFA in water and solvent B was 0.1% v/v of TFA in MeOH	254	25 min	Basar *et al.*, 2014	3
crude herbs	Gradient: solvent A (1.0% v/v aqueous acetic acid) and solvent B (1.0% v/v acetic acid in acetonitrile).	254	-	Chang-Seob, 2012.	1
Licorice powder	Gradient: 25 mM phosphate buffer (pH 2.5)–acetonitrile featuring gradient elution.	254	63.93 min	Wang & Yang, 2007.	1.2
Glycyrrhiza uralensis	Gradient: water (with 0.1% formic acid, A) and acetonitrile (with 0.1% formic acid, B).	254	9.25 min	Zhang *et al.*, 2013.	0.8
Formulation of 9 herbs	Gradient: Acetonitrile-water with 0.03% phospho-ric acid	250		Lee *et al.*, 2009.	1

Table 4.31: Summary of some reported phytochemical studies on Kaempferol estimation using HPLC

Sample (Kaempferol)	Mobile Phase	Wavelength (nm)	Flow Rate (ml/min)	Rt	Refereneces
Hippophae rhamnoides	methanol– acetonitrile–water (40:15:45, v/v/v) containing 1.0% acetic acid	279 nm	1	10.9 min	Zu *et al.*, 2006
Convolvulus pilosellifolius	Buffer methanol (90: 10)	258 nm	0.4	4.43 min	Al-Rifai *et al.*, 2015
Sorbus species	0.5%, v/v solution of orthophosphoric acid in water	370 nm	1	18.46	Olszewska, 2008
Prunus spinosa	0.5% acetic acid-methanol tetrahydrofuran (75.2:16.6:8.2, v/v/v)	280 nm	0.3	53.8 min	Owczarek, 2017
Schisandra chinensis	acetonitrile– aqueous 0.05% ortho-phosphoric acid 40:60 v/v	260 nm	0.5	7.92 min	Sladkovský *et al.*, 2001

Table 4.32: Summary of some reported phytochemical studies on Liquiritigenin estimation using HPLC

Sample (liquiritigenin)	Mobile Phase	Wavelength (nm)	Flow Rate (ml/min)	Rt	Refereneces
Glycyrrhiza uralensis	25 mM phosphate buffer (pH 2.5): acetonitrile (Gradient mobile Phase)	254 nm	1.2	28.97 min	Yuan-Chuen Wang ∗, Yi-Shan Yang, 2007

G. uralensis and *G. glabra*	water (1% acetic acid): acetonitrile (74: 26)	254 nm	0.6	6.3 min	Makio Shibano *et al*., 2009
Glycyrrhiza uralensis	acetonitrile:0.5% acetic acid in water	276 nm	1.0	8.43 min.	Jing Wang *et al*., 2013
Dalbergia odorifera	Acetonitrile and methanol	275 nm	1.0	12.40 min	Rongxia Liu *et al*, 2005

Table 4.33: Summary of some reported phytochemical studies on Naringenin estimation using HPLC

Sample (Naringenin)	Mobile Phase	Wavelength (nm)	Flow Rate (ml/min)	Rt	Refereneces
Commercial grapefruit	Acetonitrile/water	210nm	1	15.15 min	Ribeiro & Ribeiro, 2008
Citrus maxima	0.1% Orthophosphoric acid & acetonitrile (70:30)	289 nm	1	21 min	Sowmya *et al*., 2019
Naringenin	Methanol: Mili-Q Water (70:30)	288 nm	1	4.7 min	Jha *et al*., 2020
Dendrobium officinale & *Dendrobium devonianum*	acetonitrile & 0.2% formic acid	270 nm	1	66.21 min	Ye *et al*., 2017
Naringenin	Methanol	290 nm	1	12.3 min	Gaggeri *et al*., 2011

Table 4.34: Summary of some reported phytochemical studies on Quercetin estimation using HPLC

Sample (Quercetin)	Mobile Phase	Wavelength (nm)	Flow Rate (ml/min)	Rt	Refereneces
Quercetin	mixture of ACN and HPLC grade water (pH 2.6, adjusted with 2%w/v glacial acetic acid) in an isocratic elution mode	346 nm	1	2.80 min	Chaudhary *et al*., 2020
Hippophae rhamnoides	methanol– acetonitrile–water (40:15:45, v/v/v) containing 1.0% acetic acid	279 nm	1	7.3 min	Zu *et al*., 2006
Fagopyrum spp.	methanol:water:acetic acid (100:150:5)	360 nm	1.3	8.132 min	Jan *et al*., 2022
Quercetin anhydrous obtained from Sigma	acetonitrile and 2% v/v acetic acid (pH 2.60) (40%:60% v/v)	370 nm	1.3	32.195 min	Ang *at al*., 2014
Camellia sinensis	Methanol	370 nm	1	2.42 min	Savic *et al*., 2013

For the development of an HPLC technique, a review of the literature revealed a variety of solvent solutions for the estimation of Liquiritigenin, quercetin, naringenin, genistein, kaempferol, apigenin, glabridin and glycyrrhetinic acid. However, none of the papers indicated the simultaneous identification of these marker compounds, and some of the documented techniques for the simultaneous assessment of multiple marker compounds resulted in a poor peak shape and were unsuitable for the specified plant extracts. Therefore, these techniques were not implemented. We have devised a single identification and validation procedure for these marker compounds.

Developing a distinct approach for each marker was a simple but laborious task for identifying these chemicals. We have aimed to design a single approach for the simultaneous determination of all marker compounds. By modifying the proportions of the organic and aqueous phases, the mobile phase was optimised to achieve a speedy and simple assay technique with an acceptable run time, reasonable retention time, and sharp peak. During this stage of research, it was determined that the ratio of organic modifier, i.e. acetonitrile, was essential for a successful separation. Also, the acidification of the buffer with orthophosphoric acid performed a significant effect in improving the shape of the peak (Hajimehdipoor *et al*., 2012).

In a series of experiments, it was found that a gradient solvent system consisting of 0.1% phosphoric acid buffer, methanol, and acetonitrile on a C18 column provided the best chromatographic separation for Liquiritigenin, quercetin, naringenin, genistein, kaempferol, apigenin, and glabridin 18 alpha and beta glycyrrhetinic acid. The total playtime was calculated to be 50 minutes. The developed method's chromatogram is shown in the figure. Some of the standards were quite near to other compounds in the chromatogram, although separation was good for all of the standards. It was decided that this mobile phase would be the best for identifying and quantifying the aforementioned nine marker chemicals in plant extracts. Since the wavelength greatly affects the resolution and sensitivity of compounds, it was detected at 254 nm where it has the highest absorbency, and an adequate response was obtained. The kaempferol and apigenin was coming at the same Rt but after spectral confirmation, apigenin was considered.

Earlier Manglorkar in 2014 has reported the presence of Lupeol, β-sitosterol and Stigmasterol using HPLC and GCMS analysis in the roots of *T. cuneifolia*. Khan *et al*., 2012 also reported previously that the *T. cuneifolia* roots contained lupeol and ***β*-**sitosterol. However, the validation is still not done for this plant. In the current work, an effort was made to standardise and validate the β-sitosterol and Stigmasterol in the chloroform and methanolic root extract of *T. cuneifolia* (TAC & TAV MEOH) and methanolic extract of *G. glabra* (GG MEOH). The review of the literature revealed a variety of solvent system for the estimation of β-

sitosterol and Stigmasterol in several plant extracts and some of these systems are included in the table 4.34 & 4.35.

Table 4.35: Summary of some reported phytochemical studies on Stigmasterol estimation using HPLC

Sample (Stigmasterol)	Mobile Phase	Wavelength (nm)	Flow Rate (ml/min)	Rt of	Refereneces
brassicasterol, stigmasterol, campesterol and β-sitosterol	methanol	210 nm	1	19.745 min	Yi Sheng Xiao-Bin Chen, 2009
Adhatoda vasica	28%v/v of 0.1% formic acid in water (A) and 82%v/v of methanol (B)	208 nm	0.8	18.26	Nandhini & Ilango, 2020
Bambusa bambos	methanol: acetonitrile in the ratio (90:10)	208 nm	1	19.13 min	Sandhiya Sriraman *et al.*, 2015
Butea monosperma	Methanol: Water (98:2% v/v)	220 nm	1	15 min	Modh & Pandya, 2019
Momordica charantia	petroleum ether:ethanol	methanol: water (97.5:2.5, 98:02 and 98.5:1.5, v/v)	1	5.6 min	Desai *et al.*, 2020

Table 4.36: Summary of some reported phytochemical studies on β- sitosterol estimation using HPLC

Sample (β-sitosterol)	Mobile Phase	Wavelength (nm)	Flow Rate (ml/min)	Rt	Referenece
Adhatoda vasica	28%v/v of 0.1% formic acid in water (A) and 82%v/v of methanol (B)	208 nm	0.8	20.72 min	Nandhini & Ilango, 2020
Bambusa bambos	methanol: acetonitrile in the ratio (90:10)	208 nm	1	(21.16 min)	Sandhiya Sriraman *et al.*, 2015
Bombax ceiba	acetonitrile and 0.05% acetic acid	254 nm	1	4.527 min	Chauhan *et al.*, 2018
Momordica charantia	methanol: water (97.5:2.5, 98:02 and 98.5:1.5, v/v)	203, 204 and 205 nm	1	11.870 min	Desai *et al.*, 2020
MarkHerb	Methanol:acetonitrile (9:1 v/v)	202 nm	1.5	13 min	Khonsa *et al.*, 2022
β-sitosterol enriched phytosterol	MeOH	210 nm	1	21.743 min	Yi Sheng Xiao-Bin Chen, 2009

The identification of β-sitosterol and Stigmasterol was carried out in HPLC using methanol: acetonitrile in an isocratic system on a C18 column at 205nm wavelength. Rest other instrument parameters is mentioned in the table. The Rt of β-sitosterol and Stigmasterol 9.82 and 10.88 respectively.

Table 4.37: Identification result of selected standards in plant sample

Plant	Sugar markers tested											
	Apigenin	**Glabridin**	**Genistein**	**Glycyrrhizin**	**18-Alpha Glycyrrhetinic acid**	**18-beta Glycyrrhetinic acid**	**Kaempferol**	**Liquiritigenin**	**Naringenin**	**Quercetin**	**Stigmasterol**	**Beta-Sitosterol**
***T. cuneifolia* roots (MeOH ext)**	+	-	+	+	-	-	-	-	-	-	+	+
***T. cuneifolia* roots (CHCl3 ext)**	+	-	+	+	-	-	-	+	-	-	+	+
***G. glabra* roots (MeOH ext)**	+	+	+	+	-	-	-	+	-	-	+	-

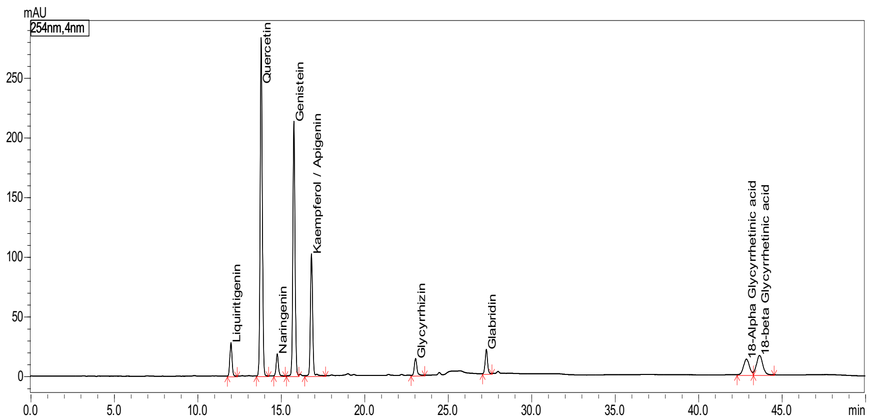


Figure 4.33: Simultaneous Method development for separation and estimation of standards using HPLC technique

Following the technique development for the marker chemicals, the *T. cuneifolia* methanol extract (TAV MEOH), *T. cuneifolia* chloroform extract (TAC), and *G. glabra* methanol extract (GG MEOH) were injected. The TAV MEOH showed the presence of genistein, apigenin, and glycyrrhizin, while the TAC showed the presence of liquiritigenin, quercetin, apigenin, and glycyrrhizin. The GG MEOH extract shown the presence of liquiritigenin, genistein, apigenin, glycyrrhizin, and glabridin. Figure 4.33 & 4.34 represents the chromatogram for the presence of above-mentioned standards in the samples.

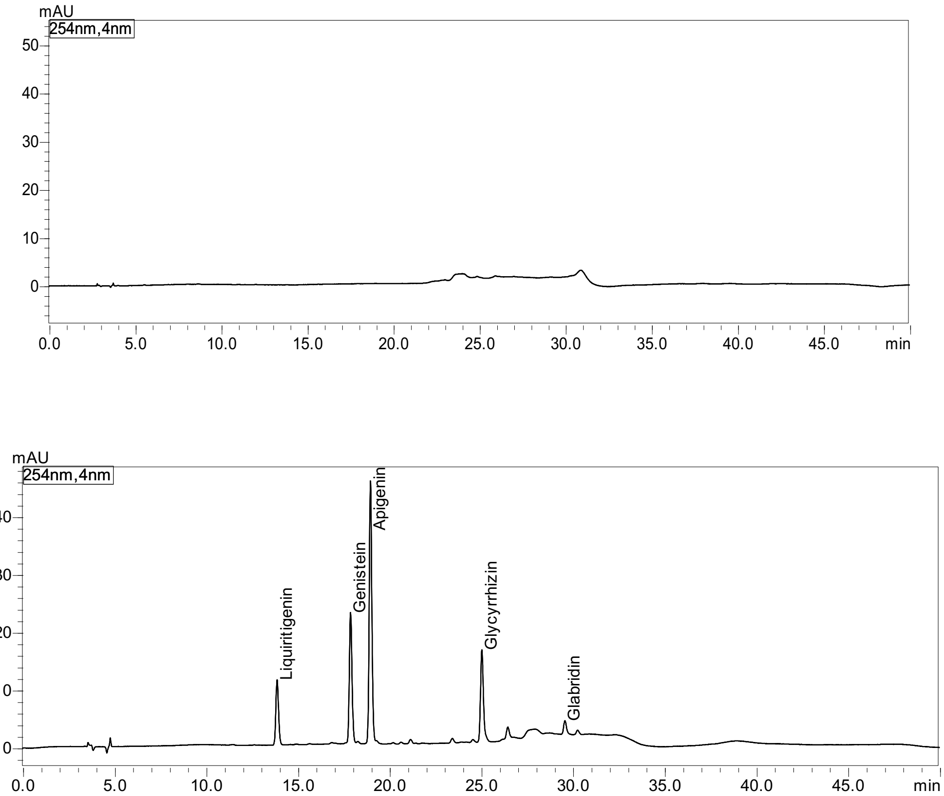


Figure 4.34: Simultaneous Method development for separation and estimation of standards using HPLC technique (A) Blank (B) standards coming in the samples

The chromatogram for the blank and plant extracts is given for stigmasterol and β-sitosterol is given in figure 4.35.

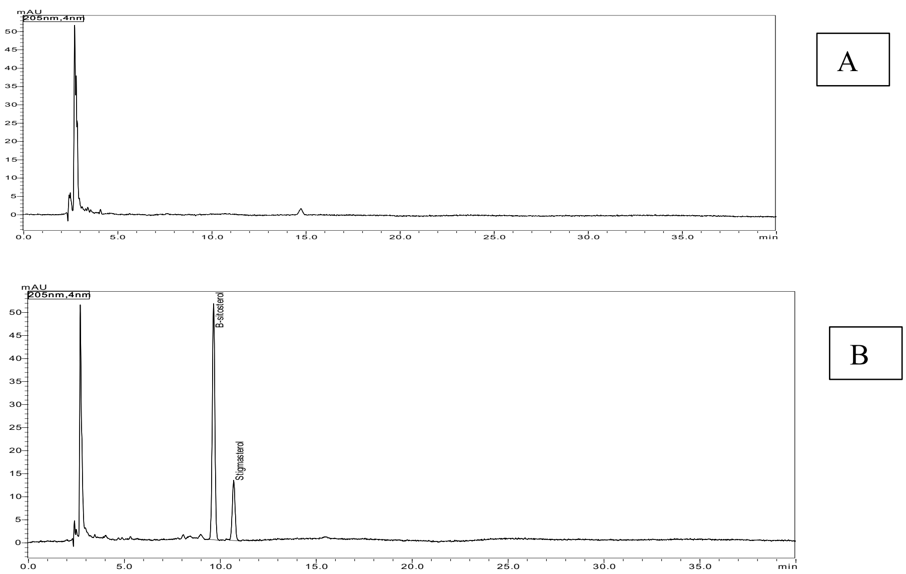


Figure 4.35: Simultaneous Method development for separation and estimation of stigmasterol and β-sitosterol using HPLC technique (A) Blank (B) Standards

