Phytochemical Fingerprinting of *Terminalia* Species from North Central Corridor of Western Ghats using HP-TLC Analysis

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For the award of the degree of

Doctor of Philosophy

In the Faculty of Pharmacy

By

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(Registration No: KLEU/Ph.D/2019-20/DO1219009)

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December 2023

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I hereby declare that the thesis entitled **"Phytochemical Fingerprinting of *Terminalia* Species from North Central Corridor of Western Ghats using HP-TLC Analysis"** *is a bonafide and original research carried out by me under the guidance of* ***Prof. (Dr.) Pramod J. Hurkadale,*** *Department of Pharmacognosy, KLE College of Pharmacy, Belagavi, KAHER, Belagavi-590010 and Co-guide* ***Dr. Harsha V. Hegde****, Scientist-E, ICMR-National Institute of Traditional Medicine (NITM) Belagavi-590010. The thesis or any part thereof has not formed the basis for the award of any degree/fellowship or similar title to any candidate of any University.*

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LIST OF ABBREVIATIONS

%	**:** Percentage
%RSD	**:** Percent Relative Standard Deviation
@	**:** At
°	**:** Degree
°C /min	**:** Degree Celsius per Minute
<	**:** Less than
>	**:** Greater than
±	**:** Plus, or minus
3D	**:** Three dimensional
µg	**:** Micro gram
µg/kg	**:** Microgram per Kilograms
µg/L	**:** Microgram per Litre
µL	**:** Micro Litre
µm	**:** Micro meter
Abs	**:** Absorbance
AAS	**:** Atomic absorption spectroscopy
ASR	**:** Anisaldehyde Sulphuric acid
APCI	**:** Atmospheric pressure chemical ionization
APIs	**:** Active Pharmaceutical Ingredients
As	**:** Arsenic
Ca	**:** Calcium
Co	**:** Cobalt
Cd	**:** Cadmium
Cr	**:** Chromium

Cu : Copper

CAS : Chemical Abstracts Service

c-GMP : Current Good manufacturing processes

Conc. : Concentration

Cm : Centimeter

CV : Coefficient of Variance

CFR : Code of Federal Regulations

DAD : Diode-Array Detection

DPPH : 2,2-diphenyl-1-picrylhydrazyl

ESI : Electrospray Ionisation Process

FDA : Food and Drug Administration

FAO : Food and Agriculture Organization

Fe : Iron

Fig : Figure

FSSAI : Food Safety and Standards Authority of India

g/ gm : Gram

g/L : Grams per Litre

g/mol : Gram per Mole

GC : Gas Chromatography

GCMS : Gas Chromatography Mass Spectroscopy

GAP : Good Agricultural Practises

GFCP :Good Field Collection Practise

GLP : Good Laboratory Practice

GMP : Good Manufacturing Practice

Hrs./ hrs. : Hours

H_2O	**:** Water
HCL	**:** Hydrochloric acid
Hg	**:** Mercury
HNO_3	**:** Nitric acid
H_2SO_4	**:** Sulphuric acid
H_2O_2	**:** Hydrogen peroxide
HAE	**:** Hydroalcoholic extract
HMs	**:** Heavy metals
HPLC	**:** High Performance Liquid Chromatography
HPTLC	**:** High Performance Thin Layer Chromatography
ICH	**:** International Council for Harmonisation
ICMR	**:** Indian Council of Medical Research
ICP-OES	**:** Inductively Coupled Plasma Atomic Emission Spectroscopy
ICP-MS	**:** Inductively Coupled Plasma Mass Spectrometry
IP	**:** Indian Pharmacopeia
ISO	**:** International Organization for Standardization
L	**:** Litre
LC	**:** Liquid Chromatography
LCMS	**:** Liquid Chromatography Mass Spectroscopy
LC-DAD-MS	**:**LIQUID Chromatography Diode array detector Mass spectroscopy
LDR	**:** Linear Dynamic Range
LOD	**:** Limit of Detection
LOQ	**:** Limit of Quantification
M	**:** Molar
M.W	**:** Molecular Weight

MF **:** Molecular Formula

m/z **:** Mass by Charge Ratio

Mn **:** Manganese

λ_{max} **:** Maximum Lambda /Absorption Maxima

MeOH **:** Methanol

Mg/mg **:** Milligram

mg/kg **:** Milligram per Kilograms

$MgCl_2$ **:** Magnesium chloride

Min **:** Minute

mL : Milli Litre

ml/min **:** Milliliter per Minute

mm/s **:** Millimeter per second

MS **:** Mass Spectroscopy\Spectrometry

MSD **:** Mass Spectrometer Detector

mix **:** Mixture

N **:** Number of trials

N_2 **:** Nitrogen

NaOH **:** Sodium hydroxide

Ng **:** nanogram

nm/ Nm **:** Nanometer

nm/min **:** Nanometer per Minute

P **:** Phosphorus

Pb **:** Lead

pH **:** Potential of Hydrogen

PDA **:** Photo diode array

Pvt. Ltd : Private Limited

ppb : Parts per Billion

ppm : Parts per Million

ppt : Parts per Trillion

PTWI : Provisional Tolerable Weekly Intake values

QA : Quality Assurance

QC : Quality Control

r^2 : Correlation coefficient

R^2 : Coefficient of Regression

Rf : Retention Factor

RPM/rpm : Rotations per minute

RP-TLC : Reverse Phase Thin Layer Chromatography

RMRC : Regional Medical Research Center

RSD : Relative Standard Deviation

Rt : Retention time

S : Seconds

SOP : Standard Operating Procedure

Std : Standard

Ta : *Terminalia arjuna*

Tb : *Terminalia bellirica*

Tc : *Terminalia chebula*

Tct : *Terminalia catappa*

TCM : Traditional Chinese Medicine

TLC : Thin Layer Chromatography

TTC : Twin Trough Chamber

USA **:** United States of America

USFDA **:** United States Food and Drug Administration

USP **:** United States Pharmacopoeia

UV **:** Ultra-Violet Spectroscopy

UPLC **:** Ultra-performance liquid chromatography

V **:** Volt

v/v **:** Volume by Volume

VSR **:** Vanillin Reagent

w/v **:** Weight by Volume

w/w **:** Weight by Weight

WHO **:** World Health Organization

z **:** Zinc

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ABSTRACT

Background: Traditional system of medicine practise and utilized by a sizeable section of the population around the globe, particularly in developing nations. The need for quality control procedures to assess their chemical composition along with their therapeutic potential and stability of these resources has been increased as a result of the rising demand for herbal raw materials and their products. The specific plant species, including *Terminalia arjuna*, *Terminalia bellirica*, *Terminalia chebula*, and *Terminalia catappa*, possess ecological and medicinal value in the bio-diversified North Central corridor of Western Ghats. Hence, these species are routinely being used for centuries for their multi-spectrum medicinal properties. The stringent quality control analysis is being required by Phyto-pharmaceuticals and related sectors with a perspective of recognizing medicinal potential of such species. In the domain of product analysis and quality assessment, the fundamental principle lies in ensuring the maximum stability of medicinal products. This mission is upheld through stringent adherence of developed methods with regulations set forth by National and International regulatory authorities which encompasses systems for the identification and purity of products, with a keen focus on conformity to the standards established by globally recognized bodies such as the USP, ICH, and ISO. By implementing these established standards and guidelines, a comprehensive approach to product analysis ensures the highest levels of quality and safety. This approach goes beyond geographical boundaries and impacting a worldwide perspective, ensuring that the analysis is conducted with a global perspective, thereby strengthen the confidence in the products integrity and performance. The proposed study displays the complex phytochemical and elemental content of various *Terminalia* species which is carried out through the application of state-of-the-art analytical techniques, including HP-

TLC, LCMS/MS, AAS, ICP-OES, and ICP-MS which conducted in strict compliance with international regulatory requirements and their standards.

Objectives: Primary: To develop novel regulatory compliant fingerprint profiles for Identification of simultaneous multi phytochemical composition and validate the developed protocol in four *Terminalia* species by HP-TLC.

Secondary: 1. To perform phytochemical profiling using hyphenated chromatographic and spectrophotometric tools like LC-MS/MS. **2.** Screening of elemental profile in four selected *Terminalia* species for evaluating its safety in routine extracts by using AAS, ICP-MS and ICP-OES.

Methodology: The aim of the research is focused on standardization and chemical profiling for selected *Terminalia* species and the methods developed and validated were complying of international regulations like USP, ISO & ICH as well. In the proposed research work, a wider spectrum of instrumental techniques, with hyphenation of chromatography coupled with advanced spectroscopy is been highlighted. This analytical set-up has been developed cconsidering the current requirement is urgent for developing alternative methods which are fast, reproducible and economic compared to existing time-consuming tools is ever surging. The sstandardization of selected species of *Terminalia* is performed using in-detailed planar chromatographic evaluation utilizing USP-21 CFR compliant HP-TLC system from CAMAG, Merck stationary phases and high pure grade solvents were employed for the study. The confirmatory analysis of *Terminalia* species and purity profiles in comparison with standards was carried out by using LCMS/MS followed by trace elemental analysis utilizing AAS, ICP-OES and ICP-MS.

Results: The HP-TLC study has provided a suitable scientific evidence on quality control method for screening of fingerprinting analysis, which, after derivatization, showcased significant bands confirming to flavonoids, phenols, tannins. An HP-TLC-DPPH assay also identified antioxidants, illustrating the technique's efficiency and effectiveness. The HP-TLC delivered excellent, reproducible results with good polynomial regression limits of 99.998% for all four species of *Terminalia* subjected for study and reported. The proposed protocol has been developed and validated in accordance with International Council for Harmonization guidelines, and all parameters had been found to be within the specified limits. The aadvanced hyphenated techniques like LC-MS provided confirmatory evidences of phyto-components identified and quantified from fingerprint profiles of HP-TLC study. The LC-MS studies further supported the data obtained from HP-TLC results and the outcomes further aided in confirming the purity of standard phytochemical components identified and estimated in *Terminalia* species. The preliminary spectroscopic analysis of atomic absorption spectroscopy revealed the presence of essential macro nutrients like Zinc, Iron, Calcium whereas, advanced spectroscopic tools like Inductive Coupled Plasma and ICP-MS (Mass Spectroscopy) assisted in quantifying micro nutrients like Magnesium, Phosphorus, Copper, along with heavy metal profile as well.

Conclusion: Thus, the validated method protocols, developed in proposed study in accordance with international standards, undoubtedly stand as a benchmark for the analysis and standardization of *Terminalia* species along with their traditional products. The results of the validation process assure that these methods hold great promise as cost-effective alternatives to establish but expensive techniques such as gas chromatography and high-performance liquid chromatography. Hence, the

elemental analysis conducted within this study plays a pivotal role in understanding the elemental role along with physiological processes and therapeutic potential of these plants. By performing the fingerprinting profile and elemental composition of these commercially significant *Terminalia* species, we not only explored their quality and authenticity but also uncover potential health benefits in addition to their safety profiles as well. This comprehensive exploration not only enhances their utility in traditional medicine but also supports the sustainable management of ecosystems that opens the door to new avenues for advanced analysis of complex phytochemical.

Keywords: *Terminalia*; HP-TLC; USP; *Terminalia* species; Western Ghats; ICP-OES; ICP-MS

Chapter 1 - Introduction and Background of Study

Natural resources are enriched sources of medicinal plants that constitute an enhanced rich therapeutic value. The interest lies in effective utilization of medicinal herbal plants, which are refined sources of abundant therapeutic values. This usefulness has been increasing over time due to their numerous documented implementations and by-products [1, 2]. The growing demands for the requirement of these plants as raw materials is worthy due to their easy availability, cultural acceptance, holistic nature, wider scope, and comparatively lower cost [3]. The structural composition of natural products offers a wide spectrum of opportunities for drug discovery and the development of lead molecules [4]. Inspite of the fact that, Natural products have long been recognized for their role in maintaining human health, their chemical composition has not been fully explored and defined until the 19th century [5]. In the 20th century, natural product research gained momentum as a means of developing new lead molecules for treatments of diverse health conditions [6,7]. Despite their remarkable potential, secondary metabolites from indigenous natural sources originating from North Western corridor of India have not been extensively studied globally for the production of newly discovered phyto- medicines [8,9]. Natural medicines currently account for approximately 35% of the total therapeutic market, equivalent to around 385 billion US dollars [10].

The investigation of medicinal plants listed in various herbal pharmacopoeias around the world has been initiated to search for novel potent therapeutic agents, especially considering the increasing prevalence of non-communicable diseases responsible for 70% of global mortality [11,12]. In accordance with the norms of World Health Organization (WHO), additionally than 60% of India's rural population rely upon herbal medicines. Traditional medicinal systems, including herbal remedies, are easily

accessible, safe, affordable, and therapeutically potent, which explains their widespread usage [13]. In addition to their therapeutic benefits, many plants are also widely used as food supplements [14], healthcare properties [15], veterinary medications [16], and other related products. In urban contexts, traditional medicine and herbs are often used as self-medication and as an alternative for modern systems of medicine and routine healthcare, especially in less privileged areas. The adoption of a holistic approach to human health forms the basic foundation of "traditional" or regional medicinal systems [17]. These medicinal systems often recommend complex herbal constituents and polyherbal extracts to give treatment for a multiple medical condition, including of persistent and degenerative diseases [18, 19].

Humanity still prefers the framework of traditional medication; however, currently there are no appropriate standardization strategies to determine its consistency, quantity and quality. Chromatographic and spectrophotometric methods are recommended for standardization and quantification of vital biomarkers of polyherbal medicinal and preparations by the WHO guidelines [20]. Quality control and standardization of raw ingredients and traditional medicinal plants are the prime conditions for their effectiveness in Western medicinal treatments. These measures ensure the right of customers to receive high-quality, tested, effective, healthy and essential solutions [21]. As per specified global regulatory protocols manufacturers must assess the quality of traditionally utilized medicinal plant according to current good manufacturing practice (c-GMP). As a safety precaution certified laboratory must evaluate the identification, content, and integrity of constituents in crude medication sources as well as processed final products. The prerequisite for this process is the development of scientifically validated methods [20, 22-24]. Pharmacopoeias and other official documents provide ready procedures and conditions for quality control of various herbs used in preparation of

conventional medicines and convene c-GMP requirements [25, 26]. However, such data arefinite and available only for individual herbal medicinal substances and fewer herbal preparations or products [27].

One of India's biodiversity hotspots is the Western Ghats, which is home to a well-known plant called *Terminalia*. The National Institute of Traditional Medicine (NITM), a division of the Indian Council of Medical Research (ICMR), has claimed diverse components of *Terminalia* species such as (fruits, bark, seeds, leaves, stem, etc.) possess spectrum of medicinal properties. These species as per survey of NITM has been widely distributed in North Central corridor of Western Ghats [28, 29].

Terminalia, belonging to the Combretaceae family, constitutes one of the largest genera worldwide, comprising over 250 species distributed across South Asia, the Himalayas, South Africa, Madagascar, and Australia. Among them, more than 50 *Terminalia* species are utilized as health supplements [30]. *Terminalia* species have significant nutraceutical value and provide numerous health benefits, including the treatment of specific ailments [31]. Ayurvedic system of medicine in India is well-known for their widespread use of *Terminalia* species enriched with ethnopharmacological properties [32]. *Terminalia* species consist of both unexplored and well-known established constituents, including glycosides, tannins, phenols, carbohydrates, flavonoids, saponins, and proteins [33]. Due to their varying quantities of diverse phytoconstituents, with medicinal potential, ethnobotanical utilization, and bioactivity, *Terminalia* species present interesting research opportunities and promise many path forwards for research of new drug lead molecules [34].

In this research, *T. arjuna, T. bellirica, T. chebula,* and *T. catappa* are four species of trees belonging to the *Terminalia* genus selected for in depth exploration. Throughout diverse cultures, these species have a rich history of being utilized for their medicinal

and practical advantages. Each species offers distinct properties and applications, making them valuable in traditional systems of medicine and other practical domains. *Terminalia* species hold historical significance as they have been used for various purposes throughout history. In the realm of traditional medicine, encompasses practices like Ayurveda, Traditional Chinese Medicine (TCM), and African medicine, the therapeutic properties of *Terminalia* species have been extensively utilized. The historical use of *Terminalia* species can be traced back to ancient texts, folklore, and indigenous knowledge systems, making them an integral part of cultural practices and traditional remedies. *Terminalia* species have a wide range of traditional uses across different cultures worldwide. For their medicinal and therapeutic benefits, various fragments of the tree, the fruits, leaves, stem, roots, and bark, are utilized. These species have traditionally been used to treat a variety of diseases, including disorders of digestion, respiratory conditions, skin ailments, and wound healing. Additionally, *Terminalia* species have been used as a source of dyes, tannins, and timber in various industries. *Terminalia* species are extensively used in the formulation of traditional medicinal and herbal remedies. Their bioactive compounds, including tannins, flavonoids, triterpenoids, and phenolic acids, contribute to their pharmacological properties. These species have been incorporated into medicinal preparations for their antioxidant, anti-inflammatory, antimicrobial, anti-diabetic, hepatoprotective, and anti-cancer activities. *Terminalia arjuna*, *Terminalia chebula*, and *Terminalia bellirica* are among the most well-known species used in medicinal formulations [35-38].

Terminalia arjuna (Roxb.) Wight & Arn., a widespread species abundantly situated in the Asian subcontinent, is an indigenous medicinal plant. It is a deciduous and evergreen tree which grows up to 20–30 m (height) above the ground, and there are about 24 species of it in India [39]. It has been discovered that the various plant parts of *T.*

arjuna, including the bark [13], fruit [40], seed [41], leaf [42], and root [43], each have unique therapeutic characteristics. It is one of the folklore plants that are most frequently utilized in Ayurveda Siddha, and Unani medical systems. Arjuna/Arjun (Hindi), Sadaru (Marathi), Yellamaddi (Telugu), Neermatti (Kannada), Arjhan (Bengali), Sadad (Gujarati), and Marudhu (Tamil and Malayalam) are some of the local/ regional names for *T. arjuna* used in India [44-46]. The bark contains a wide range of phytoconstituents such as arjunic acid, arjunolic acid, gallic acid, ellagic acid [47, 48]. Among the medicinal properties of *T. arjuna*, its barks are found to contain various beneficial attributes. One of the important bioactive components in the heartwood of *T. arjuna* (Roxb.) Wight & Arn. is arjunolic acid, a triterpenoid of the oleanane class, as proven in studies [49]. The treatment of bark reduces symptoms by lowering oxidative stress, pro-inflammatory cytokines, and chemokine production [50]. The (stem-bark) of *T. arjuna* has a high concentration of antioxidant components such as tannins, flavonoids, glycosides and inorganic minerals, are present. The terpenoids found in the bark: β-sitosterol, terminic acid [51], terminosideA and arjunaphthanoloside. Terminoside A and arjunaphthanoloside are specifically important because of their potential medical properties. Terminoside-A prevents the formation of nitric oxide and lowers its concentration in lipopolysaccharide-activated macrophages, while arjuna naphthanoside has a strong antioxidant effect [52, 53]. Compared to other plants, *T. arjuna's* (bark) contains a significant sum of antioxidant flavonoid components [54, 55]. Among these flavonoids arjunolone, bicalein, flavones, kampferol, pelorgonidin, quercetin, and luteolin are major antioxidants [56, 57]. *T. arjuna* exhibits a range of pharmacological characteristics when used in treatment of various clinical problems including heart failure, ischemia, cardiomyopathy, atherosclerosis, myocardial necrosis, tumors, viral infections, ulcers, and more.[13]In addition to numerous other bioactive properties [58], hepato-protective [43],

cardio-protective [39, 59] , anti-inflammatory [60], anti-tumor [61], and anti-atherosclerotic [62], it has been highlighted that the phytochemicals extracted from *T. arjuna* consist of glycosides, phenols, flavonoids, tannins, and triterpenoids [63].

Terminalia bellirica (Gaertn.) Roxb., one of the oldest species in the Indian subcontinent and including neighbouring countries such as Bangladesh, Nepal, Southeast Asia, Africa and America, has traditionally been utilized for its pharmacokinetic and pharmacodynamic properties, as well as its biotherapeutic potential, preventive purposes, and characteristics [64, 65]. It has various regional common names: (Beleric Myrobalan in English; Bhomora, Bhaira, Bhomra in Assam; Bahedam, Bahera in Hindi; Baheda in Marwari; Tare, Shantikayi, Tarekayi, Shanti in Kannada; Vibhitakami, Tani in Telugu Tannikai, Tanni in Malayalam; Baheda in Gujarati; Baheda, Bhara in Oriya; and Vibhita, Aksa, Aksaka, Bibhitaki in Sanskrit [66]. The fruits contain a wide range of phytoconstituents, including glycosides, flavonoids, tannins, phenols, saponins, carbohydrates, and proteins [34]. Additionally, the fruits contain a greenish-yellow oil, gallo-tannic acid, coloring agents, and resins [67]. Other compounds found in *T. bellirica* include glucoside (bellericanin) [68], tannins, ellagic acid [69], gallic acid, lignans, β-sitosterol, mannitol, glucose, fructose, rhamnose [68, 70], galloyl glucose, chebulagic acid [71], chebulinic acid [65], ethyl gallate (phenyllemblin)[72], and hexahydroxydiphenic acid ester [71] (termilignan and thannilignan). Furthermore, Anolignan B and 7-hydroxy-3',4-hydroxy-3' (methylenedioxy) flavone [73], as well as luteoline [69], are also present.

The green fruit (decoction) is employed in the treatment of coughs, whereas the fruit pulp is utilized for conditions like piles, dysenteric-diarrhea, leprosy, and dropsy. The half-ripe fruit acts as a purgative, and the kernel of the fruit has narcotic properties. In Khagrachari, the fruits are utilized to treat menstruation irregularities, while the seed oil

is exercised for rheumatism. The bark functions as an immune-modulator, while the gum of the bark possesses purgative and demulcent properties [74]. *T. bellirica* fruits are known for their traditional medicinal benefits, including antioxidant [75], antifungal [76], antimicrobial [77], analgesic [78], anti-diabetic [79], anti-depressant [80], anti-diarrheal [81], anti-fertility [82], anti-helminthic [83], anti-androgenic [82], anti-hypertensive [84], anti-HIV-1 [73], anti-pyretic [78], anti-salmonella [85], anti-secretory [86], anti-spasmodic, bronchodilator [87], antithrombotic, thrombolytic [88], anti-ulcer [89], anti-plasmodial [90], hepatoprotective [91], anti-alzheimer's [92], anti-atherogenic [93] properties. They are also used for treating cough, spleen-related issues, wound healing, gastrointestinal disorders, flatulence, clearing bowels, and dysentery [94]. The triterpenoids found in the fruits exhibit strong antimicrobial [78] properties. The entire plant acts as a β-lactamase inhibitor [95] and possesses anticancer [96], anti-inflammatory [74], antibiofilm [97], and anti-mutagenic [98] properties.

Terminalia chebula Retzius, a medium-to-large-sized tree, is widespread all through Asia, specifically in countries such as India, Nepal, Vietnam, Bangladesh, Pakistan, China, Thailand, Taiwan, Sri Lanka, and Myanmar [99-102]. It is known by various common names, including "black myrobalan", "ink tree", otherwise in English "chebulic myrobalan"; "Haritaki" in Bengali and Sanskrit; "Harad" in Hindi; "Harada" in Gujrati and Marathi; "Karkchettu" in Telugu; and "Kaduk-kaya" in Tamil. *T. chebula* is often called the "King of Medicine" in. Tibet owing to its reputation for curing various illnesses or its association with the god Shiva, known as "haritaki" (Hara) in Indian tradition. According to the legend, the plant is said to have sprouted from the ambrosia i.e. (Amrita) that fell to the ground after the god Indra shattered it. Ayurvedic therapies commonly use *T. chebula* to treat digestive issues, act as astringent, anti-pyretic, expectorant, spasmolytic, anti-viral, tonic, antiasthmatic, and anti-viral

hypoglycemic disorders [103]. The seeds, leaves, fruits and bark of *T. chebula* are widely used in traditional folk medicine. It has anti-inflammatory, antibacterial, antioxidant, anti-fungal, antidiabetic, anti-tumor, anti-cancer, anti-HIV and anti-aging properties [104-113]. It contains bioactive substances such as tannins, alkaloids, resins, phenols, amino acids, flavonoids, sterols, and fructose. The fruit of *T. chebula*, also known as myrobalan, is considered one of the richest sources of ascorbic acid. It contains intense tannins such as ellagic acid, terflavin A, arjunic acid, galloyl glucose, gallic acid, chebulic acid, corilagin, Chebulagic acid, punicalagin, chebulinic acid, and tannic acid. Flavonoids like quercetin, catechin, and kaempferol are also present. The fruit contains saccharides like D-glucose and fructose, shikimic acid and quinic acid [114-116].

The *T. chebula* (fruits) possess tonic, digestive, expectorant, and anti-dysenteric properties. They are used as rejuvenators, laxatives (when unripe), astringents (when ripe), anthelmintics, nervines, expectorants, tonics, carminatives, and hunger appetizers. They are prescribed to treat various ailments and symptoms, including anorexia, diarrhea, chronic intermittent fever, piles, leprosy (including skin illnesses), anaemia, narcosis, cough, and excessive mucus secretion. When powdered, the fruit is useful for treating chronic ulcers, tooth decay, and bleeding. The bark acts as a diuretic and a heart tonic [117]. *T. chebula* fruit contains 14 essential oils, with primary compounds such as palmitic acid, 5-methyl-furfural, phenylacetaldehyde, and furfural [118].

India ranks second in the world for cancer deaths among women and first for deaths from breast cancer. *T. chebula* is used in homoeopathic preparations for treating breast cancer and other forms of cancer. Additionally, it has been used to treat diabetes mellitus, a prevalent disease affecting the global population [119]. *T. chebula* is frequently employed in traditional Indian and Iranian medicine to address dementia, constipation,

and diabetes [120]. Triphala, a popular Ayurvedic remedy, is made from the powder of two *Terminalia* species, including *T. chebula* and *T. bellirica*. Moreover, *T. bellirica* (Gaertn.) Roxb. since ancient times it has an extended legacy of utilization in traditional and Thai-folk medicine to treat and prevent diseases that commonly affect the elderly population [121]. In Ayurvedic and Thai folklore medicine, *T. bellirica* and *T. chebula* (fruits) are frequently combined to produce Triphala, a conventional poly-herbal preparation [32].

Terminalia catappa Linn is a naturally occurring tree that is extensively spread around in the sub-tropical and tropical zones of the Indian Oceans, Southeast Asia, and Pacific. In numerous countries, it is widely cultivated as an ornamental tree, adding to its aesthetic appeal. Traditional medicinal use of certain *Terminalia* species in East and West African countries to treat infectious diseases has been reported [122, 123]. *T. catappa* exhibits an abundant profile of macro and micronutrients. According to research evidence, the extracts of *T. catappa's* roots, bark, fruits, and leaves, when combined with petroleum jelly, demonstrate antioxidant, anti-diabetic, anti-inflammatory, antimicrobial, antibacterial, and anticancer properties. The medicinal efficacy of plants is attributed to phytochemicals such as (ellagic acid, Punicalagin, Chebulagic acid, granitin B, gallic acid, Punicalin, and geranin), terpenoids (Ursolic acid, Asiatic acid), and flavonoids (Isovitexin, Rutin, and Vitexin) [124]. *T. catappa* matured ripe fruits, commonly referred to as "tropical almonds" or "Indian almonds," can be consumed raw fresh or roasted. They are a good resource for fatty-acids with a nutrient density and potential cardiovascular properties. *T. catappa* phenolic extracts consist of compounds such as quercetin, epicatechin, caffeic acid, ellagic acid, quercitrin, gallic acid, iso-quercitrin, catechin, chlorogenic acid, rutin, and kaempferol [125].

Studies on the bioactivity of *T. catappa* leaves and bark extracts have revealed their potential anti-inflammatory, anti-HIV reverse transcriptase, anti-cancer and hepato-protective effects. Extracts of *T. catappa* fruits using petroleum ether, methanol, and aqueous solutions have exhibited anti-diabetic activity. Additionally, the seed (kernel) of *T. catappa* has been associated with aphrodisiac properties [126]. This tree has a rich history of traditional use, with the leaves being utilized in traditional medicine for their antibacterial, antifungal, anti-inflammatory, and analgesic properties. They are often applied externally to treat skin infections, wounds, and various dermatological conditions [123]. Moreover, the seeds of *T. catappa* are consumed as a food source and are known for their potential anti-diabetic and anti-hyperlipidemic effects [127]. The figure (Fig. 1) illustrates the study's flow, detailing the phytochemical investigation and therapeutic activity found in four selected species of *Terminalia* [52-61, 75-79, 104-111, 124,125].

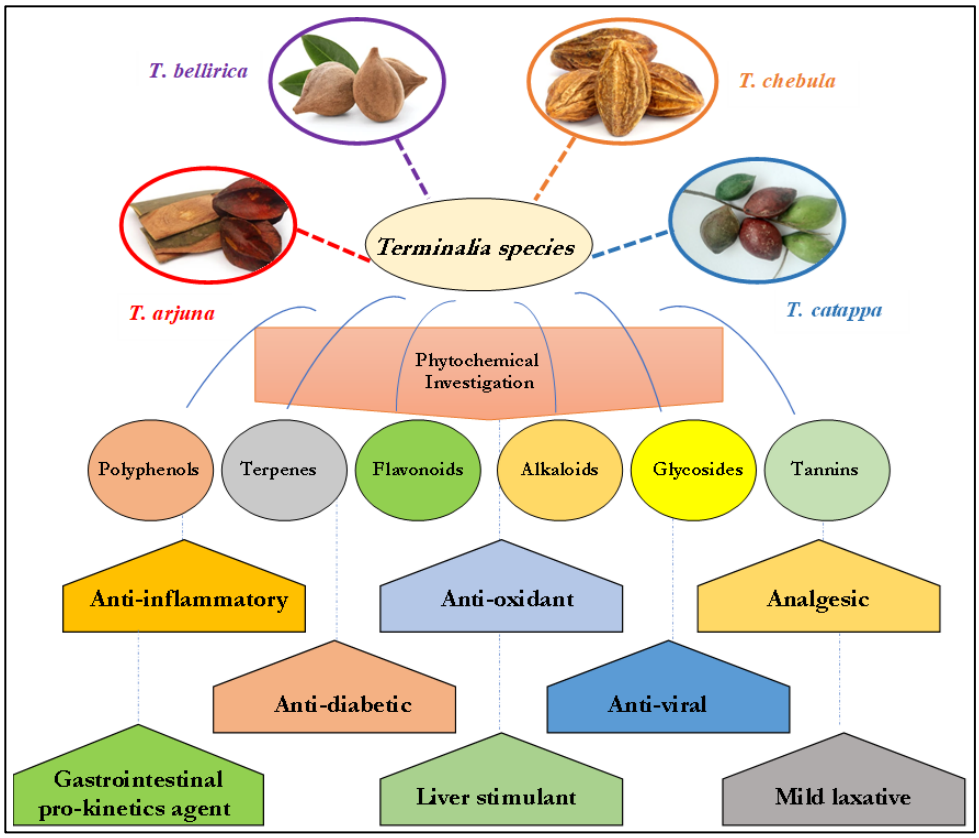


Fig.1: Phytochemical investigation and therapeutic activity present in *Terminalia*

They have gained recognition and attention not only in traditional systems of medicine but also in modern regulatory guidelines. The therapeutic potential of *Terminalia* has been acknowledged by the ICH and the WHO and they have issued guidelines for its quality control and standardization. The WHO studies/monographs on selected medicinal plants include *Terminalia* species being *T. chebula*; *T. arjuna;* and *T. bellirica.* These monographs provide comprehensive information on their botanical description, traditional uses, chemical constituents, and quality control parameters. Similarly, the ICH guidelines emphasize the importance of botanical identification, phytochemical profiling, and quality control measures for herbal medicines containing *Terminalia*. These guidelines ensure the safety, efficacy, and consistency of *Terminalia* based formulations. The inclusion of *Terminalia* in the WHO and ICH guidelines reflects the growing recognition and acceptance of *Terminalia* as a valuable medicinal plant, promoting its responsible use and standardization in modern healthcare practices **11, 128-130**.

High performance thin layer chromatography (USP-HP-TLC) densitometric fingerprinting is a systematic analytical approach employed to comprehensively analyze and assess the quality of complex mixtures, such as herbal medicinal and plant extracts. USP-HP-TLC fingerprinting involves the separation, identification and detection of multiple compounds in a sample, generating a unique chromatographic pattern or "fingerprint" that serves as a characteristic identifier. As per the global guidelines, it enables the simultaneous analysis of multiple/numerous compounds, making it easier to identify and quantify essential constituents in complex samples. The generated fingerprint can be used for quality control, batch-to-batch consistency, and authentication purposes. HP-TLC fingerprinting has gained significant attention in recent years, with numerous studies focusing on its applications in botanical analysis,

quality control of herbal products, and determination of adulterations [131-133]. HP-TLC fingerprinting has gained recognition and been incorporated into guidelines by regulatory bodies such as the United States Pharmacopeia (USP) and the International Council for Harmonisation of Technical Requirements of medicinal products for Human Use (ICH). These guidelines emphasize the impact of HP-TLC fingerprinting as a quality-control instrument for herbal medicinal and botanical outcomes. The inclusion of HP-TLC fingerprinting in the USP and ICH guidelines showcases its significance in ensuring standard quality, safety, and efficacy of herbal products [130, 134]. Additionally, the instrumentation and equipment required for HP-TLC combines the simplicity and cost-effectiveness. These HP-TLC is generally more affordable compared to techniques like HPLC or gas chromatography (GC). This unique combination makes HP-TLC a versatile and reliable analytical tool in various scientific disciplines offering rapid separations and shorter analysis times compared to other chromatographic methods. The impact of advanced sorbents, such as silica gel of high-purity and modified stationary phases, allows for faster analyte migration. Consequently, the sample throughput is significantly increased, making HP-TLC suitable for high-throughput analysis. It requires minimal sample preparation and utilizes small amounts of solvents and stationary phases. The lower cost associated with HP-TLC makes it accessible to a wider range of laboratories and researchers. The ability to utilize multiple stationary phases and mobile phase compositions enhances separation selectivity, resulting in superior peak resolution. This makes HP-TLC an excellent choice for the analysis of compounds with similar structures or closely related chemical properties. HP-TLC offers excellent sensitivity, allowing the detection of even trace/small amounts of analytes. The development of highly sensitive detection methods, such as UV absorbance, fluorescence, and chemiluminescence, enables

identification/detection of compounds at low concentrations. Additionally, the availability of specialized sample application techniques, such as multiple sample spotting and band broadening reduction, further enhances the sensitivity of HP-TLC. It provides reliable quantitative analysis due to its compatibility with densitometric scanning. Densitometric measurements allow for precise quantification by correlating the intensity of the separated spots with the analyte concentration. HP-TLC also enables simultaneous analysis of multiple analytes within a single chromatogram and time-saving approach. HP-TLC has a wide range of applications in innumerable fields, like pharmaceutical analysis, forensic science, food analysis, and natural product research. Its versatility lies in its capacity to handle an extensive variety of sample types, encompassing solids, liquids, and complex matrices. HP-TLC can be employed for the analysis of diverse compounds, including small molecules, natural products, drugs, pesticides, and plant extracts [135-138]. It plays a vital role in identifying and analyzing adulteration in various products. By comparing the chromatographic profiles of authentic and suspected samples, HP-TLC enables the identification of adulterants or impurities present. It has been used to detect adulteration in food, herbal medicines, cosmetics, and other products [139].