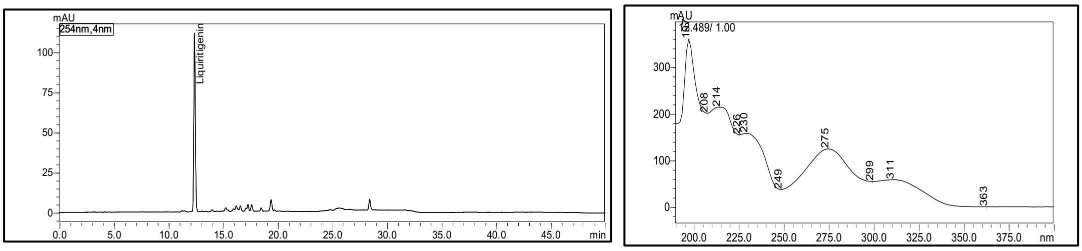


Figure 4.36: Standard chromatogram & UV spectra of liquiritigenin

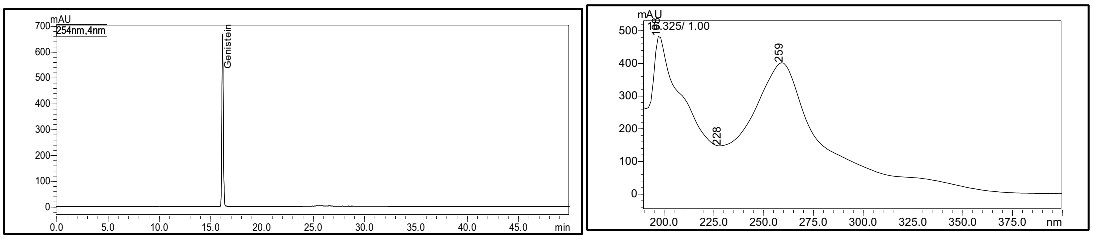


Figure 4.37: Standard chromatogram & UV spectra of Genistein

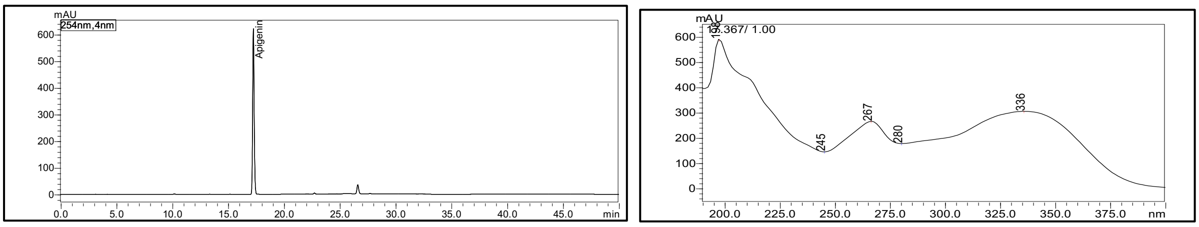


Figure 4.38: Standard chromatogram & UV spectra of apigenin

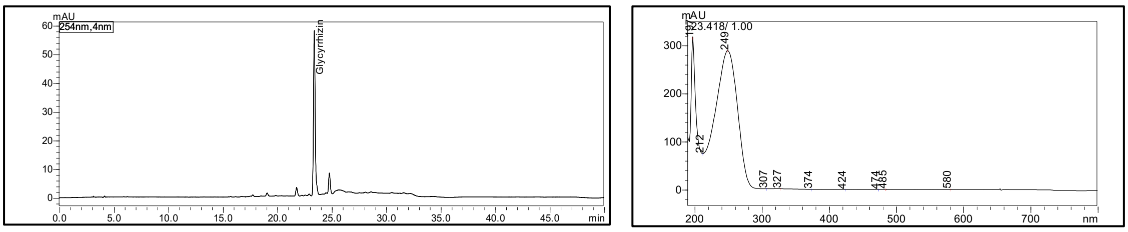


Figure 4.39: Standard chromatogram & UV spectra of glycyrrhizin

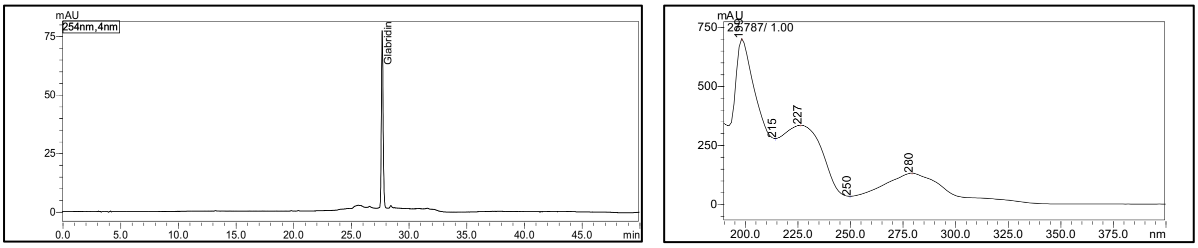


Figure 4.40: Standard chromatogram & UV spectra of glabridin

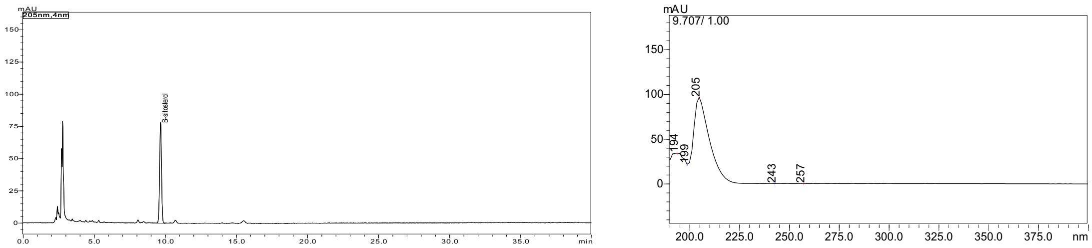


Figure 4.41: Standard chromatogram & UV spectra of β-sitosterol

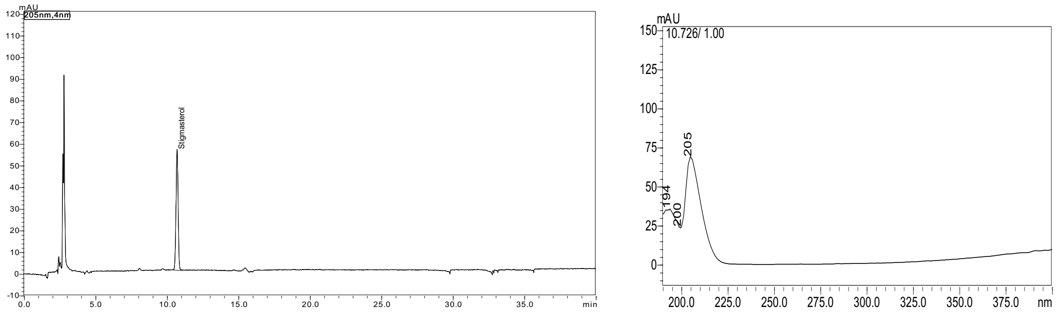


Figure 4.42: Standard chromatogram & UV spectra of stigmasterol

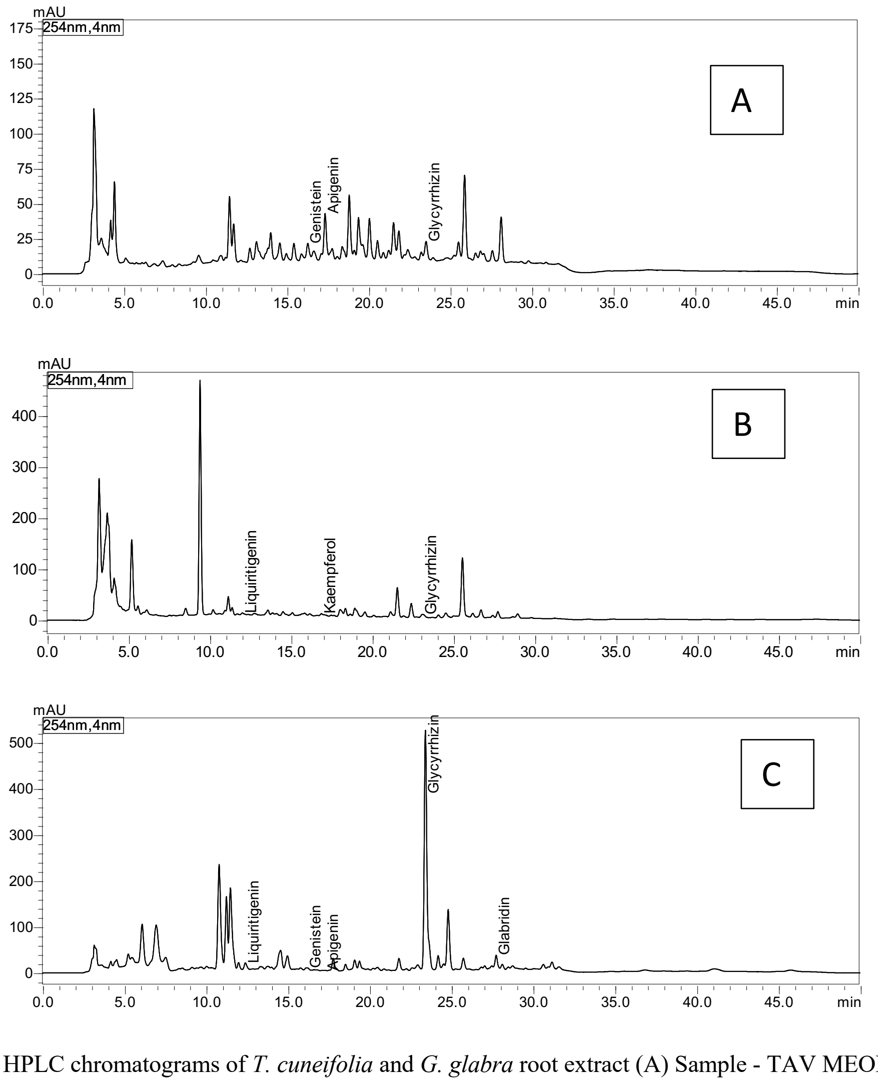


Figure 4.43: HPLC chromatograms of *T. cuneifolia* and *G. glabra* root extract (A) Sample - TAV MEOH @ 254 nm (B) Sample – TAC @ 254 nm (C) Sample - GG MEOH @ 254 nm

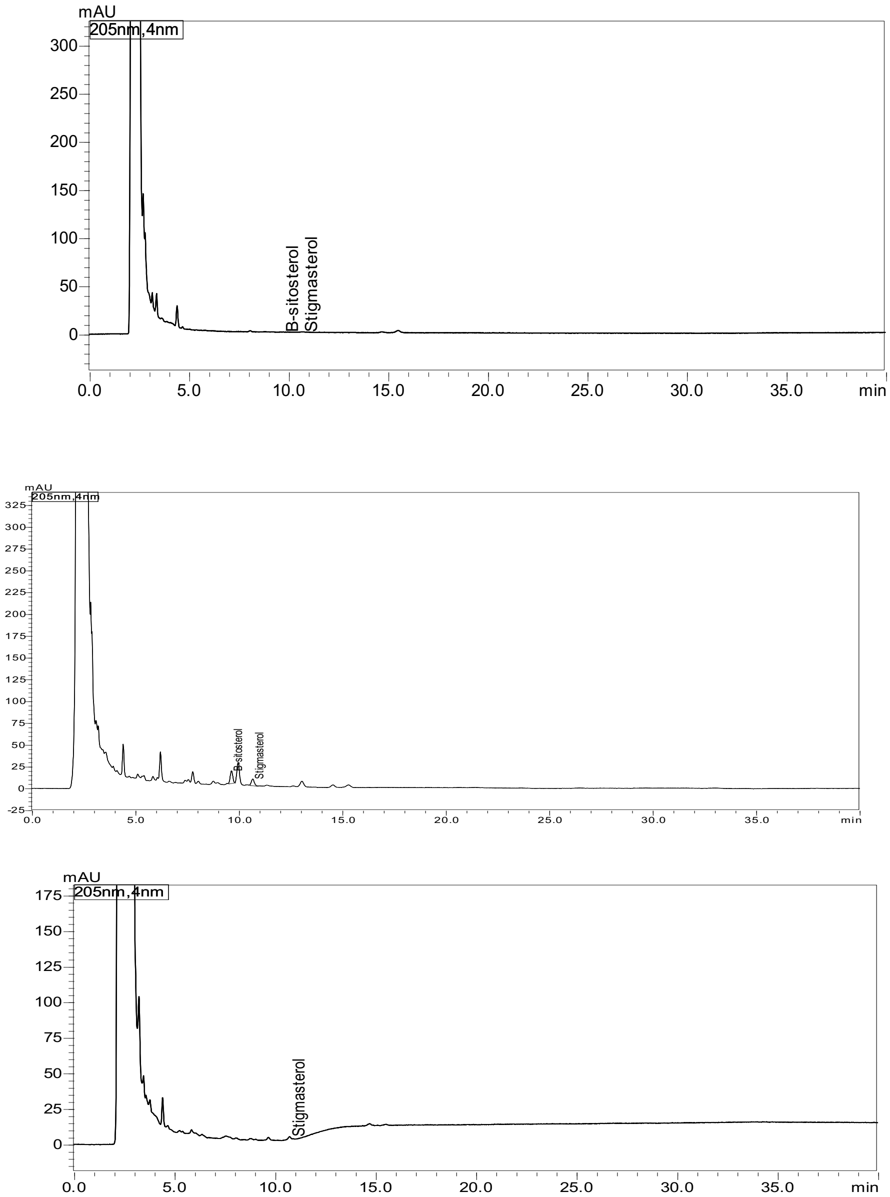


Figure 4.44: HPLC chromatograms of *T. cuneifolia* and *G. glabra* root extract showing the presence of Stigmasterol and β-sitosterol in (A) Sample - Sample – TAC @ 254 nm (B) TAV MEOH @ 254 nm (C) Sample - GG MEOH @ 254

4.3.7 Validation result

4.3.7.1 Specificity and selectivity

In this study, it was observed that the Rt values of the extracts of TAC, TAV MEOH and GG MEOH obtained by HPLC methods were comparable to that of the pure standards. The following factors were taken into account in the system's suitability in order to identify the method's optimum performance. Three conditions were met: (i) injection of mix standard preparation in duplicate; (ii) peak resolution between two peaks, which was shown to be >2.0; and (iii) the tailing factor for each analyte, which was found to be <1.5. In addition, there was no significant variance between the absorption spectra of the standard chemicals and the marker compounds found in the plant extracts, proving the method's specificity.

4.3.7.2 System suitability

System suitability tests provide an evaluation for the function of the overall system. These tests are valuable as they are a means for the verification of the reliability and reproducibility required for the analysis (ICH, 2005; Kupiec, 2004). The results of the system suitability test in terms of % RSD of RT, % RSD of Area, Tailing factor, Resolution, Theoretical plates of HPLC methods are summarized in Table 4.37 & 4.38 respectively. The system suitability tests presented CV values less than 2% for the tested parameters, thus suggesting that the chromatographic system is adequate for the analysis and meets the expectations of the analyst.

Table 4.38: Results of system suitability of HPLC methodology for simultaneous method development of standards liquiritigenin, apigenin, genistein, glabridin and glycyrrhizin

Sr. No	Parameters	Test	Limit	Liquiritigenin	Genistein	Apigenin	Glycyrrhizin	Glabridin
1	System suitability	% RSD of Rt		0.060	0.043	0.045	0.029	0.026
		% RSD of Area	NMT 2.0%	0.119	0.493	0.395	1.365	8.882
		Tailing factor	NMT 1.5	1.132	1.119	1.108	1.226	1.097
		Resolution	NLT 2.0	NA	14.255	3.927	21.348	15.365
		Theoretical plates	NLT 2000	> 2000	> 2000	> 2000	> 2000	> 2000

***NMT=Not less than; NMT=Not more than**

Table 4.39: Results of system suitability of HPLC methodology for simultaneous method development of standards β-sitosterol and stigmasterol

Sr. No	Parameters	Test	Limit	Stigmasterol	B-Sitosterol
1	System suitability	% RSD of Rt		9.829	10.887
		% RSD of Area	NMT 2.0%	0.661	0.473
		Tailing factor	NMT 1.5	1.005	0.991
		Resolution	NLT 2.0	NA	3.882
		Theoretical plates	NLT 2000	21933.800	23750.200

***NMT=Not less than; NMT=Not more than**

4.3.7.3 Sensitivity

Sensitivity is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector (Kupiec, 2004). It is generally measured in terms of limit of detection (LOD) and limit of quantitation (LOQ).

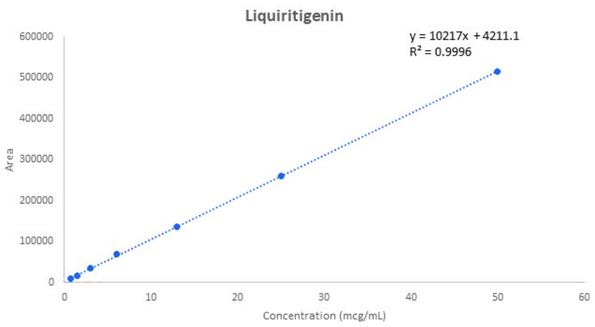
The limit of detection is the lowest amount of analyte which is detectable, but where interference from background noise occupies at least 3% (signal: noise ratio = 3) of the peak height, rendering measurement too inaccurate to be quantified. Limit of quantitation is the lowest amount of analyte at which the background noise occupies up to 10% (signal: noise ratio = 10) of the peak height, allowing for a reasonable estimate of peak area to be measured with suitable precision and accuracy (ICH, 2005). The obtained values for LOD and LOQ for markers by HPLC methods are given in Table 4.39.

Table 4.40: The chromatographic and calibration parameters, LOD and LOQ

Compound	**Rt**	**Regression equation**	**Coefficient of variation(r^2)**	**LOD (µg/mL)**	**LOQ (µg/mL)**	**% CV**
Liquiritigenin	12.305	y = 10217x + 4211.1	0.9998	0.45	0.75	0.542
Genistein	16.161	y = 37930x + 47943	0.9993	0.33	0.75	0.366
Apigenin	17.227	y = 35445x + 13823	0.9998	0.29	0.75	0.491
Glycyrrhizin	23.394	y = 4206.3x + 151028	0.9994	3.143	7.812	1.105
Glabridin	27.7	y = 7949.9x + 3968.8	0.9999	0.35	0.75	0.745
Stigmasterol	9.829	Y= 9687.3x - 683.69	0.99	0.253	0.765	0.431
β-Sitosterol	10.887	Y= 2508.8x + 398.24	0.999	0.283	0.858	0.405

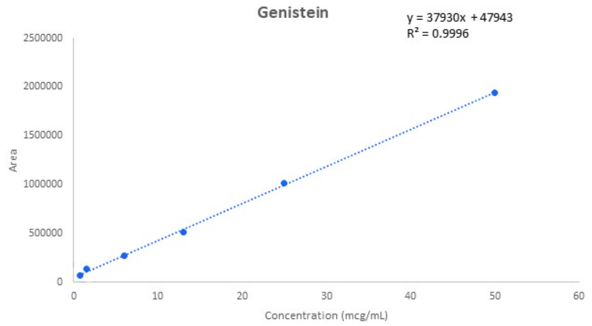
4.3.7.4 Linearity

In this study, linear correlation between peak area and concentration of standard compounds was obtained in the range of 0.75-50.0 mcg/mL for liquiritigenin, apigenin, genistein, glabridin and 7.8-500.0 mcg/mL for glycyrrhizin in HPLC, respectively. The linearity for β-sitosterol and stigmasterol were performed in the range of 3.13-50 mcg/mL. The % CV for area of the samples was found to be below 2 and the % nominal was within the acceptance range of 85-115%. Data on the response of calibrant samples of liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin, β-sitosterol and stigmasterol obtained by HPLC is given in Figure 4.46. Using this data, calibration curves of mean area versus actual concentration of standard compounds were constructed. Figure 4.46 shows the plate photo of linearity of liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin, β-sitosterol and stigmasterol by HPLC.



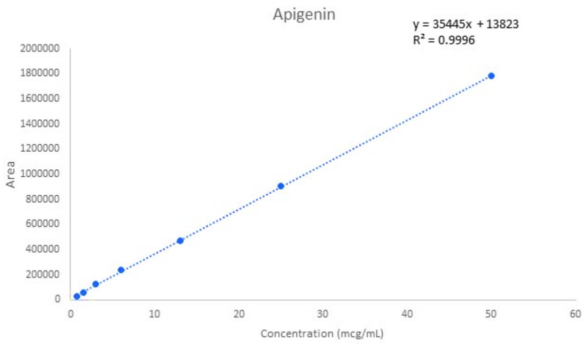
Liquiritigenin (mcg/mL)	Mean	SD	% RSD
0.75	9118	32.42	0.36
1.5	17257	153.03	0.89
3	35518	686.19	1.93
6	68747	1191.58	1.73
13	135232	1053.10	0.78
25	262410	1332.25	0.51
50	513818	7440.48	1.45

A



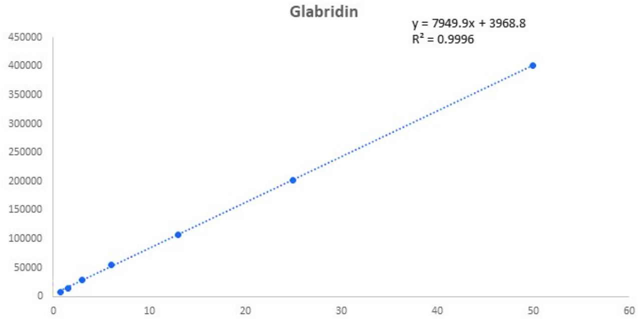
Genistein (mcg/mL)	Mean	SD	% RSD
0.75	68132	671.32	0.99
1.5	133594	1545.59	1.16
3	266490	1277.18	0.48
6	512719	1691.34	0.33
13	1011492	649.72	0.06
25	1958551	12518.11	0.64
50	3823072	72043.29	1.88

B



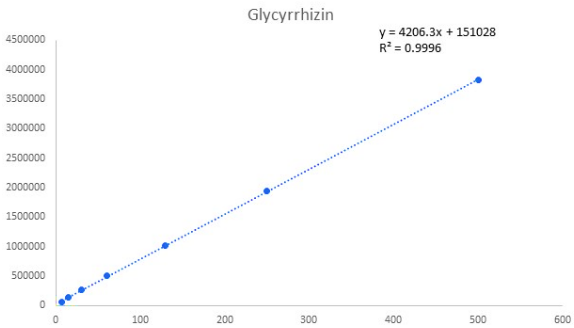
Apigenin (mcg/mL)	Mean	SD	% RSD
0.75	31260	145.43	0.47
1.5	61316	224.37	0.37
3	124062	798.54	0.64
6	240979	827.97	0.34
13	466464	2008.09	0.43
25	896824	7496.89	0.84
50	1783695	12200.97	0.68

C



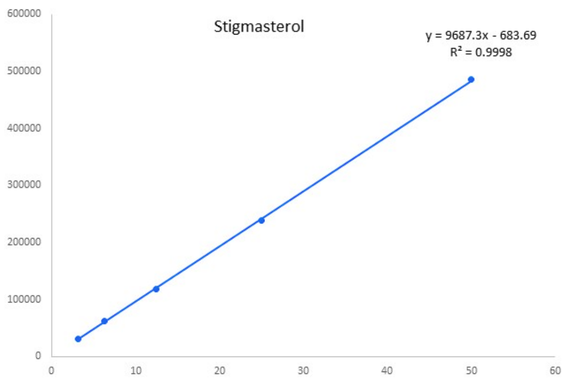
Glabridin (mcg/mL)	Mean	SD	% RSD
0.75	8159	92.82	1.14
1.5	14211	177.22	1.25
3	28476	117.24	0.41
6	54102	142.63	0.26
13	107242	616.97	0.58
25	202143	681.14	0.34
50	403593	5185.95	1.28

D



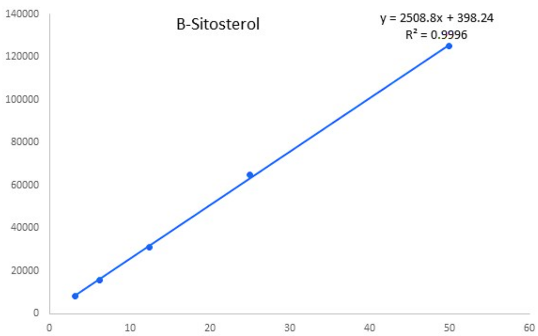
Glycyrrihizin (mcg/mL)	Mean	SD	% RSD
7.8125	46789	737.20	1.58
15.625	93079	1033.02	1.11
31.25	187215	1866.75	1.00
62.5	371644	1514.42	0.41
125	783310	2149.59	0.27
250	2978071	10259.76	0.34
500	1476020	26896.66	1.82

E



Stigmasterol (mcg/mL)	Mean	SD	% RSD
3.13	30242	227.34	0.75
6.25	62515	188.76	0.30
12.5	118134	476.06	0.40
25	239040	549.37	0.23
50	485105	2265.26	0.47

F



β-sitosterol (mcg/mL)	Mean	SD	% RSD
3.13	8365	38.63	0.46
6.25	15701	69.89	0.45
12.5	30940	69.26	0.22
25	64770	234.01	0.36
50	125257	665.11	0.53

G

Figure 4.45: calibration curves obtained by HPLC techniques (A) Liquiritigenin (B) Geinstein (C) Apigenin (D) Glabridin (E) Glycyrrhizin (F) Stigmasterol (G) β-sitosterol

4.3.7.5 Accuracy as recovery

The average recovery of each analyte was found to be 88.94%, 90.10%, 89.70%, 85.74%, 102.23%, 95.138% and 90.765% for liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin, stigmasterol and β-sitosterol. It showed the accuracy of the method for targeted analytes in ambiguous matrices. The result of the accuracy is summarised in the table 4.40, 4.41 & 4.42.