In plant analysis, reversed phase high performance thin layer chromatography (RP-HP-TLC) is commonly employed for the detection, separation, identification, and quantifying of different chemicals. It also combines the advantages of reversed phase chromatography property of stationary phase efficiently separating and analyzing complex polar plant metabolites. In RP-HP-TLC, a stationary phase with hydrophobic characteristics is employed, allowing for the moderately division of polar and non-polar compounds present in herbal extracts. This technique is particularly useful for analyzing lipophilic compounds, such as phenolic acids, flavonoids, alkaloids, and

terpenoids, which play crucial roles in the biological activities of plants. It requires minimal sample preparation and provides good resolution of complex plant extracts. The technique enables qualitative and quantitative analysis of multiple components simultaneously and has been applied in phytochemical profiling, quality control (QC) of herbal medicinal plants, and the identification of bioactive mixtures/compounds in plants. As research in the field of plant analysis continues to go deep, RP-HP-TLC remains a valuable tool for the analysis of plant metabolites, aiding in the discovery and characterization of bio-active complexes from several plant and related sources [140-142].

Liquid chromatography coupled with mass spectrometry (LC-MS/MS) is widely utilized for the identification, separation and quantification of various compounds. The separation, characteristics of liquid chromatography are combined with the highly specific and precise determination of mass spectrometry in LC-MS/MS. By enabling the examination of intricate mixtures of plant metabolites, such as phytochemicals, hormones, pesticides, and secondary metabolites. LC-MS/MS enables researchers to characterize plant compounds, elucidate metabolic pathways, and investigate the effects of environmental factors on plant metabolism. It has revolutionized plant analysis by providing high sensitivity, specificity, and throughput [143, 144].

In order to establish rapid, frugal, and coexisting analytical techniques like AAS, ICP-OES, and ICP-MS, it has become imperative to focus on evaluating elemental estimation for assessing the beneficial and toxic effects of plants. Adequate understanding of the basic components present in plants is crucial for human well-being. For foodomics and linkage toxicological studies, recent advancements have facilitated the early-stage screening of probable hazardous synthetic contaminants, such as aflatoxins and heavy metals, during prescription/medication development and the discovery of new molecules. These innovative approaches contribute to the scientific

evidence regarding the safety of plants. The increasing interest in natural processes and the chemical composition of plants for therapeutic, dietary, and environmental purposes has spurred the need for advanced studies on metal profiling related aspects. However, stringent food norms and regulations have brought attention to the entry of toxic metals into the environment, posing risks to a significant portion of the population. Plants serve as valuable sources of micro and macro essential nutrients, with various uptake mechanisms for salts, metal-ions, and minerals from the soil. These essential components play critical roles in maintaining osmotic balance, serving as crucial elements in sugars and proteins, participating in metabolic processes e.g., magnesium (chlorophylls), phosphorous (ATP), zinc (growth), copper (root metabolism), and iron (photosynthesis), and potassium (tissue cells and membranes) acts in enzyme inhibitors calcium (stress responses), potassium as a growth controller, and nickel [145].

Atomic absorption spectroscopy (AAS) is one of the routine analytical techniques in plant analysis for determining the concentration of various elements. In plant analysis, AAS finds applications in studying essential and non-essential elements in plant tissues, determining the concentration of vital nutrients like (magnesium, calcium, potassium), in addition to toxic elements estimation like (lead, cadmium, and arsenic). It provides valuable information about nutrient deficiencies, environmental contamination, and overall plant micro-macro components. By employing AAS, researchers can assess nutrient uptake, optimize fertilizer application, monitor soil quality, and investigate the impact of environmental factors on plant growth. Moreover, AAS is a technique that requires minimal sample preparation, making it suitable for routine analysis of large sample sets [146, 147].

Inductively coupled plasma optical emission spectroscopy (ICP-OES) is a systematic tool extensively exploring deepness on elemental estimations. ICP-OES utilizes an

inductively coupled plasma as the ionization source, which generates high-temperature plasma to atomize and excite ion elements in the sample. The emitted fragments are then measured to regulate the elemental configuration. In plant analysis, ICP-OES enables the simultaneous analysis of multiple elements, providing valuable information about nutrient content, heavy metal contamination, and overall plant health. It is used for quantifying essential elements like iron, manganese, and zinc, as well as non-essential elements such as cadmium and mercury. ICP-OES offers several advantages, including wide dynamic range, multi-element capability and high-sensitivity. It serves as an invaluable tool for agricultural and environmental research, enabling specific and accurate purpose of trace elements in plant samples [148, 149].

The ability to identify of trace and ultra-trace elements frequently relies on the versatile analytical technique of inductively coupled plasma mass spectrometry (ICP-MS). It coalesces the sensitivity of mass spectrometry with the potential of inductively coupled plasma to atom excitation of the sample. It enables simultaneous multi-element analysis, allowing researchers to measure an extensive range of elements in plant-based samples. By using ICP-MS, scientists can study critical and toxic elements in plants, assess nutrient uptake, investigate metal accumulation, and monitor environmental contamination. The technique offers high sensitivity, facilitating lower detection limits, and excellent precision, making it a valuable tool for comprehensive plant analysis [150].

The analysis of *Terminalia* (*arjuna*, *bellirica*, *chebula*, and *catappa*) using various analytical techniques provides valuable insights into their phytochemical composition and potential bioactive compounds. Among the distinctly diverse chromatographic techniques, for desired purpose; HP-TLC stands out as a prevailing tool for the qualitative-quantitative investigation of phytochemicals in these plant/herbal species. HP-TLC fingerprints also significantly affect the quality control (QC) profiles of a

class of different compounds (alkaloids, flavonoids, phenols, tannins, antioxidants, etc.) present in *Terminalia* species. The added detection accuracy of spectro-densitometric scanning increases resolution and effectively enables fast, robust and cost-effective performance. This confirms that HP-TLC is a versatile instrument/tool, making it the first choice for regular analysis and quality control of *Terminalia* species in all medicines.

Nevertheless, to gain a deeper understanding of the complex chemical profile and confirmation of structural elucidation of specific compounds and metal estimation, advanced techniques like LC-MS/MS,AAS,ICP-OES, and ICP-MS are undeniably crucial requisites. LC-MS/MS provides valuable information about the presence of bioactive compounds, their fragmentation patterns, and potential metabolites in *Terminalia* species. It offers enhanced sensitivity, selectivity, and the ability to identify and quantify compounds at trace levels, contributing to the outright characterization of phytochemicals responsible for their therapeutic properties. AAS, ICP-OES, and ICP-MS truly exhibit significant roles in the analysis of elemental composition and heavy metal content in any herbal and Ayurvedic formulations. These techniques enable the quantification of essential elements, trace elements, and potentially toxic metals present in plant materials. They provide the overall safety profile of these plant species, ensuring their suitability for consumption and use in herbal preparations.

Thus, with aforementioned exhaustive and comprehensive literature survey and scientific evidence-based information, a fusion of coalescence of spectro-chromatographic tools the proposed (study) research was focused on selected four *Terminalia* species of North Central Corridor of Western Ghats utilized most widely in Indian system of medicinal plants and herbs. The (HP-TLC, LC-MS/MS, AAS,ICP-OES, and ICP-MS) techniques are promising tools surely enabling researchers to

explore the phytochemical diversity, identify bioactive compounds, and assess the elemental composition of similar plant species. Such insights contribute to the understanding of their medicinal properties, quality control, *in-vitro* assay and safety evaluation, furthermore ultimatelysupporting their utilization in traditional medicine, nutraceuticals, and pharmaceutical industries and related sectors of research of National Importance.

Chapter 2 – Aims & Objectives

- **Aim:**

To design and develop a validated regulatory fingerprinting protocol for selected *Terminalia* species by chromatographic (HPTLC) tool.

- **Objectives:**

❖ **Primary Objectives:**

- ✓ To develop novel regulatory compliant fingerprint profiles for identification of simultaneous multi phytochemical composition of four *Terminalia* species by HP-TLC.
- ✓ To validate the developed protocol with HP-TLC of the four *Terminalia* species for highlighting their medicinal and therapeutic values.

❖ **Secondary Objectives**

- ✓ To perform phytochemical profiling using advanced chromatographic and spectrophotometric tools like LC-MS/MS.
- ✓ Screening of elemental profile of four selected *Terminalia* species for evaluating its safety in routine extracts by using AAS, ICP-MS and ICP-OES.

Chapter 3 – Review of Literature

As part of this thesis research, a comprehensive and systematic literature survey was conducted, focusing on the traditional medicinal uses of *Terminalia* species, their phytoconstituents, and therapeutic applications. Additionally, an innovative USP-HP-TLC technique were developed, approved and validated in accordance with ICH-21guidelines and USP monographs. Detailed instrumental analysis, including HP-TLC, followed by LC-MS/MS, AAS, ICP-OES, and ICP-MS, was carried out to obtain in-depth information on the method used for the development and validation of phytoconstituents, including validation parameters and the overall process.

❖ Therapeutic Potential and Pharmacological Significance:

Amongst the diverse array of medicinal plants in the Western Ghats, *Terminalia arjuna*, *Terminalia bellirica*, *Terminalia chebula*, and *Terminalia catappa* have gained significant attention for their remarkable therapeutic potential, prompting their selection over other species. This choice is evaluated by their extensive historical use in traditional medicinal systems and their substantiated pharmacological properties, as highlighted by recent literature.

The studies documented for, *Terminalia arjuna*, have beenrecognized for its cardioprotective attributes, has been a focal point due to its role in managing cardiovascular disorders. Studies have demonstrated its effectiveness of *T. arjuna* in improving cardiac function and alleviating conditions like heart failure and hypertension [39, 59]. *Terminalia bellirica*, acknowledged for its potent antimicrobial and antioxidant properties, has exhibited broad-spectrum antibacterial activity against pathogens [75-77]. *Terminalia chebula*, renowned for its versatile pharmacological effects, including antioxidant, anti-inflammatory, and antimicrobial activities, has

been investigated for its role in various therapeutic applications [104-113]. *Terminalia catappa*, valued for its anti-inflammatory and analgesic potential, has been explored for its traditional use in pain management and inflammation-related disorders [124]. The four selected *Terminalia* species possess a wealth of bioactive compounds such as flavonoids, tannins, and polyphenols, responsible for their diverse medicinal actions. Moreover, the plants prevalence of these species in the Western Ghats, a biodiversity hotspot, has propelled their selection, as their study aligns with efforts to conserve and utilize the region's rich flora [151].

The literature review focused by Kannan M. et al. emphasized on the quality control analysis of Thiripala chooranam, a traditional medicine used for ulcer and skin diseases, along with its plant extract. The importance of standardization and quantification in Siddha medicine to ensure product quality, safety, and efficacy, especially for diabetes treatment. The study identified starch grains in the medicine, suggesting its potential in managing pre-diabetes (IGT) and along with this HPLC analysis was also conducted for further evaluation [152]. Tiwari M. et al. highlighted the phytochemicals present in *T. chebula* and its medicinal formulations, which are widely used for treating various ailments such as diarrhea, gastroenteritis, asthma, ulcers, and more. The plant exhibited multiple beneficial effects, including antibacterial, anti-diabetic, antiviral, hypocholesterolemic, anti-plasmodial, and antinociceptive properties [153].

In the case of *Terminalia bellirica* fruit, Bharathi V. et al. performed a phytochemical analysis, identifying various bioactive substances with potent antibacterial potential in its ethanolic and methanolic extracts. The study confirmed the presence of tannins, saponin, steroids, alkaloids, polyphenols, flavonoids, anthraquinone, terpenoids,

triterpenoids, coumarins, and glycosides. The extracts demonstrated strong antibacterial activity against harmful bacterial strains, suggesting their potential use in treating bacterial related illnesses. However, the aqueous extract showed limited effectiveness against the tested microbiological strains [154].

To verify the pharmaco-therapeutic effects and biological properties of various plant species belonging to the *Terminalia* genus, Das G. et al. undertook a systematic review. The review highlighted the importance of assessing and confirming the healing potential of these plants, which have long been utilized in conventional medicine and incorporated into numerous herbal formulations. *Terminalia* species are known to be utilized intreating a range of conditions such as fever, headache, pneumonia, flu, cancer, memory enhancement, cough, back pain, and leprosy. The information gathered from this review can contribute to the development of potential medicinal applications, including the treatment of viral infections [155].

- **Phytochemical constituents Fingerprinting by HP-TLC and HPLC-MS:**

The exploration of phytoconstituents and fingerprinting through High Performance Thin-Layer Chromatography (HP-TLC) has gained significant attention in recent years due to its potential in assessing the therapeutic potential of medicinal plants. *Terminalia arjuna*, *Terminalia bellirica*, *Terminalia chebula*, and *Terminalia catappa*, endemic to the Western Ghats, have been used in traditional medicine for their diverse health benefits. Conducting HP-TLC phytochemical analysis and fingerprinting of these plants could provide valuable insights into their chemical composition, aiding future studies and applications. HP-TLC offers a robust method for the identification and quantification of phytoconstituents within plant extracts. By utilizing this technique, researchers can identify bioactive compounds like flavonoids,

tannins, and alkaloids present in *Terminalia* species, elucidating their potential pharmacological effects. Furthermore, HP-TLC fingerprinting allows for the comparison of chemical profiles among different plant samples, aiding in quality control and standardization of herbal medicines [156].

Given the rich biodiversity of the Western Ghats, analyzing these *Terminalia* species can provide a comprehensive understanding of their regional variation in phytochemical composition. This could lead to the discovery of novel bioactive compounds and facilitate the development of new pharmaceuticals or nutraceuticals. These *Terminalia* species possess a rich repository of bioactive compounds such as flavonoids, tannins, and alkaloids, which are responsible for their traditional medicinal properties. HP-TLC enables the identification, quantification, and profiling of these phytoconstituents, facilitating their isolation and characterization. Furthermore, the technique's ability to generate reproducible and standardized fingerprints of plant extracts aids in quality control and formulation development.

The utilization of advanced analytical techniques, such as High-Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry (MS), for phytoconstituent analysis and fingerprinting of *Terminalia* species, represents a critical approach in understanding their chemical composition and medicinal potential. This choice is substantiated by the intricate nature of their bioactive compounds and their diverse therapeutic applications. HPLC-MS offers unparalleled sensitivity and specificity in detecting and quantifying a wide range of phytochemicals, including flavonoids, alkaloids, and polyphenols, present in these *Terminalia* species. This technique enables the identification of unique bioactive constituents, contributing to a comprehensive profile of their chemical composition.

Additionally, fingerprinting through HPLC-MS aids in assessing the overall quality and consistency of herbal preparations, pivotal for standardization and efficacy [157, 158].

Conducting HPTLC based phytoconstituents analysis and fingerprinting of *Terminalia arjuna*, *Terminalia bellirica*, *Terminalia chebula*, and *Terminalia catappa* from the Western Ghats not only promises a fruitful avenue for future research but also holds immense potential. This methodology enhances our comprehension of their chemical composition, thereby facilitating the development of innovative therapeutic interventions. Simultaneously, it underscores the significance of conserving the rich biodiversity within the Western Ghats ecosystem. In sum, this HP-TLC based analysis offers a profound insight into the chemical makeup of these *Terminalia* species, ultimately advancing our understanding of their therapeutic prospects and reinforcing the need for their preservation within the bio diverse Western Ghats environment. Munawar TM. et al. presented a methodology for optimizing parameters and quantifying chebulinic acid in medicinal plants using HPLC. By understanding the chebulinic acid content in different medicinal plant extracts, valuable insights were obtained for potential therapeutic applications, aiding in determining weight dosages, pH, and time parameters [159].

Despite significant advancements in chemical synthesis, there is still a strong belief in herbal medicines and Phyto-pharmaceuticals among scientists, as one-third of all pharmaceuticals sold worldwide are made from plants. Herbal and traditional ayurvedic medicines have been used since ancient times. Noviana E. et al. conducted a study on herbal medicine standardization and quantification to ensure efficacy, safety, and consistent quality. Fingerprinting analysis using HP-TLC and LC-MS/MS emerged as a valuable technique for standardizing and validating quality control and

biomarkers in herbs [160]. In another study, Senjaya et al. focused on the leaves ethanolic extract of *T. catappa* and identified active compounds through metabolic profiling analysis using UPLC-MS [161]. The study conducted sheds light on the promising antioxidant properties by Kanbarkar N. et al., on Acacia suma, expanding our understanding of its potential applications. One of the novel techniques utilized in their study was HP-TLC-DPPH, which proved to be effective in determining the antioxidant activity of different compounds found in herbal extracts [162]. Another study on *T. arjuna* bark acts as a cardio-protective agent and rich source of Phyto-constituents. Tulsi JR. et al., aimed to identify marker compounds, arjunetin and arjungenin, and detect possible adulterants in *T. arjuna* bark powder by HP-TLC [163]. Li Y. et al., Proposed a research study to address the technical challenge of rapidly screening and identifying bio-active compounds, focusing on *Terminalia chebula* fruits. The study developed a method combining UPLC-qTOF-MS/MS in which seventeen compounds, including gallotannins and ellagitannins, were identified using MS/MS spectra this detected/ identified compounds for the purpose of antimicrobial activity [164]. Sowmya TN. et al., examined isolated compounds by UHPLC-MS/MS analysis revealed a high concentration of ellagitannins, polyphenols, and alkaloids in the fraction of *T.catappa* (acetone-extract) [165].

Nandanwadkar S. et al., Introduced a novel method for the formulation and evaluation of nano-particles, specifically focusing on the determination of Capsaicin obtained from the natural source of paprika. The author and team developed an accurate, cost-effective, and precise analytical protocol for the identification of Capsaicin API through an analytical fingerprint profile of the formulated nano-particles, known as Transferosomes, using an HP-TLC system. The study concluded that the developed and validated HP-TLC protocol was not only innovative and rapid but also

emphasized the significance of HP-TLC in routine quality control of various herbal formulations [166]. For chemical fingerprint Rab R. et al., developed a research protocol on Itrifal and its quantitative analysis by assay method of HP-TLC and UPLC-MS/MS. The study demonstrated that the Itrifal formulation provided accurate, linear, and rapid results when applied with these biomarkers (catechin, quercetin, gallic acid and ellagic acid) [167].

HPLC-PDA as a quality control tool for the determination of phytochemical characterization in *Terminalia arjuna* was demonstrated by Shengule SA. et al., *Terminalia arjuna*, an Ayurvedic formulation, is widely used for treating dyslipidemia, cardiac disorders, and diabetes. The study focused on characterizing *Terminalia arjuna* in Ayurvedic formulations, namely Arjunarishta (AA) and Arjunaghritah (AG), using the HPLC-PAD method. Arjunetin and Arjungenin were employed as phytochemical markers in AG and AA formulations, respectively. The authors concluded that quality assurance of AG and AA formulations is crucial for the society, especially during manufacturing processes [168].

By focusing on HPLC-MS analysis, researchers can unravel the complex phytochemical interactions within these species, shedding light on their traditional uses and enabling the discovery of novel bioactive compounds. This approach aligns with contemporary interest in harnessing natural resources for innovative therapeutic intervention.

- **Quality Evaluating Contaminants, Guiding Practices for Medicinal Plant Cultivation and Harvesting:**

The study conducted by Bisht and Uniyal raises concerns about the potential presence of toxicants, particularly heavy metals (HMs), nitrates, pesticides, and other

xenobiotics, in medicinal plant materials harvested under various conditions. Due to sources including thermal power plants, industrial, vehicles and waste incinerators, and agricultural production, heavy metals are frequently present in the environment. These metals can be harmful to human health [169]. The research emphasizes the importance of assessing the quality parameters of herbal raw materials traded in the Indian marketplace, as they may contain foreign matter, adulterations, inferior quality, or be contaminated with fungi or insects. Trees and plants, in general, can act as barriers against the spread of heavy metals [170]. Bisht et al. examined a total of 183 samples from distinct herbal raw materials and species for the finding of (Hg, Pb, As, Cd) to measure the level of heavy metal adulteration in raw products sold in the Indian market. They related the results with permissible limits set by Ayurvedic Pharmacopoeia of India (API), USFDA, and WHO [171]. Indeed, putting the WHO's Good Agricultural Practises (GAP) and Good Field Collection Practise (GFCP) recommendations into practise is an essential first step in raising the standard of herbal raw materials and guaranteeing customer safety. These guidelines provide a framework for sustainable and responsible practices in the cultivation, harvesting, and collection of medicinal plants. By following these guidelines, the potential risks of heavy metal contamination and other toxicants can be minimized, leading to better quality herbal products.

Good Agricultural Practices involve various aspects, such as selecting suitable planting sites, using quality seeds or planting materials, managing water resources efficiently, using natural fertilizers and pest control methods, and practicing proper waste disposal. By adhering to these practices, the overall health and vitality of the plants can be enhanced, reducing their susceptibility to accumulating toxic substances. On the other hand, Good Field Collection Practices pertain to the sustainable

harvesting of wild medicinal plants. This includes ensuring that harvesting is done at the right time, using proper tools and techniques, and leaving enough plants in the wild to allow for regeneration and continued growth of the population. Responsible harvesting practices can help maintain the ecological balance and prevent over exploitation of valuable plant species.

By integrating these guidelines into the production and collection processes of herbal raw materials, producers can not only enhance the quality of their products but also contribute to the sustained existence of botanical medicines and environmental conservation. Ultimately, consumers can have greater confidence in the security and effectiveness of the herbal products they use, encouraging the responsible and beneficial use of traditional medicine in healthcare [170]. Extensive research conducted by JJ Quesada-Granados et al., to explore various plant species in India commonly used in cuisines like desserts, culinary dishes, and herbal medicine. The ICP-OES and ICP-MS study of the mineral, using appropriate reference standard materials also examined the potential of these plants for the nutritional supplements industry, considering their antioxidant and mineral composition. The distribution of polyphenols and minerals in different seed parts of *T. arjuna a*nd *T. chebula* was discussed, highlighting their suitability and usability for use in nutritional supplements [172]. A research on *Terminalia* trees done by Gharib. et al., cultivated in Egypt for their windbreak and wood purposes. The *Terminalia species* resulted in increased content of potassium, magnesium, calcium, iron (leaves) and phenol, tannin, flavonoid, in presence of DPPH [173]. Nandanwadkar et al., Envisaged via ground breaking research, exploring the potential applications of natural phyto-pigments in diversified medical treatments, focusing on their fast-elemental analysis of micronutrients by HP-TLC and ICP-OES. The state-of-the-art ICP-OES techniques

provided a plausible explanation for the anti-cancer properties of phyto-pigments and detection of necessary elements like iron, zinc and calcium [145]. Gunasekaret al., research on *T. chebula* to quantitatively determined elements such as Ca, Zn, Mn, and Fe in the extracts, with Ca exhibiting the best results by ICP-OES. The study highlighted that factors such as soil nature, climatic conditions, and time of harvest could impact the elemental profile [174].

The choice of AAS, ICP-OES, and ICP-MS employed in their study for metals and heavy metal estimation is rooted in their accuracy, sensitivity, and ability is essential to quantify a wide range of elements. These methods offer a holistic understanding of the elemental makeup of *Terminalia* species, ensuring the safety and quality of herbal products. However, it is essential to consider various factors such as sample preparation, calibration standards, and quality assurance to ensure reliable results.

Chapter 4 – Need for the Study

Terminalia, a genus well known as ***"KING"*** of Ayurveda; Indian system of medicine, forms an integral part and one of the widely preferred choice of traditional medicinal systems for many centuries offering a broad spectrum of phyto-chemicals with medicinal properties serving as treatments of numerous diseases, including cardiovascular, bacterial infections, conjunctivitis, abdominal disorders, headaches, colds, sore throats, jaundice, diarrhea, gastric ulcers, hypertension, heart diseases, leprosy, edema, pneumonia, and skin diseases. *Terminalia* species is also enriched with biological properties like anti-fungal, anti-oxidant, anti-cancer, etc. in addition to therapeutic effects like wound healing and other Nutraceutical benefits [175]. The need for the study is indexed with a three-point strategic approach which is as follows:

- ✓ Although the characterization of medicinal plants by Spectro-densitometric technique is having a long history, especially with regards to assessing quality of plant, there seems to be need for streamlined direction via regulatory compliant digitalized software's for authentication of research outcomes.
- ✓ Availability of limited scientific evidence-based protocols and methods as well as research publications when ethnomedicinal plants of north western corridor is enriched with major potent bio-therapeutic medicinal properties are concerned.
- ✓ Finally, yet importantly, need for developing a rapid, frugal and sensitive analytical tool, overcoming the shortcomings of conventional sophisticated methods. Nevertheless, it is essential to establish and document the developed methods for fingerprinting profiles, method development, and validation, as well as heavy metal testing using advanced instrumental techniques, in accordance with global regulatory norms. These methods should be documented as monographs and incorporated as reference methods in herbal pharmacopeias.

Chapter 5 – Materials and Methods

This section elucidates the study that lists the materials employed in overall study to perform the entire proposed research work. The section is endorsed with the list of advanced State of Art instruments utilized in experimentation, additionally involving certified reference materials (CRM's) present in selected phyto-medicinal plants etc. The study highlights use of cutting-edge technologies for extraction, isolation, characterization, and elemental estimation. The study's prime focus was on developing a novel validated USP-HP-TLC protocol for selected *Terminalia* species (Fig.2). Coupled Technique like (LC-MS) Liquid Chromatography mass spectroscopy, tools were included as confirmatory evidence and compliance with regulatory dossiers with a sincere effort to ensure developed method becomes an integral part of routine phyto-chemical investigation. Elemental analysis like ICP-OES, Atomic absorption spectrophotometer and ICP-MS were conducted to measure elements at trace levels.

MATERIALS

1. Plant Drug Profile :

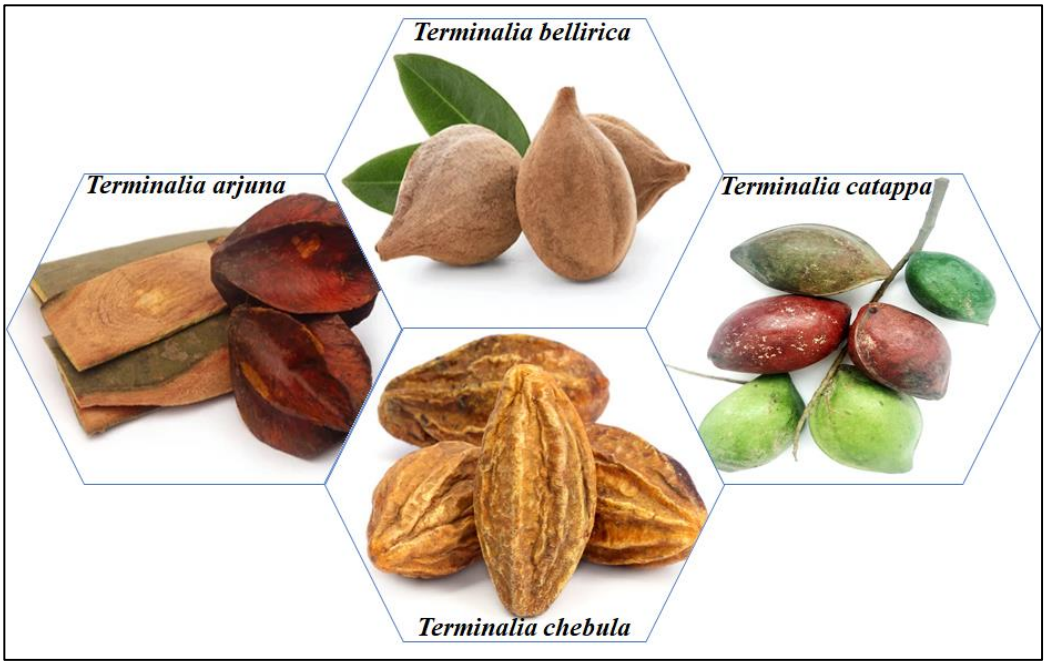


Fig. 2: Selected fruits and bark of *Terminalia* species

The scientific classification of *Terminalia* species, parts of plants, phytoconstituents present as per literature, bioactive phytoconstituents procured, drug profile of phytoconstituents, Solvents, derivatizing reagents, instruments utilized in the present research work are represented in Table 1-9.