Table 4.41: Recovery of quality control samples of standards from *G. glabra* via HPLC techniques

Sample	Amount of Compounds in sample (µg/mL)	Spiked amount (µg/mL)	Theoretical value (µg/mL)	Experimental value (µg/mL)	Recovery (%)	Average recovery (% recovery)
Liquiritigenin						
G. glabra in methanol (GG MEOH)	12.10	16 20 24	28.1 32.1 36.1	26.326 20.997 33.448	88.910 88.970 88.950	88.943
Genistein						
G. glabra in methanol (GG MEOH)	0.70	16 20 24	16.7 20.7 24.7	15.156 9.690 22.324	90.350 89.900 90.100	90.117
Apigenin						
G. glabra in methanol (GG MEOH)	0.40	16 20 24	16.4 20.4 24.4	14.539 9.414 22.143	88.366 90.139 90.595	89.700
Glycyrrhizin						
G. glabra in methanol (GG MEOH)	847.20	100 125 150	947.20 972.2 997.2	932.342 954.645 976.385	85.142 85.956 86.123	85.740
Glabridin						
G. glabra in methanol (GG MEOH)	25.40	16 20 24	41.4 45.4 49.4	41.755 35.650 49.874	102.221 102.495 101.973	102.230

Sample	Amount of Compounds in sample (nano gram)	Spiked amount (µg/mL)	Theoretical value (µg/mL)	Experimental value (µg/mL)	Recovery (%)	Average recovery (% recovery)

Stigmasterol						
***G. glabra* in methanol (GG MEOH)**	**0.1 0.1 0.1**	**40 50 60**	**40.1 50.1 60.1**	**38.001 48.140 56.849**	**94.753 96.079 94.581**	**95.138**
B-Sitosterol						
***G. glabra* in methanol (GG MEOH)**	**0.268 0.268 0.268**	**40 50 60**	**40.268 50.268 60.268**	**36.241 45.314 55.630**	**89.932 90.092 92.270**	**90.765**

Table 4.42: Recovery of quality control samples of standards from *T. cuneifolia* chloroform extract via HPLC techniques

	Amount of Compounds in sample (µg/mL)	Spiked amount (µg/mL)	Theoretical value (µg/mL)	Experimental value (µg/mL)	Recovery (%)	Average recovery (% recovery)
Liquiritigenin						
TAC	**0.22**	**16 20 24**	**16.22 20.22 24.22**	**14.61 20.09 23.21**	**89.95 99.36 95.79**	**95.03**
Genistein						
TAC	**0.02**	**16 20 24**	**16.02 20.02 24.02**	**14.41 19.51 22.70**	**89.95 97.46 94.49**	**93.97**
Apigenin						
TAC	**0.05**	**16 20 24**	**16.05 20.05 24.05**	**16.25 17.90 23.99**	**101.25 89.25 99.74**	**96.75**
Glycyrrhizin						
TAC	**1.51**	**100 125 150**	**17.51 21.51 25.51**	**17.92 20.78 25.33**	**102.56 96.36 99.25**	**99.39**
Glabridin						

TAC	0	16 20 24	16 20 24	16.20 19.86 23.22	101.25 99.29 96.74	99.09

Table 4.43: Recovery of quality control samples of standards from *T. cuneifolia* methanol extract via HPLC techniques

	Amount of Compounds in sample (µg/mL)	Spiked amount (µg/mL)	Theoretical value (µg/mL)	Experimental value (µg/mL)	Recovery (%)	Average recovery (% recovery)
Liquiritigenin						
TAV MEOH	-	16 20 24	16 20 24	15.80 18.85 23.51	98.74 94.25 97.97	96.99
Genistein						
TAV MEOH	0.22	16 20 24	16.22 20.22 24.22	16.12 19.15 23.34	99.35 94.65 96.32	96.77
Apigenin						
TAV MEOH	0.66	16 20 24	16.66 20.66 24.66	17.03 19.99 23.30	102.3 96.63 94.32	97.75
Glycyrrhizin						
TAV MEOH	1.99	100 125 150	17.99 21.99 25.99	17.95 22.64 25.26	99.74 103.25 96.96	99.98
Glabridin						
TAV MEOH	-	16 20 24	16 20 24	16.04 19.85 22.83	100.26 99.23 95.14	98.21

4.3.7.6 Precision

In this experiment, intermediate precision was studied to investigate intra-day and inter-day variations in the HPLC methods for three times on the same day and three different days using three different concentration levels of standards. The repeatability precision of standards was determined based on measuring the peak area and retention times. The low coefficient of variation values of intra-day and inter-day precision and the % nominal values between 85-115% revealed that the proposed method is precise with respect to the criteria of the intermediate precision. The results of intra-day and inter-day precision using HPLC are listed in Table 4.43 & 4.44.

Table 4.44: Results of intermediate precision of standards by HPLC technique (Day 1, Day 2 & Day 3)

Intermediate Precision (Day-1) (Content in %)							
Sr. No.	**STD Name**	**Replicate-1**	**Replicate -2**	**Replicate -3**	**Mean**	**SD**	**% RSD**
1	Liquiritigenin	0.123	0.126	0.122	0.124	0.002	**1.683**
2	Genistein	0.007	0.007	0.007	0.007	0.000	**0.829**
3	Apigenin	0.004	0.004	0.004	0.004	0.000	**2.500**
4	Glycyrrhizin	8.261	8.566	8.412	8.413	0.153	**1.813**
5	Glabridin	0.252	0.251	0.254	0.252	0.002	**0.605**
6	Stigmasterol	0.109	0.106	0.106	0.107	0.002	**1.619**
7	B-Sitosterol	0.288	0.281	0.286	0.285	0.004	**1.265**

Intermediate Precision (Day-2) (Content in %)							
Sr. No.	**STD Name**	**Replicate -1**	**Replicate -2**	**Replicate -3**	**Mean**	**SD**	**% RSD**
1	Liquiritigenin	0.131	0.128	0.129	0.129	0.002	**1.181**
2	Genistein	0.009	0.009	0.009	0.009	0.000	**1.111**
3	Apigenin	0.004	0.004	0.004	0.004	0.000	**4.225**
4	Glycyrrhizin	8.863	8.906	8.793	8.854	0.057	**0.644**
5	Glabridin	0.259	0.261	0.262	0.261	0.002	**0.586**
6	Stigmasterol	0.112	0.111	0.115	0.113	0.002	**1.848**
7	B-Sitosterol	0.296	0.286	0.291	0.291	0.005	**1.718**

Method Precision (Interday) (Content in %)							
Sr. No.	**STD Name**	**R1**	**R2**	**R3**	**Mean**	**SD**	**% RSD**
1	Liquiritigenin	0.135	0.131	0.133	0.133	0.002	**1.504**
2	Genistein	0.008	0.008	0.008	0.008	0.000	**1.250**
3	Apigenin	0.004	0.004	0.004	0.004	0.000	**3.787**
4	Glycyrrhizin	8.021	8.017	8.102	8.047	0.048	**0.596**
5	Glabridin	0.269	0.272	0.266	0.269	0.003	**1.115**
6	Stigmasterol	0.110	0.119	0.116	0.115	0.005	**3.985**
7	B-Sitosterol	0.279	0.278	0.280	0.279	0.001	**0.358**

Table 4.45: Results of intraday precision of standards by HPLC technique

Method Precision (Intraday) (Content in %)							
Sr. No.	**STD Name**	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Mean**	**SD**	**% RSD**
1	Liquiritigenin	0.139	0.140	0.139	0.139	0.001	**0.414**
2	Genistein	0.007	0.007	0.007	0.007	0.000	**1.657**
3	Apigenin	0.004	0.004	0.004	0.004	0.000	**1.353**
4	Glycyrrhizin	8.761	8.695	8.799	8.752	0.053	**0.601**
5	Glabridin	0.260	0.263	0.264	0.262	0.002	**0.794**
6	Stigmasterol	0.115	0.113	0.112	0.113	0.002	**1.322**
7	B-Sitosterol	0.275	0.267	0.265	0.269	0.005	**2.004**

4.3.7.7 Robustness

It was observed that the chromatographic separation of kaempferol was not affected when the experiment was performed by different analysts. Moreover, % CV values calculated for peak area, Rt were less than 2 and no significant variation was observed between the actual and the altered conditions with % mean difference values in the range of ± 5.0. The Rt was shifted slightly in HPLC due to the altered flow rate and mobile phase composition. Nonetheless, the % CV and % mean difference values were found to be within the acceptable limits. The findings of the study thus suggest that the method is robust and suitable for use.

4.3.8 Assay of liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin and stigmasterol and β-Sitosterol from plant extracts

The proposed method was utilised to assess the concentration of liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin, stigmasterol and β-Sitosterol in plant root extracts from TAC, TAV MEOH and GG MEOH. The summary of the result is given in table 4.45. This will be the first report of a validated technique for rapid detection and quantification of liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin, stigmasterol and β-Sitosterol in TAC, TAV MEOH root extract versus GG MEOH root extract.

Table 4.46: Results from the quantitative analysis of phytocomponents detected in *T. cuneifolia* & *G. glabra*

Sr. No.	**Compound name**	***G. glabra* in methanol (GG MEOH)**		***T. cuneifolia* in methanol (TAV MEOH)**		***T. cuneifolia* in chloroform (TAC)**	
		% Content	**Content in (µg/mg)**	**% Content**	**Content in (µg/mg)**	**% Content**	**Content in (µg/mg)**
1	Liquiritigenin	0.121	1.21	**ND**	ND	0.022	0.22
2	Genistein	0.007	0.07	0.022	0.22	0.002	0.02
3	Apigenin	0.004	0.04	0.066	0.66	0.005	0.05
4	Glycyrrhizin	8.472	84.72	0.199	1.99	0.151	1.51
5	Glabridin	0.254	2.54	**ND**	ND	**ND**	ND
6	Stigmasterol	0.01	0.10	0.12	1.2	0.01	0.10
7	β-Sitosterol	ND	ND	0.28	2.8	0.01	0.10

Thus, these methods represent a reliable procedure for simultaneous detection, separation and quantification of liquiritigenin, apigenin, genistein, glabridin and glycyrrhizin; Stigmasterol and β-Sitosterol from the chloroform and methanolic extracts of *T. cuneifolia* and *G. glabra*. The complete validation of the methods showed satisfactory results for the tested parameters. The results of the study indicate that the methods were rapid, simple, reliable, accurate, linear, selective, sensitive as well as economical and reached suitable recovery and good precision. Thus, through these validation studies, the methods' ability to provide good quantification in the laboratory was confirmed.

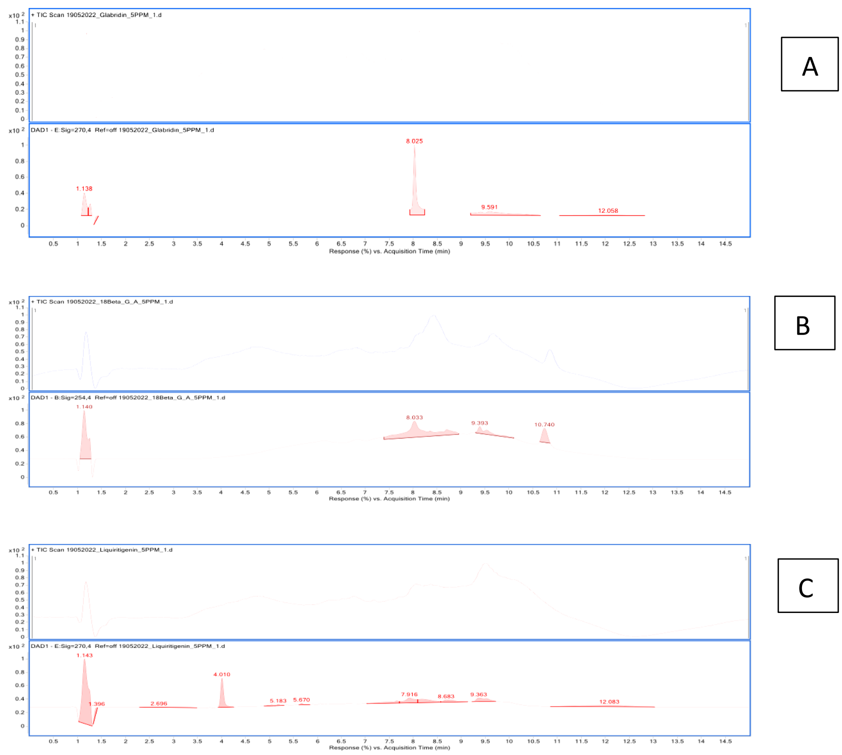
4.3.9 LC-MS/MS-Q-TOF analysis of Targeted metabolites

The previous HPLC method validation of Liquiritigenin, Naringenin, Kaempferol, Apigenin, Glycyrrhizin, Glabridin, Glycyrrhetinic acid and Stigmasterol in the *T. cuneifolia* root extract has been done in comparison with *G. glabra* wherein, the result showed the presence of only five compounds i.e., liquiritigenin, apigenin, genistein, glycyrrhizin & stigmasterol which are present in common. To further verify this, the *T. cuneifolia* root samples extracted in hexane : ethyl acetate (HXEATAV), in Methanol **(**MEOHTAV), in chloroform (CHTAV) were subjected for LC-MS/MS-Q-TOF analysis. For comparison, the chloroform extract of *G. glabra* (CHGG) were also taken into consideration. The analysis showed the following result:

Table 4.47: Characterization of standard compounds by LC-MS/MS-Q-ToF

Sr. No.	**Proposed Compounds**	**Molecular Formula**	**RT (min)**	**Ionization ESI (+)**	**Molecular Weight**	**Observed (*m/z*)**
1	Apigenin	C15 H10 O5	11.22	(M+NH4)+	270.0541	288.0883
2	Glabridin	C20 H20 O4	7.926	(M+H)+	324.1390	325.1435
3	Glycyrrhizin	C42H62O16	5.845	(M+H)+ (M+NH4)+ (M+Na)+	822.4008	823.4111 840.4196 845.3899
4	Glycyrrhetinic acid	C30 H46 O4	11.589	(M+H)+ (M+Na)+	470.3385	471.3478 493.3198
5	Kaempferol	C15 H10 O6	4.547	(M+H)+	286.0469	287.0543
6	Liquiritigenin	C15 H12 O4	5.795	(M+H)+	256.0718	257.0788
7	Naringenin	C15 H12 O5	6.425	(M+H)+ (M+Na)+	272.0685	273.0760 295.0576
8	Stigmasterol	C29 H48 O	10.022	(M+H)+	412.3705	413.3787

They are widespread secondary plant metabolites and have promising health effects. A total of 8 compounds were tentatively characterized and MS/MS used for the confirmation of their fragmentation pattern (table 4.45).



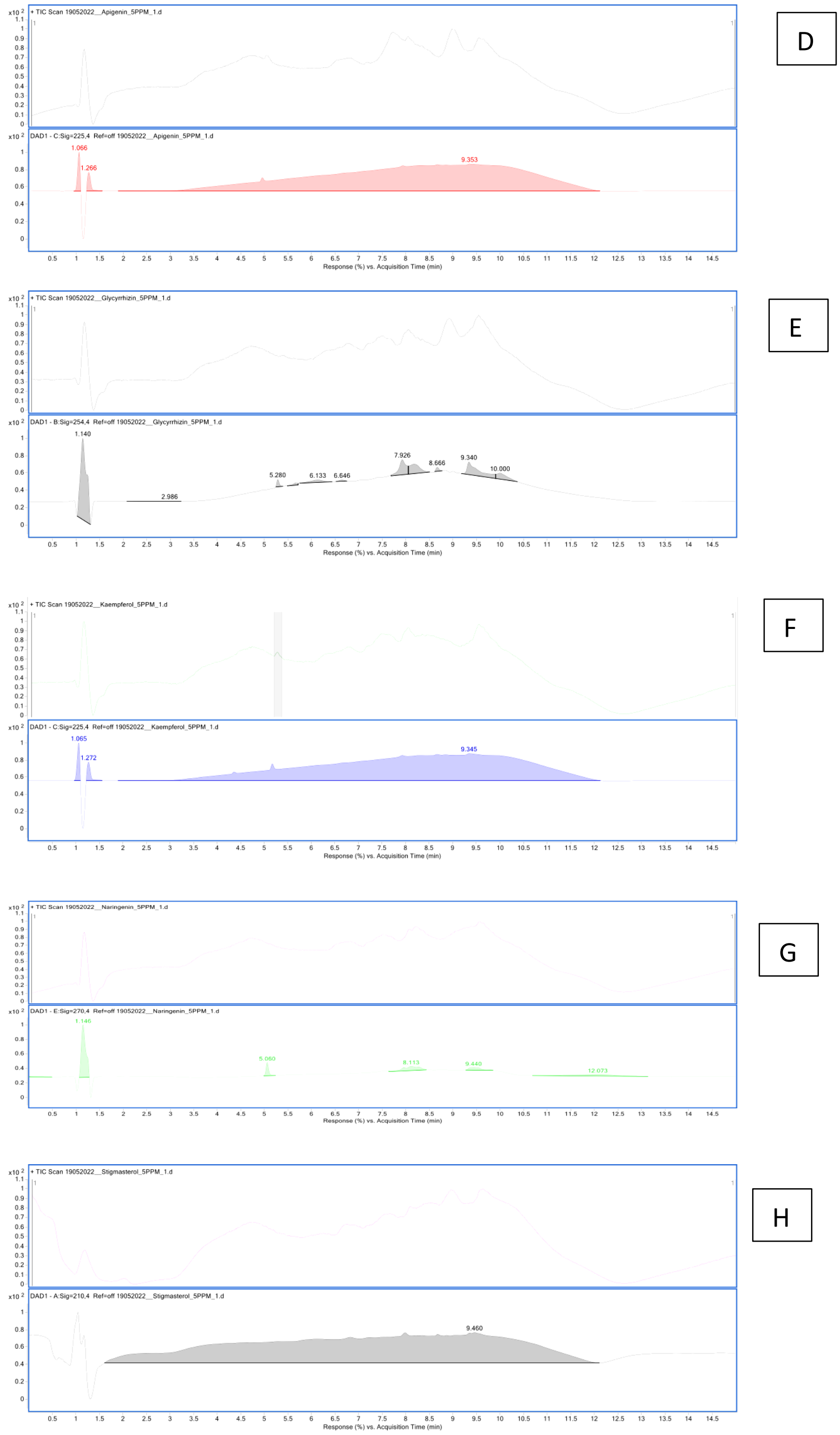


Figure 4.46: Total ion chromatogram and (TIC) its spectra from diode array detector of the standard compounds (A) Glabridin_standard_5PPM (325.1437) (B) Glycyrrhetinic acid_standard_5PPM (471.3476) (C) Liquiritigenin_standard_5PPM (257.0729) (D) Apigenin_standard_5PPM (271.0597) (E) Glycyrrhizin_standard_5PPM (823.4100) (F) kaempferol_standard_5PPM (287.0544) (G) Naringenin_standard_5PPM (272.0684) (H) Stigmasterol_standard_5PPM (412.37052).