2. Botanical description for *Terminalia* species:

Table 1: Scientific classification of *Terminalia* species

Taxonomy	
Kingdom	Plantae
Sub kingdom	Tracheobionta
Division	Magnoliophyta
Sub Division	Spermatophyta
Class	Magnoliopsida
Order	Myrtales
Family	Combretaceae
Genus	*Terminalia*
Species	*Terminalia arjuna*,*Terminalia bellirica*, *Terminalia chebula*, and*Terminalia catappa*

3. Parts of *Terminalia* plant selected :

Table 2: Selected parts of the *Terminalia* species

Terminalia species	Parts of plants	ICMR-NITM Specimen number
Terminalia arjuna (Roxb.) Wight & Arn	Bark	RMRC-1634
Terminalia bellirica (Gaertn.) Roxb	Fruit	RMRC-1633
Terminalia chebula Retzius	Fruit	RMRC-1632
Terminalia catappa Linn	Fruit	RMRC-1635

4. Phytoconstituents present in *Terminalia* :

Table 3: Phytoconstituents present in *T. arjuna*, *T. bellirica*, *T. chebula* and *T. catappa*

Terminalia species	Phytoconstituents
Terminalia arjuna	Arjunolic acid, Arjunic acid, Arjungenin, Arjunglucosides I, II and III, Ellagic acid, Gallic acid, Arjunolone (6,4′ dihydroxy, 7 methoxy flavone), baicalein, Arjunetin, Oleanolic acid, and arjunolitin, Terminoside A, β-Sitosterol,Olean-3β,22β-diol-12-en-28β-D-glucopyranoside-oic acid, Olean-3α,5α,25-triol-12-en-23,28-dioic acid-3α-D-glucopyranoside [63].
Terminalia bellirica	Gallo-tannic acid, Tannins, ellagic acid, galloyl glucose, chebulagic acid, ethyl gallate, hexahydroxydiphenic acid ester, β-sitosterol, mannitol, glucose, fructose, rhamnose, Glucoside (bellericanin), Ellagic acid, gallic acid, lignans(termilignan and thannilignan), 7-hydroxy 3'4' (methylenedioxy) flavone and anolignanB, luteoline [178].
Terminalia chebula	Gallic acid, ellagic acid, ethyl gallate, methyl gallate, chebulagic acid, tannic acid, chebulic acid, chebulinic acid, chebulanin, corilagin, neo-chebulinic acid, 1,2,3,4,6-penta-O-galloylβ-D-glucose, 1,6-di-O-galloyl-D-glucose, 3,4,6-tri-O-galloylD-glucose, terchebulin, phyllanemblinin E, 10-O-methyl neochebulinate, neochebulgic acid, phyllanemblinin F, 1,2,3,6-tetra-O-galloylβ-D-glucose, tellimagrandin, vanillic, caffeic, p-coumaric, ferulic, shikimic [179].
Terminalia catappa	gallic acid, catechin, chlorogenic acid, kaempferol, ellagic acid, rutin, quercetin, quercitrin, iso-quercitrin, epicatechin, caffeic acid, chebulagic acid, Punicalagin, Punicalin, geranin, granitin B, Vitexin, Isovitexin, Ursolic acid, Asiatic acid [123-125]

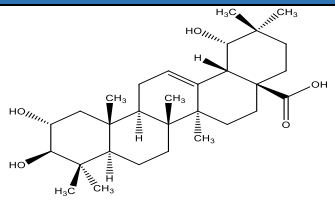
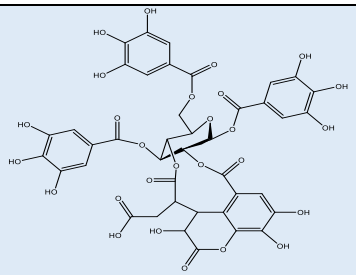
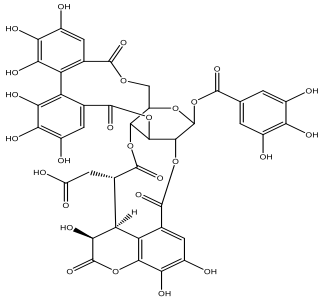
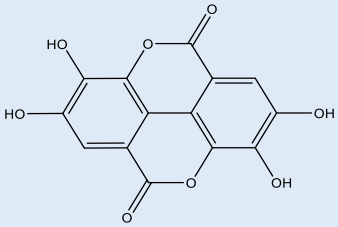
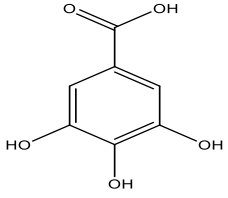
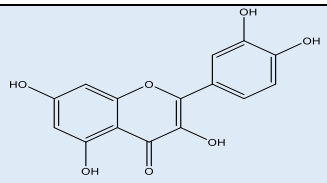
5. Bioactive Phytoconstituents selected for the Study (Biomarkers) :

Table 4: Phytoconstituents in *Terminalia* species

Sr. No	Phytoconstituents	Supplier	Make
1.	Arjunic acid	Natural remedies Pvt. Ltd	Bengaluru, India
2.	Chebulinic acid	Natural remedies Pvt. Ltd	Bengaluru, India
3.	Chebulagic acid	Natural remedies Pvt. Ltd	Bengaluru, India
4.	Ellagic acid	Himalaya Drug Company	Bengaluru, India
5.	Gallic acid	Himalaya Drug Company	Bengaluru, India
6.	Quercetin	Himalaya Drug Company	Bengaluru, India

6. Phytochemical constituents Drug profile :

Table 5: Phytoconstituents entities structural representation, molecular formula and weight

Sr. No	Phytoconstituents	Structure	Description
1.	Arjunic acid		MF:$C_{30}H_{48}O_5$ MW:**488.70(g/mol)**
2.	Chebulinic acid		MF:$C_{41}H_{32}O_{27}$ MW: **956.68 (g/mol)**
3.	Chebulagic acid		MF:$C_{41}H_{30}O_{27}$ MW: **954.67 (g/mol)**
4.	Ellagic acid		MF:$C_{14}H_6O_8$ MW: **302.19 (g/mol)**
5.	Gallic acid		MF:$(HO)_3C_6H_2CO_2H$ MW: **170.12 (g/mol)**
6.	Quercetin		MF:$C_{15}H_{10}O_7 \cdot 2H_2O$ MW: **338.27 (g/mol)**

7. Solvents used :

Table 6: List of Solvents

Sr. No.	Solvents	CAS No.	Manufacturer
1	Ethanol	64-17-5	Merck, Germany
2	Water	7732-18-5	Merck, Germany
3	Methanol	67-56-1	Merck, Germany
4	Glacial Acetic Acid	64-19-7	Merck, Germany
5	Isopropyl Alcohol	67-63-0	Merck, Germany
6	Ethyl Acetate	141-78-6	Merck, Germany
7	Toluene	108-88-3	Merck, Germany
8	Triethylamine	122-44-8	Merck, Germany
9	Formic Acid	64-18-6	Merck, Germany
10	Cyclohexane	110-82-7	Merck, Germany
11	Chloroform	67-66-3	Merck, Germany
12	n-Hexane	110-54-3	Merck, Germany
13	Hydrochloric acid	7647-01-0	Merck, Germany
14	Nitric acid	7697-37-2	Merck, Germany
15	Sulphuric acid	7664-93-9	Merck, Germany
16	Hydrogen Peroxide	124-43-6	Merck, Germany

8. Plates used for HP-TLC analysis :

Table 7: TLC plates utilized during HP-TLC analysis

Sr. No	TLC Plates	Make/ Manufacturer
1.	TLC Silica gel 60 F_{254}	Merck, Germany
2.	TLC Silica gel 60 RP-18 $F_{254}S$	Merck, Germany

9. Reagents used :

Table 8: List of Derivatizing Reagents

Sr. No.	Reagents	CAS No.	Manufacturer
1	Anisaldehyde solution	SRA1	Merck, Germany
2	Natural Product Reagent	524-95-8	Carl Roth, Germany
3	Vanillin	121-33-5	Merck, Germany
4	Ferric chloride/ Iron chloride	7705-08-0	Merck, Germany
5	2,2-Diphenyl-1-picrylhydrazyl	1898-66-4	Merck, Germany

10. Instruments used :

Table 9: List of Instruments with model and make

Sr. No	Instrument Name (Model)	Make/ Manufacturer
1.	U.V. Spectrophotometer	Shimadzu, Japan
2.	HP-TLC System	CAMAG, Switzerland
3.	Electronic Balance	Shimadzu, Japan
4.	Rotary evaporator	IKA Laboratories, Bangalore
5.	Micro centrifuge	Remi Motors Pvt. Ltd, India
6.	Digital ultrasonic cleaning baths	Branson Ultrasonic, India
7.	LC-MS/MS	Perkin Elmer, Germany
8.	AAS	Motras Scientific, India
9.	ICP-OES	Perkin Elmer, Germany
10.	ICP-MS	Perkin Elmer, Germany

Methodology

1. Collection and authentication of Plant Material :

Healthy stem of *T. arjuna and Fruits of T. bellirica, T. chebula, T. catappa were* collected from Jamboti forest Khanapur, Belgaum, Karnataka. The material was identified and authenticated verified by certified Taxonomist from ICMR-National Institute of Traditional Medicine, Belagavi, Karnataka.

2. Sample Extraction Process :

Based on literature review hydroalcoholic method for extraction by soxhlation process was selected for *Terminalia* species with the solvents in the ratio of (70:30) ethanol: water to obtain the maximum yield and phytoconstituents of interest (Fig.3).

a. The dried bark of *T. arjuna* and the fruits of *T. bellirica, T. chebula,* and *T. catappa* were finely powdered and sieved through a mesh size of #40. The powdered plant material was then subjected to extraction using a hydro-alcoholic solution through the Soxhlet extraction process at a temperature of 80˚C [180,181].

b. The solvent was evaporated using a rotary evaporator, with vacuum system, a rotating assembly, and a condenser with a collecting flask. Extract was stored at 4˚C [182].

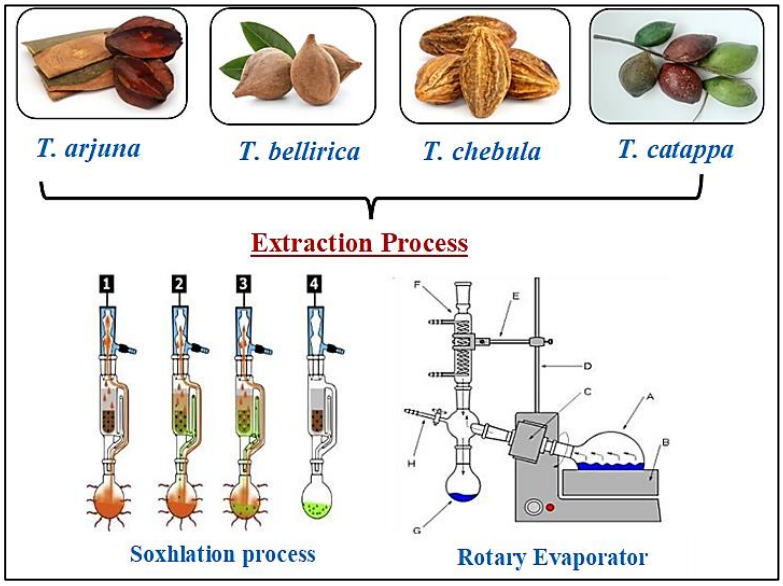


Fig. 3: Extraction process of *Terminalia* plant material

3. Preliminary/ Quality Evaluation:

A. Phytochemical Screening:

The phytochemical analysis utilized the hydroalcoholic extract (HAE) of *Terminalia* (*arjuna*, *bellirica*, *chebula*, and *catappa*) as a component, employing standard methods to assess its qualitative chemical composition shown in (Table 10) [183, 184].

Table 10: Preliminary Qualitative Phytochemical tests

Sr. No	Chemical Test	Procedure
1	**Alkaloids**	A 0.5 g of sample was weighed to which 8 ml of 1% HCl was added. The mixture was then heated and filtered. Subsequently, 2 ml of the resulting filtrate were treated with the reagents. The presence of alkaloids was determined by observing the turbidity or precipitate formed.
A	**Mayer's Test**	When a few drops of Meyer's reagent were added to 2 ml of the filtrate, a cream-colored precipitate formed.
B	**Dragendorff's Test**	When a few drops of Dragendorff's reagent was added to 2 ml, the formation of a reddish-brown precipitate was indicated.
C	**Wagner's test**	When 2 ml of a solution was mixed with a few drops of Wagner's reagent, the result was the formation of a reddish-brown precipitate.

D	**Hager's test**	When a few drops of Hager's reagent were added to 2 ml of the solution, the formation of a yellow-colored precipitate was indicated.
2	**Test for Carbohydrates**	
A	**Molisch's Test (General test)**	A few drops of alpha-naphthol solution were added to a 2ml aqueous sample solution, shaken, and then concentrated $H_2SO_4$was introduced along the sides of the test tube. The presence of carbohydrates was indicated by the formation of a violet color at the interface between the two liquids.
3.	**Test for Flavonoids**	
A	**Shinoda Test**	5 ml of 95% ethanol, a few drops of concentrated HCl, and 0.5 g of magnesium turnings were added to the dry powdered sample. The confirmation of the presence of flavonoids was indicated by the appearance of colors ranging from orange to pink, and red to purple.
		1ml of sodium hydroxide (NaOH) was added to the dry powdered sample, resulting in the development of an intense yellow color. Subsequently, a few drops of concentrated H_2SO_4 were added, causing the color to change to colorless.
4	**Tannins**	The samples, which weighed 0.25 g, were dissolved in 10 ml of distilled water and then filtered. To the resulting filtrate, 2 ml of a 10% w/w lead acetate solution was

		added. The presence of tannins in the test samples was indicated by the formation of an intense yellow-colored precipitate.
5	**Terpenoids (Salkowski test)**	0.5 g of the samples was mixed with 2 ml of chloroform, and then 3 ml of concentrated H_2SO_4 was carefully added into it. At the interface, a reddish-brown coloration indicated a positive result for the presence of terpenoids.
6	**Steroids (Salkowski test)**	The samples, weighing 0.5 g, were mixed with 2 ml of chloroform, and then 3 ml of concentrated H_2SO_4 was added carefully. At the interface, the formation of a layer that is either greenish-yellow or reddish-brown indicated a positive result for the presence of steroids.
7	**Phenols**	To the samples weighing 0.5 g, 2 ml of distilled water was added, and then a few drops of a 10% aqueous ferric chloride solution were carefully introduced. The presence of phenols was indicated by the formation of a blue or green color.
8	**Saponins (Froth test)**	The samples, weighing 0.5 g, were vigorously mixed with 5 ml of distilled water and then filtered. The presence of saponins was indicated by the formation of foam.
9	**Liebermann-Burchard's test for triterpenoids**	The extract was treated with a few drops of acetic anhydride, then boiled and allowed to cool. Afterward, concentrated sulfuric acid was carefully added from the sides of the test tube. The presence of tri-terpenoids was

		indicated by the appearance of a brown ring at the interface of the two layers and the formation of a deep red color.
10	**Lead acetate test for flavonoids**	A few drops of 10% lead acetate solution were added to the alcoholic solution of the extract. The presence of flavonoids was indicated by the appearance of a yellow precipitate.
11	**Legal's test for lactones**	For Sodium Nitroprusside and Pyridine Test: Sodium nitroprusside and pyridine were added to the extract mixtures then treated with NaOH. The presence of lactones was determined by observing the development of a deep red color.
		Ferric Chloride Test for Phenolic Compounds and Tannins: A test tube containing 2 mL of the extract was prepared. Ferric chloride solution was added drop by drop to the test tube. The presence of phenolic compounds and tannins was identified by the formation of a bluish-black precipitate.
12	**Salkowski reaction test for Phytosterols**	To the chloroform extract (0.5 mL) in a test tube, 1 mL of concentrated H_2SO_4 was carefully added from the sides of the test tube. The presence of phytosterols was indicated by the appearance of a reddish-brown color in the chloroform layer.

13	**Ninhydrin test for proteins**	A few drops of ninhydrin were added to the extract. The presence of amino acids was indicated by the appearance of a blue color, although proteins may rarely yield a positive result.
14	**Keller-Killani test for glycosides**	To the extract, a total of 1 mL of glacial acetic acid, a few drops of ferric chloride solution, and concentrated H_2SO_4 were added (added slowly through the sides of the test tube). The presence of de-ox sugars was indicated by the appearance of a reddish-brown ring at the junction of the liquids.

B. Physicochemical Investigation:

The physicochemical investigation of the crude powder from four *Terminalia* species was conducted to determine the qualitative chemical composition using established standardized available protocol (Table 11) [185, 186].

Table 11: List of tests for physicochemical investigation

Sr. No	Tests	Procedure
1.	**Ash Value**	
	Total Ash	A precise weight of 2 grams was taken for the sample, which was subsequently stirred in a crucible and heated in a muffle furnace at a temperature of 500 to 600°Cuntil carbon-free ash was achieved. After cooling the crucible, it was weighed again, and the percentage of total ash was calculated relative to the weight of the air-dried drug. $$\% \text{ Total Ash} = \frac{\text{Wt. of total ash}}{\text{Wt. of crude drug}} \times 100$$
	Acid-insoluble Ash	The obtained ashes were boiled with 25 ml of hydrochloric acid (70 g/litre) for 5 minutes and then filtered using ashless filter paper. The insoluble material on the filter paper was washed with hot water, and subsequently, the filter paper was dried to a constant weight in a muffle oven. The percentage of acid-insoluble ash was calculated relative to the weight of the air-dried drug. $$\% \text{ Acid insoluble ash} = \frac{\text{Wt. of acid insoluble ash}}{\text{Wt.of crude drug}} \times 100$$
	Water soluble Ash	The ashes obtained previously were boiled with 25 ml of water for 5 minutes. The insoluble material collected on the filter paper, free from ash, was washed with hot water and then ignited in a muffle oven for 15 minutes at a temperature below

		450°C. The weight difference between the resulting ash and the weight of the water-insoluble material yielded the weight of water-soluble ash. The percentage of water-soluble ash was calculated relative to the weight of the air-dried powder. % water soluble ash = $\frac{\text{Wt.of total ash} - \text{Wt.of waterinsoluble ash}}{\text{Wt.of crude drug}}$ x 100
2	**Extractive value**	
	Alcohol Soluble Extractive	Using precise weighing, a 4 g sample was macerated in a conical bottle with 100 ml of alcohol for 24 hours, with continuous shaking at 6-hour intervals. Subsequently, it was left undisturbed for 18 hours and then rapidly filtered to prevent any deterioration. For preservation, the 25 ml filtrate was evaporated until completely dried in a porcelain dish, and then further dried to a constant weight at 105°C. The percentage of alcohol-soluble extracts was calculated relative to the weight of the air-dried material. % Alcohol soluble extractive= $\frac{\text{wt.of extract}}{\text{wt.of plant material}}$ x 100
	Loss on Drying	The mass loss resulting from heating was represented as a weight percentage. A precisely weighed 10 g sample was poured onto a Petri dish and then introduced into a hot air oven at 105°C for a duration of 5 hours. The decrease in mass of the specimen was calculated with respect to the initial weight. % Loss on drying= $\frac{\text{final volume}}{\text{Initial volume}}$x 100

4. UV-Vis Spectrophotometer :

UV spectroscopy, also known as ultraviolet-visible spectroscopy or UV-Vis spectroscopy are commonly utilized analytical technique in the field of chemistry and biochemistry. The technique involves the measurement of the absorption of ultraviolet (UV) or visible light by molecules in a sample. The principle behind UV spectroscopy is based on the interaction between light and matter, particularly the absorption of light by electrons in molecules [187].

- **Spectroscopic Conditions:**

UV-Vis double beam spectrophotometer from Shimadzu UV model 1800 (Fig. 4) equipped with matched 10mm quartz cuvettes was employed in the study. AR grade Methanol was utilized in the study.

- **Wavelength Determination :**

The range of 200–400 nm was determined for the wavelength for standards concentrations of arjunic acid, chebulinic acid, gallic acid, ellagic acid, and quercetin.

- **Preparation of Standard Solution [187]:**

To detect the wavelengths of phytomarkers in *Terminalia* species, a solution was prepared by dissolving 10 mg of the standard in 10 ml of methanol, resulting in a concentration of 1000 µg/ml. In order to create the secondary stock solution, 1ml was withdrawn from the primary stock solution and diluted to 10ml to give 100µg/ml. The solution was then analyzed using a UV spectrophotometer for spectral analysis.

Fig. 4: UV-Vis Spectrophotometer

5. TLC Method Development and Validation Process :

TLC (Thin Layer Chromatography) plays a crucial role in preliminary Quality Control analysis by providing a rapid and effective separation technique for complex mixtures. Thin Layer Chromatography (TLC) is significant tool for fixing a suitable solvent system during method development by trial and error of different solvent combinations, TLC allows rapid evaluation of the separation efficiency, selectivity, and resolution of the target compounds. This helps in optimizing the chromatographic conditions for subsequent analysis [188].

➢ **Schematic representation :**

1. • ***Terminalia* species**
2. • **Study of Phyto-constituents**
3. • **Solvent Selection**
4. • **Sample Preparation**
5. • **Serial Dilutions**
6. • **Application in form of spot/ band on Merck TLC Silica Gel F_{254}**
7. • **Optimized Sample Preparation**
8. • **Method Development**
9. • **Densitometric scanning and Evaluation**
10. • **Spectral Documentation**

6. High Performance Thin Layer Chromatography :

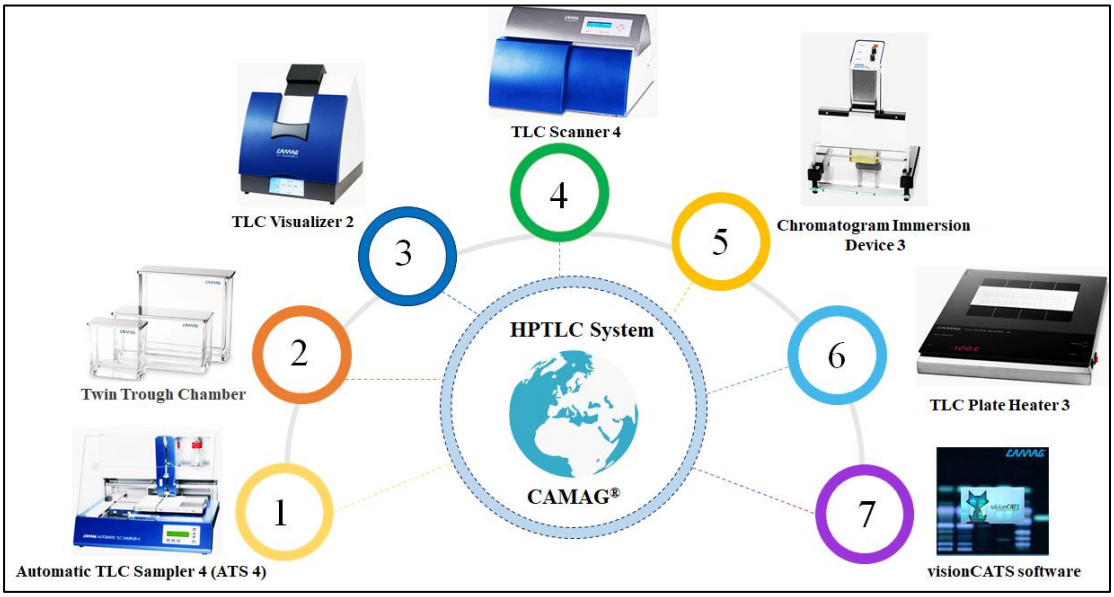


Fig. 5: Schematic representation of HP-TLC system

- **Methodology for HP-TLC [188] :**

HP-TLC (High Performance Thin Layer Chromatography) is a technique that involves the separation and analysis of complex mixtures. The complete system of CAMAG HP-TLC is shown in Fig.5. It utilizes a thin layer of stationary phase, such as silica gel or cellulose, on a plate. The sample is applied as a small band on the plate, which is then developed in a suitable mobile phase. After development, the plate is dried by heater, and the separated components are visualized using different detection techniques. HP-TLC offers high resolution, sensitivity, and reproducibility, making it widely applicable in various industries for quality control and analysis purposes.

HP-TLC is performed on 20x10 cm HP-TLC aluminum plates coated with silica gel 60F254 sample application, chromatogram development, derivatization, and documentation (Table 12 and 13).

NOTE: Record Temperature and Relative Humidity in the laboratory

Table 12: Steps to be followed for HP-TLC analysis

Preparation of Plates	➢ Obtain HP-TLC plate silica gel 60 F 254 (20x10 cm). Record the batch number. ➢ Inspect plate under UV 254 nm for any damage of the layer. If damage is detected discard plate. ➢ Pour 20 ml of developing solvent over the filter paper into the rear trough ensuring complete wetting. Pour a sufficient amount of developing solvent into the front trough to have a level of 5 mm.
Sample Application	Select the following application parameters on the application device: ➢ Band length 8 mm ➢ Number of tracks ➢ Sample solvent type: methanol ➢ Apply the application volumes as according to the standardized procedure for selected samples of interest. ➢ Plate conditioning (manual development only) ➢ Let the mobile phase ascend until it reaches the mark. ➢ Open the lid and remove the plate. Place it upright in a rack under a fume hood. ➢ Dry plate with cold air from a hair dryer for 5 min.
Automatic Development	Use the following settings of the automatic chamber: ➢ Enable pre-drying ➢ Saturation with filter paper 20 min ➢ Humidity control 10 min with MgCl2 ➢ Migration distance 70 mm ➢ Drying time 5 min ➢ 10 ml of developing solvent ➢ 25 ml of saturation solvent *NOTE: if no humidity control is available follow step 4*
Derivatization	Examples: **4A. FLAVONOIDS – Derivatization by Dipping** ➢ Heat the dry plate for 5 min at 100°C. ➢ While hot dip plate for 1 sec into a solution of 0.5% NP reagent in ethyl acetate. **4B. FLAVONOIDS - Derivatization by Automatic Spraying** ➢ Heat the dry plate for 5 min at 100°C. ➢ While hot spray the plate with 3.5 ml of a solution of 1% NP reagent in methanol ➢ Then with a solution of methanol.
Photo Documentation	30 min after the second derivatization step, take an image of the derivatized plate under UV 366 nm.
Reporting	Create a copy of software-based report or use own reporting document.

Table 13: Specifications of HP-TLC

Parameters	Description
Instrument	CAMAG HP-TLC
Stationary Phase	Silica gel F_{254} HPTLC pre-coated plates
Sample applicator	CAMAG LINOMAT V/ Automatic TLC sampler (ATS)
Band width	8.0 mm
Syringes	CAMAG Linomat Syringe (100 µL)
Volume of mobile phase	20-30 ml
Development mode	CAMAG Twin Trough Chamber (Ascending development)
Development distance	70 mm
Chamber saturation time	15-20 mins
Densitometer	Scanner 4
Scanning wavelength	254nm, 366 nm, 540 nm
Software	visionCATS Version 3.1
Lamp	Deuterium (D2), Mercury (Hg), Tungsten (W)
Measurement modes	Visible light, Absorbance & Fluorescence
Photo documentation	CAMAG TLC Visualizer 2

- **Sample Preparation for Fingerprinting Analysis by HP-TLC :**

HP-TLC fingerprinting is a valuable technique in analyzing medicinal plants. It allows for the identification and quality assessment of complex plant extracts by comparing their characteristic chromatographic fingerprints. This method offers several benefits, including rapid analysis, high resolution, reproducibility, and cost-effectiveness. HP-TLC fingerprinting can aid in authentication, standardization, and quality control of herbal medicines, ensuring their safety and efficacy. It also enables the detection of adulterants and variations in plant material, contributing to the overall quality assurance of medicinal plants and their derived products. The sample preparation for fingerprinting analysis is depicted in Fig.6 [189].

Fig. 6: Sample preparation for HP-TLC Fingerprinting Analysis

- **Derivatization Solution for detection of different class of compounds and their activity by HP-TLC:**

  The procedures and observations of the reagents that are used for the derivatization of developed HPTLC plates are reported in Table 14 [162, 190-192].

  Table 14: List of reagents for detection of phytochemicals

Derivatizing Reagents	Procedure of Derivatizing Reagents	Observations
Anisaldehyde Sulphuric acid (ASR)	The Anisaldehyde reagent solution was prepared freshly by mixing 1 mL of Anisaldehyde with 20 mL of glacial acetic acid, followed by 170 mL of methanol and 10 mL of concentrated Sulphuric acid. The resulting colorless solution was stored in a refrigerator at 2-8˚ C. It is important to note that the reagent has limited stability, and if a pink discoloration appears, it should be discarded.	Dark blue, yellow brown, blue violet zones confirmed the presence if phenols, saponins and triterpenoids
Vanillin Reagent (VSR)	The acidified vanillin reagent was prepared by weighing 3g of vanillin and mixing it with 1.5mL of concentrated Sulphuric acid (H_2SO_4) and 100 mL of absolute ethanol.	Dark Blue, Green, Red, Brown coloured bands confirmed the presence of essential oils and bitter compounds.

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical Scavenging activity	The DPPH was obtained by dissolving 8mg of DPPH in 200 mL of ethanol in a dark environment. To protect it from light, the solution was prepared in an amber-colored bottle. For the detection of antioxidants, a 0.2% (w/v) DPPH solution was prepared in methanol, stored at 2-8 ◦C, and kept away from light.	Lemon-yellow dots on a bluish-purple background confirmed presence of Anti-oxidants
Ferric chloride ($FeCl_3$)	Dissolve 2g of $FeCl_3$ in 10mL of water and then dilute it to a final volume of 200mL with ethanol.	Dark Blue colour bands confirmed the presence of Tannins and Phenols
Natural Product Reagent	Dissolve 1gm of (2-aminoethyl diphenyl borinate) dissolved in 200ml of ethyl acetate. Preheat the plate before derivatization and derivatize it with natural product reagents.	Florescent compounds, Red fluorescence confirmed Flavonoids, plant pigments whereas chlorophyll pigments

- **HP-TLC Validation Parameters are as follows:**

The validation parameters of HP-TLC analysis are reported in Table 15 [162, 180].

Table 15: Parameters for HP-TLC Validation

Sr.No	HPTLC Validation parameters	
1	Linearity	To establish the standard calibration for linearity, a series of increasing concentrations of the reference standard from stock solutions were applied to TLC plates (n=6) after the plate was prewashed. The plates were then developed, dried, and scanned following the aforementioned procedure. A calibration curve was created by plotting the average peak area (Y-axis) against the concentration (ng/spot) (X-axis).
2	Sensitivity	The Limit of Detection (LOD) was determined based on the analyte concentration at which the Signal-to-Noise (S/N value 3). Similarly, the Limit of Quantification (LOQ) was determined based on the analyte concentration at which the (S/N value 10).
3	Specificity	Specificity refers to the ability to accurately measure a specific analyte in the presence of other components in a sample. To determine the specificity of the method, the standard and sample drugs were compared. The confirmation of bands for the reference standard in *Terminalia* species was done by comparing the Rf values and overlaying peak purity spectra with those of the standard.

4	Precision	Precision of the developed method was evaluated through a study of inter-day, intra-day, and repeatability. The precision of the method was assessed by calculating the percent relative standard deviation (%RSD) of peak area. The inter-day study was conducted for three consecutive days to determine the precision. The findings of repeatability, intra-day, and inter-day precision were expressed as %RSD [193].
5	Reproducibility	The reproducibility of the method was assessed by measuring the precision between the obtained results. The method involved applying six aliquots of a (same concentration) standard solution containing spots of reference standards onto TLC plates, which were then developed and measured to obtain the final results. Reproducibility was expressed as the coefficient of variation (CV %) of the measured concentrations at each calibration level.
6	Accuracy/ Recovery	Recovery studies were conducted by adding known amounts of the biomarker, corresponding to 80%, 100%, and 120% of the reference standards, to the hydro-alcoholic extracts. Each level was analyzed in triplicates. The recovery of the reference standards in the hydro-alcoholic extracts of *Terminalia* species was calculated.

7. Liquid chromatography-tandem mass-spectrometry (LC-MS/MS) :

Liquid chromatography-tandem mass-spectrometry (LC-M.S/MS) is a highly effective technique for plant analysis, providing comprehensive molecular profiling of plant samples. By combining the separation capabilities of liquid-chromatography (LC) with the sensitive and selective detection of tandem mass spectrometry (MS/MS), LC-MS/MS allows for in-depth exploration of the intricate chemical composition of plants. It enables the identification and quantification of diverse plant metabolites, making it indispensable in plant research areas such as metabolomics, plant biochemistry, and natural product studies.

- **Principle :**

The principle of LC-MS/MS involves the integration of two interconnected analytical techniques. In LC, the sample is separated into its components using a liquid mobile phase that interacts with a stationary phase, allowing for the separation based on various physicochemical properties. The eluted compounds are then introduced into the mass spectrometer, where they undergo ionization and subsequent fragmentation. The resulting ions are selectively detected and quantified based on their mass-to-charge ratio (m/z) and characteristic fragmentation patterns, providing both qualitative and quantitative information about the analyzed compounds.

- **Experimental :**

Chromatographic separation was carried out using a PerkinElmer LC–MS/MS system (Fig.7), and detection was achieved utilizing a PerkinElmer Q-Sight 220 MS/MS detector equipped with a dual ionization ESI and APCI source. ESI and APCI operated independently through two separate inlets. Instrument control,

data acquisition, and data processing were all managed through Intuitive software provided by PerkinElmer. Detailed information regarding the LC and MS source parameters can be found in Table 16-18 [194].

- **Preparation of standard solution :**

  Standard solutions of arjunic acid, chebulagic acid, chebulinic acid, betulinic acid, ellagic acid, gallic acid, rutin and quercetin were prepared in the concentration of 1000 µg/mL in Methanol. Subsequently, volume injected 10 µL of each standard into the LC-MS/MS for analysis.

- **Method for sample preparation:**

  A 10 mg sample was initially extracted with 10 ml of methanol. From this stock solution, 1 ml was diluted to 10 ml, resulting in a second stock solution with a concentration of 100 µg/ml. The aliquot was subsequently subjected to centrifugation, and the supernatant was collected and further diluted for LC-MS/MS analysis. To mitigate the risk of contamination, high quality glass wares and accessories was employed throughout the sample preparation process. Finally, 10 µl of the prepared sample was injected into the LC-MS/MS system for analysis.

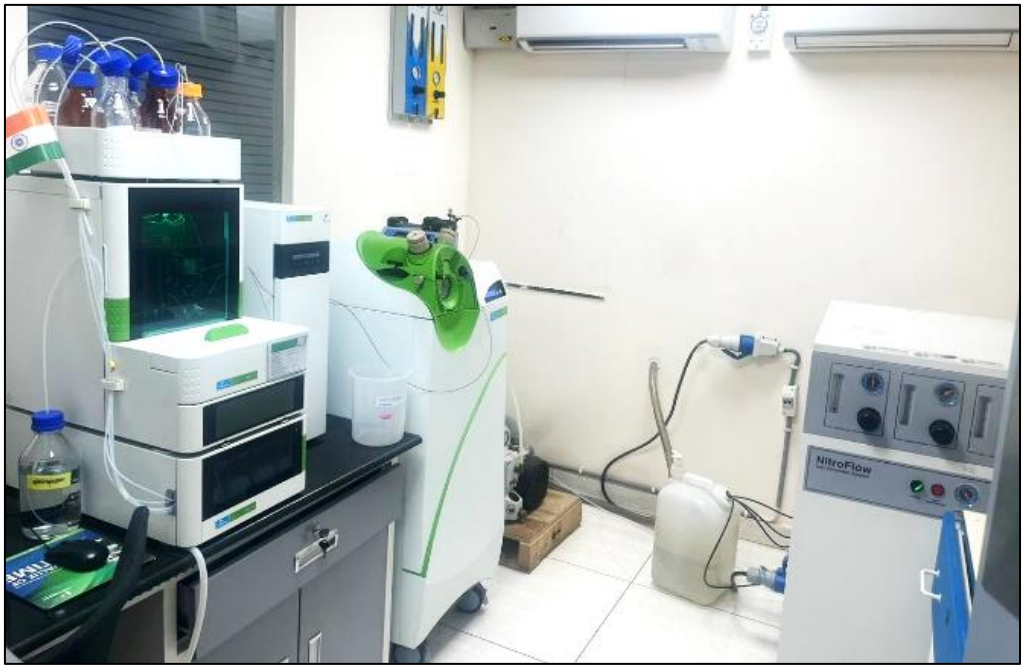


Fig.7: Liquid chromatography-tandem mass-spectrometry (LCMS/MS)

- **Liquid Chromatography Conditions:**

Table 16: Liquid Chromatography conditions used on the QSight ® 220 instrument

Instrument	LC QSight ® 220
Software	Intuitive
Sample preparation	Methanol
Flow rate	0.5ml/min
Injection volume	1 µl
Temp. oven	40˚ C
EV	-90 V
Sweeps/ Reading	12
Replicates	3

- **Mass Spectrometry Conditions**: The LC-MS/MS analysis was performed using the QSight® 220 triple quadrupole mass spectrometer.

Table 17: Mass Spectrometry conditions used on the QSight ® 220 instrument

Instrument	MS QSight® 220
ESI Voltage (V)	5850
HSID Temp (°C)	200
Nebulizer Gas Setting	120
Drying Gas Setting	70
Source Temp. (°C)	300
Dwell Time (MS)	20
Pause time (MS)	5
Electro spray	5500
Entrance voltage	40
Collision cell lens 2	-130
Collision energy	-30

Table 18: Tuning recommended source parameter values

Parameter	Values
Drying gas	50
HSID temperature	320
Nebulizer gas	80
ESI voltage	5000
Source temperature	0

8. Atomic Absorption Spectrophotometer (AAS) [195, 196] :

AAS offers sensitivity-selectivity, and accuracy in determining elemental composition in plants. This information is vital for assessing mineral composition, detecting contaminants, and monitoring environmental impacts on nutritional safety and toxicity. AAS aids in plant physiology research, agriculture, and environmental monitoring by efficiently analyzing major elements in plant samples.

- **Principle:**

  Atomic Absorption Spectroscopy (AAS) is based on the absorption of characteristic wavelengths of light by free atoms in their ground state. A hollow cathode lamp is used as the radiation source, emitting light at specific wavelengths corresponding to the elements being analyzed. The sample is ionized in a flame or graphite furnace, vaporizing and exciting the elements. A monochromator selects the desired wavelength, and a detector measures the absorbance of the radiation after passing through the ionized sample. The absorbance is directly proportional to the element's concentration, enabling quantitative determination.

- **Experimental:** The analysis was carried on AAS of Motras scientific plus system for the detection of metal present in selected four *Terminalia* species (Fig.8 and Table 19).

- **AAS standard preparation:**

  To prepare the standard solutions, 1000µg/ml of the stock solution was added to 100 ml of 2% HNO_3, resulting in a concentration of 10 ppm. From this 10 ppm solution, aliquots of 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml were withdrawn and added

to 100 ml of water to obtain standard solutions with concentrations of 1 ppm, 2 ppm, 3 ppm, 4 ppm, and 5 ppm, respectively [195, 196].

➢ **AAS Sample Digestion Procedure:**

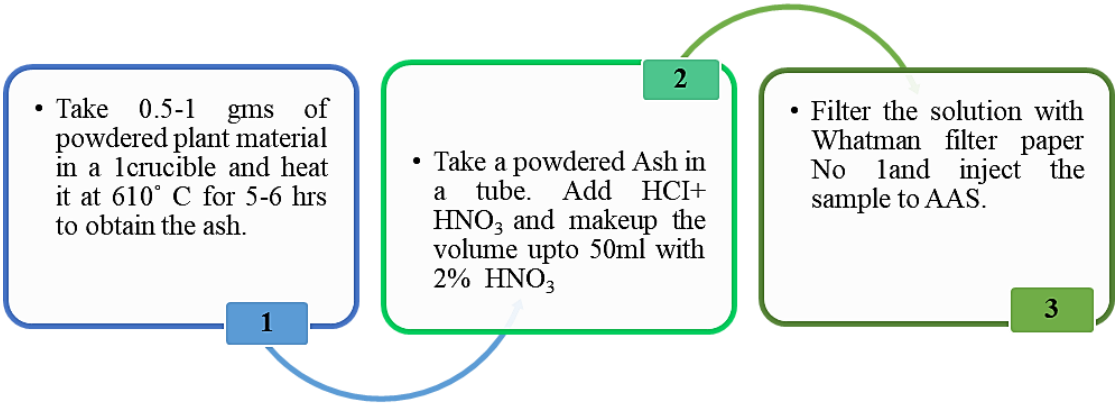


Fig. 8: Atomic Absorption Spectrophotometer (AAS)

➢ **<u>AAS Instrument Specifications:</u>**

Table 19: Specifications of Atomic Absorption Spectrophotometer(AAS)

Instrument	AAS Motras Scientific
Model	AAS Plus-ADS1000 FSX
Lamp	Fe, Ca and Zn
AAS Software	AAS Analyzer
Variable Slit	0.0 to 2.5nm
Air	Acetylene Burner
Gas	Acetylene (C_2H_2)
Replicates	(n=3)
Air Compressor	10
Sample Calculation	PPM
Modulation	Ultra-fast 1024 Hz
Band width	0.0 nm- 2.5 nm

9. Inductively Coupled Plasma Optical Emission Spectrometry (ICP- OES):

ICP-OES is a robust method for elemental investigation in plants. With its, broad dynamic range, and fast analysis, this versatile technique is essential for studying the elemental composition of plants, monitoring nutrient uptake, and assessing the effects of environmental factors on plant nutritional composition.

- **Principle of ICP-OES:**

ICP-OES operates by introducing a finely aerosolized sample into an argon plasma generated by high-frequency induction. The high temperature of the plasma ionizes the sample, promoting the emission of characteristic optical radiation. This radiation is then dispersed by a spectrometer, which separates the emitted wavelengths using a diffraction grating. The intensities of the emitted wavelengths are measured by photomultiplier detectors, providing qualitative and quantitative information about the elemental composition of the sample.

- **Instrumentation :**

Within the realm of instrumentation, a Titan MPS microwave digestor, a substantial power of 950 W along with meticulous temperature and pressure monitoring and control, assumed a pivotal role in the sample digestion process. Aligning with the rigorous standards set by the U.S. Environmental Protection Agency (EPA), the microwave underwent calibration procedures involving the controlled heating of precise water volumes at designated power levels. Drawing from cumulative experience, it was advised to maintain sample weights at or below the stipulated values to prevent potential over pressuring and venting hazards during the microwave digestion sequence. Consequently, the integration of these constituents in the analysis warranted cautious

adjustments, such as opting for reduced sample sizes and lower power levels, to prevent any risk of over pressuring.

The bottom of the analysis was delivered to the Perkin-Elmer ICP AVIO 200 inductively coupled plasma-optical emission spectrometer (Fig.9), which boasted an axial standard torch coupled with a cross-flow nebulizer. Augmenting precision, an auto sampler featuring a quartz sample probe was employed, effectively mitigating cross-contamination between samples. Detailed specifics regarding the operational parameters of the ICP-OES Avio 200 and the optimal conditions for the plasma can be found in the provided table. The selection of the ICP AVIO 200 was done because of its sensitivity and precision, particularly in the domain of trace metal detection. An additional merit lay in its simultaneous measurement capability, thereby circumventing any compromise on sample throughput even when performing measurements across multiple wavelengths of an element. As a strategic approach, multiple emission lines were concurrently assessed for each element to ensure the validation of analytical outcomes. In the context of *Terminalia* species sample analysis, the methodology pivoted around an acid matrix. The identification of optimum background correction points (BGC) and peak windows constituted a crucial prelude to the analysis, their determination being contingent on the intrinsic attributes of the sample. A pre-analysis calibration aligned the standard concentrations with the projected levels found within the diluted samples, establishing a firm foundation for subsequent analysis endeavors [197].

- **Standard Preparation :**

  The metal contents are determine based on the presence or absence of elements in selected plant materials, as specified in the literature review.

  For our research we took 0.1 ml of the metal standard from 1000 mg/L (1000ppm) as standard stock and dilute it to 10 ml with 2% HNO_3 (i.e. 10 ug/ml or 10 ppm). From this stock, prepare aliquots by taking 1 ml, 3 ml, and 5 ml, and dilute each to 10 ml with 2% HNO_3, resulting in aliquots of 1 ug/ml (1 ppm), 3 ug/ml (3 ppm), and 5 ug/ml (5 ppm), respectively.

- **Sample preparation for Inductively Coupled Plasma-Optical Emission Spectroscopy Analysis Utilizing the Avio 200 System by PerkinElmer :**

1. Take 0.4 gm of dried powdered sample of the selected four *Terminalia* species into a digestion vessel.
2. Add 5.0 ml Nitric Acid (HNO_3 -70%) and 3 ml H_2O_2 - Hydrogen peroxide.
   Mix well and wait for 5-10 mintues before closing the digestion vessel.
3. Close the vessel with stopper and run the following program **(Table 20)**. **Note:**To avoid foaming & splashing wait until the vessel has cooled down to room temp. about 20 minutes.
4. Carefully open the digestion vessel in a fume hood wearing hand, eye, and body protection since a large amount of gas is produced during the digestion process.
5. This solution is analyzed by ICP-OES for major and minor elemental composition (Table 21)

Table 20: Optimize conditions for digestion system

Step	Target temp.	Pressure Max (bar)	Ramp time-Min	Hold time -Min	Power-%
1	150	30	10	5	20
2	190	35	5	15	90
3	50	35	1	10	0

Fig.9: ICP-OES AVIO 200 and Sample Titan MPS Digestor

Table 21: Optimization Conditions for ICP-OES

Optimum Conditions	Parameters
Plasma power	1350- 1500 Watts
Gas flow	0.2(Nebulizer)/ 0.8 Auxiliary (Liters/Minutes)
Coolant	287.78 °C(550 F)
Nebulizer type	Cross flow
Pump Speed	6.2 RPM
Stabilization Time	60 Seconds
No. of probes for each Measuring	3
Plasma observation	Axial (Low Concentration), Radial (High Concentration)
Integration time	5 s

10. Inductively Coupled Plasma Mass Spectrometry (ICP-MS):

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is a highly effective technique for elemental analysis in plants. By combining the sensitivity and wide elemental coverage of mass spectrometry with the robust plasma source of inductively coupled plasma, ICP-MS enables accurate determination and quantification of trace elements in plants. This capability has revolutionized plant analysis, allowing for investigations into nutrient uptake, metal accumulation, and the influence of environmental factors on plant health.

- **Principle:**

ICP-MS operates by ionizing and atomizing the sample using an inductively coupled plasma source. The plasma source generates temperatures, resulting in the complete ionization and excitation of elements in the sample. The ions produced in the plasma are then introduced into the mass spectrometer, where they are separated based on their mass-to-charge ratios (m/z) and detected by sensitive detectors. The abundance of ions at specific m/z values provides qualitative and quantitative information about the elements present in the sample.

- **Instrumentation:**

The equipment employed for this study is a NexION 2000 ICP-MS (PerkinElmer Inc., Connecticut, USA), featuring a Quadrupole ion deflector designed to concentrate the ion beam onto the dual-mode detector. Alongside its remarkable sensitivity, the NexION 2000 ICP-MS integrates a helium gas collision system, effectively mitigating spectral interference attributed to argon. Subsequent data processing utilized the Syngistix ™ Software (PerkinElmer Inc.) shown in Fig.10 [197, 198].

- **Calibration Procedure and Analytical Approach :**
- **Methodology :**

Calibration solutions used for ICP-MS analysis were accurately prepared, involving the specific dilution of commercially sourced multi-element standards with 1% nitric acid-ultrapure water to achieve targeted concentrations. The elemental standards encompassed atomic masses such as 53Cr, 57Fe, 59Co, 60Ni, 63Cu, 66Zn, 75As, 111Cd, 202Hg, and 208Pb. The quantification of heavy metals including Cr, Fe, Co, Ni, Cu, Zn, As, Cd, Hg, and Pb within medicinal plant samples was meticulously conducted through ICP-MS analysis, consistently repeated in triplicate. Employing the Perkin Elmer NexION 2000 ICP-MS model, the analytical procedure for heavy metal assessment adhered to the specifications outlined in Table 22 [197, 198].

➢ **Sample Preparation :**

1. Weigh about 0.1 g of sample into digestion vessel
2. Add 5 mL of nitric acid and 1 mL of hydrogen peroxide to the sample and keep it for minimum of 10 minutes in the fume hood
3. Sample digest in Microwave sample preparation system.
4. After digestion open the vessel carefully and left it as such until brown fumes were no longer visible.
5. Rinse the lid and the walls with Milli-Q-water and Transfer into a 50 mL Volumetric flask and make up to 50 mL with Milli-Q-water and aspirate into ICP-MS.

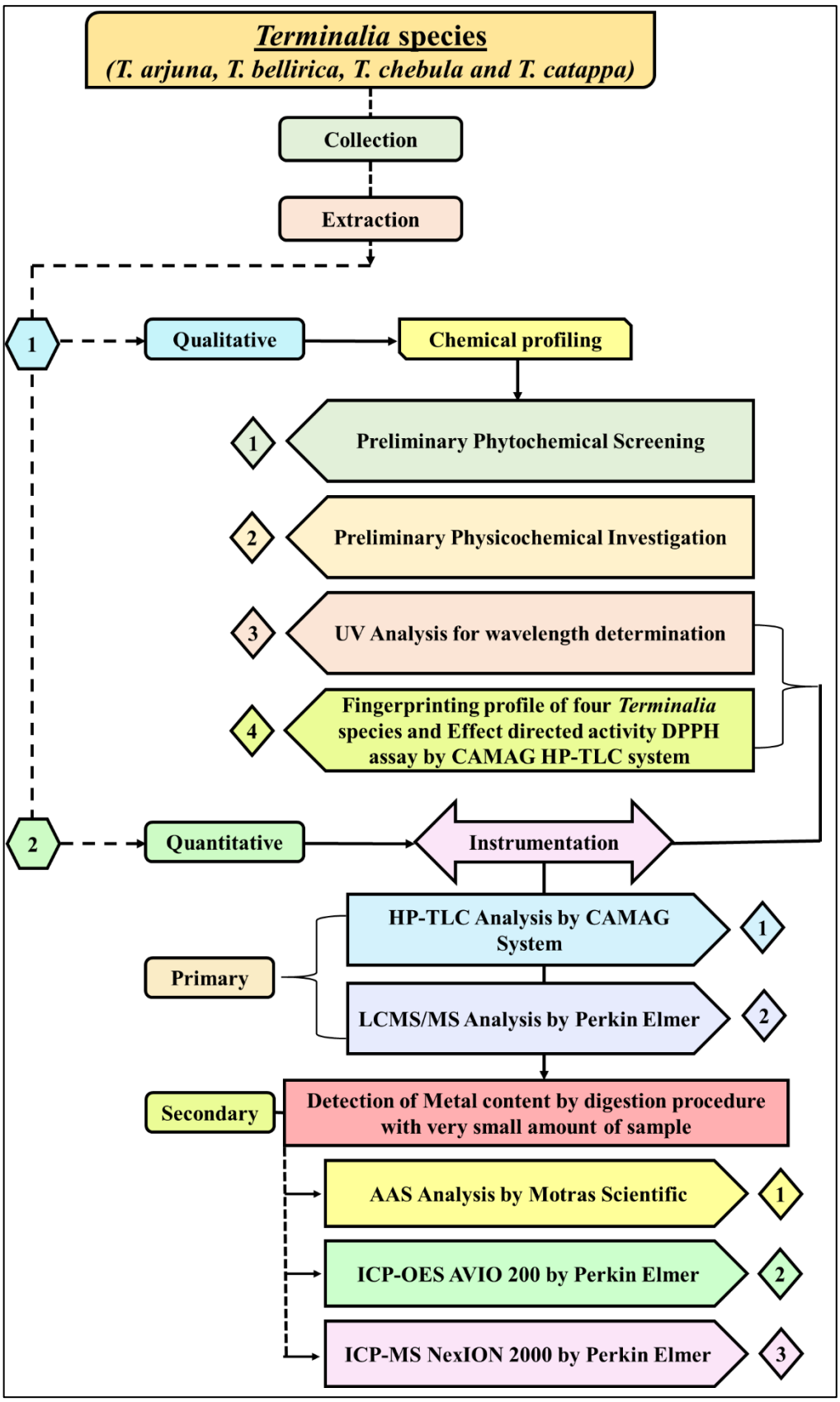
Fig. 10: Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

- **A Summary of Instrument conditions:**

Table 22: Optimized conditions for ICP-MS

Instrument	NexION 2000 (Perkin Elmer)
Instrument Software	SYNGISTIX
RF Power	1600 W
Plasma Gas Flow	18 L/min
Auxiliary Gas Flow	1.2 L/min
He Flow	On
He Flow Rate	4.0 mL/min
Nebulizer Glass	Type C
Nebulizer Flow	< 2% optimized for oxide
Read delay and Analysis Speed	-35.0 rpm and -50.0 rpm
Sweeps/Reading	30
Readings/Replicate	1
Number of Replicates	3
Dwell Time	50 ms
Scan Mode	Peak Hopping
RP_a	0.00
RP_q	0.25
Detector Mode	Dual

Study Flow Chart



Chapter 6 – Results

1. Botanical Evaluation:

1.1. Macroscopic characteristics :

The results for macroscopic analysis of selected *Terminalia* species provide valuable insights into the composition of each plant part, offering information on purity (foreign matters), drying efficiency (loss on drying), inorganic content (total ash and acid insoluble ash), moisture levels, and solubility characteristics (water and alcohol soluble extractives). Additionally, the pH values of the 10% aqueous solutions indicate the acidity or alkalinity of the plant parts.Interpretation of these results (Table 23) in the context of established quality standards and specifications is essential for assessing the suitability of the plant materials for their intended applications.

Table 23: Macroscopic characteristics of the selected *Terminalia* species

Parameters	*T. arjuna*	*T. bellirica*	*T. chebula*	*T. catappa*
Part used	Dried bark	Fruits	Fruits	Fruits
Shape	Fibrous-woody and smooth-skinned	ovoid	Round to ovoid	flattened-egg-shaped
Size	8.5 cm length x 6.3 cm width	2-2.5 x 1.8 cm	4 cm in length x 2.5 cm wide	5-7 cm x 3-3-5.5 cm
Color	pinkish-gray	yellowish brown	yellowish brown	green (unripe), yellow or red (ripe)
Odor	characteristics	aromatic	unpleasant	pleasant smell
Taste	acrid bitter taste	astringent	bitter	slightly acidic

2. Evaluation parameters :

2.1. Physico-chemical evaluation :

The moisture content, extractive values, total ash value, acid insoluble ash value, and water-soluble ash value of each *Terminalia* species were assessed, and all parameters were found to meet the specified limits (Table 24).

Table 24: Average percentage of physicochemical parameters in four *Terminalia* species

Parameters	Results			
	T. arjuna	***T. bellirica***	***T. chebula***	***T. catappa***
Foreign Matters	1 % w/w	1 % w/w	2.5 % w/w	1.3% w/w
Loss on Drying	7.08 %	12.27 %	2.51%	5.3%
Total Ash	7.2 %	3.46 %	8.56%	7.9 %
Moisture Content	1 %	0.8 %	8.59%	1.95 %
Acid Insoluble Ash	1.72 %	0.56 %	0.20%	1.047%
Water Soluble Extractives	64.54 %	1.91 %	0.93%	0.38 %
Alcohol Soluble Extractives	36.32 %	15.20 %	17.28%	23.66 %
pH (10 % aqueous solution)	3.967%	4.66%	7.45%	4.7%

2.2. Chemical evaluation :

The chemical evaluation of *T. arjuna*, *T. bellirica*, *T. chebula*, and *T. catappa* involved a detailed phytochemical analysis to identify various phytoconstituents represented in Table 25 and Fig. 11. This comprehensive phytochemical analysis provides valuable insights into the diverse chemical composition of the selected plant parts, laying the foundation for understanding their potential pharmacological and therapeutic properties. The variations in phytoconstituents among the plant species highlight their distinct biochemical profiles, which may contribute to their varied medicinal uses.

- **Phytochemical analysis :**

Table 25: Preliminary phytochemical investigation of four *Terminalia* species

+: Presence in low concentration; ++: Present in high concentration

Phytoconstituents	Tests	*T. arjuna*	*T. bellirica*	*T. chebula*	*T. catappa*
Phytosterols	Salkowski reaction	++	+	+	+
Triterpenoids	Liebermann-Burchard's test	+	+	+	+
Saponins	Foam test	+	+	+	+
Alkaloids	Dragendorff test	+	++	+	+
Carbohydrates	Molisch's test	+	+	+	+
Flavonoids	Lead Acetate test	++	++	++	++
Lactones	Legal's test	++	+	+	+
Phenols	5% $Fecl_3$ Test	+	++	++	+
Tannins	5% $Fecl_3$ Test	++	++	++	++
Proteins	Ninhydrin test	+	+	+	+
Glycosides	Keller-Killani test	++	+	+	+

.

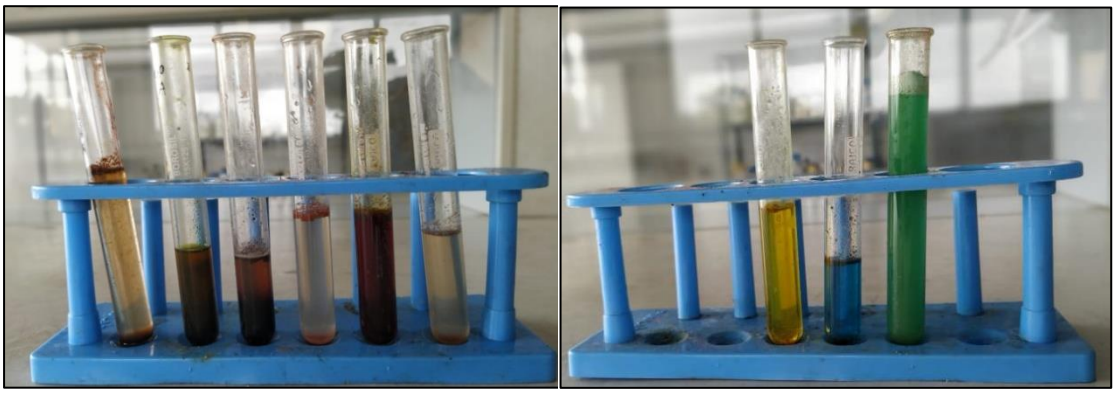


Fig.11: Phytochemical tests for *Terminalia* species

3. Extraction :

The fruits of *T. arjuna*, *T. bellirica*, *T. chebula*, and *T. catappa* were subjected to extraction using the specified procedure, and the percentage yield of each extract was determined based on the weight of air-dried plant material. The extraction process involved Soxhlet extraction with 70% alcohol. The resulting extracts were concentrated using a rotary evaporator and utilized for various Pharmacognostic screening tests. A total of 150 grams of each *Terminalia* species was thoroughly extracted, and the resulting extract was evaluated to determine the percentage yield (Table 26).

Table 26: Percentage yield of Hydro-alcoholic extracts of *Terminalia* species

Crude Drug	Percentage Yield (%)
Terminalia arjuna	26.75
Terminalia bellirica	25.43
Terminalia chebula	22.07
Terminalia catappa	27.54

4. **UV data for Qualitative analysis / Confirmation :**

These specific λ values represent the wavelengths at which each phytoconstituent demonstrates maximum absorbance during UV spectral analysis (Fig. 12). The information on the absorption maxima is crucial for characterizing and quantifying these phytoconstituents, aiding in the identification and quality assessment of the plant materials.The UV data results for the identified phytoconstituents in the selected plant parts are presented below in Table 27, indicating the range of maximum spectral scan (λ):

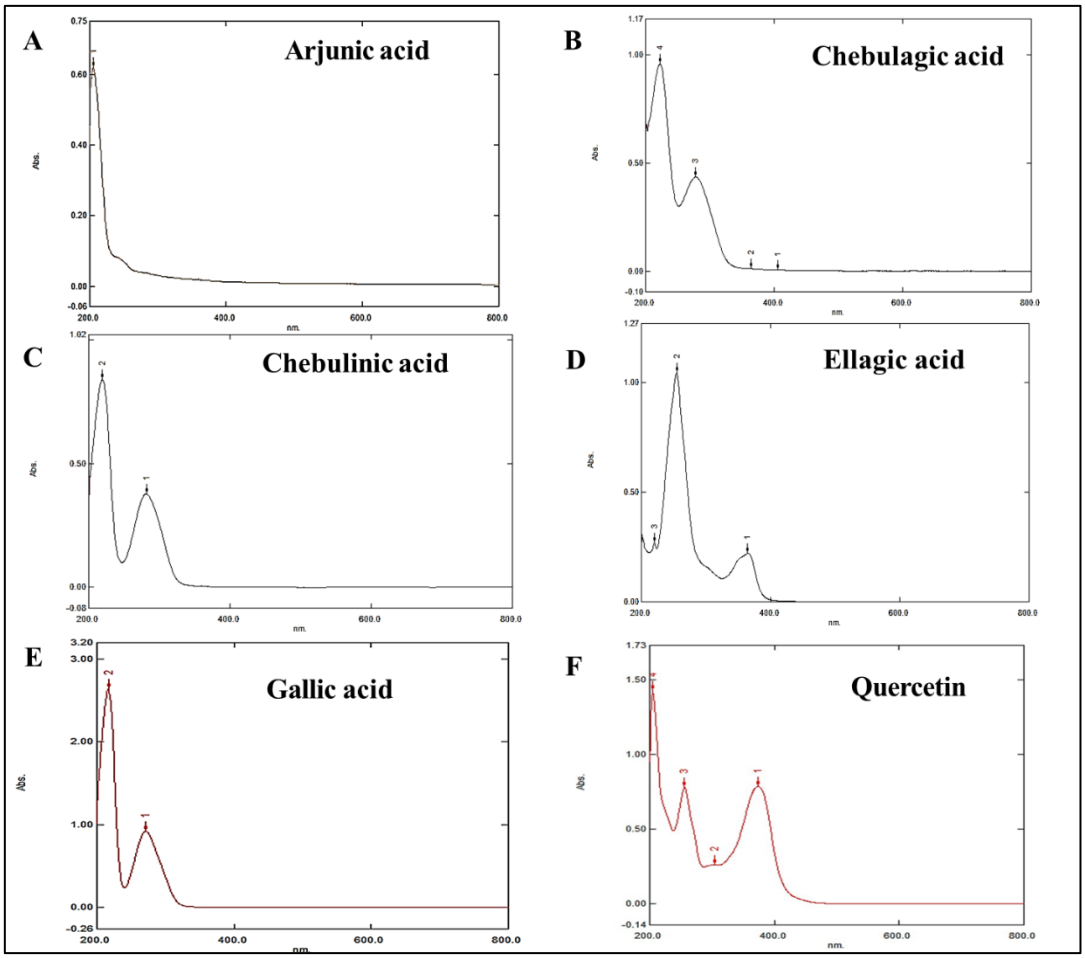


Fig.12: UV Spectral scan of Phytoconstituents present in selected *Terminalia* species

Table 27: Spectral Data for phytoconstituents present in four *Terminalia* species

Phytoconstituents	Range of maximum spectral scan (λ)
Arjunic acid	205 nm
Chebulagic acid	222.5 nm
Chebulinic acid	281 nm
Gallic acid	271 nm
Ellagic acid	254.5 nm
Quercetin	374 nm

5. Fingerprint Profiling by HP-TLC :

HP-TLC fingerprinting analysis was conducted on selected *Terminalia* species to detect classes of compounds, including flavonoids, phenols, tannins, and antioxidants (Table 28). The analysis utilized an optimized solvent system and various derivatizing reagents (Table 29). The results were documented through photo documentation and analyzed using densitometric and fluorescence detectors at wavelengths of 254 nm, 366 nm, and 540 nm.

Table 28: Sample preparation for Fingerprinting Analysis

Sample	Name	Concentration (Ethanol)
A	*Terminalia arjuna (Ta)*	100 mg/ 10 ml
B	*Terminalia bellirica (Tb)*	100 mg/ 10 ml
C	*Terminalia chebula (Tc)*	100 mg/ 10 ml
D	*Terminalia catappa (Tct)*	100 mg/ 10 ml
Plate type	TLC Silica gel F254s	(20 X 10) cm

Table 29: HP-TLC fingerprinting analysis optimized solvent system for different class of compounds (Phytoconstituents)

Class of compounds	Solvent System
Flavonoids	Ethyl acetate: Water: Formic Acid: Acetic Acid (100: 26: 11: 11) v/v/v/v
Phenols	Cyclohexane: Ethyl acetate: Formic Acid (4:6:1) v/v/v
Tannins	Toluene: Ethyl acetate: Formic Acid (6:4:0.3) v/v/v
Antioxidants	n-Butanol: Glacial acetic acid: Water (4:4:1, v/v/v)

5.1. Flavonoid contents :

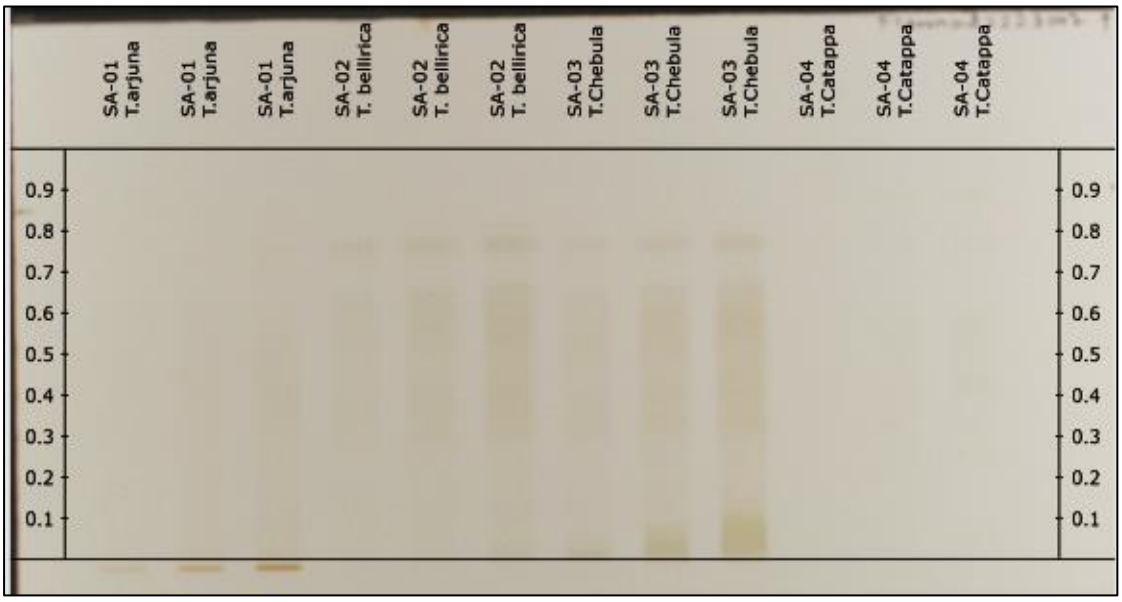


Fig.13: HP-TLC Fingerprinting profile for flavonoids

(Plate Image @540 nm)

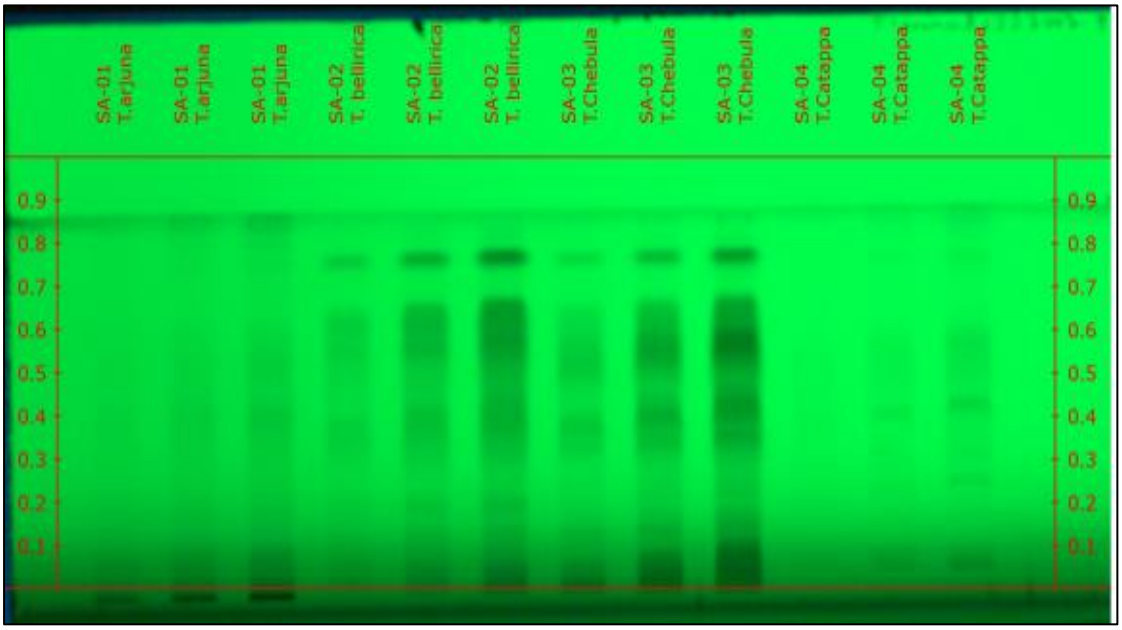


Fig.14: HP-TLC Fingerprinting profile for flavonoids

(Plate Image @254 nm)

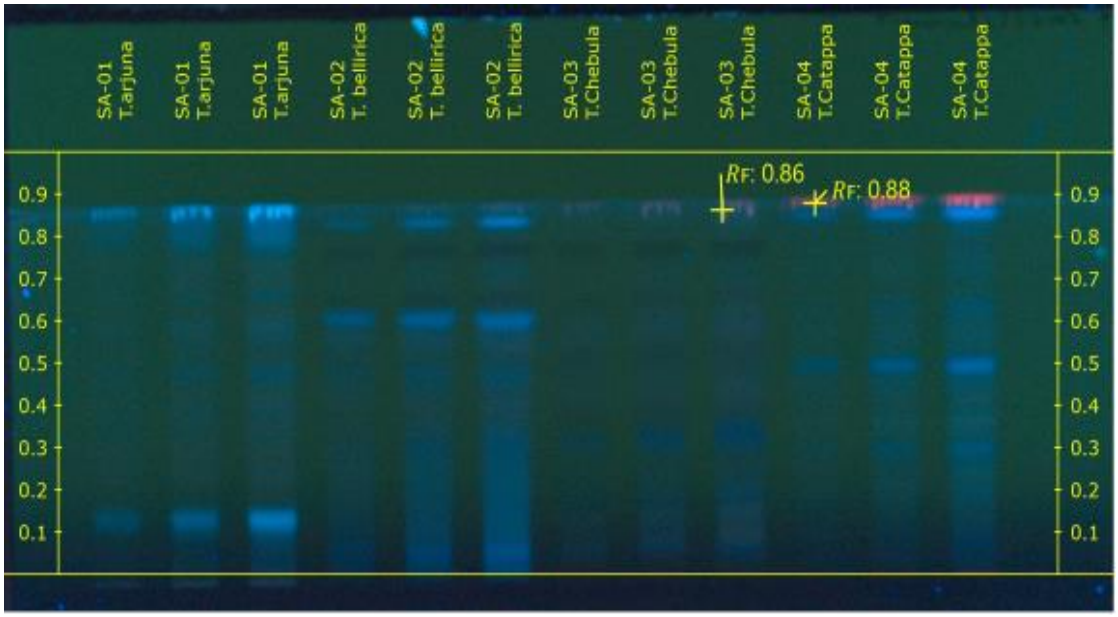


Fig: 15: HP-TLC Fingerprinting profile for flavonoids

(Plate Image @366nm)

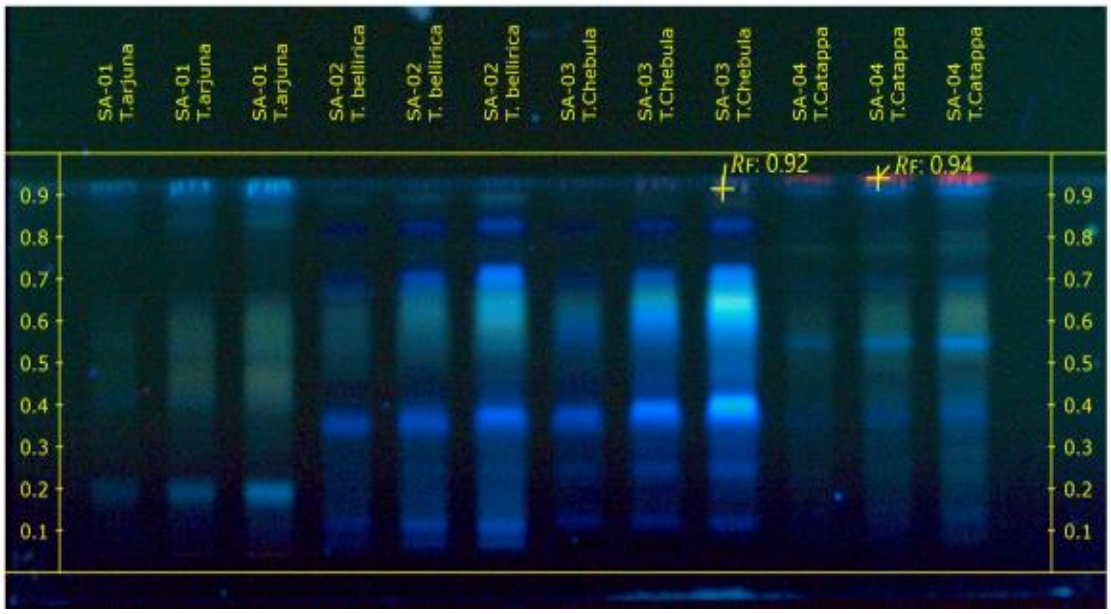


Fig. 16: Derivatized plate with Natural Product reagent with confirmation of flavonoids (Plate image @366 nm)

The fingerprinting plate for flavonoids was developed and photo documented at Fig. 13-15. The fingerprinting exhibited Fig. 16 (two blue and two green bands-*Ta*); (one dark green, two dark blue, one green and two blue bands-*Tb*); (six dark blue, two

blue bands-*Tc*); (six green, one red and two blue bands-*Tct*) after derivatization with Natural Product reagent corresponding to flavonoid compounds.