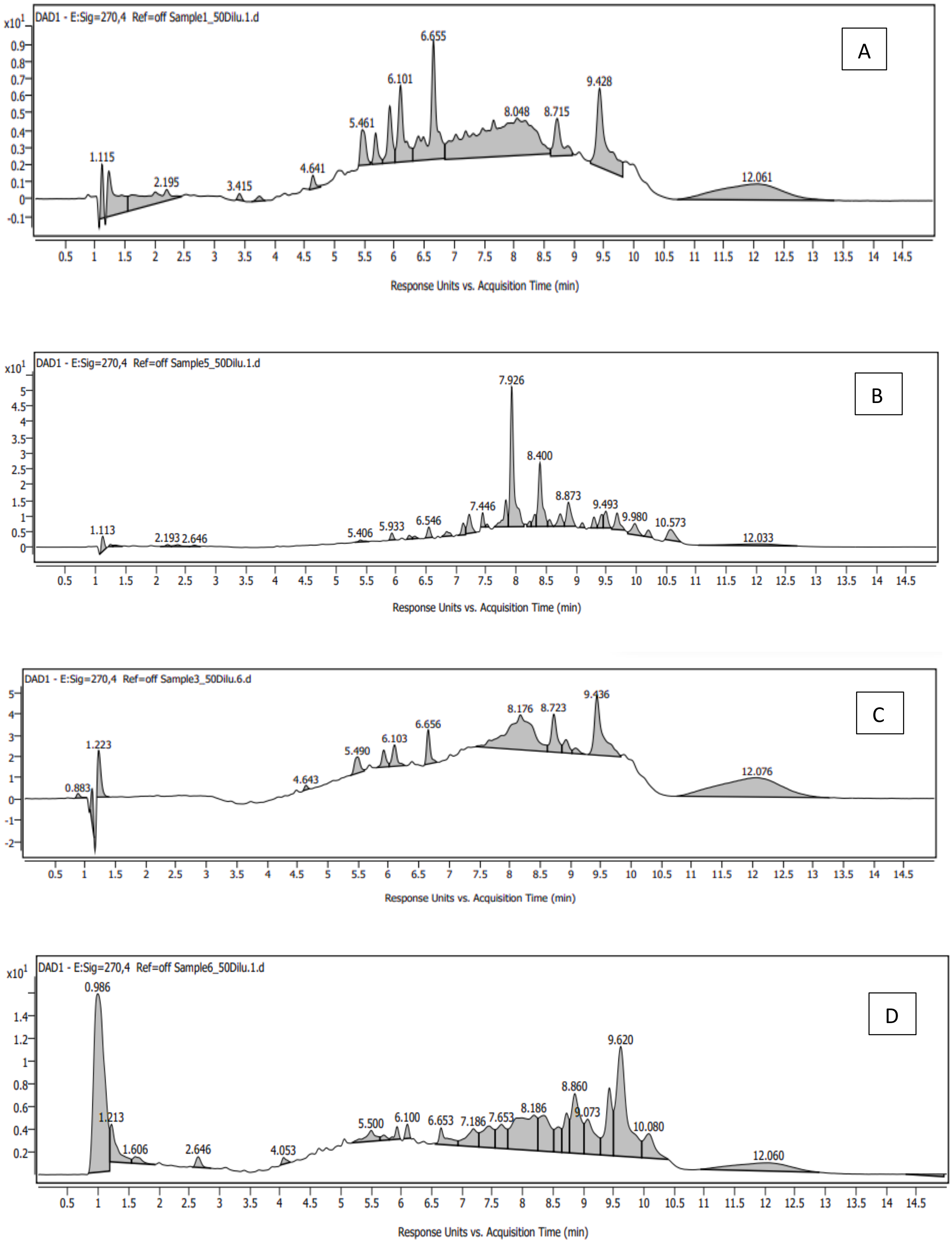


Figure 4.47: Chromatogram of the samples (A) HXEATAV, (B) MEOHTAV, (C) CHTAV and (D) CHGG

Table 4.48: Result of the characterisation of aforementioned compounds in the plant samples

Sr. No.	Proposed Compounds	Molecular Formula	HXEATAV sample	MEOHTAV sample	CHTAV sample	CHGG sample
1	Apigenin	C15 H10 O5	ND	ND	ND	ND
2	Glabridin	C20 H20 O4	+	+	+	+
3	Glycyrrhizin	C42 H62 O16	+	+	ND	+
4	Glycyrrhetinic acid	C30 H46 O4	+	+	+	+
5	Kaempferol	C15 H10 O6	+	ND	+	ND
6	Liquiritigenin	C15 H12 O4	+	ND	ND	+
7	Naringenin	C15 H12 O5	ND	ND	+	+
8	Stigmasterol	C29 H48 O	ND	+	ND	+

*ND=Not detected

4.3.10 Discussion

Standardization and validation are most important parameter for phyto-characterisation. Licorice essentially known for bioactive compound glycrrhizin was an initial target to be confirmed in *Taverniera cuneifolia*. Previous HPTLC studies by Zore *et al.* (2008) and Awad *et al.* (2011) have stated the presence of glycyrrhizin with no validation. In contrast, Mangalorkar (2014) was uncertain about the presence of glycyrrhizin. Thus, in order to standardize and validate the presence of glycyrrhizin, HPLC and LC-MS/MS studies were done in comparison with *G. glabra*. The LC-MS/MS analysis of glycyrrhizin in GG and TC showed a concentration of 8681997.68 ng mL^{-1} and 153072.85 ng mL^{-1} respectively (Table 4.26, Figure 4.32). Though glycyrrhizin was present in *T. cuneifolia*, the concentration was very low as compare to *G. glabra*.

With the dispute of glycyrrhizin being standardized and validated the second aspect was to standardize and validate the presence of other active phytoconstituents of *T. cuneifolia* in comparison with *G. glabra*. This was achieved by developing HPLC based simultaneous method for twelve phytoconstituents of which ten i.e, three triterpene glycosides (glycyrrhizin, 18 alpha and beta glycyrrhetinic acid), one isoflavan (glabridin), one isoflavone (Geinstein), two flavanone (naringenin and liquiritigenin), two Flavone (kaempferol and apigenin), and one flavonol (quercetin) were analysed together (Fig 4.33 & 4.34) while the rest two triterpenoid phytosterols (stigmasterol and β-sitosterol) were segregated separately (Fig 4.34). Out of the 10, only 3 in methanolic extract and 4 in chloroform extract could be developed in simultaneous method (Fig 4.33, A and B) while rest were not detected in this method. In contrast in GG methanolic extract revealed the presence of 5 components (Fig 4.33, C). Though there are various extraction techniques developed for each of these phyto-constitiuent discussed (cf. table 4.26, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32 & 4.33); there are no simultaneous techniques of this kind developed till now which could resolve all the 10 phyto-components in single method. Simultaneous method showed that glycyrrhizin content in *G. glabra* methanolic extract was substantially higher than *T. cuneifolia* methanolic extract (Table 4.45), whereas, the concentrations of apigenin, genistein, and stigmasterol were greater in TC MEOH then GG MEOH. Glabridin was not detected in this method, however, the LC-MS/MS-Q-ToF data showed the presence of glabridin (cf. table 4.45 & 4.46).

Simultaneous method developed for stigmasterol and β-sitosterol showed that stigmasterol was present in both *G. glabra* and *T. cuneifolia* while β-sitosterol was not detected in GG MEOH. However, earlier studies on LCMS (Khalaf et. al., 2011, Suman et. al., 2009) have recorded stigmasterol and β-sitosterol wherein the mobile phase was prepared from methanol and acetonitrile 30:70 (v/v) and 90:10 (v/v) isocratic elution.

The aforementioned LCMS/MS and HPLC data was also validated through LC-MS/MS-Q-ToF. To do so, hexane: ethyl acetate, methanol, and chloroform extracts of *T. cuneifolia* were taken and compared to chloroform extract of *G. glabra* (CHGG) (table 4.46, figure 4.48). Targeted 8 phytoconstituents were analyzed, of which 6 (other than Apeginine and Kaempferol) of *G. glabra* were detected in chloroform extract, 5 in hexane extract of *T. cuneifolia* (other than Naringenin, Apigenin and Stigmasterol), 4 in methanol extract of *T. cuneifolia* (other than Apigenin, Kaempferol, Liquiritigenin and Naringenin) and chloroform extract (other than Apigenin, Glycyrrhizin, Liquiritigenin and Stigmasterol). Apigenin was not located in any of the sample. However, it was recorded in HPLC studies while comparing with standards. In earlier untargeted LC-MS/MS studies of Mangalorkar (2014) Kaempferol tetraacetate, Naringenin trimethyl ether, Apigenin, Naringenin triacetate were detected in *T. cuneifolia*. Interestingly glabridin that couldn't be detected in HPLC studies were detected in LC-MS/MS-Q-ToF studies.

The content of glycyrrhetinic acid and apigenin simultaneously quantified using TLC densitometric method was found to be 0.65 ± 0.059 and 0.0074 ± 0.0004 % w/w respectively HPLC, LC-MS/MS, LC-MS/MS-Q-ToF studies clearly indicated the presence of glycyrrhizin, Liquiritigenin, Genistein, Apigenin, Glabridin, Stigmasterol and β-Sitosterol in various concentration of different solvents. Though there were conflicts with respect to Apigenin and Glabridin in the various techniques adopted. The content of glycyrrhetinic acid and Apigenin simultaneously quantified using TLC densitometric method was found to be 0.65 ± 0.059 and 0.0074 ± 0.0004 % w/w respectively in *G. glabra* by Rathee *et al*., (2010) while in HPLC studies of GG and TC the concentration was 0.004 and 0.066.

4.4: Purification of the active fractions by Column chromatography Column chromatography

Secondary metabolites with potential pharmacological effects have long been derived from plants (Russell & Duthie, 2011). Due to their potential anti-oxidant, anti-inflammatory, and cancer-preventive activities, polyphenolic (flavonoids) chemicals, which are abundant in plant-based diets, offer a variety of health benefits (Li *et al*., 2014). Separation and characterisation of complicated mixtures are of utmost relevance in a variety of disciplines that demand very high separation power. Using column chromatography, isolating a particular component from crude plant extract and purifying the sample is a straightforward procedure. Absorbent (stationary phase) and solvent (mobile phase) selections have the greatest impact on the purity of the target chemical (Tang *et al*., 2014). Thin layer chromatography (TLC) offers partial separation of both organic and inorganic substances using thin-layered chromatographic plates. This technique is particularly effective for determining the purity of fractions.

Gradient solvent system (non-polar to highly polar solvent system) allows optimal elution and separation of organic components from any plant organic extract. The ratio of gradient solvent to be utilised in column chromatography is summarised in table 4.47.

Table 4.49: Gradient solvent system used in column-chromatography for the isolation of compounds from extract.

Sr. No.	Solvent System	Ratio (in %)	Fraction	Volume (after drying) (in ml)	Analysis done by
1	Hexane	100	2	1	GC-MS
2	Hexane: Ethyl acetate	9:1	5	1	GC-MS
3	Hexane: Ethyl acetate	8:2	6	2	GC-MS
4	Hexane: Ethyl acetate	6:4	5	2	GC-MS
5	Hexane: Ethyl acetate	2:8	6	2	GC-MS
6	Ethyl acetate	100	4	1.5	LC-MS-Q-ToF
7	Ethyl acetate: Methanol	9:1	8	2	LC-MS-Q-ToF
8	Ethyl acetate: Methanol	8:2	7	2	LC-MS-Q-ToF
9	Ethyl acetate: Methanol	7:3	8	2	LC-MS-Q-ToF
10	Ethyl acetate: Methanol	6:4	8	2	LC-MS-Q-ToF
11	Ethyl acetate: Methanol	5:5	9	2	LC-MS-Q-ToF
12	Ethyl acetate: Methanol	3:7	9	2	LC-MS-Q-ToF
13	Ethyl acetate: Methanol	1:9	9	2	LC-MS-Q-ToF
14	Methanol	100	6	1	LC-MS-Q-ToF

The visible spots of the components on the chromatoplate are denoted, and the Rf value of each spot is determined using the following formula:

Rf = distance travelled by the sample (cm) / the distance travelled by the solvent (cm)

Those fractions which were giving the same Rf value were pooled together in a vile as one. There were few fractions which were giving salts and later converting into a thick mass were again subjected for further separation.

4.4.1 GC-MS Analysis result

Gas chromatography-mass spectrometry (GC-MS) is a combined analytical method used to detect and identify chemicals in plant samples (Uma & Balasubramaniam, 2012). GC-MS is crucial for the phytochemical investigation and chemotaxonomic research of medicinal plants with bioactive constituents (Hethelyi *et al.*, 1987). GC–MS is one of the most efficient, precise, and rapid methods for detecting numerous chemicals, such as alcohols, alkaloids, nitro compounds, long chain hydrocarbons, organic acids, steroids, esters, and amino acids (Razack *et al*., 2015), and only needs a minimal amount of plant samples. Therefore, in the current investigation, GC–MS was used to detect and identify non polar components contained in the *T. cuneifolia* leaves, seeds and roots.

(I) GCMS Analysis of Column fraction of root

Gas chromatography-mass spectrometry (GC–MS) analysis of *T. cuneifolia* root column fractions revealed the presence of six major bioactive compounds as shown in table (above). From the results from GC–MS spectra, Diisooctyl phthalate, hexadecanoic (Palmitic acid), Dodecane, 2,6,11-trimethyl- are the most abundant in occurrence while methyl tetradecanoate (Myristic acid methyl ester), 9-dodecanoic acid, methyl ester E and Methyl-18-methyl - nonadecanoate were in lower concentration. These bioactive compounds has been reported to play crucial roles in disease and general metabolisms of humans. Diisooctyl Phthalate was earlier Isolated from Rourea mimosoides, for its A Partial PPARγ Agonist Potently Blocks Adipocyte Differentiation. It showed the ability to enhance the glucose uptake in C2C12 myoblast cells as well as active to promote the transcription of PPAR-γ. I. (Adenan *et al*., 2022), Hexadecanoic acid (Palmitic acid) and methyl ester possesses antioxidant, anti-inflammatory, antihyperlipidemic, and antibacterial properties. Dodecane, 2,6,11-trimethyl has been reported from the rhizome of kali-musli- Curculigo orchioides.(Daffodil *et al*., 2012). The GCMS analysis of the fraction is given in the table 4.48 & 4.49:

(II) GCMS Analysis of *T. cuneifolia* leaves and seed extract

The GCMS analysis of leaf oil, leaf oil residue, seed oil and seed oil residue has been done. Details are as following.

4.4.1.1 GC Analysis root (Nonpolar fraction)

For GCMS analyses, samples of hexane: ethyl acetate with five distinct ratios were analysed. This resulted in the identification of 58 compounds. Maximum fractions were reported in a mixture of hexane and ethyl acetate with a ratio of 55:45. In nearby ratio fractions, a number of related chemicals were identified. The mixture of hexane and ethyl acetate with a ratio of 55:45 eluted the greatest number of distinct chemicals. The GCMS analysis of column chromatography of root extract detected a total of 27 different chemicals. Other than that, 16 compounds have been identified in leaf extract (11 saturated and 5 unsaturated); only two saturated fatty acids have been identified in leaf oil residue; 14 compounds have been identified in seed oil (5 saturated and 9 unsaturated) and 15 compounds have been identified in seed oil residue (6 saturated and 9 unsaturated) (table 4.48 & 4.49).The data incorporated here includes column chromatography fractions (highlighted in yellow), leaf oil (highlighted in green color), leaf oil residue (highlighted in orange color), seed oil (colorless) and seed oil residue (highlighted in blue color). The details of the phytocomponents detected is as follows:

All the saturated and unsaturated fatty acid identified from GCMS analysis are fatty acid or fatty acyls. Fatty acids are both essential dietary sources of fuel for animals and essential cellular structure components. Fatty acids and acyls serve as the primary source of cellular energy and influence further biological processes. GCMS analysis of saturated fatty acid oil identifies Dodecane, 2,6,11-trimethyl-, n-Hexadecanoic acid (Palmitic acid) from the 10EA+90HEN-4 and 50EA+50HEN-1 column fractions are even detected in Seed oil and leaf oil. Stearic acid, Myristic acid and Stearic acid are identified from seed oil whereas Lauric acid derivative has been identified from leaf oil of *T. cuneifolia*. Earlier, the study done by Manglorkar in 2014 has also mentioned the presence of saturated fatty acids such as Caproic acid, Myristic acid, Lauric acid, Palmitic acid, Stearic acid and unsaturated fatty acid - Linoleic acid and Oleic acid in the roots of *T. cuneifolia* by GCMS analysis. The saturated fatty acids consist of palmitic acid (20.72%) and stearic acid (5.01%), while the unsaturated fatty acids consist of oleic acid (16.99%) and linoleic acid (omega-6-fatty acid - 51.70%). Even the fatty acid content of Glycyrrhiza uralensis included 61.84 % linoleic acid, 25.51 % -α-linolenic acid, 3.02 % stearic acid, and 7.98 % palmitic acid. The fatty acid profile of *T. cuneifolia's* oil is comparable to that of *Sesamum indicum, Madhuca indica, Carthamus tinctorius*, and *Prunus amygdalus*. The fatty acid profile of *T. cuneifolia* differs from that of Crotolaria juncea, Medicago spp., Arachis hypogea, and Glycine max. The research revealed evidence that *T. cuneifolia* seed oil contains PUFA, MUFA, and SFA. Sunflower oil and safflower oil include omega-6 fatty acids (Linoleic acid), which are proven to lessen the risk of cardiovascular disease (Chaiyasit *et al*., 2007). Linoleic acid, ethyl ester, Hexadecanoic acid, Ethyl palmitate, Ethyl linoleate, γ-Octalactone, Propyl p-hydroxybenzoate, Hexadecanoic acid, ethyl ester, Linoleic acid ethyl ester, 9,12,15-Octadecatrienoic acid,ethyl ester, (Z,Z,Z)-, 4-Methyl-γ-lactones are some of the fatty acids which has been identified in G. graba plant (Farag & Wessjohann, 2012; Zhao *et al*., 2013; Frattini *et al*., 1977; Kameoka et. al., 1987; Kinoshita *et al*., 1987). There are some steroidal compounds as well which are listed in figure 4.62 identified by GCMS analysis.

Table 4.50: List of saturated fatty acids identified in GCMS analysis of column fraction, leaf oil, leaf oil residue, seed oil, and seed oil residue

Peak No.	Fractions	Saturated fatty acid Compounds (From Roots)	MF	MW	Rt	Area	Area %	Height	Types of Lipids
4	10EA+90HE N-4	Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$	212.41	9.684	368219	8.32	168582	Fatty Acyls
1	10EA+90HE N-6	Nonane, 4,5-dimethyl-	$C_{11}H_{24}$	156.31	5.921	437810	10.46	160333	Fatty Acyls
2	10EA+90HE N-6	2,6-Dimethyldecane	$C_{12}H_{26}$	170.33	6.545	389586	9.31	123574	Fatty Acyls
3	10EA+90HE N-6	Eicosane	$C_{20}H_{42}$	282.5	9.062	505434	12.08	254569	Fatty Acyls
2	50EA+50HE N-1	Decane, 4-ethyl-	$C_{12}H_{26}$	170.33	5.917	675673	4.2	309702	Fatty Acyls
11	50EA+50HE N-1	Succinic acid, 2-ethoxyethyl octadecyl ester	$C_{26}H_{50}$	442.67	12.237	633289	3.94	201448	Fatty Acids
18	50EA+50HE N-1	n-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256.42	17.223	573568	3.56	147707	Fatty Acids
Leaf Oil									
1		2-Methyltetracosane	$C_{25}H_{52}$	352.7g/mol	10.137	286453.20	0.202	87,47,846	Fatty Acyls
2		Undecane, 3,8-dimethyl-	$C_{13}H_{28}$	184.36g/mol	10.686	150557.60	0.106	59,46,260	Fatty Acyls
4		Dodecanoic acid, TMS derivative (Lauric acid)	$C_{15}H_{32}O_2Si$	272.5g/mol	12.049	111746.40	0.079	49,51,558	Fatty Acids
11		Decane, 2,9-dimethyl-	$C_{12}H_{26}$	170.33g/mol	12.623	239818.60	0.169	81,74,356	Fatty Acyls
15		Undecane, 3,7-dimethyl-	$C_{13}H_{28}$	184.36g/mol	13.11	206734.40	0.146	74,48,188	Bromolipids
21		Myristic acid, TMS derivative	$C_{17}H_{36}O_2Si$	300.6g/mol	14.412	194197.90	0.137	62,05,677	Fatty Acids
23		2-Bromotetradecane	$C_{14}H_{29}Br$	277.28g/mol	15.343	162228.60	0.114	38,29,700	Bromolipids
24		Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.5g/mol	15.584	178017.1	0.126	42,61,108	Fatty Acids
25		1-Iodo-2-methylundecane	$C_{12}H_{25}I$	296.23g/mol	16.015	146106.10	0.103	43,81,183	Fatty Acyls
26		n-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256.42g/mol	16.577	34792192.00	24.552	233431808.0	Fatty Acids
28		Palmitic Acid, TMS derivative	$C_{19}H_{40}O_2Si$	328.6g/mol	17.601	1572279.900	1.110	2,37,62,100	Fatty Acids
Leaf Oil residue									

2		Octadecane, 2-methyl-	$C_{19}H_{40}$	268.5g/mol	22.209	27459158.00	69.373	12,82,83,328	Fatty Acyls
3		Dodecane, 2,6,10-trimethyl-	$C_{15}H_{32}$	212.41g/mol	22.351	6213486.5	15.698	5,19,85,596	Fatty Acyls
Seed Oil									
49		Heptadecane, 9-hexyl-	$C_{23}H_{48}$	324.6g/mol	22.869	29,904.50	0.101	691645	Fatty Acyls
52		[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-,	$C_{21}H_{38}O_2$	322.5g/mol	23.751	53,808.00	0.182	8,76,594	Fatty Acids
53		2,2,4,4-Tetramethyl-6-(1-oxo-3-phenylprop-2-enyl)-cyclohexane-1,3,5-trione	$C_{19}H_{20}O_4$	312.4g/mol	24.072	19,328.70	0.065	5,67,284	Fatty Acyls
82		E-9-Tetradecenoic acid (Myristic acid)	$C_{14}H_{26}O_2$	226.35g/mol	29.031	44,615.30	0.151	16,80,928	Fatty Acids
93		Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366.7g/mol	30.635	40,459.80	0.137	11,10,445	Fatty Acyls
Seed Oil Residue									
1		Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366.7g/mol	20.475	31,688.30	0.103	8,84,506	Fatty Acyls
12		Hexadecenoic acid, Z-11- (Palmitic acid derivative)	$C_{16}H_{30}O_2$	254.41g/mol	21.222	2,28,659.20	0.745	57,27,398	Fatty Acids
15		Nonadecane, 2-methyl-	$C_{20}H_{42}$	282.5g/mol	22.955	56,60,736.50	18.448	3,85,25,392	Fatty Acyls
		Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	$C_{16}H_{48}O_7Si_8$	577.2g/mol		23,402.90	0.076	4,68,712	
48		Octadecane, 6-methyl-	$C_{19}H_{40}$	268.5g/mol	26.848	2,15,768.40	0.703	23,95,936	
94		Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis-	$C_{39}H_{80}O_2$	581.1g/mol	29.963	39,618.90	0.129	12,89,333	

Table 4.51: List of unsaturated fatty acids identified in GCMS analysis of column fraction, leaf oil, leaf oil residue, seed oil, and seed oil residue

Peak No.	**Fractions**	**Unsaturated Fatty acids** **Compounds (From Roots)**	**MF**	**MW**	**Rt**	**Area**	**Area%**	**Height**	**Types n of Lipids**
5	45EA+55HEN-20	1-Dodecene	$C_{12}H_{24}$	168.32	7.809	399540	2.39	235041	Fatty Acyls
6	45EA+55HEN-24	1-Heptadecene	$C_{17}H_{34}$	238.5	13.036	98068	4.86	42492	Fatty Acyls
Leaf Oil									

29		E-1,8-Dodecadiene	$C_{12}H_{22}$	166.3g/mol	18.471	90,059.90	0.064	19,51,009	Fatty Acyls
32		9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278.4g/mol	20.364	83067704	58.62	1.75E+08	Fatty Acids
33		à-Linolenic acid, TMS derivative	$C_{21}H_{38}O_2Si$	350.6g/mol	21.024	4190645	2.957	19488726	Fatty Acids
34		4,9-Decadienoic acid, 2-nitro-, ethyl ester	$C_{12}H_{19}NO_4$	241.28g/mol	21.376	929430.1	0.656	55,29,546	Fatty Acids
37		Undec-10-ynoic acid, heptyl ester	$C_{18}H_{32}O_2$	280.4g/mol	22.19	123795.7	0.087	14,16,404	Fatty Acids
Seed Oil									
37		E-10-Dodecen-1-ol propionate	$C_{15}H_{28}O_2$	240.38g/mol	20.617	20,783.50	0.07	5,85,735	Fatty Acids
42		9-Octadecen-12-ynoic acid, methyl ester	$C_{19}H_{32}O_2$	292.5g/mol	22.011	58,251.30	0.197	10,46,429	Fatty Acids
44		10-Heptadecen-8-ynoic acid, methyl ester, (E)-	$C_{18}H_{30}O_2$	278.4g/mol	22.172	37,785.90	0.128	7,13,045	Fatty Acids
55		trans-13-Octadecenoic acid (Stearic acid)	$C_{18}H_{34}O_2$	282.5g/mol	24.608	10829275	36.624	55418324	Fatty Acids
64		9,12-Hexadecadienoic acid, methyl ester	$C_{17}H_{30}O_2$	266.4g/mol	26.749	51,712.40	0.175	15,35,375	Fatty Acids
72		Z,Z,Z-4,6,9-Nonadecatriene	$C_{19}H_{34}$	262.5g/mol	27.649	44,573.90	0.151	13,09,320	Fatty Acyls
78		cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	310.5g/mol	28.445	20,416.00	0.069	9,23,460	Fatty Acids
79		cis-5,8,11,14,17-Eicosapentaenoic acid	$C_{20}H_{30}O_2$	302.5g/mol	28.507	21,608.50	0.073	7,01,819	Fatty Acids
80		Arachidonic acid	$C_{20}H_{32}O_2$	304.5g/mol	28.717	314211.2	1.063	37,92,764	Fatty Acids
Seed Oil Residue									
5		Methyl Z-11-tetradecenoate	$C_{15}H_{28}O_2$	240.38g/mol	20.63	14,84,159	34,256.20	0.112	Fatty Acids
7		10-Methyl-E-11-tridecen-1-ol propionate	$C_{17}H_{32}O_2$	268.4g/mol	20.722	17,80,621	32,646.30	0.106	Fatty Acids
14		Methyl 10,12-octadecadiynoate	$C_{19}H_{30}O_2$	290.4g/mol	22.684	52,43,886	470998.8	1.535	Fatty Acids
36		5,8,11,14-Eicosatetraynoic acid, TBDMS derivative	$C_{26}H_{38}O_2Si$	410.7g/mol	25.133	7,19,602	25,366.80	0.083	Fatty Acids
38		trans-13-Octadecenoic acid (Stearic acid)	$C_{18}H_{34}O_2$	282.5g/mol	25.293	21,268.70	0.069	6,95,565	Fatty Acids
50		Z-8-Methyl-9-tetradecenoic acid	$C_{15}H_{28}O_2$	240.38g/mol	27.039	22402.4	0.073	11,40,256	Fatty Acids
63		17-Pentatriacontene	$C_{35}H_{70}$	490.9g/mol	27.933	180336.9	0.588	21,60,014	Fatty Acyls
77		9-Octadecene, 1-[3-(octadecyloxy)propoxy]-, (Z)-	$C_{39}H_{78}O_2$	579g/mol	28.877	21,007.90	0.068	13,83,702	Fatty Acyls

Saturated Fatty acid chromatogram from column chromatography

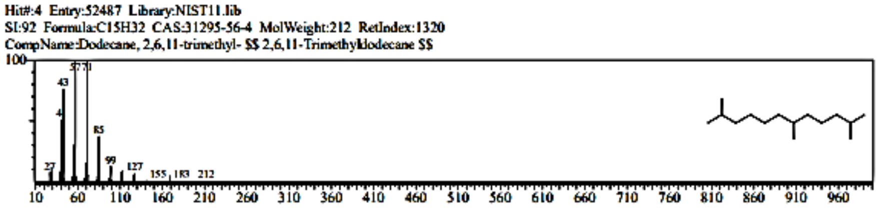


Figure 4.48: GCMS chromatogram of Dodecane, 2,6,11-trimethyl-

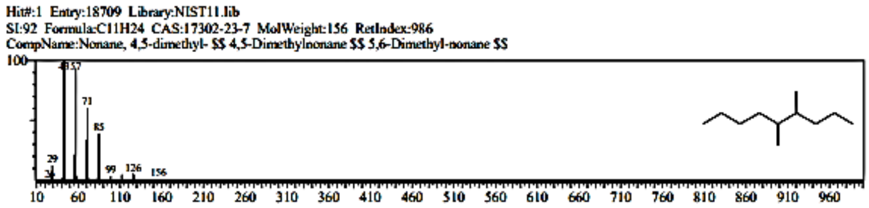


Figure 4.49: GCMS chromatogram of Nonane, 4,5-dimethyl-

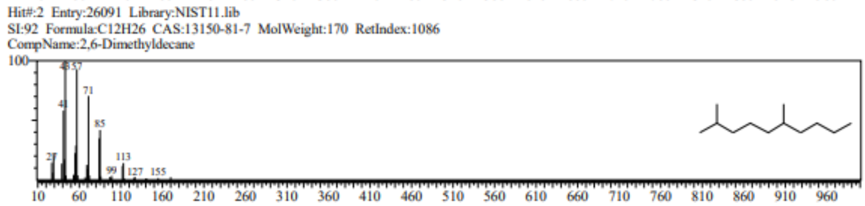


Figure 4.50: GCMS chromatogram of 2,6-Dimethyldecane

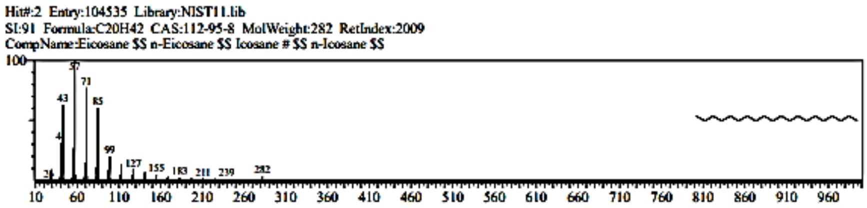


Figure 4.51: GCMS chromatogram of Eicosane

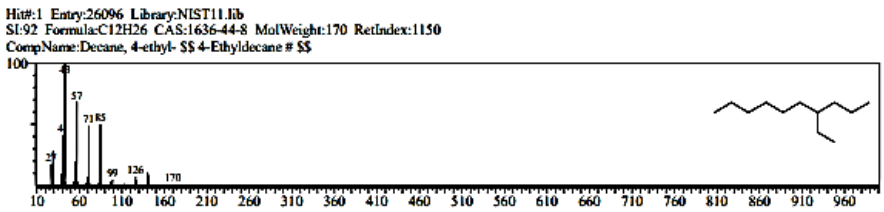


Figure 4.52: GCMS chromatogram of Decane, 4-ethyl-

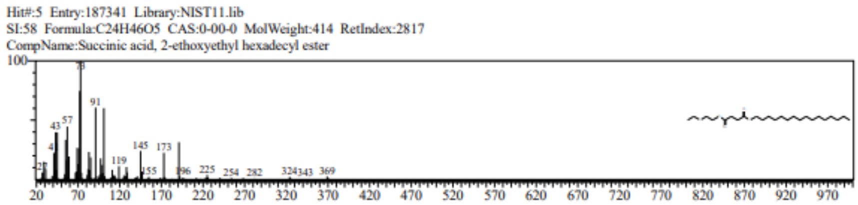


Figure 4.53: GCMS chromatogram of Succinic acid, 2-ethoxyethyl octadecyl ester

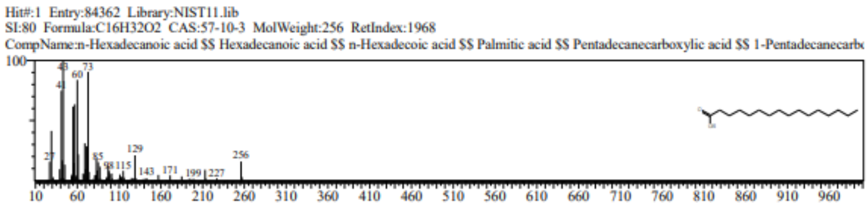


Figure 4.54: GCMS chromatogram of n-Hexadecenoic acid

Unsaturated Fatty Acid chromatogram from column fraction

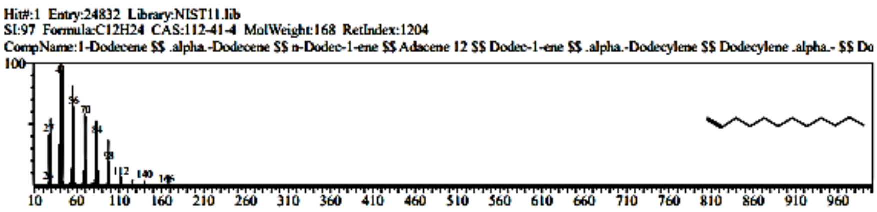


Figure 4.55: GCMS chromatogram of 1-Dodecene

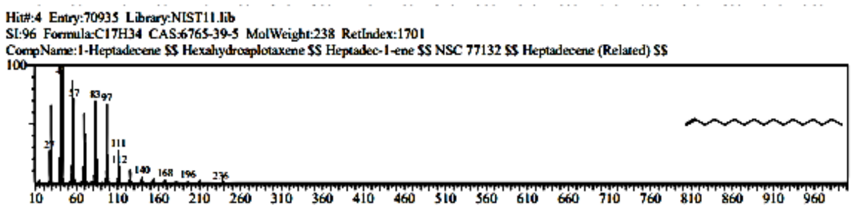


Figure 4.56: GCMS chromatogram of 1-Heptadecene

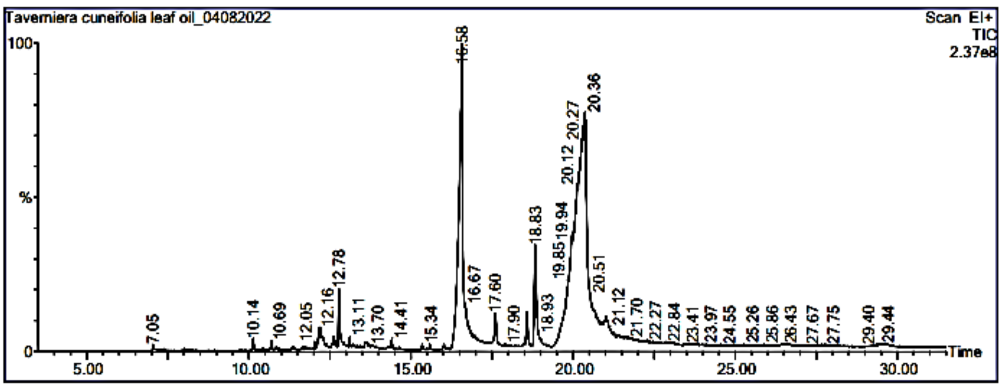


Figure 4.57: GCMS chromatogram of *T. cuneifolia* leaf oil

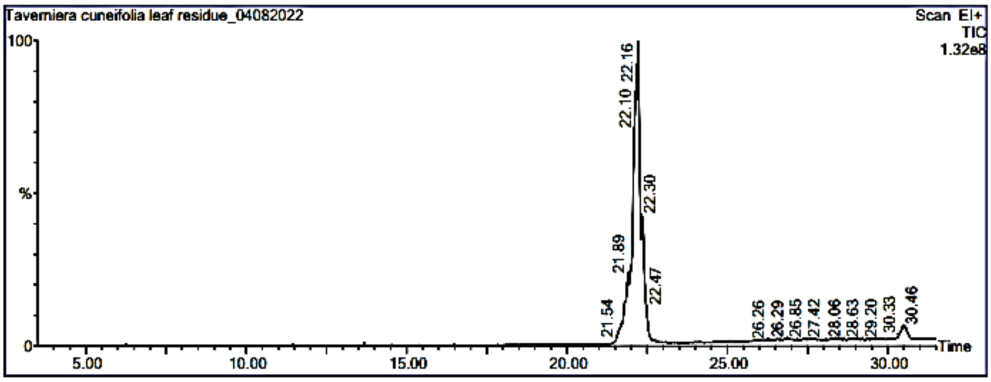


Figure 4.58: GCMS chromatogram of *T. cuneifolia* leaf residue

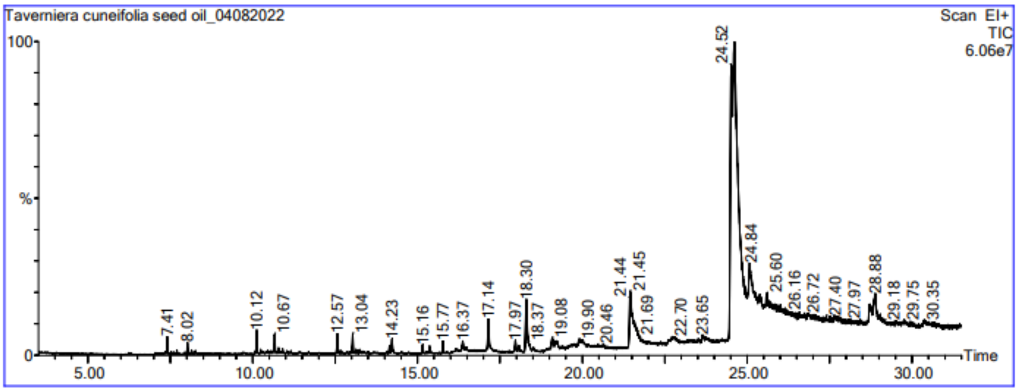


Figure 4.59: GCMS chromatogram of *T. cuneifolia* seed oil

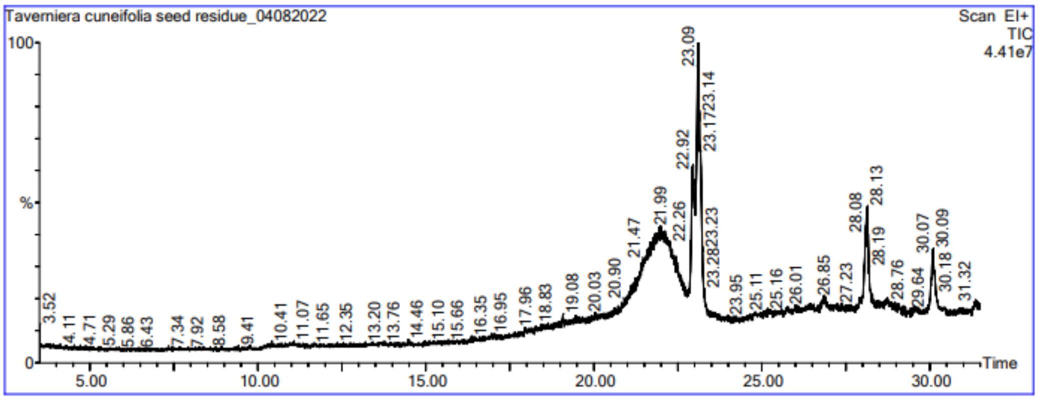


Figure 4.60: GCMS chromatogram of *T. cuneifolia* seed oil residue

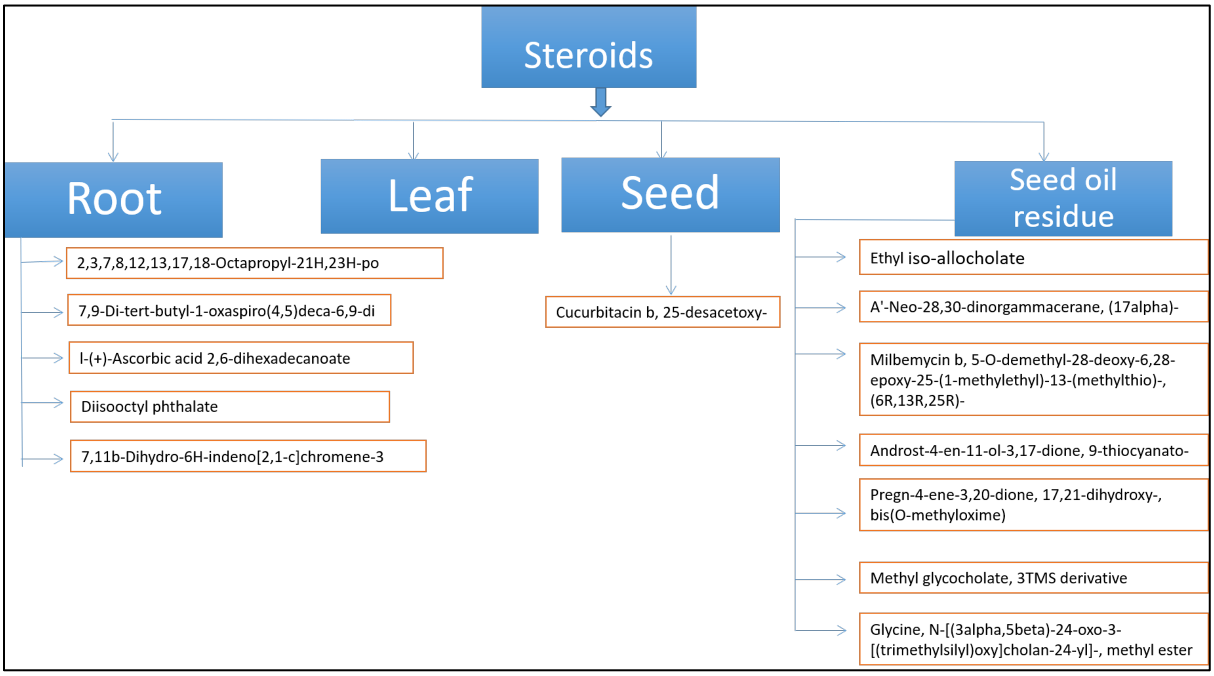


Figure 4.61: Steroidal compounds identified by GCMS analysis

4.4.2 LC-MS-Q-ToF for Metabolomics study of *T. cuneifolia*

Metabolomic techniques provide exhaustive profiling of the cell metabolome or "Library of metabolites," which offers chemical markers of cell dynamics and metabolic activity. Identification of metabolites is accomplished by a variety of analytical methods. Metabolic profiling is often classified as either targeted or nontargeted. In the targeted strategy, profiles of particular metabolites with known identities are generated. Nontargeted profiling is the simultaneous assessment of as many metabolites as feasible in a biological material using NMR or MS. In the majority of metabolomics investigations, methods based on mass spectrometry (MS) are used. Modern MS systems, such as those that include time-of-flight mass analyzers, provide very high mass resolution and mass accuracy.

Combining these MS instruments with high-resolution chromatographic methods has enabled the resolution of literally hundreds of distinct tiny compounds. The great mass precision of these techniques enables detection of peaks using databases like as METLIN, HMDB, and KEGG.

For the metabolomic profiling, *T. cuneifolia* methanolic root extract and fifth fraction from column chromatography (ethyl acetate: methanol, 80:20) were selected as this fraction was giving totally different TLC pattern and eluted in a salt form. Table 4.52, 5.53, 4.54, 4.55, 4.56, 4.57 & 4.58 represents the metabolite profile of *T. cuneifolia* root and column extract analysed by non-targeted LC-MS-Q-ToF with ESI operating in positive ionisation mode. Table 4.52, 5.53, 4.54, 4.55, 4.56, 4.57 & 4.58 contains the names of the detected and identified compounds, the IUPAC names of the compounds, the molecular formula, the PubChem compound identification number, the retention time (Rt), the experimental mass (m/z), the height of the peak, and the area covered by each peak for the identified compounds.

The total ion current (TIC) base peak chromatogram (BPC) of *T. cuneifolia* root extract obtained from extracted ion chromatogram in positive ionisation mode is shown in Figure 4.63, 4.64, 4.65, 4.66 & 4.67. Using the Mass Hunter software from Agilent Technologies, a total of 39 metabolites were discovered using LC-QToF-MS and listed in Table 2. 14 of the discovered and identified molecules belonged to the lipid class, followed by 9 flavonoids, 4 glycosides, 2 alkaloids, 46 triterpenes.