5.2. Detection of Phenol contents :

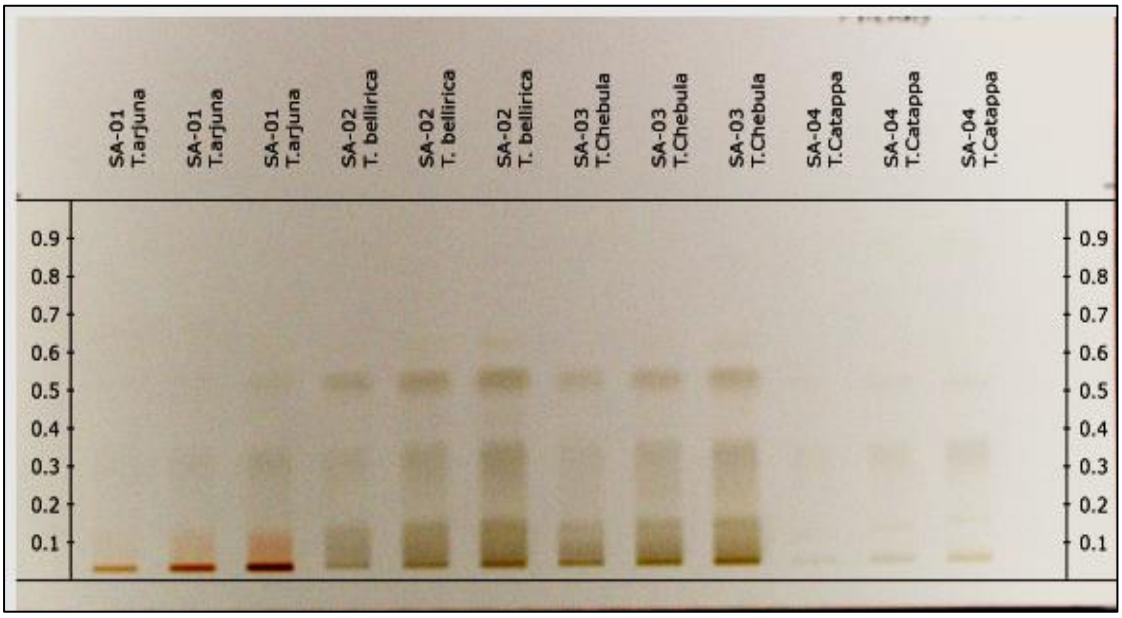


Fig. 17: HP-TLC Fingerprinting profile for phenols

(Plate Image @540 nm)

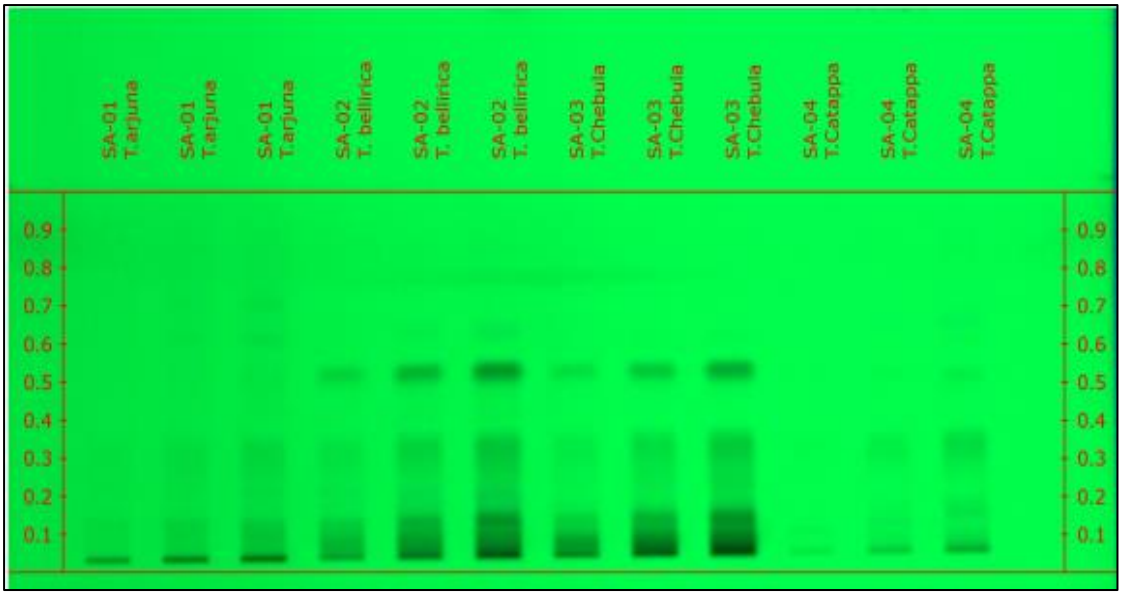


Fig. 18: HP-TLC Fingerprinting profile for phenols

(Plate Image @254 nm)

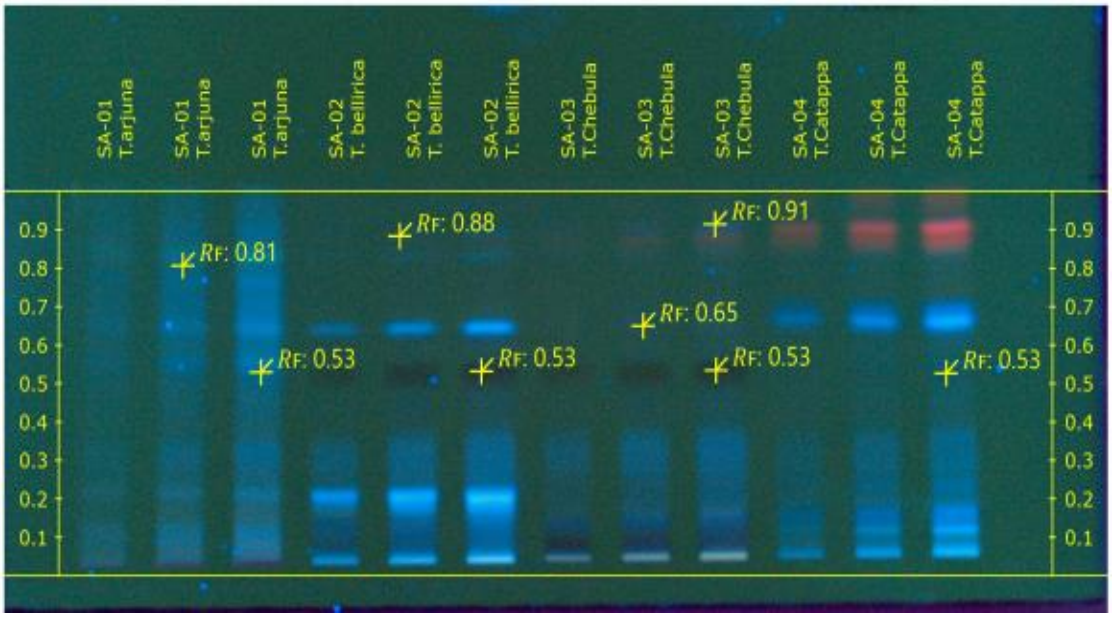


Fig.19: HP-TLC Fingerprinting profile for phenols

(Plate Image @366 nm)

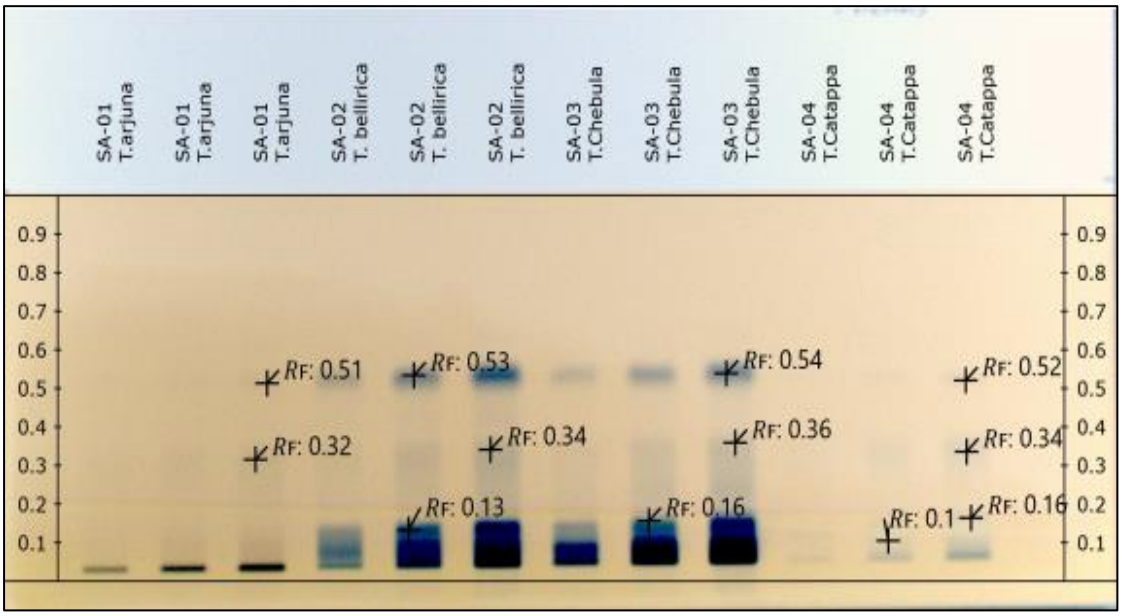


Fig. 20: Derivatized plate with alcoholic Ferric chloride reagent with confirmation of phenols (Plate image@540 nm)

The fingerprinting plate for phenols was developed and photo documented at Fig. 17-19. After derivatization with the Alcoholic Ferric chloride reagent corresponding to phenolic compounds Fig. 20 (Five dark blue bands-*Ta*); (six dark

blue bands-*Tb*); (six dark blue bands-*Tc*); (eight dark blue bands-*Tct*) were detected.

5.3. Detection of Tannin contents :

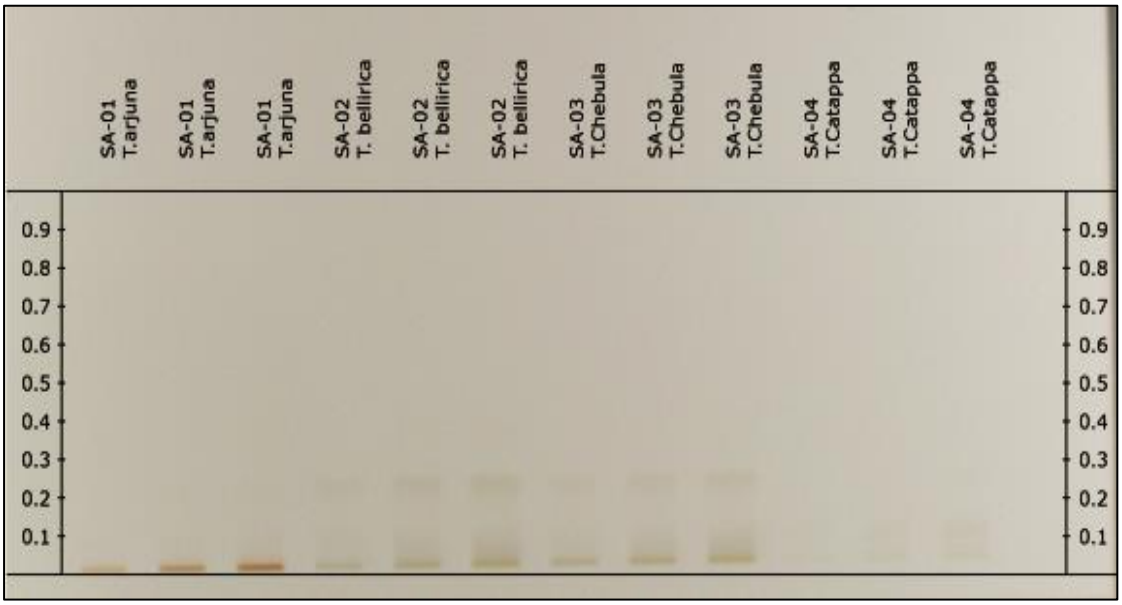


Fig. 21: HP-TLC Fingerprinting profile for tannins(Plate Image @540 nm)

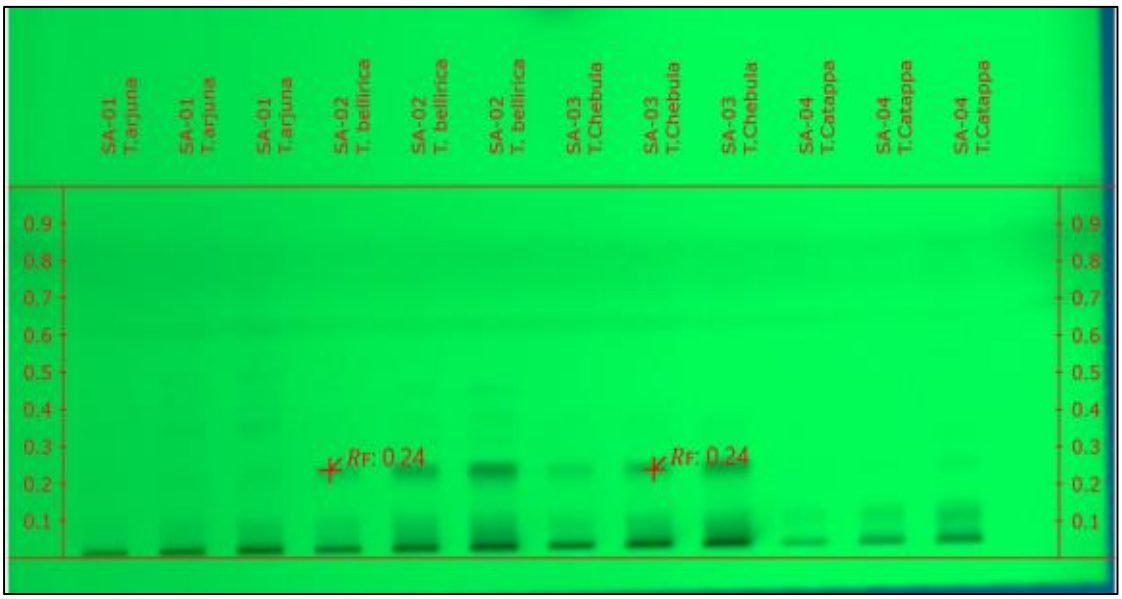


Fig. 22:HP-TLC Fingerprinting profile for tannins (Plate Image @254 nm)

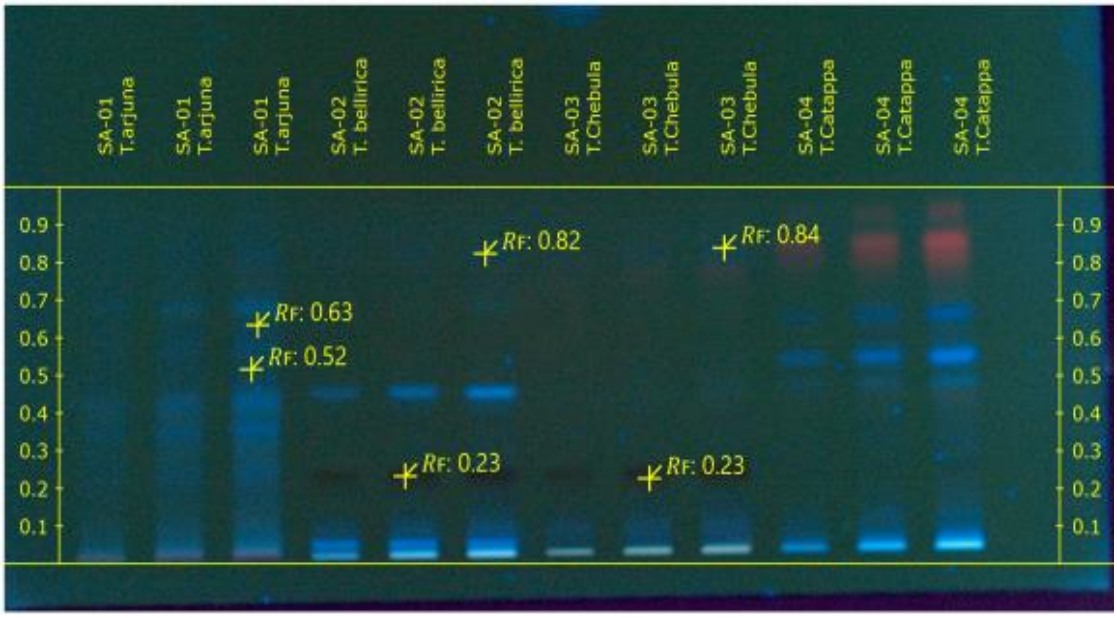


Fig. 23:HP-TLC Fingerprinting profile for tannins (Plate Image @366 nm)

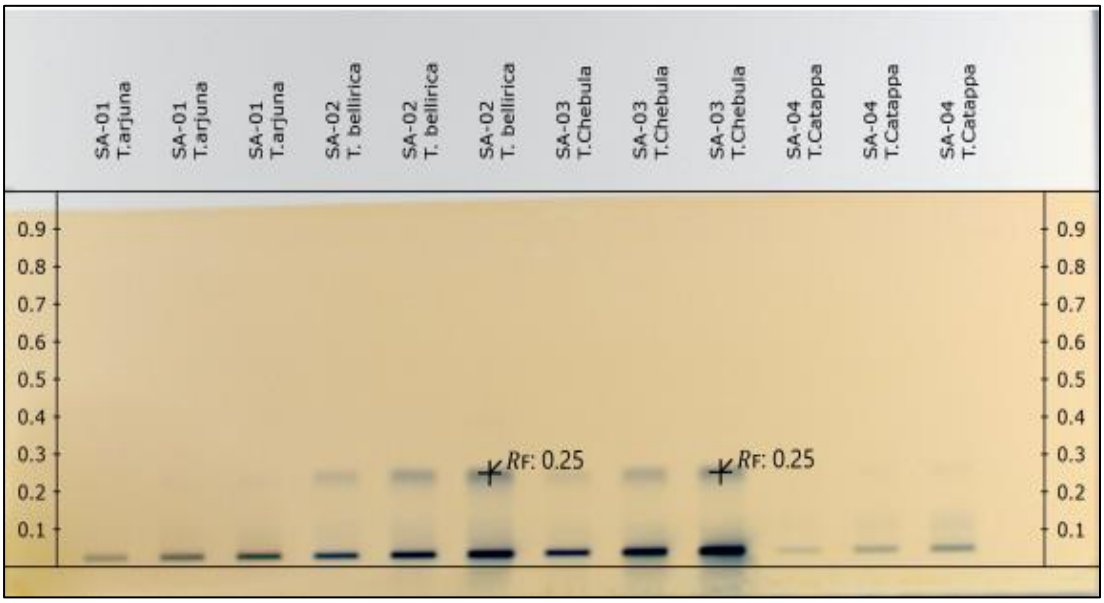


Fig. 24: Derivatized plate with Ferric chloride reagent with confirmation of tannins (Plate image @540 nm)

The fingerprinting plate for tannins was developed and photo documented at Fig. 21-23. Fingerprinting results after derivatizing with Iron Chloride ($FeCl_3$) reagent corresponding to tannin: Fig. 24 (One dark blue band-*Ta*); two dark blue bands-*Tb*); (two dark blue bands -*Tc*); (one dark blue band-*Tct*).

5.4. Detection of antioxidants by HP-TLC-DPPH :

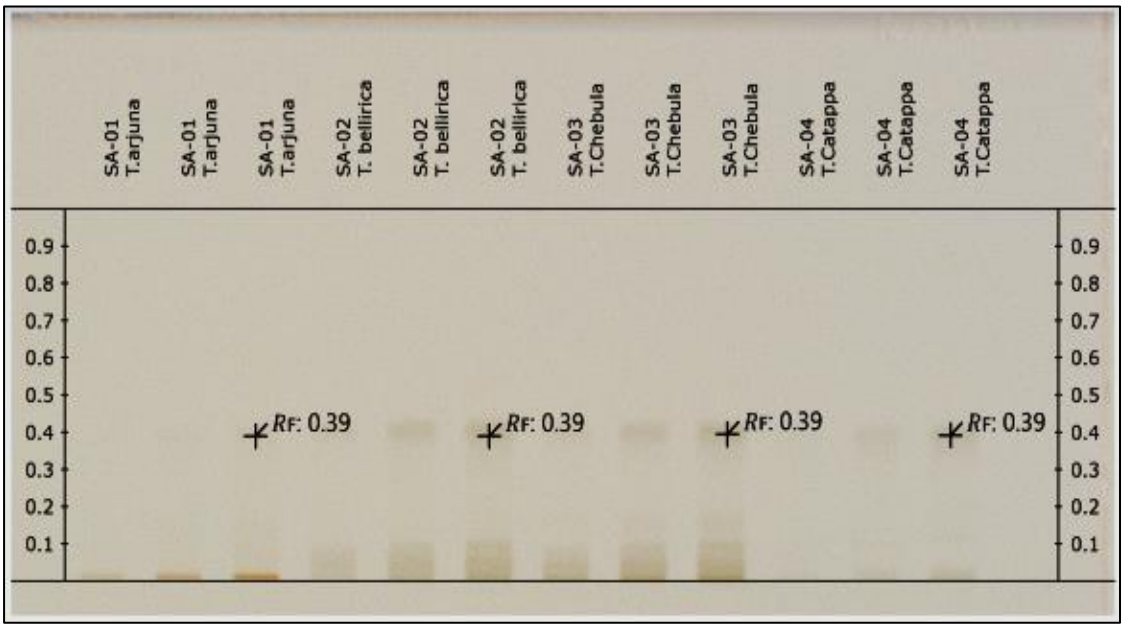


Fig. 25: HP-TLC Fingerprinting profile for effect directed DPPH activity

(Plate Image @540 nm)

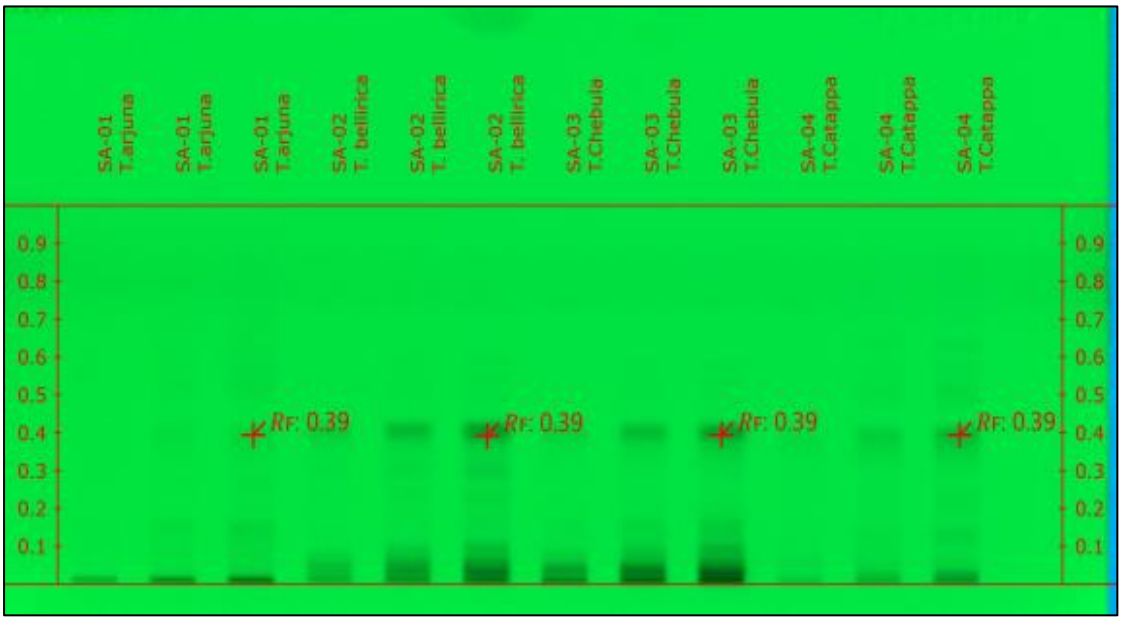


Fig. 26: HP-TLC Fingerprinting profile for effect directed DPPH activity

(Plate Image @254 nm)

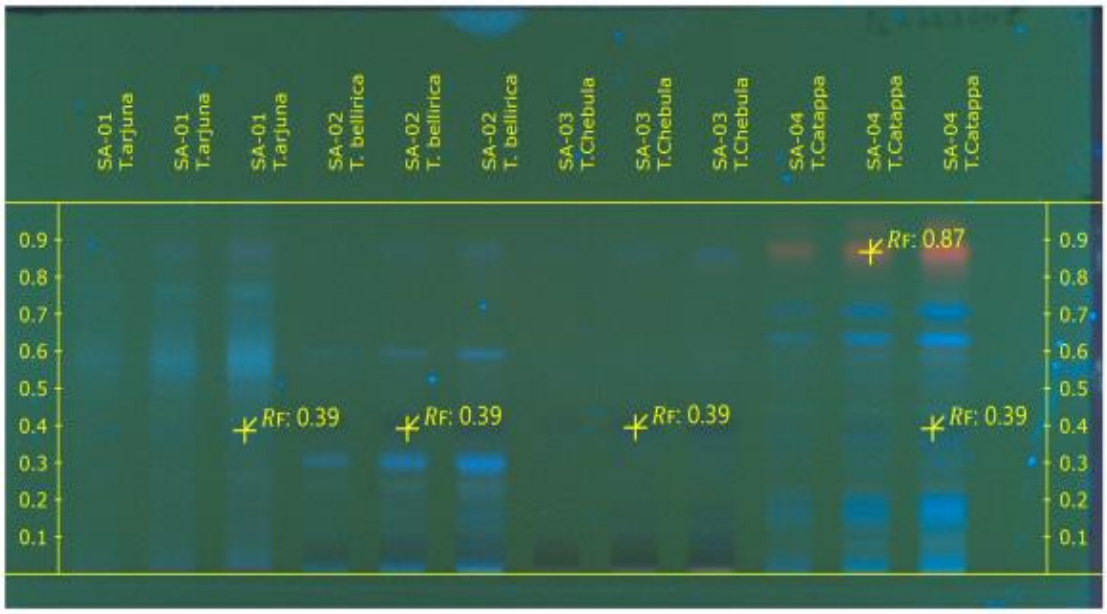


Fig. 27: HP-TLC Fingerprinting profile for effect directed DPPH activity (Plate Image @366 nm)

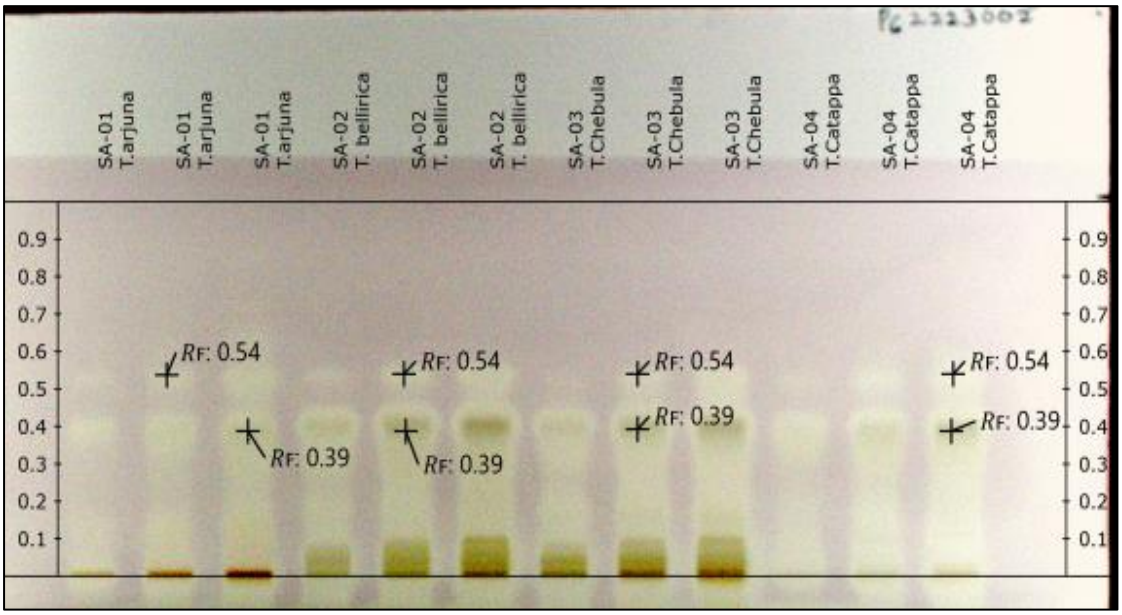


Fig. 28: Derivatized plate with effect directed HP-TLC-DPPH reagent with confirmation of antioxidants (Plate image @366 nm)

The fingerprinting plate for antioxidants was developed and photo documented at Fig. 24-26. The derivatization with DPPH reagent corresponding to antioxidants Fig. 27 (Three lemon yellow bands-*Ta*); (three lemon yellow bands-Tb); (three lemon yellow bands-*Tc*); two lemon yellow bands (*Tct*).

6. Quantification of Gallic acid and Ellagic acid in four *Terminalia* species :

The following methods validated were in accordance with the ICH recommendations stated in Table 30 contains details for standard and sample preparation. The calibration curves for Gallic acid and Ellagic acid were created in the 100-700 ng/mL range. Table 31 and Fig. 29-33 show the optimized solvent solution, Rf values, and plate images.