4.4.2.1 LC-MS/MS-Q-ToF analysis of Targeted metabolites

The LC-MS/MS-Q-ToF analysis was done for the identification of Liquiritigenin, Naringenin, Kaempferol, Apigenin, Glycyrrhizin, Glabridin, Glycyrrhetinic acid and Stigmasterol in the ColTAVEAMEOH and ColMEOHTAV column samples. The analysis showed the following result:

Table 4.52: Characterization of standard compounds by LC-MS/MS-Q-ToF

Sr. No.	**Proposed Compounds**	**Molecular Formula**	**RT (min)**	**Ionization ESI (+)**	**Molecular Weight**	**Observed (*m/z*)**
1	Apigenin	C15 H10 O5	11.22	(M+NH4)+	270.0541	288.0883
2	Glabridin	C20 H20 O4	7.926	(M+H)+	324.1390	325.1435
3	Glycyrrhizin	C42H62O16	5.845	(M+H)+ (M+NH4)+ (M+Na)+	822.4008	823.4111 840.4196 845.3899
4	Glycyrrhetinic acid	C30 H46 O4	11.589	(M+H)+ (M+Na)+	470.3385	471.3478 493.3198
5	Kaempferol	C15 H10 O6	4.547	(M+H)+	286.0469	287.0543
6	Liquiritigenin	C15 H12 O4	5.795	(M+H)+	256.0718	257.0788
7	Naringenin	C15 H12 O5	6.425	(M+H)+ (M+Na)+	272.0685	273.0760 295.0576
8	Stigmasterol	C29 H48 O	10.022	(M+H)+	412.3705	413.3787

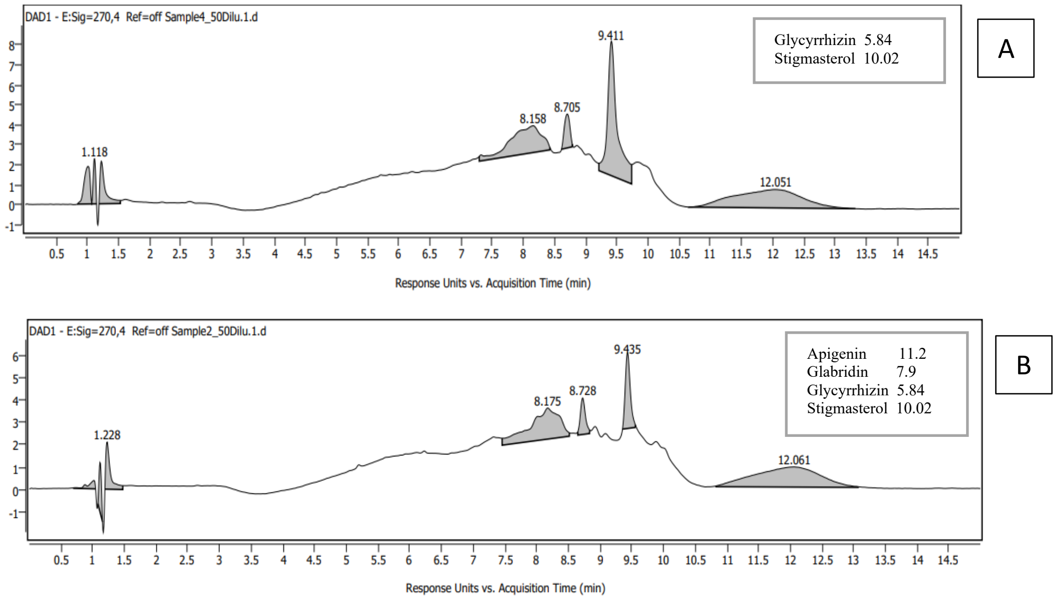


Figure 4.62: Chromatogram of the sample (a) ColTAVEAMEOH (b) ColMEOHTAV

Table 4.53: Representing the identification of standards in the column fractions

Sr. No.	Proposed Compounds	Molecular Formula	RT	ColTAVEAMEOH	ColMEOHTAV
1	Apigenin	$C_{15} H_{10} O_5$	11.22	ND	+
2	Glabridin	$C_{20} H_{20} O_4$	7.926	ND	+
3	Glycyrrhizin	$C_{42} H_{62} O_{16}$	5.845	+	+
4	Glycyrrhetinic acid	$C_{30} H_{46} O_4$	11.589	ND	ND
5	Kaempferol	$C_{15} H_{10} O_6$	4.547	ND	ND
6	Liquiritigenin	$C_{15} H_{12} O_4$	5.795	ND	ND
7	Naringenin	$C_{15} H_{12} O_5$	6.425	ND	ND
8	Stigmasterol	$C_{29} H_{48} O$	10.022	+	+

*ND=Not detected

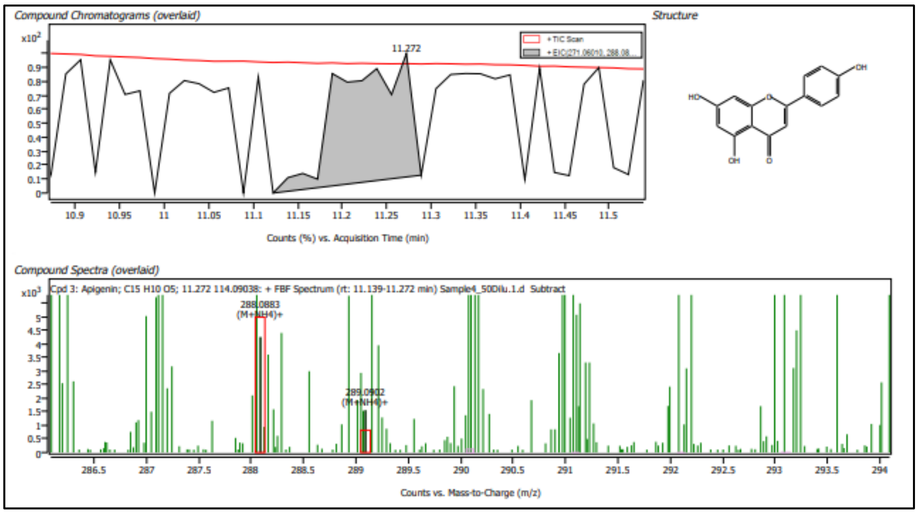


Figure 4.63: Total ion chromatogram and (TIC) its spectra from diode array detector of the standard compounds Apigenin

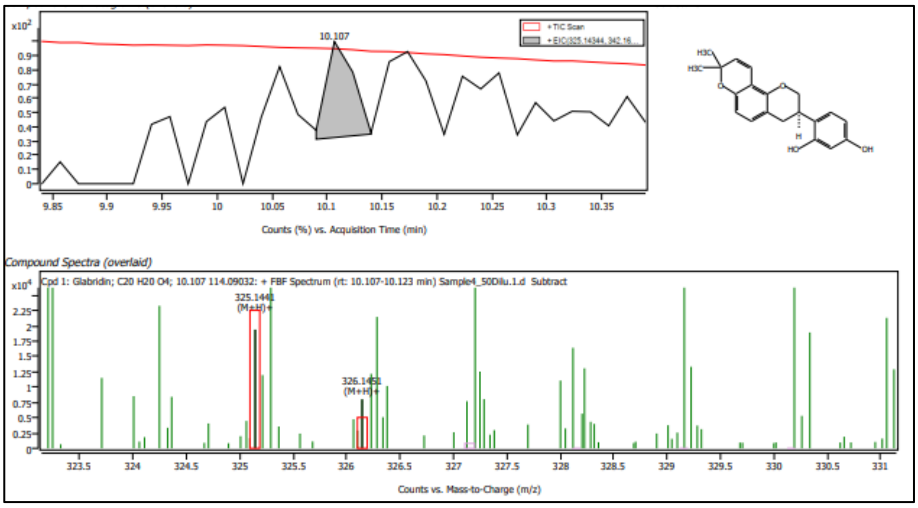


Figure 4.64: Total ion chromatogram and (TIC) its spectra from diode array detector of the standard compounds Glabridin

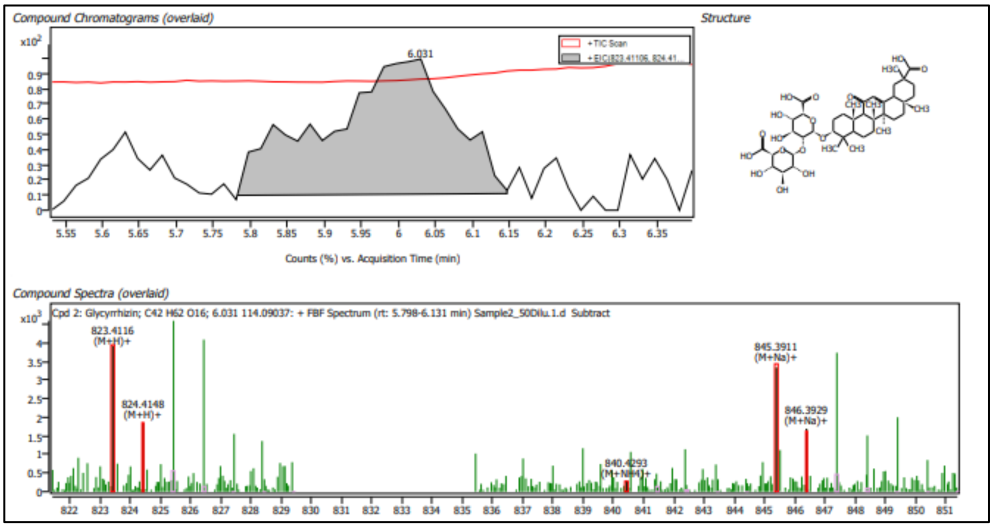


Figure 4.65: Total ion chromatogram and (TIC) its spectra from diode array detector of the standard compounds Glycyrrhizin

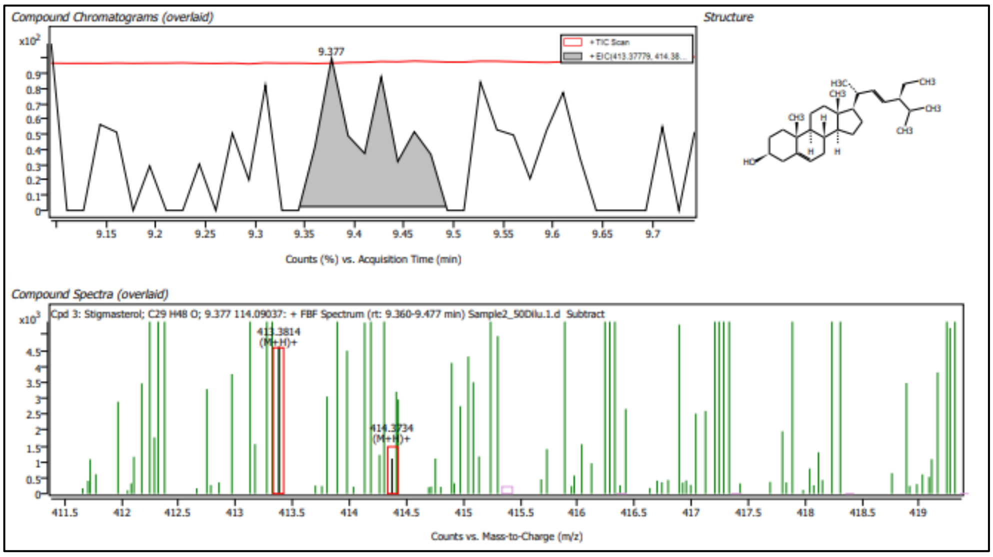


Figure 4.66: Total ion chromatogram and (TIC) its spectra from diode array detector of the standard compounds Stigmasterol

4.4.2.2 LCMS-QTOF For Untargeted Metabolomics study of *T. cuneifolia* root

In LCMS-QTOF data 90 molecules were identified out of which 65 were of natural origin with its prospective relation with Fabaceae family. This includes 19 lipids, 2 vitamin, 5 phenolic compounds, 9 terpenoidal glycosides, 1 alkaloid, 25 terpene and 4 sterols compounds (cf. Fig 4.47 and 4.48). The data incorporated here includes crude methanolic Soxhlet extract (highlighted in yellow) and biofraction of *T. cuneifolia* by column chromatography (highlighted in brown color). The details of the phytocomponents detected is as follows:

4.4.2.3 Primary metabolites detected in LCMS-Q-ToF

There are 19 lipids and 2 vitamins has been identified in the untargeted metabolomic study in the Roots of *T. cuneifolia* extract (Table 4.53 & 4.54). There were 5 fatty acid/epoxy fatty acid derivatives (17(S)-HpDoHE; 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid; 9(10)-epoxy-12Z,15Z-octadecadienoic acid; methyl 15,16-epoxy-9,12-octadecadienoate, PGA2 methyl ester) and one omega-3-fatty acid (triacontahexaenoic acid 30:6(omega3)). There were also one 20-carbon polyunsaturated fatty acids (2-glyceryl-PGE2) identified in the roots of *T. cuneifolia*. Apart from that, one aldehyde derived from stearic acid i.e., Stearaldehyde and one sterol lipid i.e., Theonellasterol B were also identified from LCMS-Q-ToF study. The Stearaldehyde are used in pharmaceutical drugs for nervous and mental conditions and Theonellasterol B are derivative of stigmasterol used in manufacture of progesterone and corticoids. Rest other compounds which are identified are Glycerophospholipids (PPA (16:0/18:1 (9Z)); PI P(16:0/20:3 (5Z,8Z,11Z)); PE (P-18:0/17:2 (9Z,12Z)); PE(17:0/20:2 (11Z,14Z))) and Glycerophosphoserines (PS(18:4 (6Z,9Z,12Z,15Z) /20:4 (5Z,8Z,11Z,14Z)); PS (O-20:0/18:2 (9Z,12Z))). Denisova *et al*. in 2007 reported that the hexane extract of *G. glabra* was found to contain 70 % neutral and 30 % polar lipids.

The earlier HPLC examination confirmed the presence of four vitamins, including B1, B2, B3, and B6, in the roots of *T. cuneifolia* (Manglorkar, 2014), meanwhile Wang, Qian, *et al*. reported the presence of six vitamins, namely B1, B2, B3, B5, E, and C, in *G. glabra* in 2015. In contrast, we have been able to identify vitamin D and vitamin K analogues (Table). Locally, the roots of *T. cuneifolia* are used to cure Ulcers. Presumably, the presence of these vitamins in *Taverniera cuneifolia* plays a significant role in ulcer therapy.

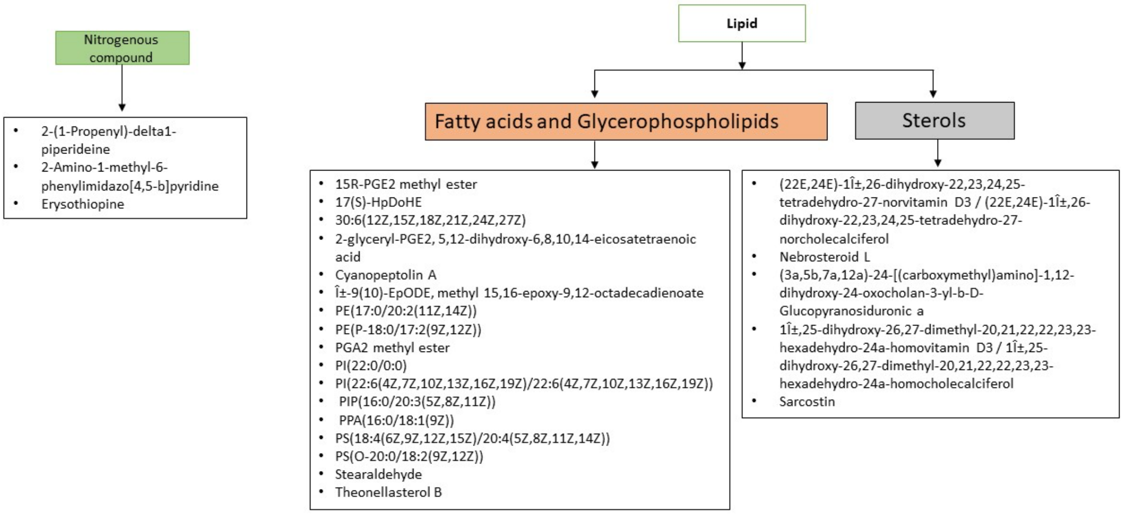


Figure 4.67: Lipids identified in LC-MS-Q-ToF analysis

4.4.2.4 Secondary metabolites

4.4.2.4.1 Phenolic compounds

The roots of *T. cuneifolia* yielded five phenolic compounds that are categorised as Simple phenols (Demethylphylloquinone, Archangelicin, Gingerdione, and Chavicol) and phenolic acid (Veratic acid) (table 4.54). Chavicol, which is present in *T. cuneifolia*, has also been discovered in *G. glabra*; however, Lopochavicol, which was detected in *G. glabra*, was not found in *T. cuneifolia*. All four phenols, with the exception of Archangelicin, were identified from the ethyl acetate: methanol fraction of column chromatography. Manglorkar (2014) found the presence of 4-phenolic acids (p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, syringic acid, o-coumaric acid (cis and trans), caffeic acid and ferulic acid) using paper chromatography and p-hydroxybenzoic acid in the roots of *T. cuneifolia* using LC-MS, respectively. Two phenols, thymol and carvacrol, were discovered solely in *G. glabra* samples (Farag & Wessjohann, 2012). The Gingerdione has a multiple use in the treatment of cough, stomach-ache, asthma, worms, leprosy, skin, gastrointestinal and respiratory diseases (Charles *et al*., 2000). The phenols are recognised as potent natural antioxidants with a variety of biological and pharmacological activities, including anti-inflammatory, anticancer, antibacterial, antiallergic, antiviral, antithrombotic, hepatoprotective, food additive, signalling molecules, and others (Kumar *et al*., 2019; Mori *et al*., 1999; Medina *et al*., 2007; Bodini *et al*., 2009).

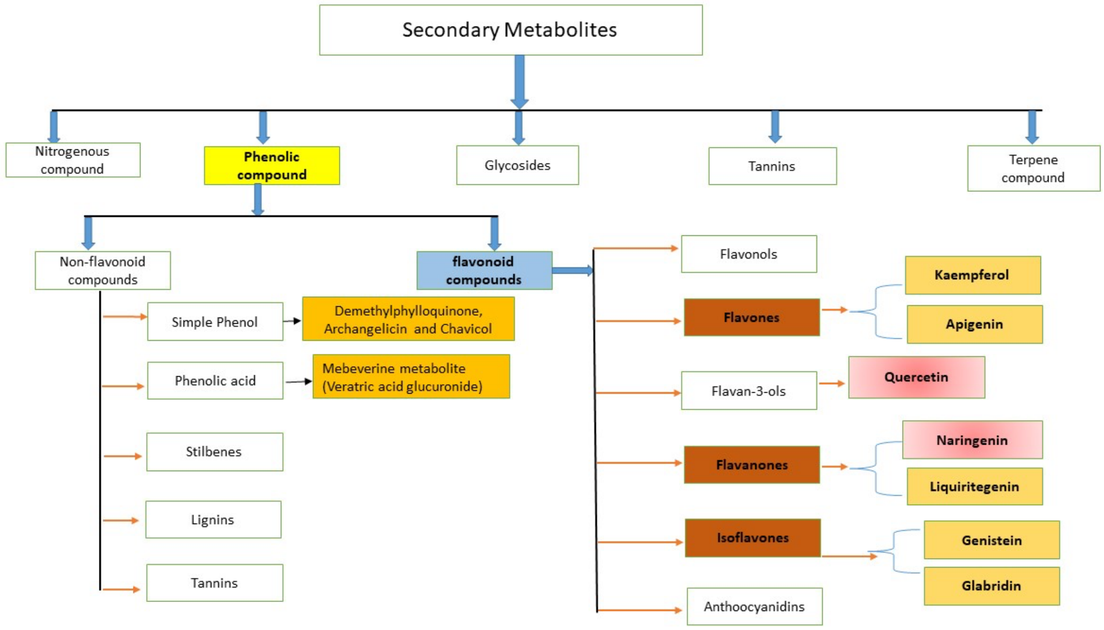


Figure 4.68: Phenolic compounds identified in LC-MS-Q-ToF analysis

4.4.2.4.2 Terpenoidal glycosides

Terpene glycosides have received a significant amount of attention as antimicrobials, taste precursors, and detergents. They are taken from plant matter or manufactured using chemical and biocatalytic processes. Triterpene glycosides, such as ginsenosides and oleanolic acid, have gained a considerable amount of attention due to their many medico-biological qualities, such as anti-viral, anti-inflammatory, and neuroprotective benefits (Moon & Lee, 2012), whilst glycyrrhizic acid imparts liquorice with a sweet flavour (Hayashi & Sudo, 2009). The production and biological effects of oleanolic acid oligoglycosides have been widely researched (Xu *et al*., 2014) because of the extraordinary bioactivities and physical features of triterpenoid saponins. Diterpene glycosides, such as steviol glycosides, are the sweeteners in stevia plant leaves (Stevia rebaudiana Bertoni). Out of 9 terpenoidal glycosides, 7 were simple terpenoidal glycosides (Apiosylglucosyl 4-hydroxybenzoate, AzI, Mabioside A, Glucosylarjunolate 3-[rhamnosyl-(1->3)-glucuronide], Cyclopassifloside VII and Saponin E. Apart from that, one terpenoidal steroid i.e., Mabiogenin 3-[rhamnosyl-(1->6)-glucoside]; one triterpenoid saponin i.e., Oleragenoside and two glycosidal compounds i.e., Apiosylglucosyl 4-hydroxybenzoate and Nigerose (Sakebiose) were also identified in the crude extract of *T. cuneifolia* (table 4.55, figure 4.70). In the previous study, LCMS analysis showed presence of two diterpenes Przewalskin and Coronarin in the roots of *T. cuneifolia* (Mangalorkar, 2014).