Table 30: Sample and Standard preparation applied on RP-HP-TLC plate

Sample	Analyte Name	Concentration (MeOH)
Std 1	Gallic acid	0.5 mg/ 10 ml (50 ppm)
Std 2	Ellagic acid	1 mg/ 10ml (100 ppm)
A	*Terminalia arjuna*	100 mg/ 10 ml
B	*Terminalia bellirica*	1 mg / 10 ml
C	*Terminalia chebula*	10 mg/ 10 ml
D	*Terminalia catappa*	100 mg/ 10 ml
Plate type	TLC Silica gel 60 RP-18 F_{254}s	(20 X 10) cm

Table 31: Optimized Solvent system and Rf values of Gallic acid and Ellagic acid

Solvent system	Methanol: Water: Formic acid: Acetic acid (80:100:4:4) v/v/v/v
Std	**Rf**
Gallic acid	0.81
Ellagic acid	0.33

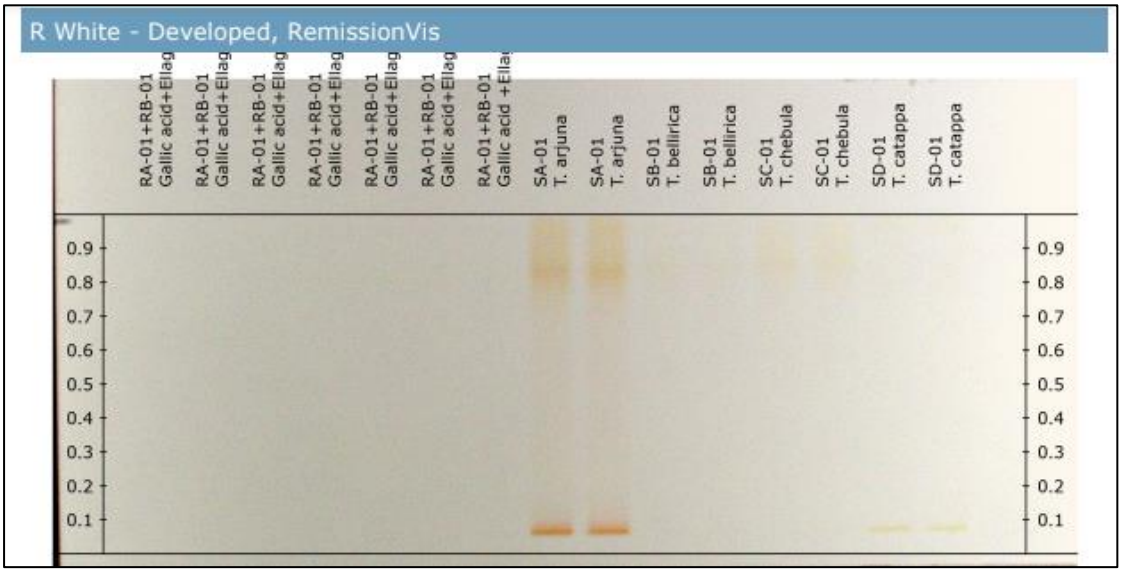


Fig. 29: RP-HPTLC Plate Image @ White Light for four *Terminalia* species

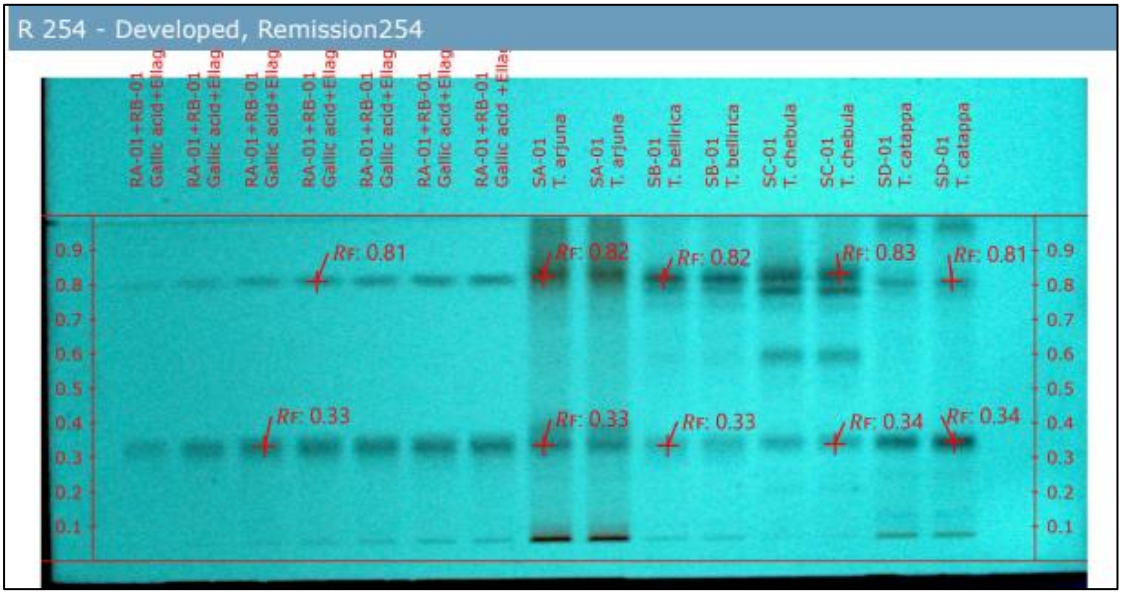


Fig. 30: RP-HPTLC Plate Image @254 nm for four *Terminalia* species

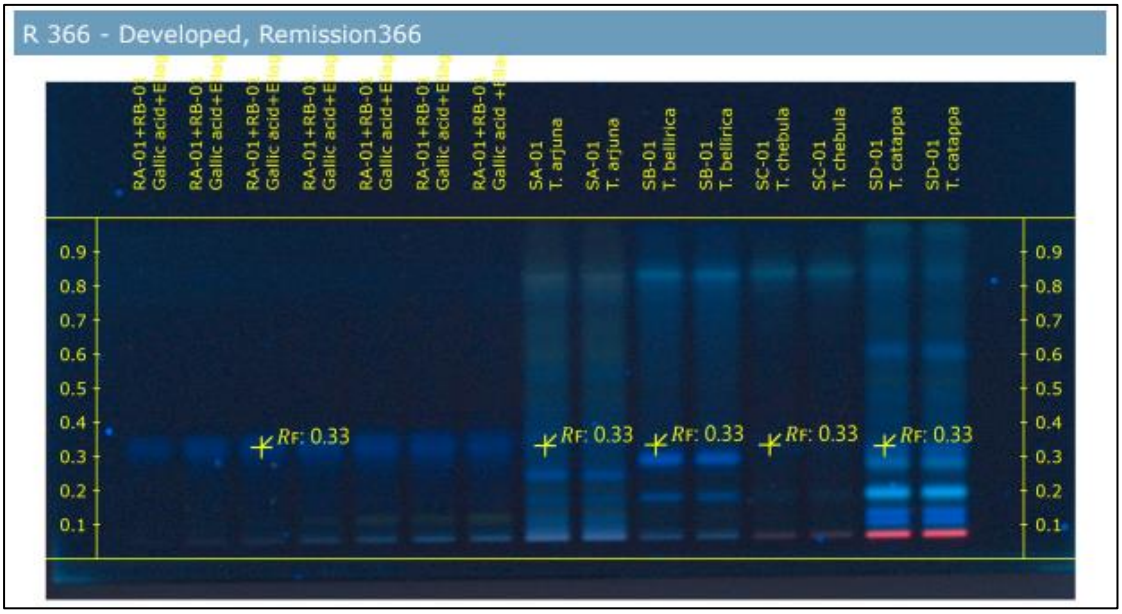


Fig. 31: RP-HPTLC Plate Image @366 nm for four *Terminalia* species

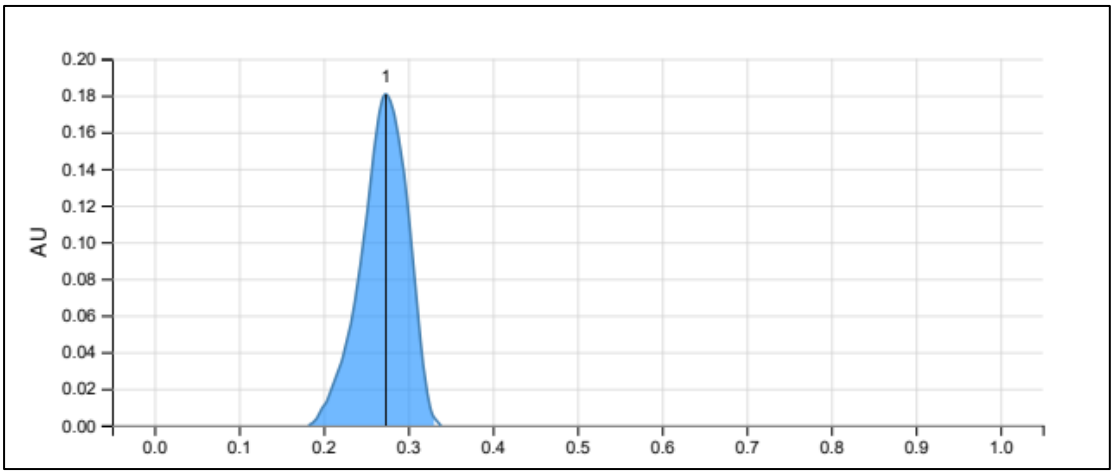


Fig.32: Chromatogram view of Ellagic acid at Rf 0.33 in four *Terminali*a species

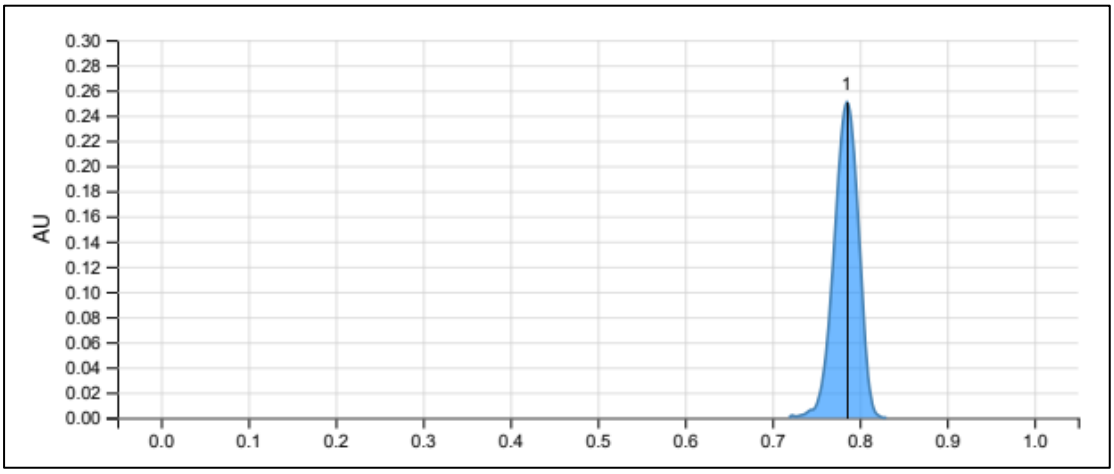


Fig. 33: Chromatogram view of Gallic acid at Rf 0.81in four *Terminali*a species

The spectra of Gallic acid and Ellagic acid were verified at 254 nm and 279 nm (Fig. 34 and 35)

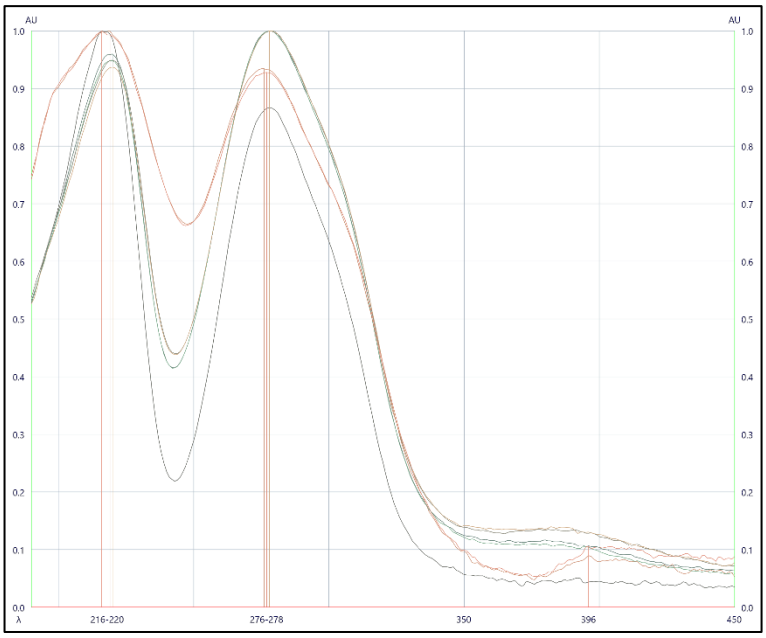


Fig. 34: 3D overlay spectra of Gallic acid @ 254 nm and confirmation in four *Terminalia* species

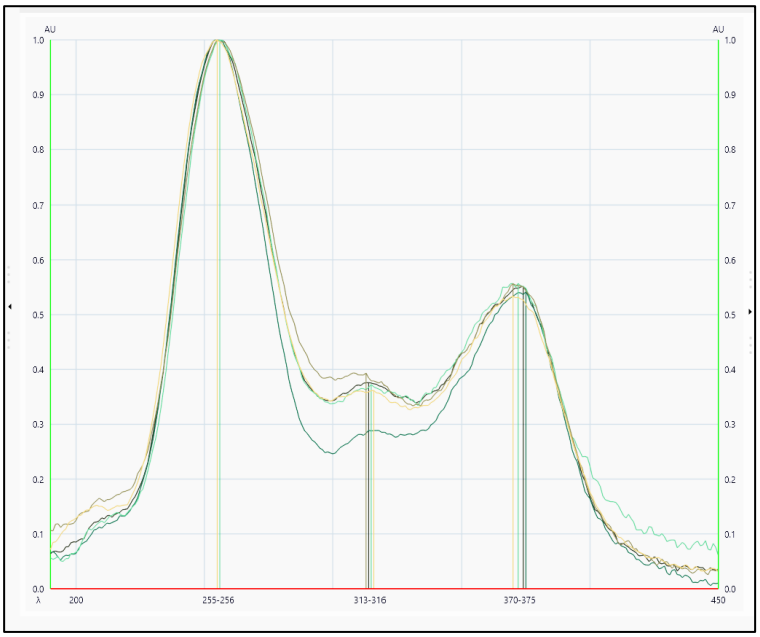
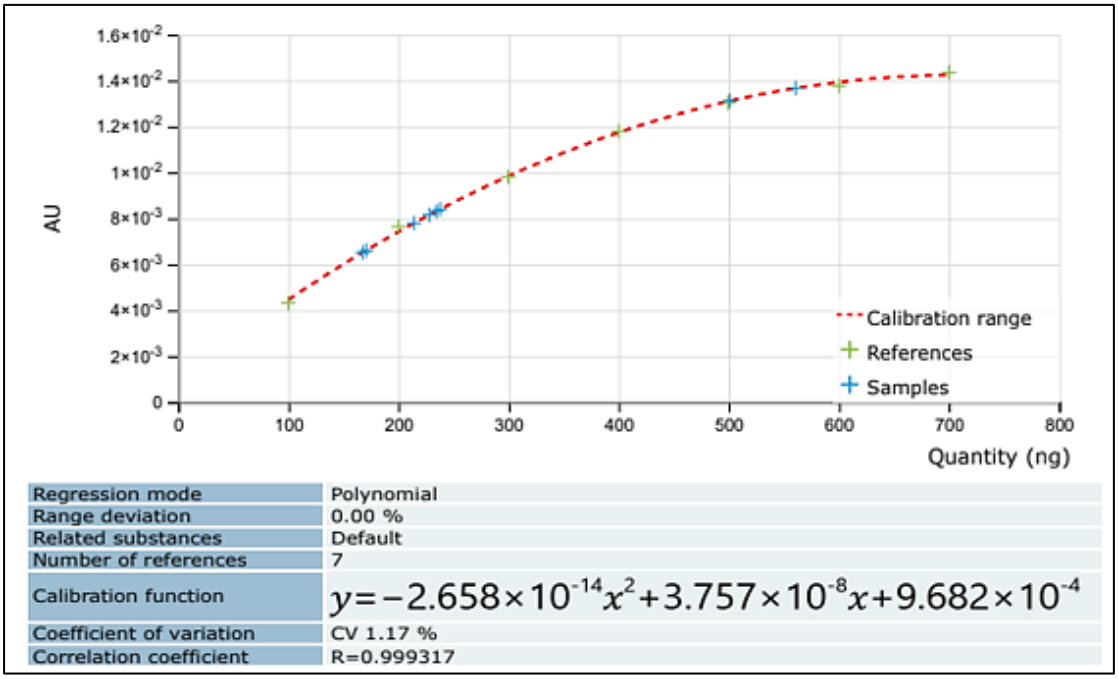


Fig. 35: 3D overlay spectra of Ellagic acid @ 279 nm and confirmation in four *Terminalia* species

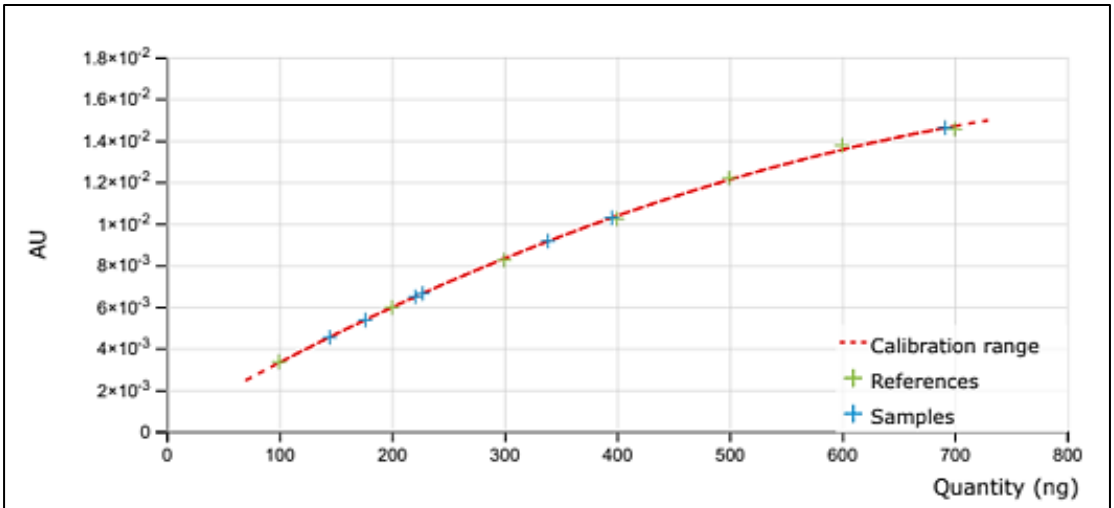
The study exhibited acceptable polynomial regression results for Gallic acid (r2 = 0.9999) and Ellagic acid (r2 = 0.9999). An average relative standard

deviation (RSD) was determined to be 1.17%, and the correlation coefficients for Gallic acid were R = 0.999556 and R = 0.999317 for Ellagic acid (Figs. 36 and 37).The quantification of gallic acid in *T. arjuna* is 32.05 µg/mL, in *T. bellirica* is 46.11 µg/mL, in *T. chebula* is 36.72 µg/mL, and in *T. catappa* is 32.12 µg/ml. On the other hand, the quantification of ellagic acid in *T. arjuna* is 24.15 µg/mL, in *T. bellirica* is 15.74 µg/mL, in *T. chebula* is 22.10 µg/mL, and in *T. catappa* is 106.2 µg/ml. The LOD-LOQ values for Gallic Acid were determined to be 75 ng and 225 ng, respectively, while for EA, the LOD-LOQ values were calculated as 80 ng and 240 ng, respectively. The %RSD for intra-day precision (repeatability) was determined to be 0.56%, while it was 0.78% for inter-day precision (intermediate). The method's precision was demonstrated by % RSD values greater than 2% for both intra-day and inter-day precision. The reproducibility was evaluated applying a total of six levels of Standard GA and EA, with GA 6µL (600 ng) and EA 3µL (300 ng) chosen as the optimal reproducibility values.



Regression mode	Polynomial
Range deviation	0.00 %
Related substances	Default
Number of references	7
Calibration function	$y=-2.658\times10^{-14}x^2+3.757\times10^{-8}x+9.682\times10^{-4}$
Coefficient of variation	CV 1.17 %
Correlation coefficient	R=0.999317

Fig.36: Calibration result of Ellagic acid present in four *Terminalia* species



Regression mode	Polynomial
Range deviation	5.00 %
Related substances	Default
Number of references	7
Calibration function	$y=-1.522\times10^{-14}x^2+3.114\times10^{-8}x+3.427\times10^{-4}$
Coefficient of variation	CV 1.17 %
Correlation coefficient	R=0.999556

Fig. 37: Calibration result of Gallic acid present in four *Terminalia* species

The co-efficient of variance for Gallic acid and Ellagic acid have 1.99% CV and 1.21% CV, specifically, in the final measured values. The sample and standard regions were compared independently at 80%, 100%, and 120%. The recovery study findings, represented as a percentage of recovery, were within acceptable limits (Table 32). The Quantification of Gallic acid and Ellagic acid in four *Terminalia* species are showed in Table 33 and 34.

Table 32: Percentage recovery (n=3) of Gallic acid and Ellagic acid in four *Terminalia* species

	% Recovery levels	Conc. of Std spiked on sample (ng/band) (n=3)	Theoretical Conc. (ng)	Observed Conc. Spot (ng)	% Recovery	Average Percentage Recovery
Gallic acid in *T. arjuna*	80 %	240	0.00664	0.00668	98.52	97.22%
	100 %	300	0.00678	0.00690	97.76	
	120 %	360	0.00712	0.00735	98.40	
Gallic acid in *T. bellirica*	80 %	240	0.01460	0.01473	96.40	98%
	100 %	300	0.01528	0.01566	98.48	
	120 %	360	0.01673	0.01698	97.12	
Gallic acid in *T. chebula*	80 %	240	0.00915	0.00920	89.58	97.96%
	100 %	300	0.01027	0.01075	94.67	
	120 %	360	0.01112	0.01126	99.63	
Gallic acid in *T. catappa*	80 %	240	0.00453	0.00458	85.44	95.64%
	100 %	300	0.00536	0.00582	97.58	
	120 %	360	0.00618	0.00632	98.91	
Ellagic	80 %	240	0.00661	0.006620	96.64	

acid in *T. arjuna*	100 %	300	0.00685	0.007495	94.41	95.46%
	120 %	360	0.00707	0.008380	98.33	
Ellagic acid in *T. bellirica*	80 %	240	0.00824	0.00830	96.28	97.16%
	100 %	300	0.00862	0.00912	95.80	
	120 %	360	0.00894	0.00995	97.42	
Ellagic acid in *T. chebula*	80 %	240	0.00779	0.00782	95.95	96.37%
	100 %	300	0.00815	0.00845	98.68	
	120 %	360	0.00895	0.00925	97.49	
Ellagic acid in *T. catappa*	80 %	240	0.01256	0.01275	97.17	98.36%
	100 %	300	0.01312	0.01345	96.51	
	120 %	360	0.01368	0.01383	98.41	

Table 33: Quantified values and %CV values of Gallic acid in four *Terminalia* species

Amount of Gallic acid detected in sample			%CV
A	*T. arjuna*	32.05µg/ml	2.07%
B	*T. bellirica*	46.11µg/ml	0.08%
C	*T. chebula*	36.72µg/ml	3.87%
D	*T. catappa*	32.12µg/ml	3.94%

Table 34: Quantified values and % CV values of Ellagic acid in four *Terminalia* species

Amount of Ellagic acid detected in sample			%CV
A	*T. arjuna*	24.15µg/ml	1.36%
B	*T. bellirica*	15.74µg/ml	1.03%
C	*T. chebula*	22.10µg/ml	4.43%
D	*T. catappa*	106.2µg/ml	4.94%

7. Phytochemical screening of *T. chebula, T. bellirica* and *T. catappa* containing Chebulagic acid and Chebulinic acid as biomarker :

The preparation of chebulagic acid and chebulinic acid, as well as *T. bellirica*, *T. chebula*, and *T. catappa*, is detailed in Table 35. The mobile phase and Rf values used for their development are provided in Table 36 and Fig. 38-41.

Table 35: Preparation of three *Terminalia* species, Chebulagic acid and Chebulinic acid

Sample	Analyte Name	Concentration (MeOH)
Std 1	Chebulagic acid	0.5 mg/ 10 ml (50 ppm)
Std 2	Chebulinic acid	1 mg/ 10 ml (100 ppm)
A	*Terminalia bellirica*	100 mg / 10 ml
B	*Terminalia chebula*	10 mg/ 10 ml
C	*Terminalia catappa*	400 mg/ 10 ml
Plate type	TLC Silica gel F254	(20 X 10) cm

Table 36: Optimized Solvent system and Rf values of Chebulagic acid and Chebulinic acid

Solvent system	Toluene: Ethyl Acetate: Formic acid: Water (6:14:8:2) v/v/v/v
Std	**Rf**
Chebulagic acid	0.34
Chebulinic acid	0.39

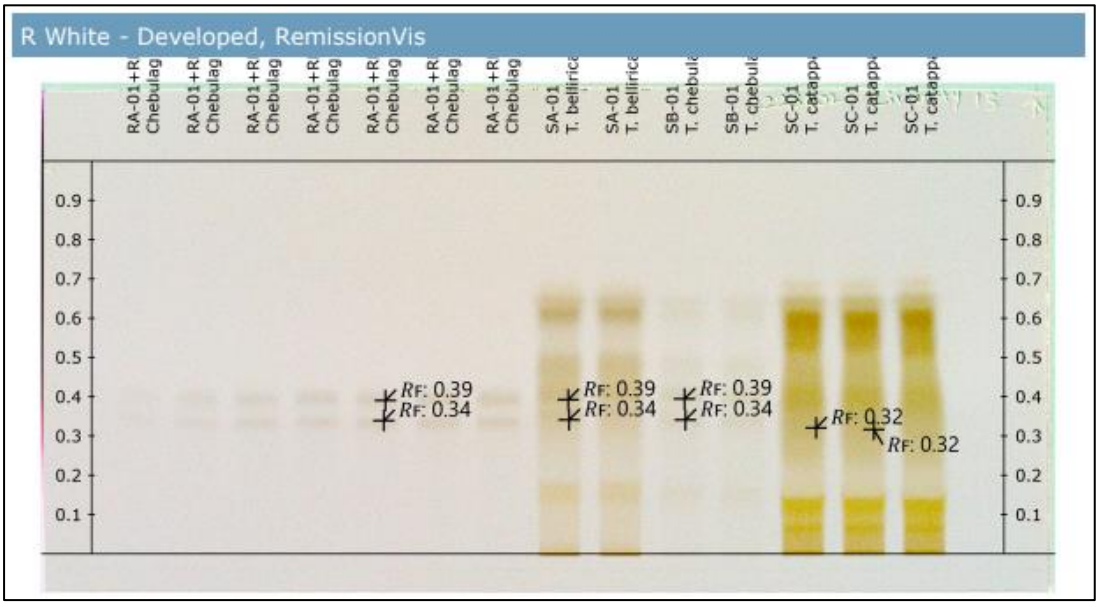


Fig. 38: Chebulagic acid and Chebulinic acid present in three *Terminalia* species (Plate Image @ 540 nm)

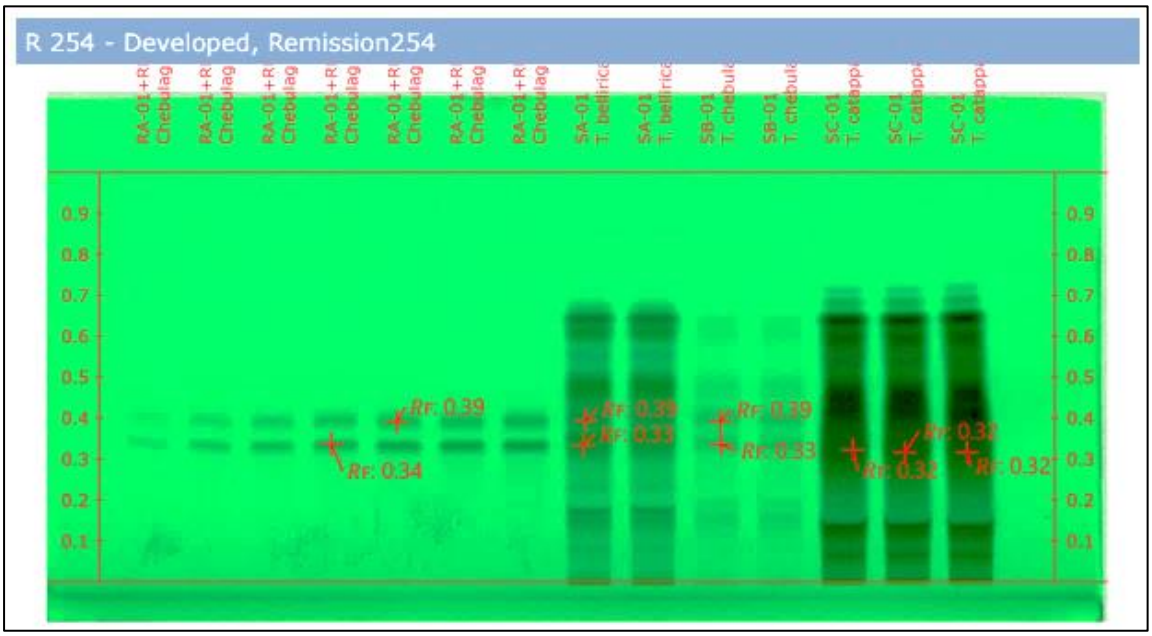


Fig. 39: Chebulagic acid and Chebulinic acid present in three *Terminalia* species (Plate Image @254 nm)

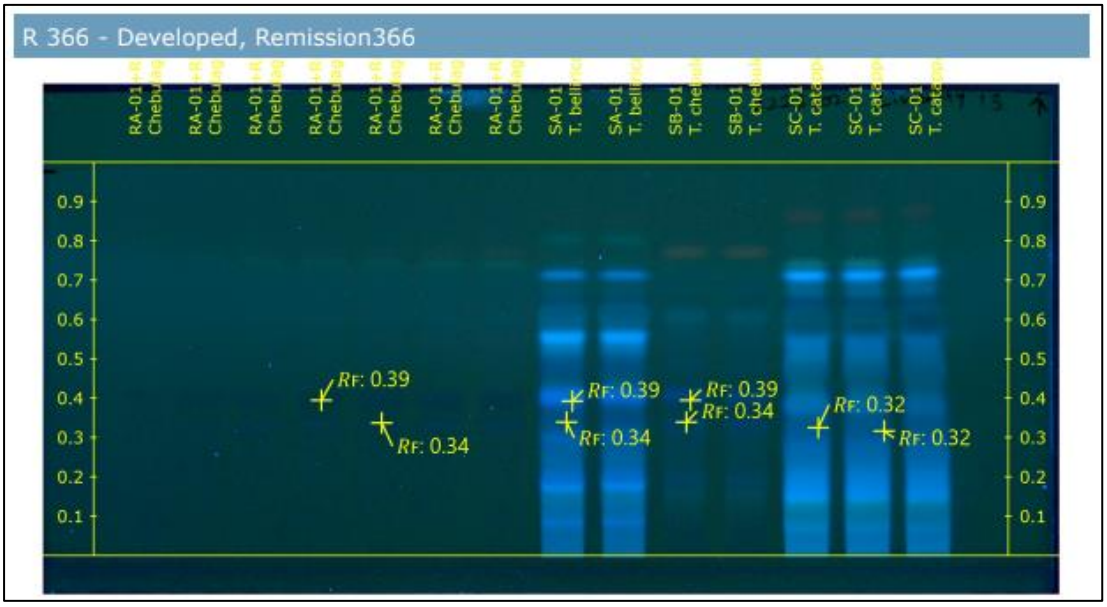


Fig. 40: Chebulagic acid and Chebulinic acid present in three *Terminalia* species (Plate Image @366 nm)

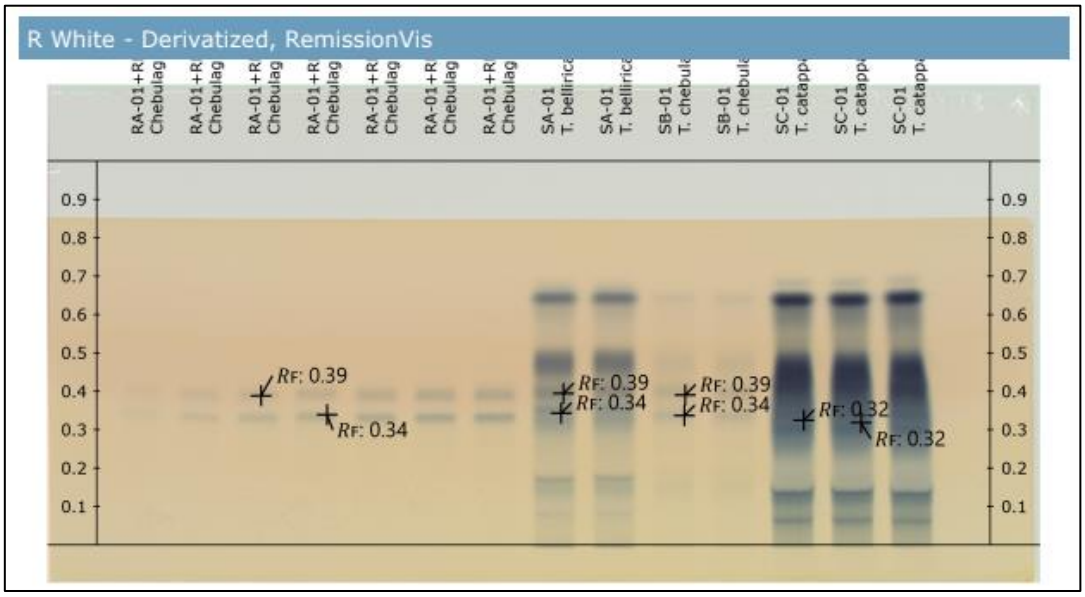


Fig. 41: Chebulagic acid and Chebulinic acid present in three *Terminalia* species after derivatization with Ferric chloride (Plate Image @ 540 nm)

Calibration curves were generated over a concentration range of 50 to 350 ng/ml, and the LOD was determined as 50 µg. The chromatograms of standards are depicted in Fig. 42 and 43. The spectra of chebulagic acid and chebulinic acid were verified at 304 nm and 285.8 nm (Fig. 44 and 45). Regression coefficients (R) were 0.9985 for chebulagic acid (Fig. 46) and 0.9990 for

chebulinic acid (Fig.47). The %RSD for intra-day precision (repeatability) was found to be 0.59%, and for inter-day precision (intermediate), it was 0.85%. Reproducibility was assessed using six levels of Standard Chebulagic acid and chebulinic acid, with both at 2 µL (2000 ng) as optimized reproducibility levels.