Along with that, she has also reported Cabraleadiol, Ganoderol B, Eichlerialactone, Beta amyrin, Limonol, Ganoderic acid SZ, Ganoderol A and F and Cedrelone some of the rare triterpenoids from *T. cuneifolia* plant. The main constituent of roots is glycyrrhizin, a triterpenoid saponin is reported from *G. glabra* roots as well as from the roots of *T. cuneifolia* (Zore 2008; Manglorkar, 2014).

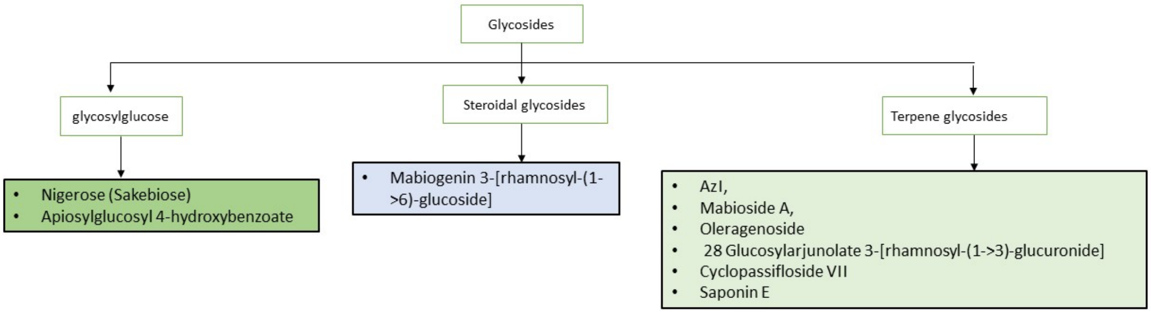


Figure 4.69: Terpenoidal glycosides identified in LC-MS-Q-ToF analysis

4.4.2.4.3 Alkaloids

Erysothiopine, an alkaloid, has been found in the roots of *T. cuneifolia* crude extract through LC-MS-Q-ToF analysis (table 4.56). Fukui *et al*., 1988 and L. Wang, Yang, *et al*., 2015 indicated the existence of alkaloids in early research of the *G. glabra* plant, but no information is currently available about the kind of alkaloid found in the *G. glabra* plant. Similarly, Manglorkar's early examination of alkaloids in the *T. cuneifolia* plant showed the absence of alkaloids.

4.4.2.4.4. Terpenes

In the LC-MS-Q-ToF analysis, 25 terpenoidal compounds have been identified in which 10 compounds were identified from the column chromatography fraction and 15 were from the crude methanolic extract of the *T. cuneifolia* root (table 4.57, figure 4.71). Out of 25 compounds, 16 was simple terpenoidal or sesquiterpenoid or monoterpenoidal compounds and rest were triterpenoid saponins. Some of the main triterpenoid saponin compounds which were identified were abrusoside A, Chikusetsusaponin V and Dianoside A. 11-Oxo-beta-amyrin were also detected which is a precursor compounds for the formation of glycyrrhizin. The Rhizomes of *G. glabra* is also reported to contain various number of triterpenes (Meena *et al*. 2010) such as betulinic acid, Liquiritic acid, β-amyrin and lupeol are some of the important triterpenes reported from the roots of *G. glabra* (Canonica *et al*., 1996; Hayashi *et al*., 1988). Several pharmacological qualities of *G. glabra*, such as anti-ulcer, anti-inflammatory, spasmolysis, anti-oxidative, contravariance, antiviral, anticancer activity, hepatoprotective, expectorant, and memory boosting actions, have been attributed to triterpenoids. Betulinic acid and -amyrin have been found in the roots of both plants, *G. glabra* and *T. cuneifolia.*

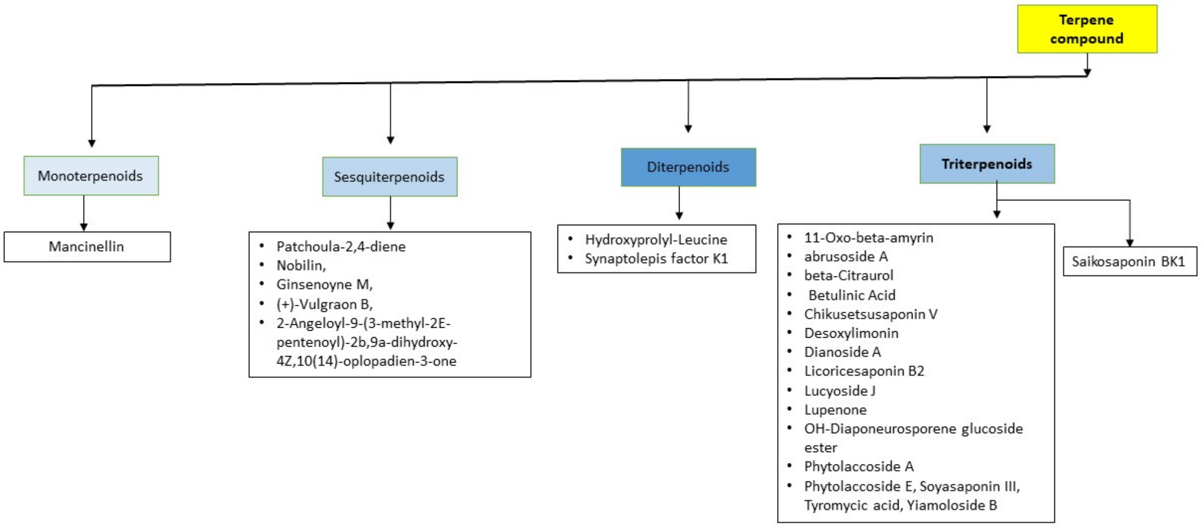


Figure 4.70: Terpenes identified in LC-MS-Q-ToF analysis

4.4.2.4.5 Sterols

Sterols, or steroid alcohols, are a vital family of chemical compounds that make up a subset of the steroids. Various organisms, including plants, animals, and fungus, produce them naturally. Phytosterols are plant-derived fatty molecules (steroids) that account for the majority of unsaponifiable substance in plant lipids (Piironen *et al*., 2003). Plant sterols and stanols, together known as phytosterols, are steroid molecules found in plants that are structurally and functionally similar to cholesterol but differ only in their carbon side chains and/or the presence or absence of a double bond. In the LCMS-Q-ToF analysis, 4 sterols has been identified from which, one sterols i.e., Nebrosteroid L were identified from the crude methanolic extract of *T. cuneifolia* whereas 3 sterols i.e., (3a,5b,7a,12a)-24-[(carboxymethyl)amino]-1,12-dihydroxy-24-oxocholan-3-yl-b-D-Glucopyranosiduronic a, 1Î±,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3 / 1Î±,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homocholecalciferol and Sarcostin (table 4.52, figure 4.72). Although β-sitosterol and stigmasterol were previously isolated from licorice roots and *T. cuneifolia* roots, respectively, by Suman *et al*. (2009) and Manglorkar *et al*. (2014), neither compound was detected by LC-MS-Q-ToF analysis.

Table 4.54: The sterols identified through LC-MS-Q-ToF analysis

Peak No.	Name (Sterols)	Formula	Phyto group	Score	Mass	RT	Height	Area
117	15R-PGE2 methyl ester	$C_{21} H_{34} O_5$	icosanoid/prostanoid	82.79	366.2402	21.353	85954	646208
113	17(S)-HpDoHE (17(S)-hydroperoxy Docosahexaenoic Acid	$C_{22} H_{32} O_4$	hydroperoxy fatty acid/docosanoid	83.5	360.2296	26.367	80639	489857
39	30:6(12Z,15Z,18Z,21Z,24Z,27Z)/ triacontahexaenoic acid 30:6(omega3)	$C_{30} H_{48} O_2$	omega-3 fatty acid	94.07	440.367	17.507	112820	1137149
6	2-glyceryl-PGE2/ prostaglandin E2 2-glyceryl ester	$C_{23} H_{38} O_7$	Eicosanoids	98.58	426.2614	15.9	147470	534825
123	5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid	$C_{20} H_{32} O_4$	fatty acid derivative	82.53	336.2299	20.676	79937	466428
138	Cyanopeptolin A	$C_{46} H_{72} N_{10} O_{12}$	cyclodepsipeptide	80.82	956.5383	19.498	424682	2571548
102	Î±-9(10)-EpODE	$C_{18} H_{30} O_3$	epoxy fatty acid	84.4	294.2217	25.82	225993	1113672
128	methyl 15,16-epoxy-9,12-octadecadienoate	$C_{19} H_{32} O_3$	Fatty Acids and Conjugates/Epoxy fatty acid	81.99	308.2374	30.579	307285	1631432
47	PE(17:0/20:2(11Z,14Z))	$C_{42} H_{80} N O_8 P$	Glycerophospholipids	92.89	757.5599	28.829	154174	2669541
49	PE(P-18:0/17:2(9Z,12Z)) Glycerophospholipids	$C_{40} H_{76} N O_7 P$	Glycerophospholipids	92.84	713.5337	28.365	255497	3460735
147	PGA2 methyl ester	$C_{21} H_{32} O_4$	lipid compounds that are derived enzymatically from fatty acids	79.44	348.2297	24.221	40942	398635

167	PI(22:0/0:0)/ *phosphatidylinositol*	$C_{31}H_{61}O_{12}P$	glycerophosphoinositol	77.45	656.3922	19.878	68926	398511
101	PI(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))/ myo-inositol	$C_{53}H_{79}O_{13}P$	Glycerophosphoinositols/ phosphatidylinositol	84.54	954.5214	20.895	105817	548467
28	PIP(16:0/20:3(5Z,8Z,11Z))/ Phosphatidylinositol Phosphate	$C_{45}H_{82}O_{16}P_2$	Glycerophospholipids	96.26	940.5064	18.792	527896	3633757
65	PPA(16:0/18:1(9Z))/ 1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-pyrophosphate	$C_{37}H_{72}O_{11}P_2$	Glycerophospholipids	90.9	754.4543	15.044	86911	452088
54	PS(18:4(6Z,9Z,12Z,15Z)/20:4(5Z,8Z,11Z,14Z))/ Glycerophosphoserines	$C_{44}H_{70}NO_{10}P$	Glycerophosphoserine	92.38	803.4717	18.945	91393	511650
61	PS(O-20:0/18:2(9Z,12Z))	$C_{44}H_{84}NO_9P$	Glycerophosphoserines	91.73	801.586	28.292	110830	882041
	Stearaldehyde	$C_{18}H_{36}O$	long chain fatty aldehyde	7.392	268.2767	638-66-4	-	77.89
48	Theonellasterol B	$C_{30}H_{46}O$	Sterol Lipids	92.86	422.3565	17.503	88178	810094

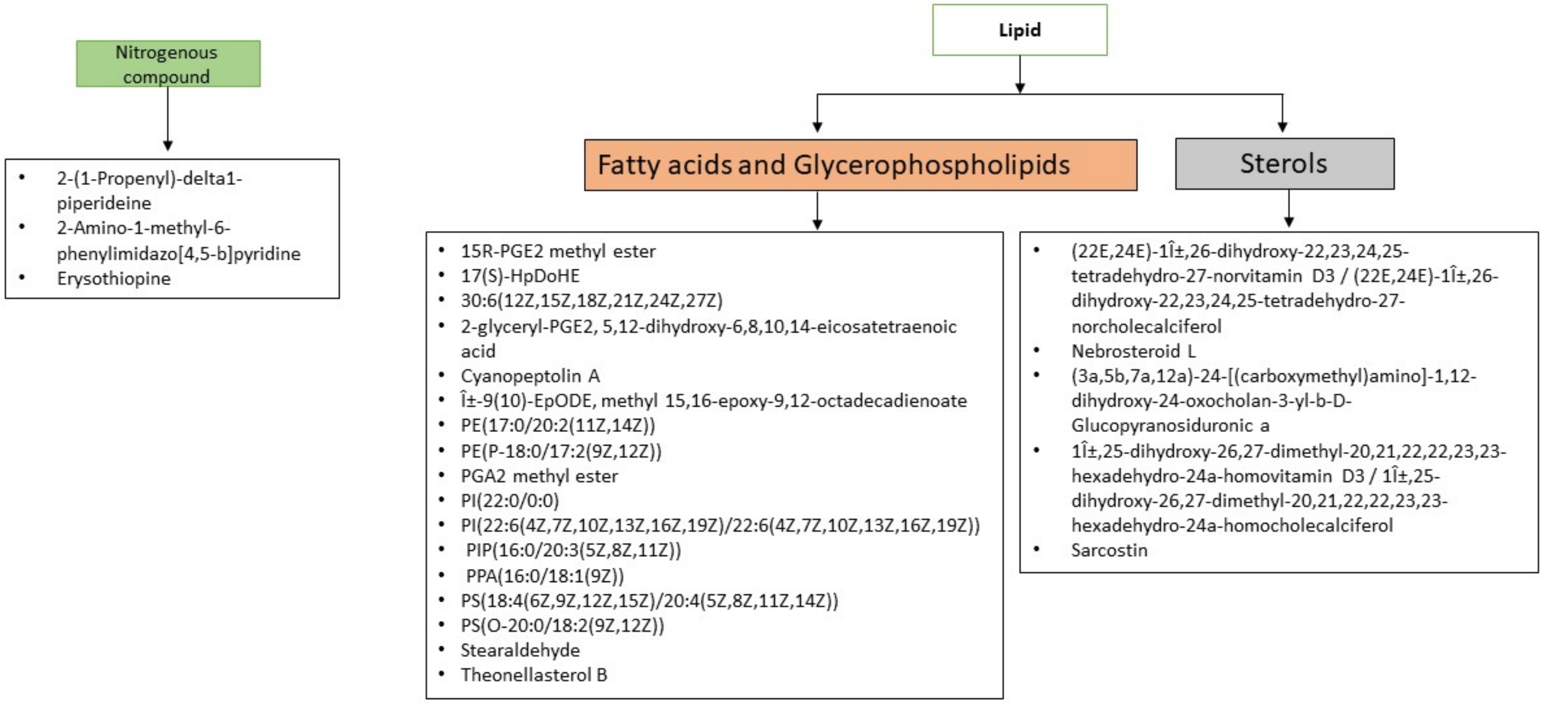


Figure 4.71: Sterols identified in LC-MS-Q-ToF analysis

Table 4.55: The Vitamins identified through LC-MS-Q-ToF analysis

Peak No.	Name (Vitamin)	Formula	Phyto group	Score	Mass	RT	Height	Area
169	(10E)-19-fluoro-1Î±,25-dihydroxyvitamin D3 / (10E)-19-fluoro-1Î±,25-dihydroxycholecalciferol	$C_{27} H_{43} F O_3$	analog of vit.D	76.85	434.3203	16.424	221500	1157231
174	Demethylphylloquinone	$C_{30} H_{44} O_2$	vitamin K1 or phylloquinone	76.07	436.3355	18.936	19973	80681

Table 4.56: The Phenols identified through LC-MS-Q-ToF analysis

Peak No.	Name (Phenols)	Formula	Phyto group	Score	Mass	RT	Height	Area
142	Mebeverine metabolite (Veratric acid glucuronide)	$C_{15} H_{18} O_{10}$	phenolic acid	78.49	m	1.146	9723	33109
	Gingerdione	$C_{17} H_{24} O_4$	phenols	8.539	292.1679	61871-71-4	-	72.96
88	Demethylphylloquinone	$C_{30} H_{44} O_2$	Phenol lipid/ketones/ precursor of vitamin K	86.32	436.3361	18.151	227369	1387609
2	Archangelicin	$C_{24} H_{26} O_7$	polyphenolic	99.16	426.1675	19.445	149314	1221520
	Chavicol	$C_9 H_{10} O$	Phenylpropene	7.613	134.0730	501-92-8	-	97.51

Table 4.57: The Terpenol glycosides identified through LC-MS-Q-ToF analysis

Peak No.	Name (Terpenoidal glycosides)	Formula	Phyto group	Score	Mass	RT	Height	Area
132	Nigerose (Sakebiose)	$C_{12}H_{22}O_{11}$	glycosylglucose	79.01	342.1171	1.042	32103	75074
93	Apiosylglucosyl 4-hydroxybenzoate	$C_{18}H_{24}O_{12}$	glycoside	85.66	432.1288	4.071	47841	827760
23	Mabiogenin 3-[rhamnosyl-(1->6)-glucoside]	$C_{42}H_{68}O_{14}$	Steroidal glycosides	96.99	796.4623	18.114	82565	430675
16	AzI	$C_{48}H_{74}O_{18}$	Terpene glycosides	97.69	938.4889	15.044	167657	910947
22	Mabioside A	$C_{48}H_{78}O_{19}$	Terpene glycosides	97.15	958.5141	15.544	79871	568189
88	Oleragenoside	$C_{42}H_{64}O_{16}$	terpene glycoside/triterpenoid saponin	86.27	824.4211	16.028	69453	574503
24	28-Glucosylarjunolate 3-[rhamnosyl-(1->3)-glucuronide]	$C_{48}H_{76}O_{20}$	Terpene glycosides	96.72	972.4942	14.268	72358	255171
62	Cyclopassifloside VII	$C_{37}H_{62}O_{13}$	Terpene glycosides	91.69	714.4216	20.444	308606	1847733
31	Saponin E	$C_{42}H_{68}O_{14}$	Terpene glycosides	95.82	796.4601	18.102	83764	596302

Table 4.58: The Alkaloids identified through LC-MS-Q-ToF analysis

Peak No.	Name (Alkaloids)	Formula	Phyto group	Score	Mass	RT	Height	Area
151	Erysothiopine	$C_{19}H_{21}NO_7S$	Alkaloids	79.01	407.1031	9.888	158312	946827

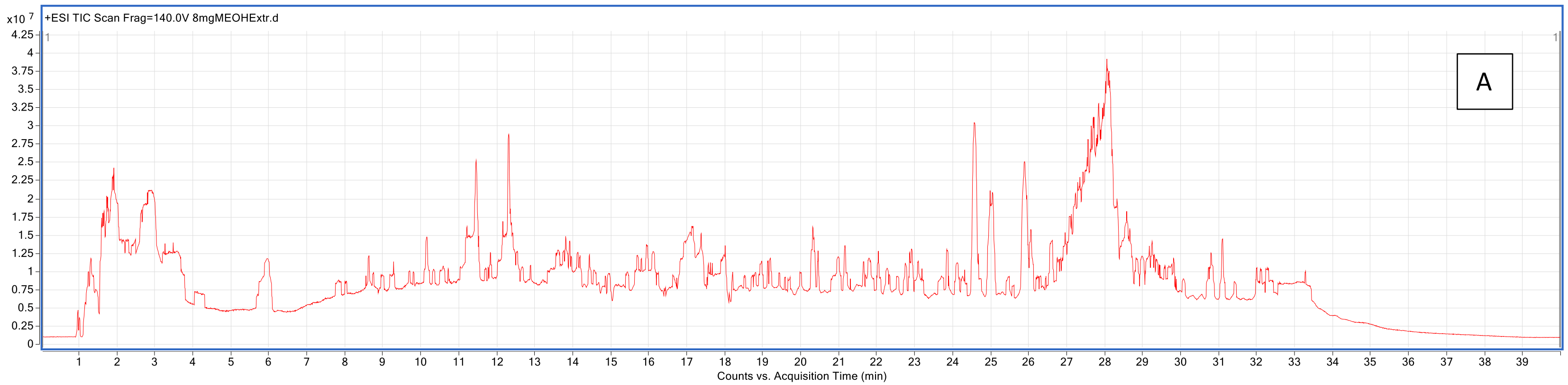
Table 4.59: The Terpenes identified through LC-MS-Q-ToF analysis

Peak No.	Name (Terpenes)	Formula	Phyto group	Score	Mass	RT	Height	Area
	11-Oxo-beta-amyrin	$C_{30}H_{48}O_2$	pentacyclic triterpenoid	5.805	440.3655		-	75.39
	abrusoside A	$C_{36}H_{54}O_{10}$	triterpenoid saponin	5.306	646.3721		-	92.68
	beta-Citraurol	$C_{30}H_{42}O_2$	triterpenoids	5.617	434.3192	57593-78-9	-	89.44
63	Betulinic Acid	$C_{30}H_{48}O_3$	pentacyclic triterpenoid	91.34	456.3623	20.321	213459	1226738
145	Chikusetsusaponin V	$C_{48}H_{76}O_{19}$	triterpenoid saponin	79.75	956.4998	15.04	105865	1036003
139	Desoxylimonin	$C_{26}H_{30}O_7$	Triterpenes/ Limonins/steroid lactone	80.8	454.199	12.553	190269	820512
41	Dianoside A	$C_{42}H_{66}O_{15}$	triterpenoid saponin	93.88	810.4424	17.375	84290	571928
	Licoricesaponin B2	$C_{42}H_{64}O_{15}$	triterpenoid saponin	5.414	808.4243	118536-86-0	-	61.59
24	Lucyoside J	$C_{42}H_{66}O_{15}$	Triterpene saponins	95.31	810.4419	16.697	120988	432858
178	Lupenone	$C_{30}H_{48}O$	triterpenoid	75.31	424.372	28.406	62910	439541
81	OH-Diaponeurosporene glucoside ester	$C_{36}H_{54}O_6$	triterpenoid.	88.75	582.3932	28.406	30592	189140
46	Phytolaccoside A	$C_{36}H_{56}O_{10}$	Triterpenoids	93.14	648.3891	19.511	144772	734479
29	Phytolaccoside E	$C_{42}H_{66}O_{16}$	Triterpenoids	95.94	826.4358	16.73	62715	734610
	Soyasaponin III	$C_{42}H_{68}O_{14}$	Triterpene saponins	5.569	796.4610	55304-02-4	-	94.23
57	Synaptolepis factor K1	$C_{36}H_{54}O_8$	diterpenoid	92.07	614.3836	20.61	66941	297378

	Tyromycic acid	$C_{30}H_{44}O_3$	2,5,6,10,10,14,21-heptamethyl-23-oxahexacyclo[19.2.1.0²,¹⁹.0⁵,¹⁸.0⁶,¹⁵.0⁹,¹⁴]tetracos-17-ene-11,22-dione	5.140	452.3284	104759-35-5	-	98.59
59	Yiamoloside B	$C_{43}H_{68}O_{15}$	triterpenoid saponin	91.86	824.4586	19.226	410058	2894608
31	Mancinellin	$C_{36}H_{52}O_8$	monoterpenoid	94.56	612.3675	18.156	154712	1149552
112	Hydroxyprolyl-Leucine	$C_{11}H_{20}N_2O_4$	dipeptide	83.52	244.1432	12.103	99935	2118363
176	Patchoula-2,4-diene	$C_{15}H_{22}$	sesquiterpenes	75.95	202.1716	1.832	92026	1086267
5	Nobilin	$C_{20}H_{26}O_5$	Sesquiterepene lactone	98.53	346.1782	24.735	2043819	16219823
100	Ginsenoyne M	$C_{32}H_{46}O_2$	Sesquiterpenes	84.56	462.3506	31.407	110240	669157
5	(+)-Vulgraon B	$C_{16}H_{24}$	sesquiterpenes	98.7	216.1877	3.626	136379	2491399
2	2-Angeloyl-9-(3-methyl-2E-pentenoyl)-2b,9a-dihydroxy-4Z,10(14)-oplopadien-3-one	$C_{26}H_{36}O_5$	sesquiterpenoid	99.3	428.2559	32.083	181097	1028158
74	Saikosaponin BK1	$C_{48}H_{78}O_{17}$	triterpenoid saponin/saikosaponin	89.85	926.5268	26.795	57049	308373

Table 4.60: The Sterols identified through LC-MS-Q-ToF analysis

Peak No.	Name (Sterols)	Formula	Phyto group	Score	Mass	RT	Height	Area
60	Nebrosteroid L	$C_{30}H_{46}O_4$	Sterol Lipids	91.76	470.3414	26.063	2226332	12342527
66	(3a,5b,7a,12a)-24-[(carboxymethyl)amino]-1,12-dihydroxy-24-oxocholan-3-yl-b-D-Glucopyranosiduronic a	$C_{32}H_{51}NO_{12}$	lipid steroid	89.52	641.343	25.95	185430	1333110
44	1Î±,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3 / 1Î±,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homocholecalciferol	$C_{30}H_{44}O_3$	Secosteroids/Steroid	92.88	452.3305	17.784	173367	1116096
120	Sarcostin	$C_{21}H_{34}O_6$	Steroids/Pregnenes	81.24	382.2345	31.569	140220	949335



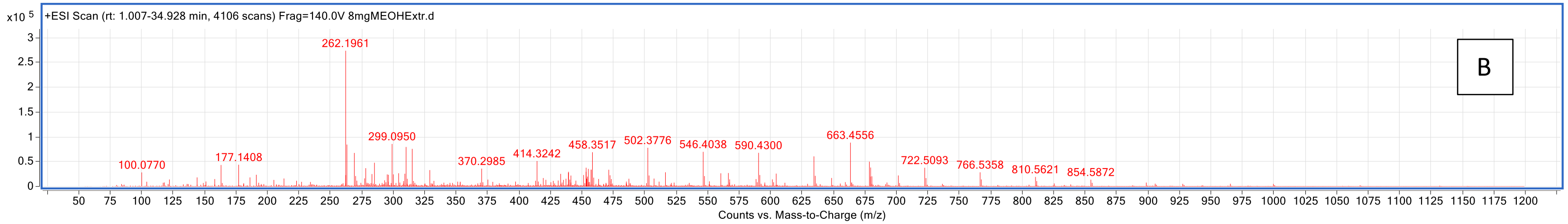


Figure 4.72: Chromatogram of LC-MS-Q-Tof of untargeted metabolite of crude extract of roots of *T. cuneifolia* (A) TIC base peak chromatogram (BPC) of *T. cuneifolia* obtained in extracted ion chromatogram in positive ionization mode (B)Mass spectrum of *T. cuneifolia* obtained in extracted ion chromatogram in positive ionization mode

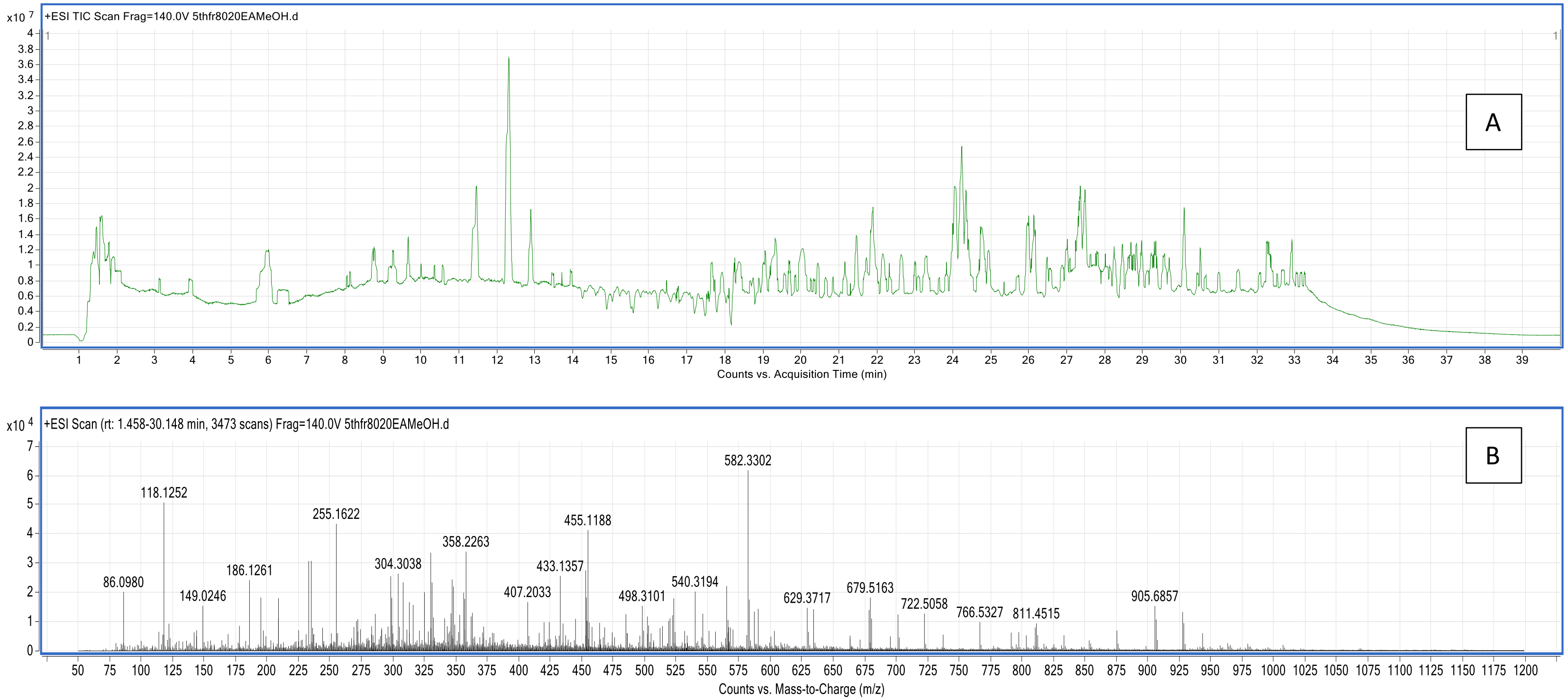
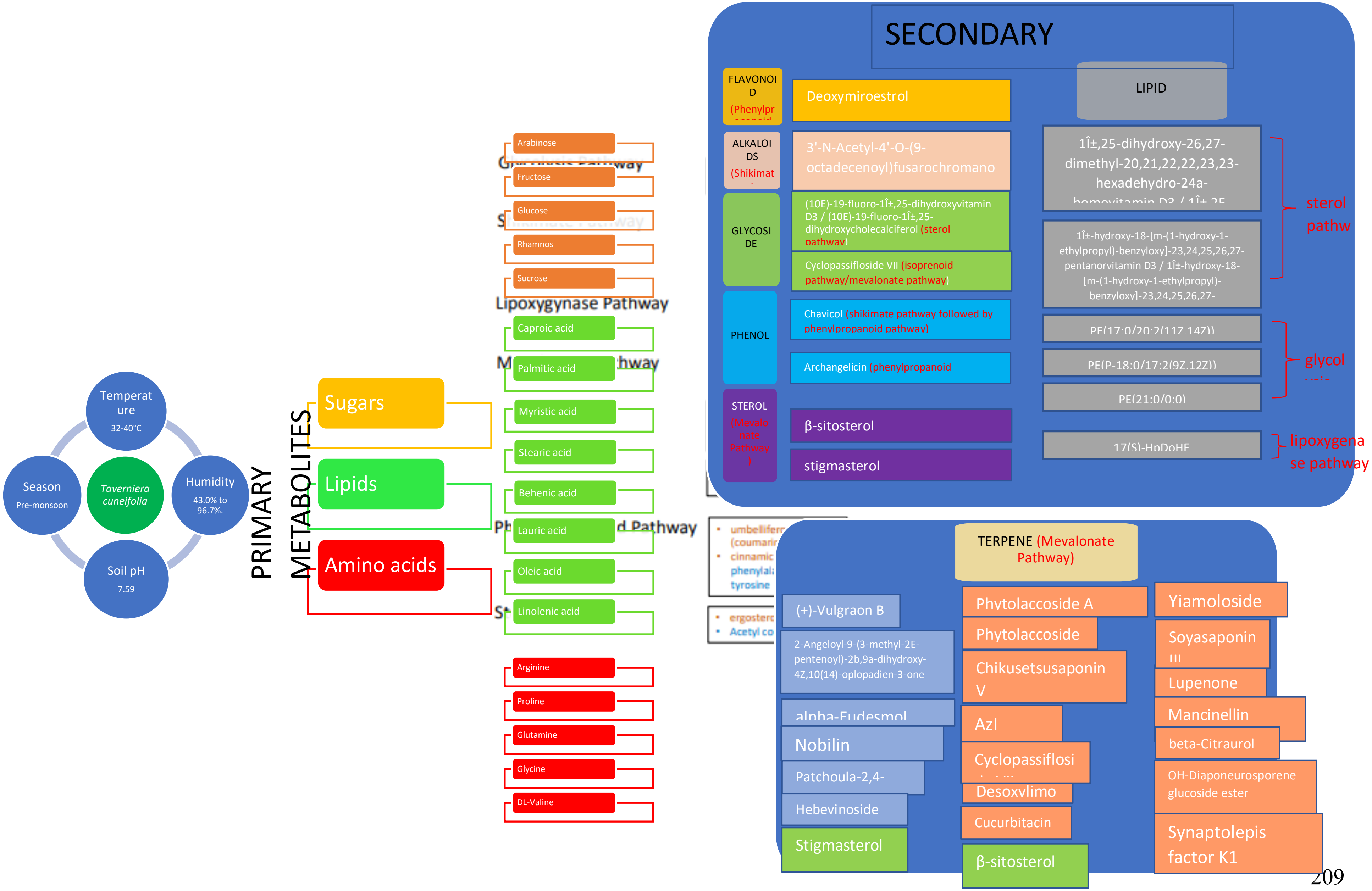


Figure 4.73: Chromatogram of LC-MS-Q-Tof oF untargeted metabolite of 5th fraction of ethyl acetate:methanol (80:20) from column chromatography of roots of *T. cuneifolia* (A) TIC base peak chromatogram (BPC) of *T. cuneifolia* obtained in extracted ion chromatogram in positive ionization mode (B)Mass spectrum of *T. cuneifolia* obtained in extracted ion chromatogram in positive ionization mode

4.5 Conclusion

1. Presence of glycyrrhizin in *Taverniera cuneifolia* was standardized and validated by HPLC and LCMS/MS studies. Though the presence of glycyrrhizin in *Taverniera cuneifolia* was less but the presence was distinctly observed.
2. 11 components (Liquiritigenin, quercetin, naringenin, genistein, kaempferol, apigenin, glabridin, glycyrrhizin, glycyrrhetinic acid, Stigmasterol and beta sitosterol) similar in *Glycyrrhiza glabra* and *Taverniera cuneifolia* were detected via LC-MS/MS-Q-ToF. Simultaneous method development of these compounds has been done via HPLC. 7 constituents could be validated of which 5 were developed by one method and 2 compounds i.e., Stigmasterol and Beta sistosterol validated by other method.
3. As the plant is consider an alternative of sugar. Out of 5 known sugars from *Taverniera cuneifolia* 3 sugars (Glucose, fructose and sucrose) with good concentration were validated via HPTLC.
4. Among other primary metabolites Amino acids and Lipids were studied also studied. Of the 5 number of known amino acids Arginine from *Taverniera cuneifolia* and Proline from *G. glabra* were detected in higher concentration in HPTLC studies which are produced in stress condition. Indicating its probable role in secondary metabolites and other prime constituents of *Taverniera cuneifolia*. Among the known fatty acids of Taverniera cuneifolia, Dodecane, 2,6,11-trimethyl-, n-Hexadecanoic acid (Palmitic acid), Stearic acid, Myristic acid, Stearic acid and Lauric acid. The fatty Lipid profile of Root, Leaves and seeds were also studied to understand the physiological link between the synthesis and sink (Figure 4.75)
5. Untageted LC-MS-Q-ToF studies showed that there are 19 lipids, 2 vitamin, 5 phenolic compounds, 9 terpenoidal glycosides, 1 alkaloid, 25 terpene and 4 sterols compounds (Figure 4.73 & 4.74).
6. Based on the phytochemical characterization and analysis of roots and vegetative parts it can be stated that plants could be used for various therapeutic values such Anti-alzheimers, Anti-oxidative activity, Anticancer, Anti-inflammatory, Anti-obesity, immuno-booster etc. (figure 4.76).
7. Overall the characteristic feature of the plant shows that the *Taverniera cuneifolia* in itself and not as an alternative of *Glycyrrhiza glabra* could be an answer to many therapeutic disorders and diseases.



SECONDARY
FLAVONOID (Phenylpr
Deoxymiroestrol
LIPID
ALKALOIDS (Shikimat
3'-N-Acetyl-4'-O-(9-octadecenoyl)fusarochromano
1α,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-
GLYCOSIDE
(10E)-19-fluoro-1α,25-dihydroxyvitamin D3 / (10E)-19-fluoro-1α,25-dihydroxycholecalciferol (sterol pathway)
Cyclopassifloside VII (isoprenoid pathway/mevalonate pathway)
1α-hydroxy-18-[m-(1-hydroxy-1-ethylpropyl)-benzyloxy]-23,24,25,26,27-pentanorvitamin D3 / 1α-hydroxy-18-[m-(1-hydroxy-1-ethylpropyl)-benzyloxy]-23,24,25,26,27-
sterol pathw
PHENOL
Chavicol (shikimate pathway followed by phenylpropanoid pathway)
Archangelicin (phenylpropanoid
PE(21:0/0:0)
glycol
STEROL
β-sitosterol
stigmasterol
17(S)-HpDoHE
lipoxygenase pathway
TERPENE (Mevalonate Pathway)
(+)-Vulgraon B
2-Angeloyl-9-(3-methyl-2E-pentenoyl)-2b,9a-dihydroxy-4Z,10(14)-oplopadien-3-one
Nobilin
Patchoula-2,4-
Hebevinoside
Stigmasterol
Phytolaccoside A
Phytolaccoside
Chikusetsusaponin V
Azl
Cyclopassiflosi
Desoxylimo
Cucurbitacin
β-sitosterol
Yiamoloside
Soyasaponin III
Lupenone
Mancinellin
beta-Citraurol
OH-Diaponeurosporene glucoside ester
Synaptolepis factor K1
Temperature 32-40°C
Season Pre-monsoon
Taverniera cuneifolia
Humidity 43.0% to 96.7%.
Soil pH 7.59
PRIMARY METABOLITES
Sugars
Lipids
Amino acids
Arabinose
Fructose
Glucose
Rhamnos
Sucrose
Lipoxygynase Pathway
Caproic acid
Palmitic acid
Myristic acid
Stearic acid
Behenic acid
Lauric acid
Oleic acid
Linolenic acid
Arginine
Proline
Glutamine
Glycine
DL-Valine

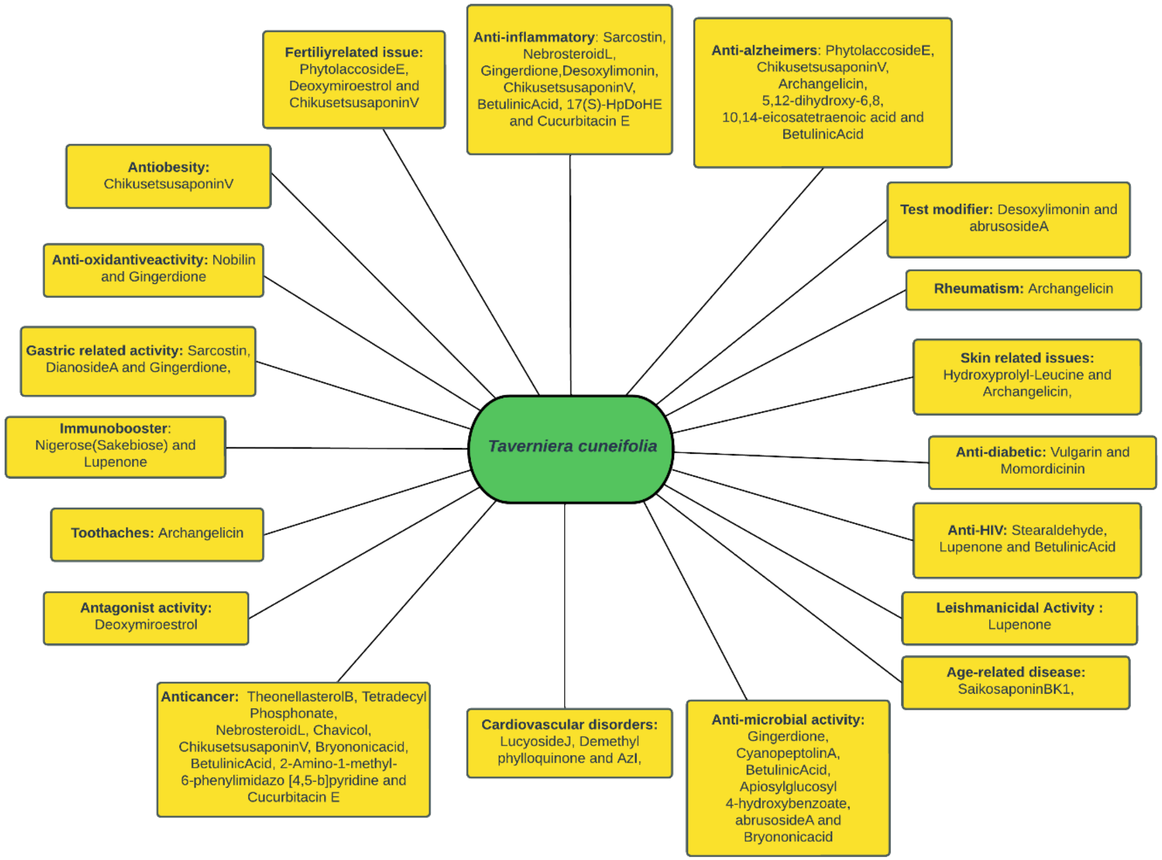


Figure 4.76: Possible therapeutic importance of various phyto-constituents found in *Taverniera cuneifolia*

4.6 Suggestions

1. Phytochemical characterization of new phytocomponents analysed in the present studies could be further validated.
2. Clinical studies on therapeutic importance judged by the overall phytochemicals need to be further verified. Docking studies followed by clinical studies could be productive for understanding therapeutic importance of compound synergistically and as an isolated molecule.
3. Unknown phytocomponents couldn’t be analysed by LC MS/Ms and GC MS/Ms should be further elucidated by NMR studies.