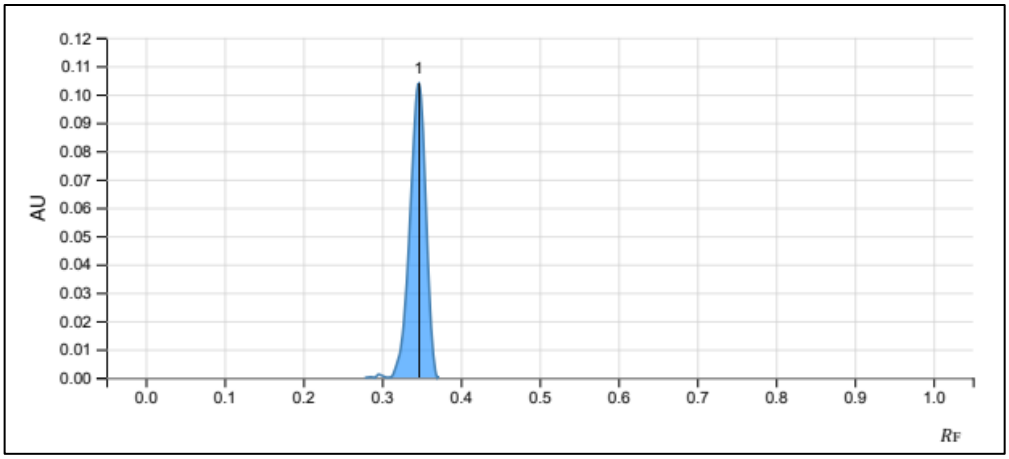


Fig 42: Chromatogram of Chebulagic acid @ Rf 0.34

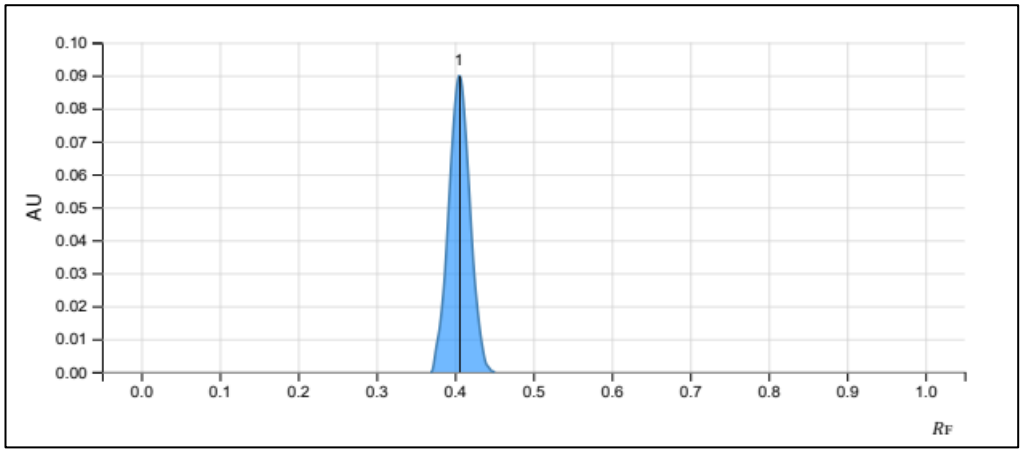


Fig 43: Chromatogram of Chebulinic acid @ Rf 0.39

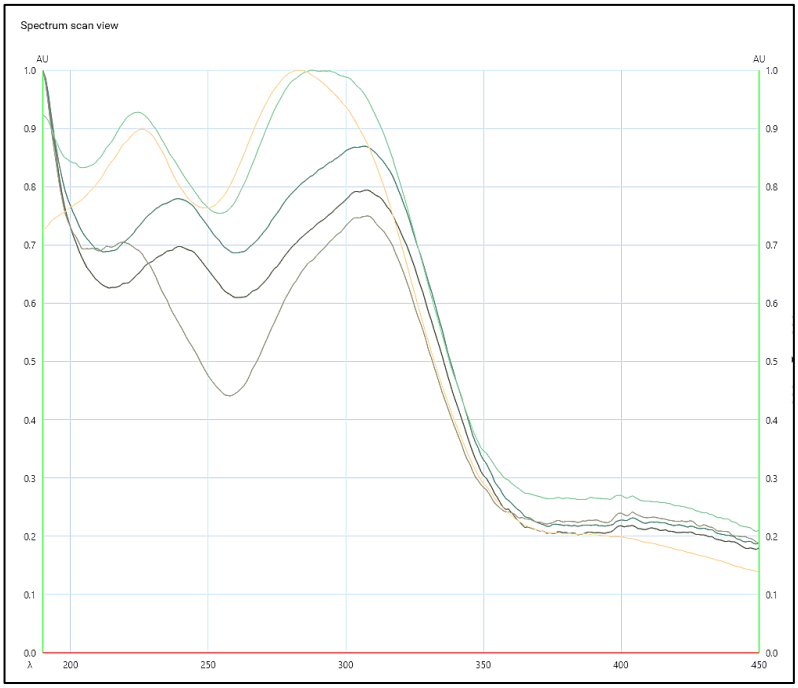


Fig. 44: Overlaying spectra of Chebulagic acid and confirmation in three *Terminalia* species

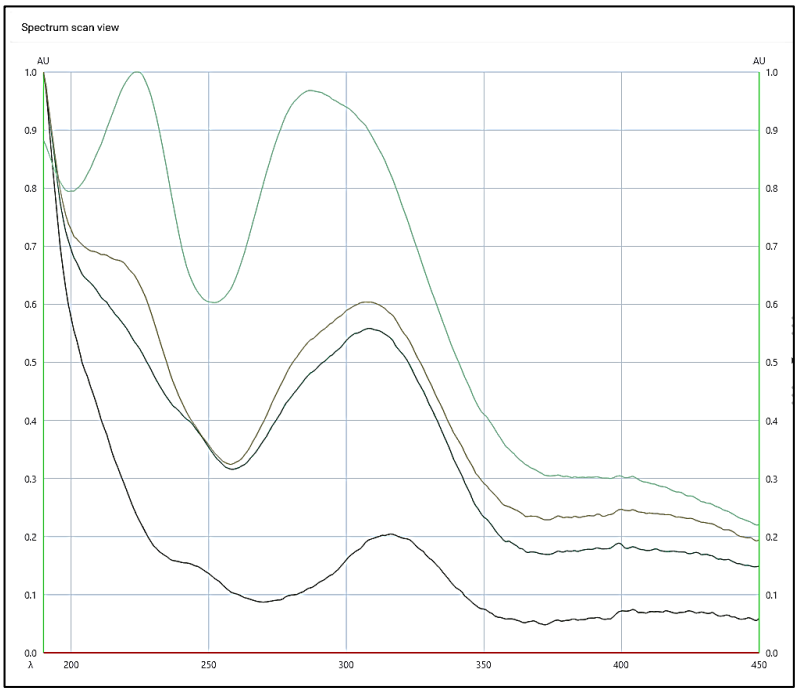


Fig. 45: Overlaying spectra of Chebulinic acid and confirmation in three *Terminalia* species

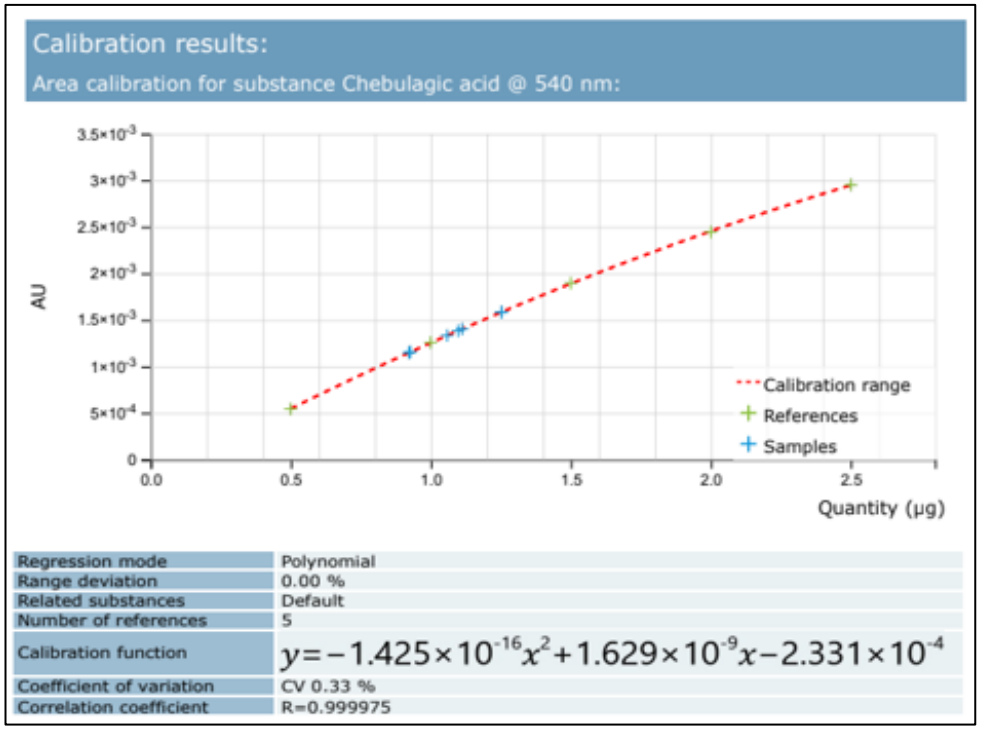


Fig. 46: Calibration result of Chebulagic acid present in three *Terminalia* species

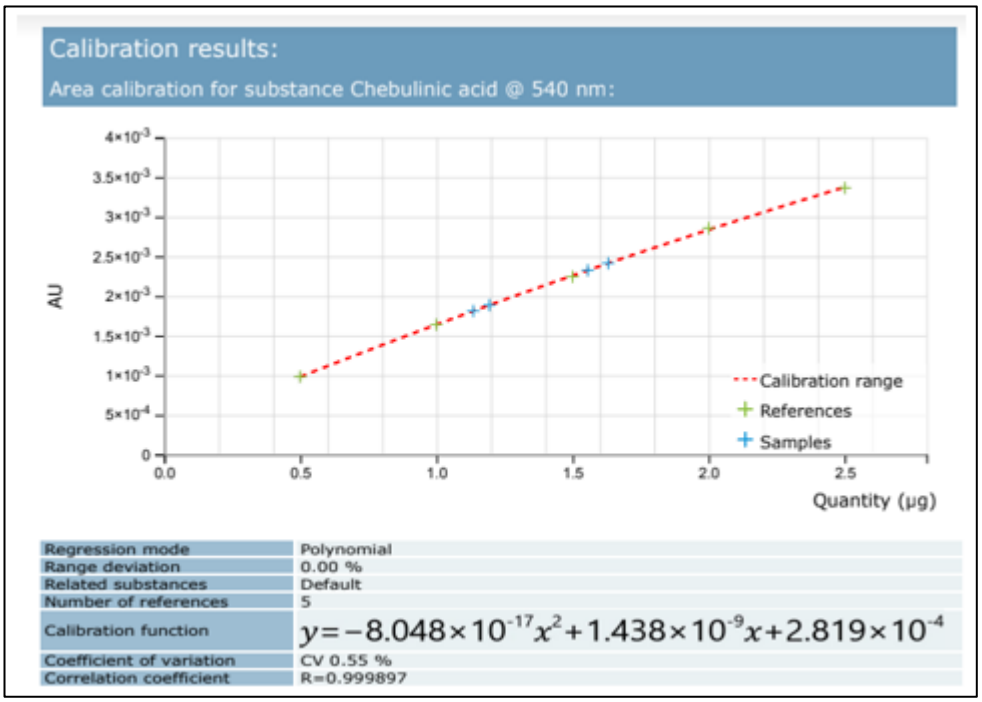


Fig. 47: Calibration result of Chebulinic acid present in three *Terminalia* species

The obtained areas of the sample and standard were compared separately at 80%, 100%, and 120%. The results of the recovery studies, expressed as

percentage recovery, were within acceptable limits (Table 37). The quantification values of coefficient of variance for Chebulagic acid and Chebulinic acid in three *Terminalia* species are showed in Table 38 and 39. The quantification of chebulagic acid concentrations in *T. bellirica* (122.5 µg/ml), *T. chebula* (122.4 µg/ml), and *T. catappa* (63.39 µg/ml), and chebulinic acid concentrations in *T. bellirica* (116.5 µg/ml) and *T. chebula* (159.4 µg/ml) were observed.The final quantified results for chebulagic acid and chebulinic acid showed 1.75% CV and 1.61% CV, respectively.

Table 37: Percentage recovery (n=3) of Chebulagic acid and Chebulinic acid present in three *Terminalia* species

	%Recovery levels	Conc. of Std spiked on sample (ng/band) (n=3)	Theoretical Conc. (ng)	Observed Conc. (ng)	%Recovery	Average Percentage Recovery
Chebulagic acid in *T. bellirica*	80 %	800	0.005310	0.006620	97.93	109.51 %
	100 %	1000	0.006185	0.007495	114.24	
	120 %	1200	0.007070	0.008380	116.38	
Chebulagic acid in *T. chebula*	80 %	800	0.000970	0.002810	109.58	112.62 %
	100 %	1000	0.001245	0.003135	113.25	
	120 %	1200	0.001350	0.003240	115.03	
Chebulagic acid in *T. catappa*	80 %	800	0.000395	0.010095	94.21	101.80 %
	100 %	1000	0.000585	0.010285	102.33	
	120 %	1200	0.000815	0.010515	108.86	
Chebulinic	80 %	800	0.000655	0.003165	72.33	

acid in *T. bellirica*	100 %	1000	0.000770	0.003280	83.34	80.88 %
	120 %	1200	0.000795	0.003305	86.98	
Chebulinic acid in *T. chebula*	80 %	800	0.001200	0.004700	90.28	96.83 %
	100 %	1000	0.001270	0.004770	99.09	
	120 %	1200	0.001760	0.005260	101.11	

Table 38: Quantified values and %CV values of Chebulagic acid in three *Terminalia* species

Amount of Chebulagic acid detected in sample			%CV
A	*T. bellirica*	122.5µg/ml	3.83%
B	*T. chebula*	122.4µg/ml	0.47%
C	*T. catappa*	63.39µg/ml	2.88%

Table 39: Quantified values and %CV values of Chebulinic acid in three *Terminalia* species

Amount of Chebulinic acid detected in sample			%CV
A	*T. bellirica*	116.5µg/ml	1.75 %
C	*T. chebula*	159.4µg/ml	1.61 %

8. Quantification of Arjunic acid in *T. arjuna* and *T. chebula* species :

In the present research work, sample and standard preparation details are provided in Table 40, along with the optimized solvent system and Rf values (Table 41 and Fig. 48-50).The 3D spectral data of arjunic acid is shown in Fig. 51.

Table 40: Preparation of samples of two *Terminalia* species and Arjunic acid

Sample	Analyte Name	Concentration (MeOH)
Std	Arjunic acid	10 mg/ 10 ml (1000 ppm)
A	*Terminalia arjuna*	100 mg / 10 ml
B	*Terminalia chebula*	100 mg/ 10 ml
Plate type	TLC Silica gel F254	(20 X 10) cm

Table 41: Optimized Solvent system and Rf values of Arjunic acid

Solvent system	Toluene: Methanol: Ethyl Acetate: Acetic acid (12:9:3:0.6) v/v/v/v
Std	**Rf**
Arjunic acid	0.83

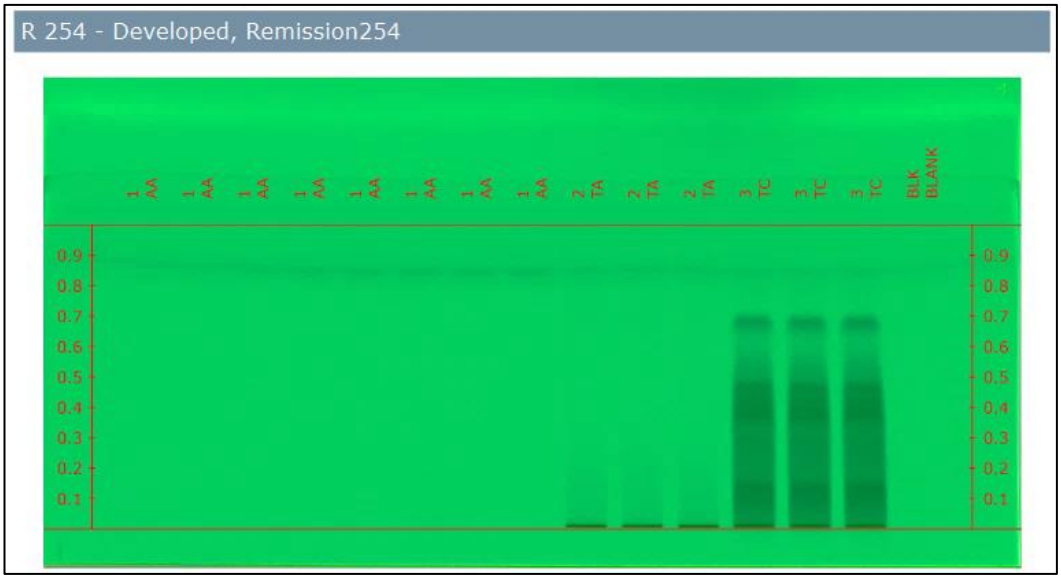


Fig. 48: Arjunic acid present in Two *Terminalia* species **(**Plate Image @254 nm)

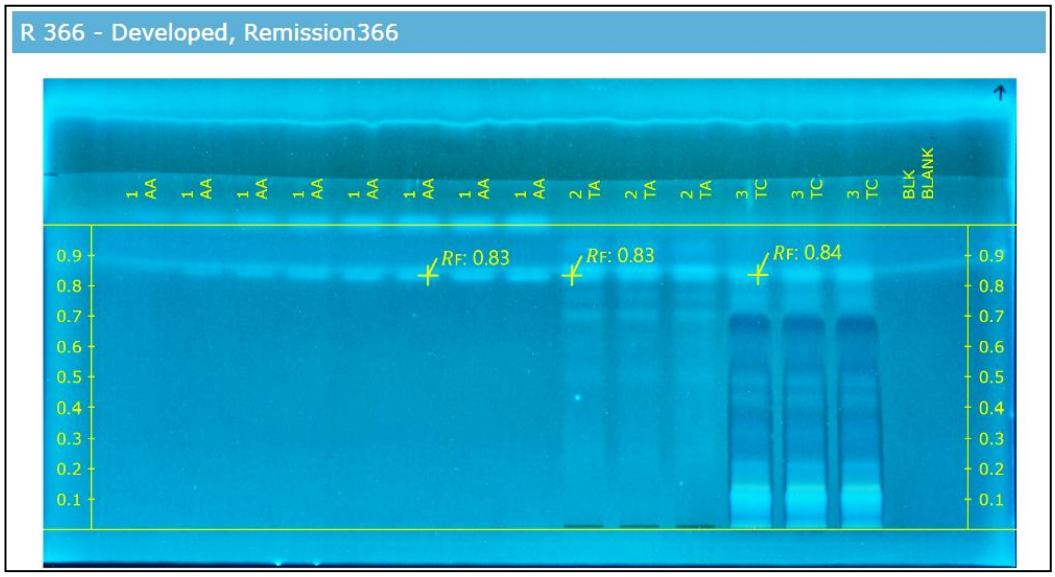


Fig. 49: Arjunic acid present in Two *Terminalia* species **(**Plate Image @366 nm)

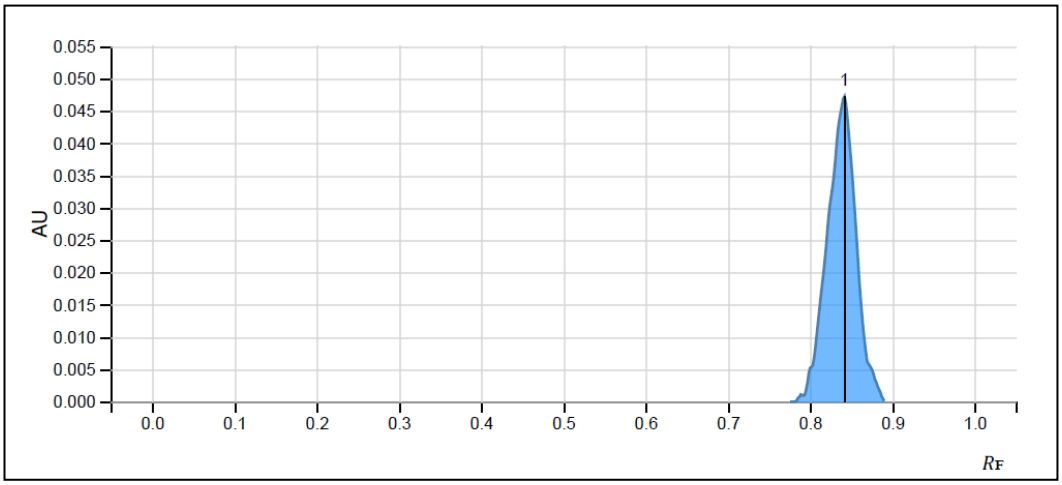


Fig. 50: Chromatogram of Arjunic acid @Rf 0.83

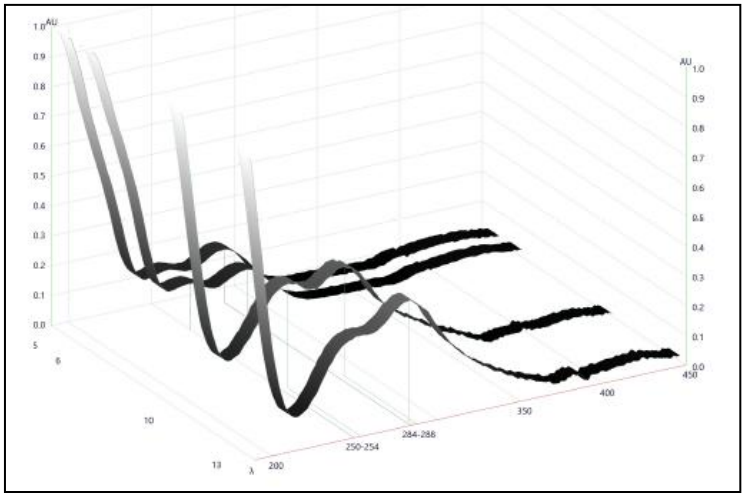


Fig. 51:3D overlaying spectra of Arjunic acid @366 nm and confirmation in two *Terminalia* species

The linearity range for arjunic acid was found to be 10-80 µg/ ml, with an LOD of 30 µg and LOQ of 96 µg. The regression coefficient (R) for arjunic acid was 0.9983 (Fig. 52). The reproducibility was assessed using six levels of Standard arjunic acid, with 2 µL (20 µg) as the optimized reproducibility level, resulting in a final quantified result of 0.79% CV.

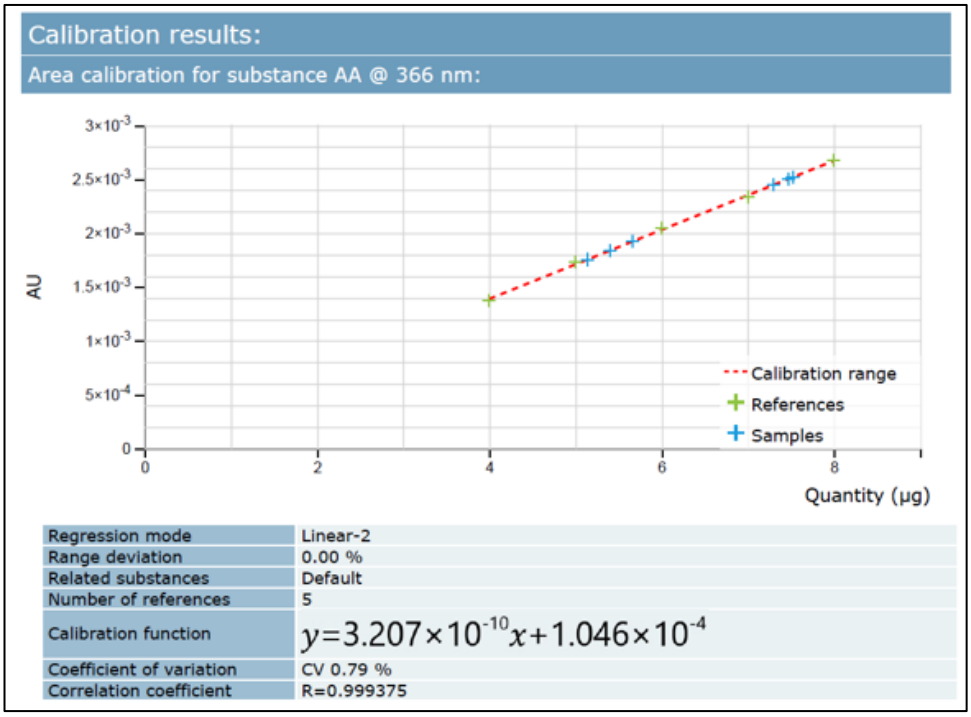


Fig. 52: Calibration result of Arjunic acid present in two *Terminalia* species

The obtained areas of the sample and standard were separately compared at 80%, 100%, and 120%. The recovery studies showed % recovery values within acceptable limits (Table 42) and their quantification values (coefficient of variance) of arjunic acid in two *Terminalia* species were shown in Table 43.The quantification of arjunic acid in *T. arjuna* was found to be 4.95 mg/ml, while in *T. chebula*, it was 0.900 mg/ml.

Table 42: Percentage recovery (n=3) of Arjunic acid in two *Terminalia* species

	%Recovery levels	Conc. of Std spiked on sample (ng/band) (n=3)	Theoretical Conc. (ng)	Observed Conc. (ng)	%Recovery	Average Percentage Recovery
Arjunic acid in *T. arjuna*	80 %	3200	0.004213	0.004233	95.47	95.41 %
	100 %	4000	0.00434	0.00437	94.69	
	120 %	4800	0.00463	0.00468	96.07	
Arjunic acid in *T. chebula*	80 %	3200	0.003063	0.003080	96.55	96.43%
	100 %	4000	0.003323	0.003330	95.21	
	120 %	4800	0.003670	0.003690	97.54	

Table 43: Quantified values and CV values of Arjunic acid in two *Terminalia* species

Amount of Arjunic acid detected in sample			%CV
A	*T. arjuna*	4.955mg/ml	1.60%
B	*T. chebula*	0.900 mg/ml	4.86%

9. Phytochemical screening of Ayurvedic formulations containing *Terminalia bellerica* and *Terminalia chebula* with respect to Gallic acid as biomarker :

In proposed research of four selected species via; *T. bellirica*, *T. chebula*, and Triphala, along with gallic acid as the reference standard, were analyzed. The preparation amounts are mentioned in Table 44. Chromatograms obtained, along with the optimized solvent system and Rf values, are provided in Table 45 and Fig. 53, 54.

Table 44: Preparation of Two *Terminalia* species, Triphala and Gallic acid

Sample	Analyte Name	Concentration (MeOH)
Std	Gallic acid	1mg in 10ml (100 ppm)
A	*Terminalia bellerica*	100mg in 10ml
B	Triphala	100mg in 10ml
C	*Terminalia chebula*	100mg in 10ml

Table 45: Optimized solvent system and Rf of Gallic acid present in two *Terminali*a species and Ayurvedic formulation

Solvent system	Benzene: Ethyl Acetate: Methanol: Acetic acid (10:7:2:1.2) v/v/v/v
Std	**Rf**
Gallic acid	0.6

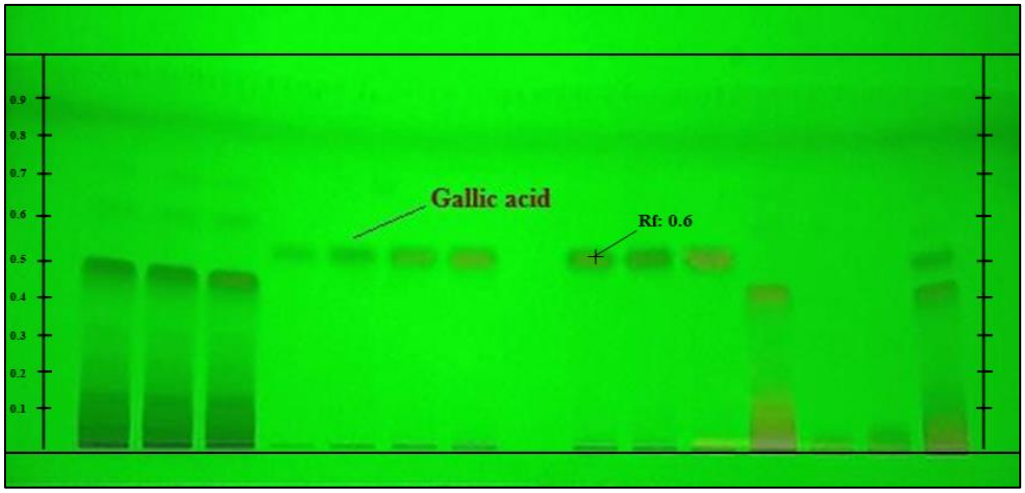


Fig. 53: Gallic acid present in two *Terminalia* species along with Ayurvedic formulation (Plate @254 nm)

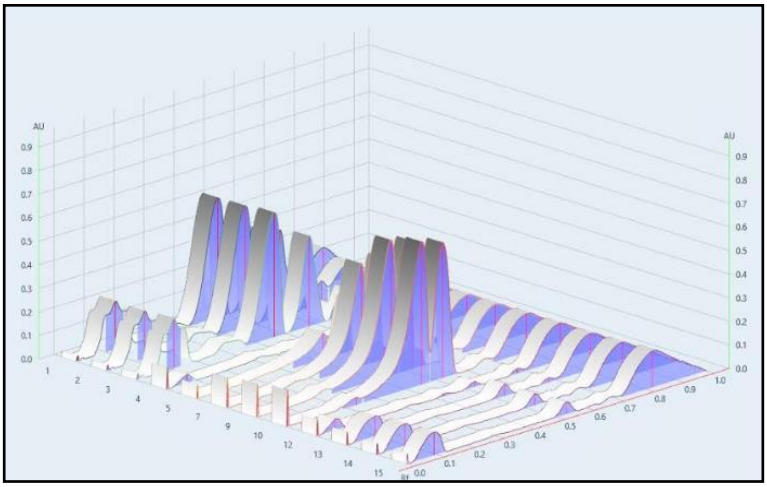


Fig. 54: 3D Chromatogram of Gallic acid @Rf 0.6 and confirmation in two *Terminalia* species along with Ayurvedic formulation

The linearity calibration range was 0.5-3.5 µg/ ml, with an LOD of 0.45 µg and LOQ of 1.5 µg. The regression coefficient (R) for gallic acid was 0.9999 (Fig. 55). The 3D spectral data of gallic acid, *T. bellirica*, *T. chebula*, and Triphala are depicted in (Fig. 56). The reproducibility was assessed using six levels of standard gallic acid, with 2 µL (2 µg) as the optimized reproducibility level resulting in a final quantified result of 0.27% CV.

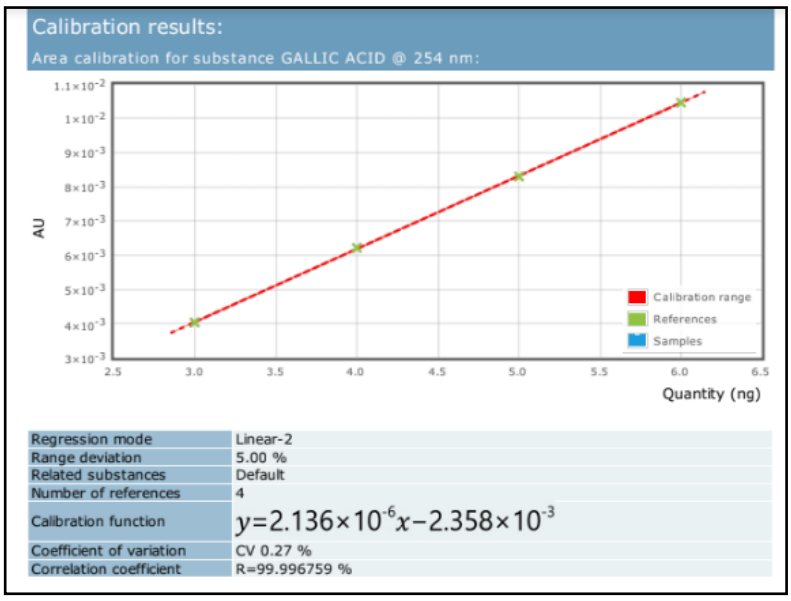


Fig. 55: Linearity, LOD and LOQ of Two *Terminalia* species along with Ayurvedic formulation and Gallic acid

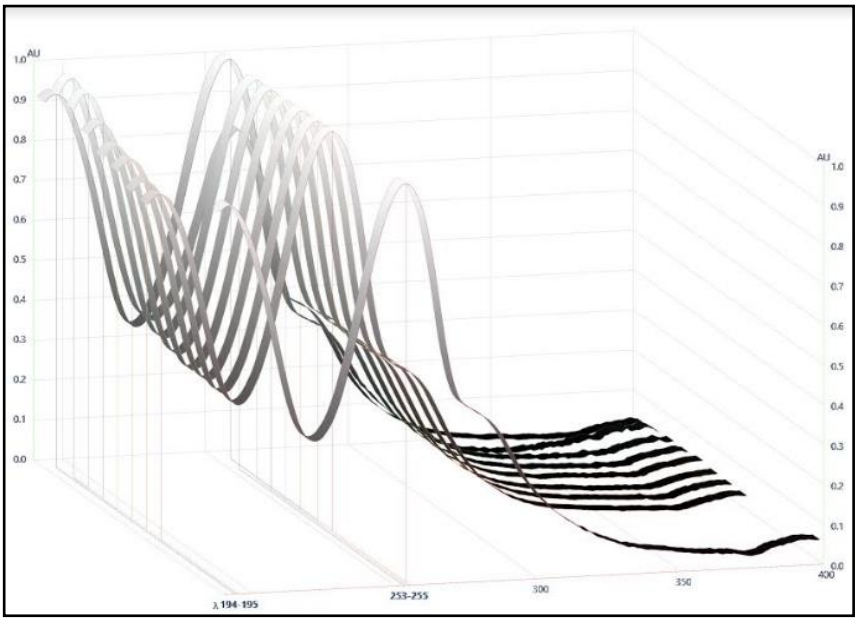


Fig. 56: 3D Spectral data of Two *Terminalia* species along with Ayurvedic formulation and Gallic acid @254 nm

The obtained areas of the sample and gallic acid were separately compared at 80%, 100%, and 120%. The recovery studies showed % recovery values within acceptable limits (Table 46). The quantification values of (coefficient of

variance) gallic acid in *T. bellirica* (150.8µg/ml); *T. chebula* (153µg/ml); and Triphala (172.9µg/ml) (Table 47).

Table 46: Percentage recovery (n=3) of Gallic acid two *Terminalia* species along with Ayurvedic formulation

	%Recovery levels	Conc. of Std spiked on sample (ng/band) (n=3)	Theoretical Conc. (ng)	Observed Conc. (ng)	%Recovery	Average Percentage Recovery
Gallic acid in *T. bellirica*	80 %	800	0.01149	0.01202	83.70	84.25 %
	100 %	1000	0.01154	0.01212	84.40	
	120 %	1200	0.01158	0.01216	84.67	
Gallic acid in *T. chebula*	80 %	800	0.01294	0.01307	80.82	81.15 %
	100 %	1000	0.01296	0.01312	81.13	
	120 %	1200	0.01299	0.01318	81.50	
Gallic acid in Triphala	80 %	800	0.01119	0.01282	91.70	92.10%
	100 %	1000	0.01123	0.01289	92.20	
	120 %	1200	0.01137	0.01292	92.41	

Table 47: Quantified values and %CV values of Gallic acid in two *Terminalia* species and Ayurvedic formulation

Amount of Gallic acid detected in sample			%CV
A	*T. bellirica*	150.8 µg/ml	2.27%
B	*T. chebula*	153 µg/ml	2.98%
C	Triphala	172.9 µg/ml	1.02%

10. Simultaneous Method development and Validation of Gallic acid and Quercetin as Bio-markers in Marketed polyherbal formulations containing *Terminalia bellirica* and *Terminalia chebula* :

The research study undertaken focuses on detecting gallic acid and quercetin in *T. bellirica*, *T. chebula*, Triphala, Vyoshadi, and Pilonil formulations. The sample and standard preparation details are provided in Table 48. The ascending plate development with optimized solvent system and Rf values is presented in Table 49 and Fig. 57-61. The compound presence was confirmed using derivatizing reagents for phenolic (Anisaldehyde), terpenoids (Vanillin), and antioxidants (DPPH), as depicted in Fig. 62.

Table 48: Preparation of two *Terminalia* species, polyherbal formulations, Gallic acid and Quercetin

Sample	Analyte Name	Concentration (MeOH)
Std 1	Gallic acid	8mg in 10ml (80 ppm)
Std 2	Quercetin	10mg in 10ml (1000 ppm)
A	Pilonil	2.682gm in 10ml
B	Vyoshadi	499.99mg in 10ml
C	Triphala	100mg in 10ml
D	*Terminalia bellirica*	100mg in 10 ml
E	*Terminalia chebula*	100mg in 10ml

Table 49: Optimized solvent system and Rf of Gallic acid and Quercetin

Solvent system	Toluene: Isopropyl alcohol: Acetic acid (7:2.5:0.5) v/v/v
Std	**Rf**
Gallic acid	0.46
Quercetin	0.67

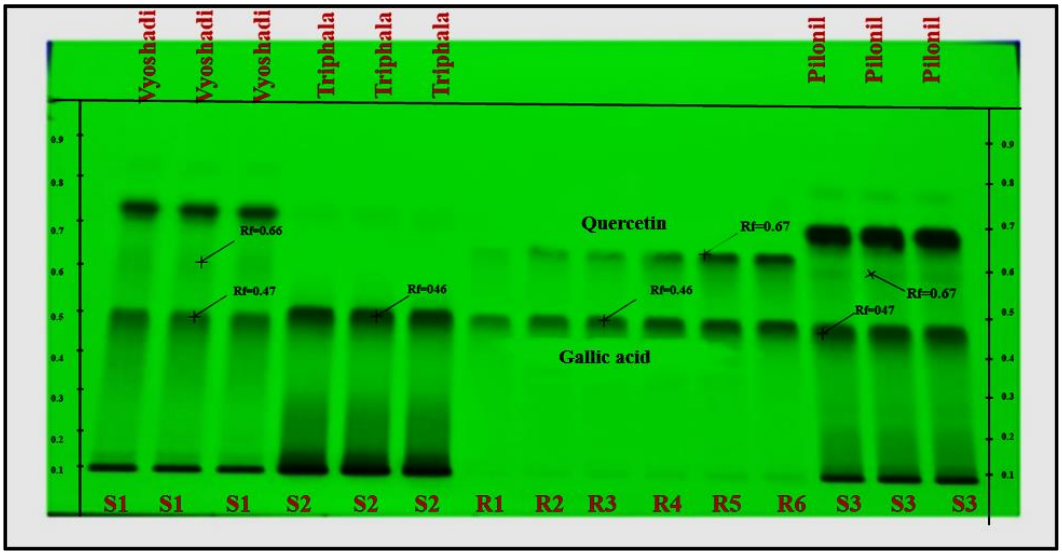


Fig.57: Gallic acid and Quercetin present in two *Terminalia* species and Polyherbal formulation (Plate Image @254nm)

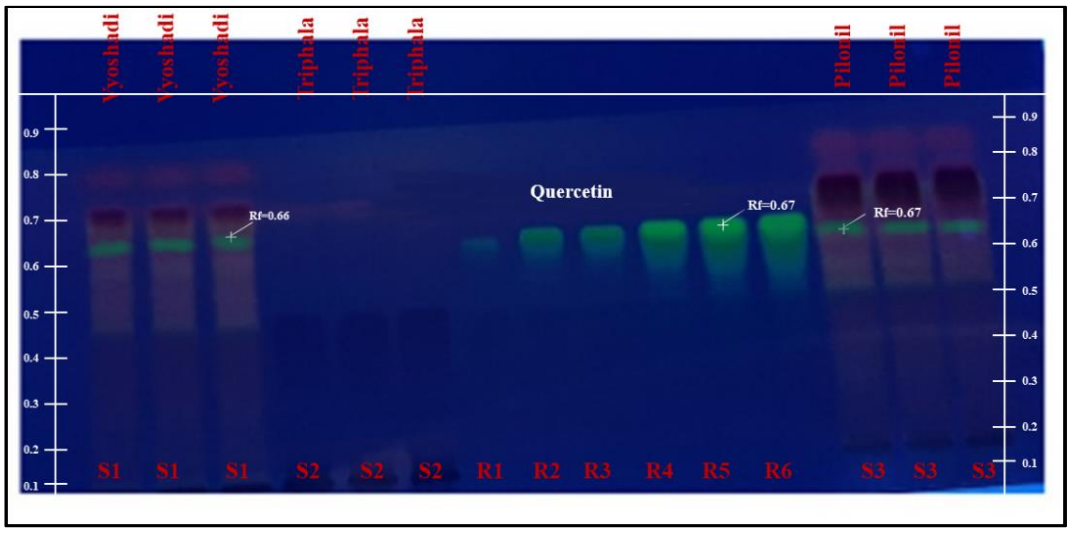


Fig.58: Gallic acid and Quercetin present in two *Terminalia* species and Polyherbal formulation (Plate Image @366nm)

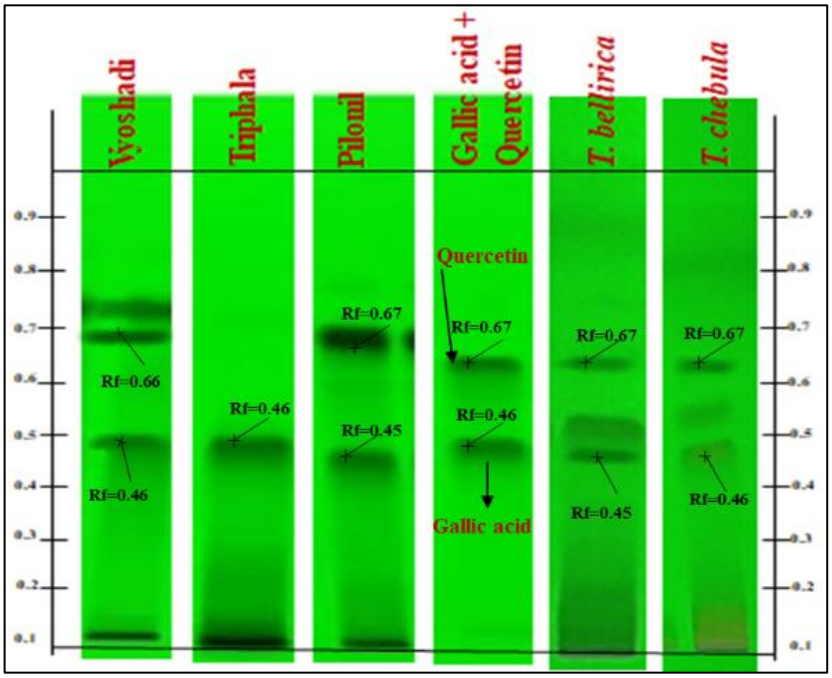


Fig.59: Plate Image @ 254nm with Gallic acid and Quercetin present in three Polyherbal formulations, *T. bellirica* and *T. chebula*

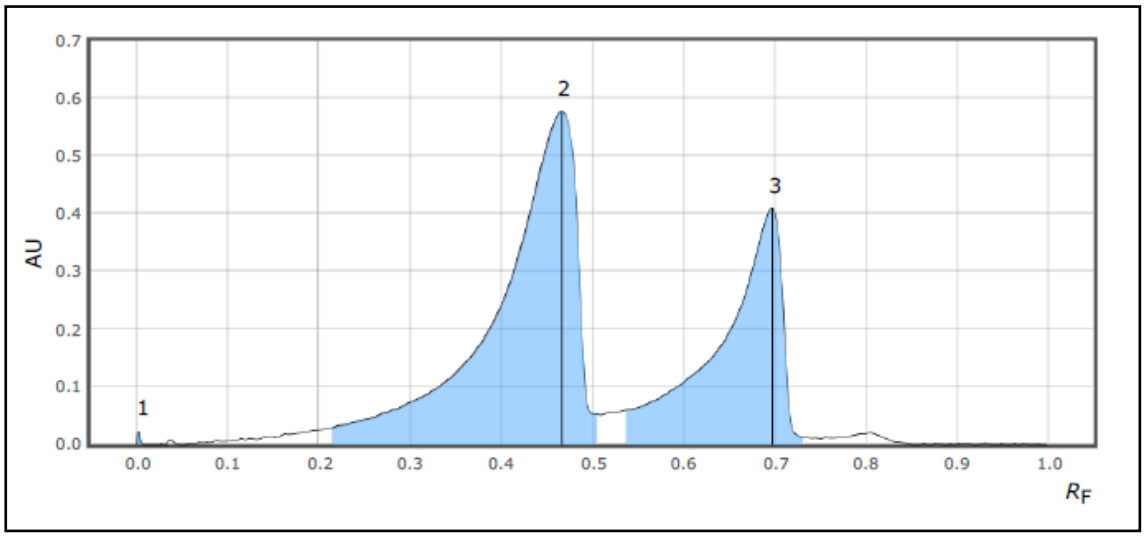


Fig. 60: Chromatogram of Gallic acid @Rf 0.46 and Quercetin @Rf 0.67

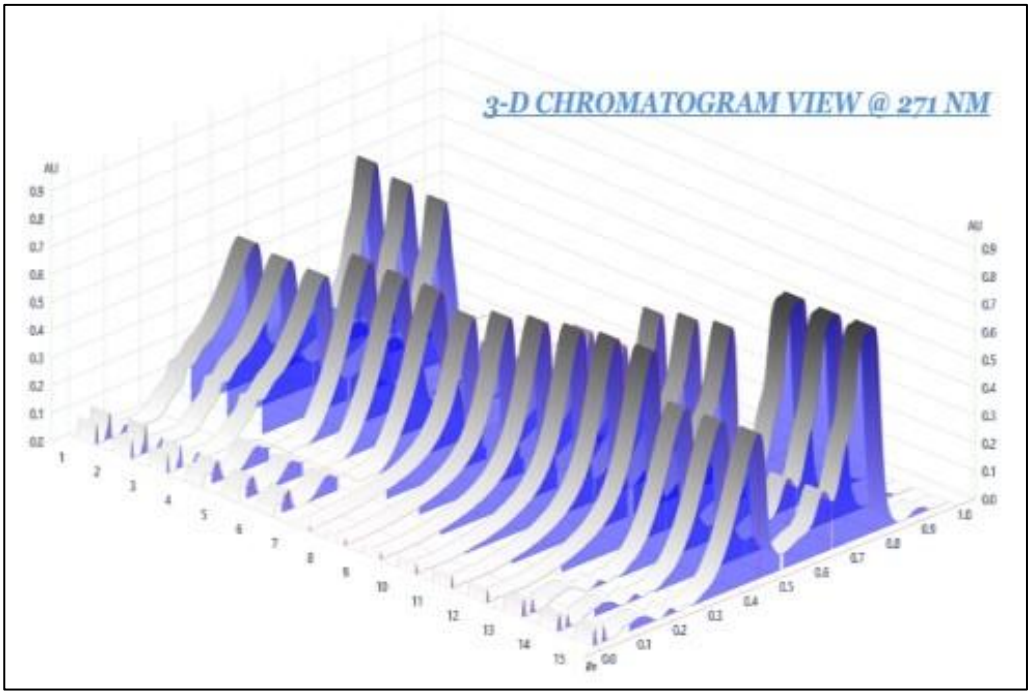


Fig. 61: 3D chromatogram data of Polyherbal Formulations with presence of Gallic acid and Quercetin

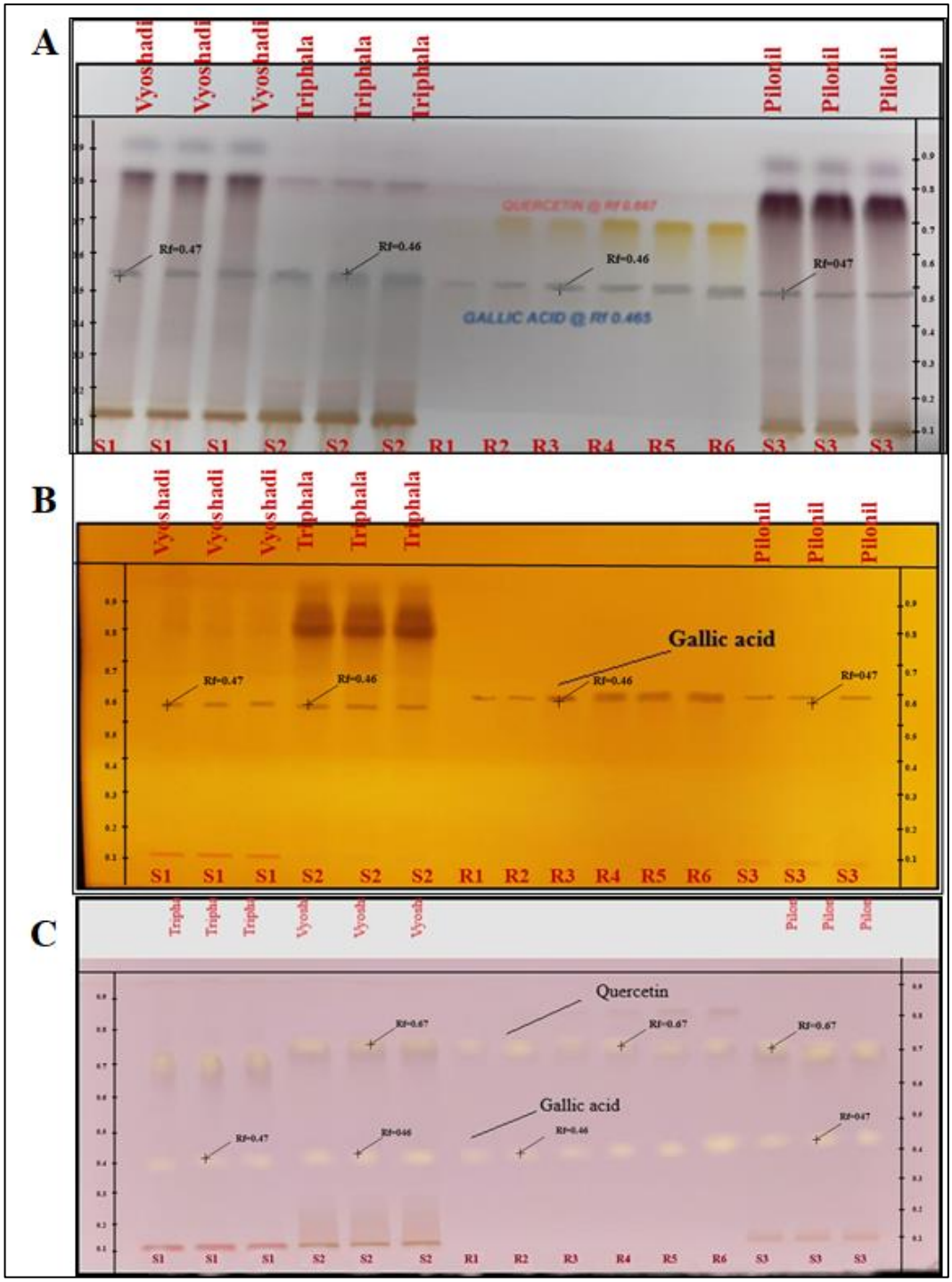


Fig.62: **A)** Plate Image @540nm plate derivatized with Anisaldehyde reagent, **B)** Derivatized with Vanillin reagent (blue color indicates presence of phenolic compound)**,** **C)** Derivatized with DPPH reagent (Yellow color indicates Antioxidant activity)

Linearity was established in the range of 5 µg/ mL to 10 µg/ mL for gallic acid and 1 µg/mL to 6 µg/mL for quercetin. The LOD and LOQ for gallic acid were 800 ppm and 2400 ppm, while for quercetin, they were 5 ppm and 8 ppm, respectively. The research achieved good regression coefficients, r2 = 0.9998 (Gallic acid) and r2 = 0.9999 (Quercetin), as shown in Fig. 63 and Fig. 64, respectively. 3D spectral data of gallic acid and quercetin with Polyherbal formulations are represented in (Fig. 65, 66).Reproducibility was assessed with 6 levels of standard (Gallic acid & Quercetin) with 5 µl each.

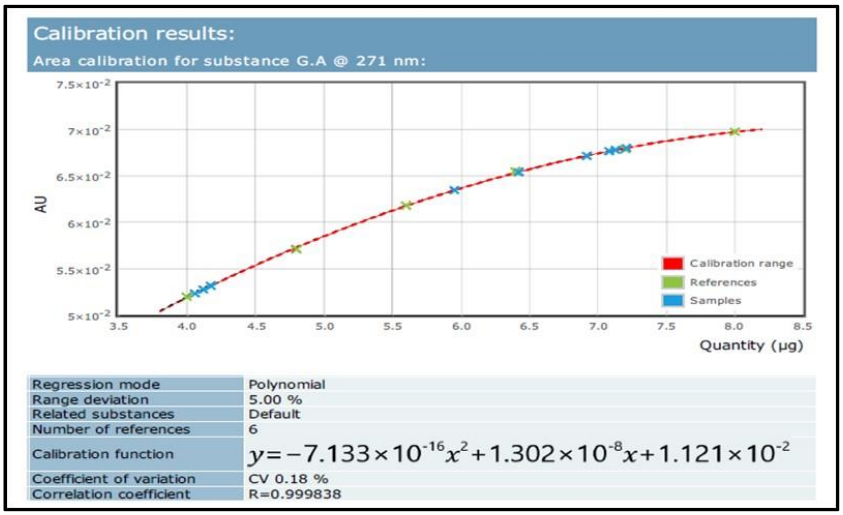


Fig. 63: Linearity, LOD and LOQ of Gallic acid along with three Polyherbal formulations

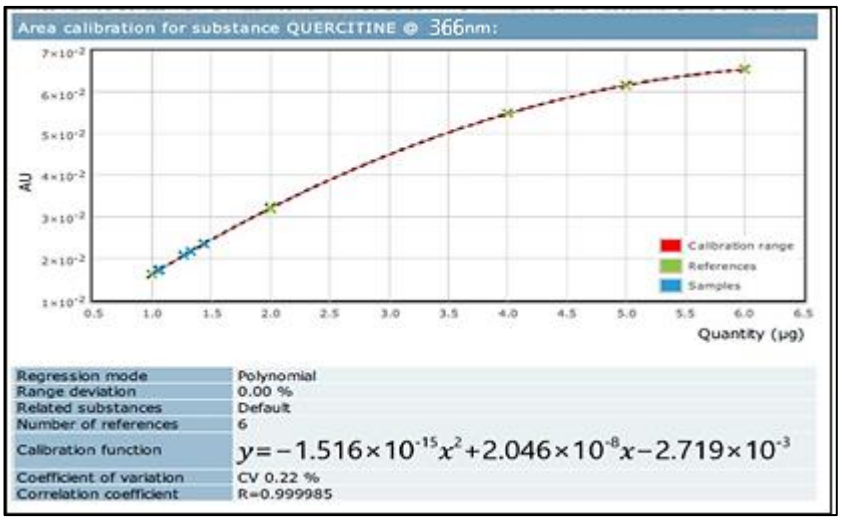


Fig. 64: Linearity, LOD and LOQ of Quercetin along with three Polyherbal formulations

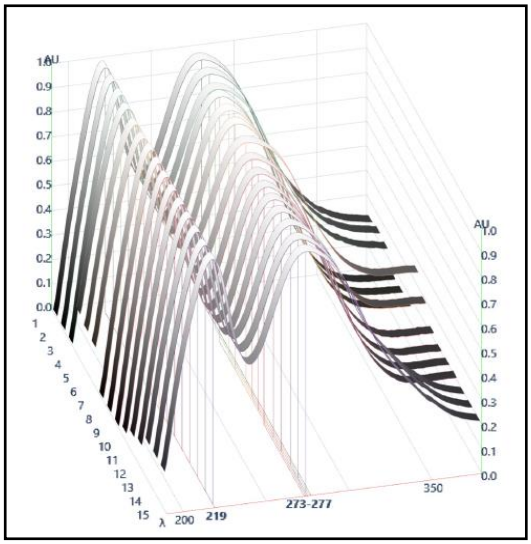


Fig. 65: 3D spectral view of presence of Gallic acid @271 nm in three Polyherbal formulations

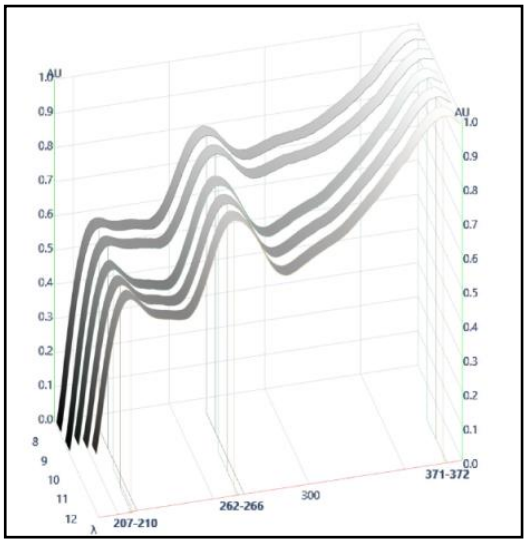


Fig. 66: 3D spectral view of presence of Quercetin @366 nm in three Polyherbal formulations

The recovery data of gallic acid, quercetin and samples were compared separately at 80%, 100 % and 120%. The recovery values are represented in Table 50. The quantification values of coefficient of variance for Gallic acid and quercetin were found in (Table 51, 52).

Table 50: Percentage recovery (n=3) of Gallic acid and Quercetin in two *Terminalia* species and three Polyherbal formulations

	%Recovery levels	Conc. of Std spiked on sample (ng/band) (n=3)	Theoretical Conc. (ng)	Observed Conc. (ng)	%Recovery	Average Percentage Recovery
Gallic acid in *T. bellirica*	80 %	3840	0.01149	0.01297	90.32	91.80 %
	100 %	4800	0.01162	0.01320	91.92	
	120 %	5760	0.01174	0.01338	93.17	
Gallic acid in *T. chebula*	80 %	3840	0.01145	0.01289	90.07	93.96 %
	100 %	4800	0.01252	0.01359	94.96	
	120 %	5760	0.01294	0.01386	96.85	
Gallic acid in Pilonil	80 %	3840	0.06345	0.07227	91.12	94.92 %
	100 %	4800	0.06542	0.07513	94.72	
	120 %	5760	0.06801	0.07846	98.92	
Gallic acid in Vyoshadi	80 %	3840	0.05232	0.06102	93.30	93.73 %
	100 %	4800	0.05276	0.06132	93.76	
	120 %	5760	0.05315	0.06158	94.15	
Gallic acid in Triphala	80 %	3840	0.06716	0.08129	96.83	97.23 %
	100 %	4800	0.06766	0.08170	97.31	
	120 %	5760	0.001760	0.08191	97.56	
Quercetin in *T. bellirica*	80 %	1600	0.03228	0.03845	95.29	96.20 %
	100 %	2000	0.03235	0.03889	96.38	
	120 %	2400	0.03247	0.03912	96.95	
Quercetin in *T. chebula*	80 %	1600	0.05226	0.05855	89.63	
	100 %	2000	0.05232	0.05891	90.18	

	120 %	2400	0.5240	0.05934	90.84	90.21 %
Quercetin in Pilonil	80 %	1600	0.02079	0.02386	91.83	93.10 %
	100 %	2000	0.02178	0.02425	93.34	
	120 %	2400	0.02357	0.02446	94.14	
Quercetin in Vyoshadi	80 %	1600	0.01717	0.01889	88.02	90.04 %
	100 %	2000	0.01727	0.01942	90.49	
	120 %	2400	0.01733	0.01966	91.61	

Table 51: Quantified values and % CV values of Gallic acid in three Polyherbal formulations and two *Terminalia* species

Amount of Gallic acid detected in sample			%CV
A	Pilonil	652.8µg/ml	9.71%
B	Triphala	352.2µg/ml	1.57%
C	Vyoshadi	411.9µg/ml	1.41%
D	*T. bellirica*	183.9 µg/ml	6.27%
E	*T. chebula*	171.2 µg/ml	2.25 %

Table 52: Quantified values and %CV values of Quercetin in two Polyherbal formulations and two *Terminalia* species

Amount of Quercetin detected in sample			%CV
A	Pilonil	134.5µg/ml	6.43%
C	Vyoshadi	106.0µg/ml	0.44%
D	*T. bellirica*	91.95 µg/ml	0.82%
E	*T. chebula*	85.6 µg/ml	1.2%

11. Phytochemical screening of Ayurvedic formulations containing *Terminalia arjuna* with respect to Gallic acid as biomarker :

This research undertaken focuses on the detection of gallic acid in *T. arjuna* and Arjunarishta Ayurvedic formulations. The standard and sample preparation details are provided in Table 53. Plate development with optimized solvent system and Rf values is presented in Table 54 and Fig. 67-70.

Table 53: Preparation of Gallic acid, *T. arjuna* and Ayurvedic formulation

Sample	Analyte Name	Concentration (MeOH)
Std	Gallic acid	4mg in 10ml (40 ppm)
A	*Terminalia arjuna* (H_2O Extract)	100mg in 10ml
B	Arjunarishta	0.5ml in 10ml
C	*Terminalia arjuna* (HA Extract)	100mg in 10ml

Table 54: Optimized Solvent system and Rf of Gallic acid

Solvent system	Isopropyl alcohol: Formic acid: Water: Acetic acid (18:0.5:0.5:0.3) v/v/v
Std	**Rf**
Gallic acid	0.75

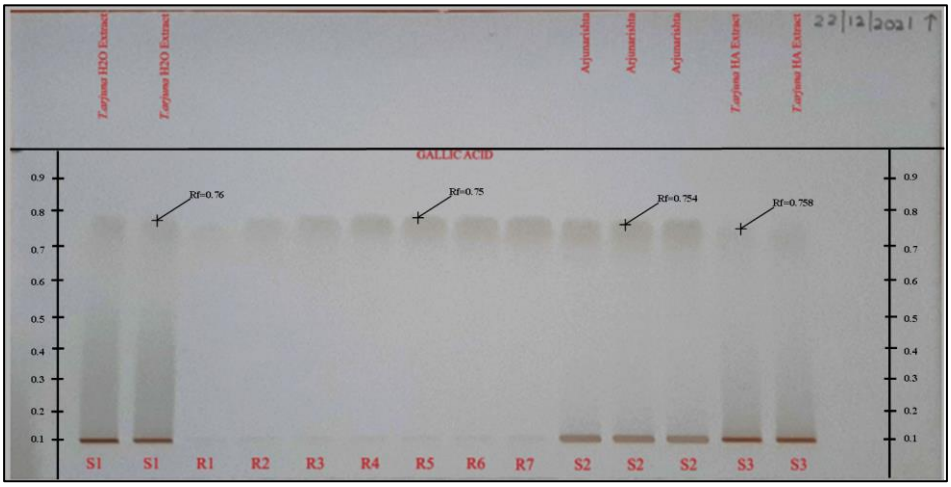


Fig. 67: Gallic acid present in *T. arjuna* and Ayurvedic formulation (Plate Image @ 540 nm)

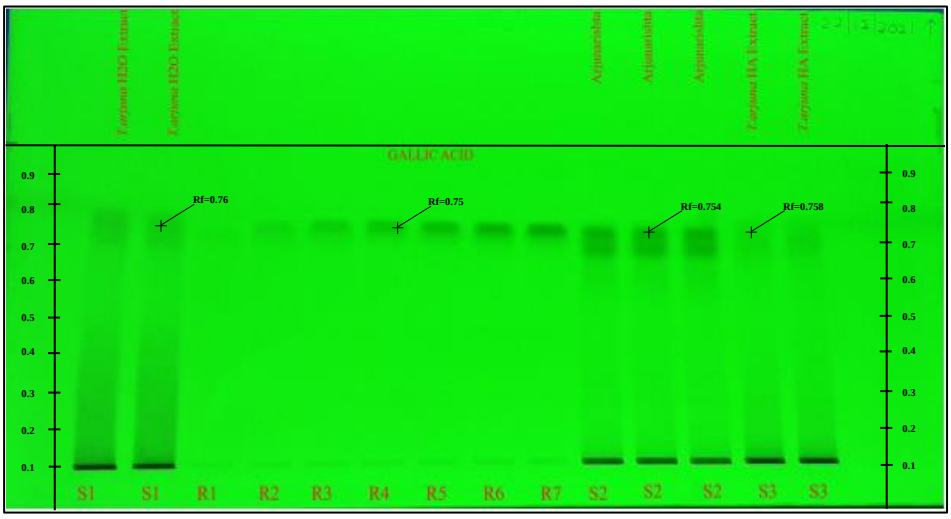


Fig. 68: Gallic acid present in *T. arjuna* and Ayurvedic formulation (Plate Image @254 nm)

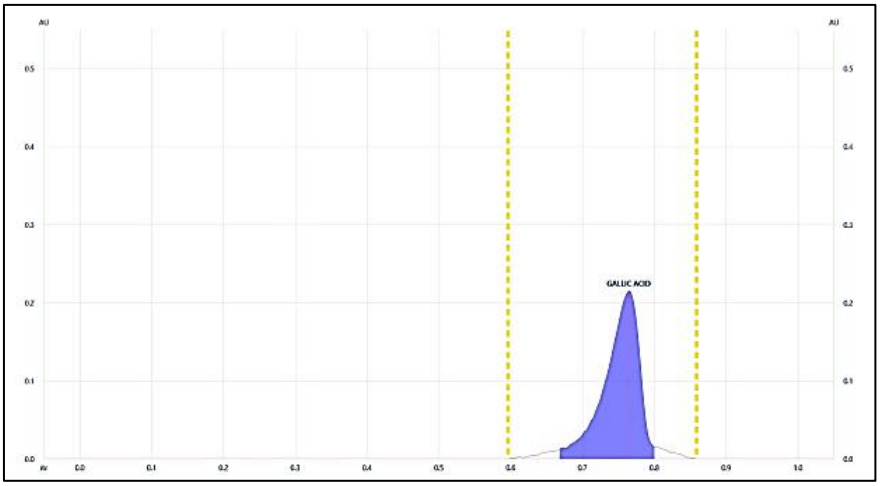


Fig. 69: Gallic acid Chromatogram @Rf 0.75

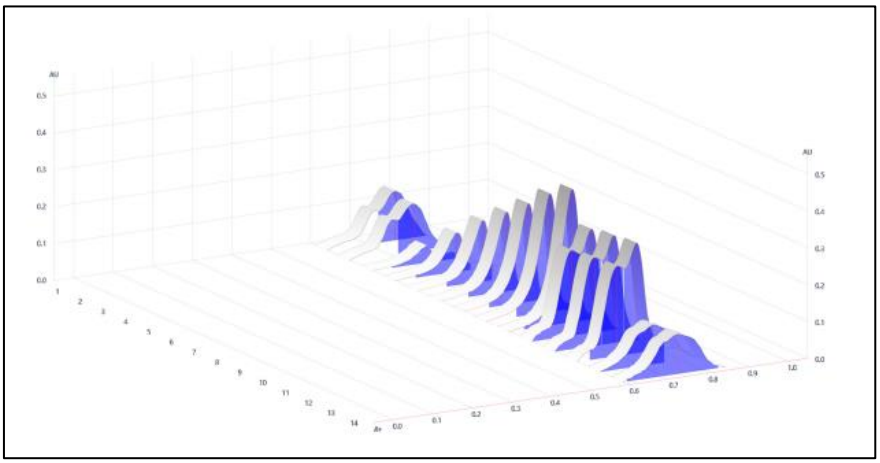
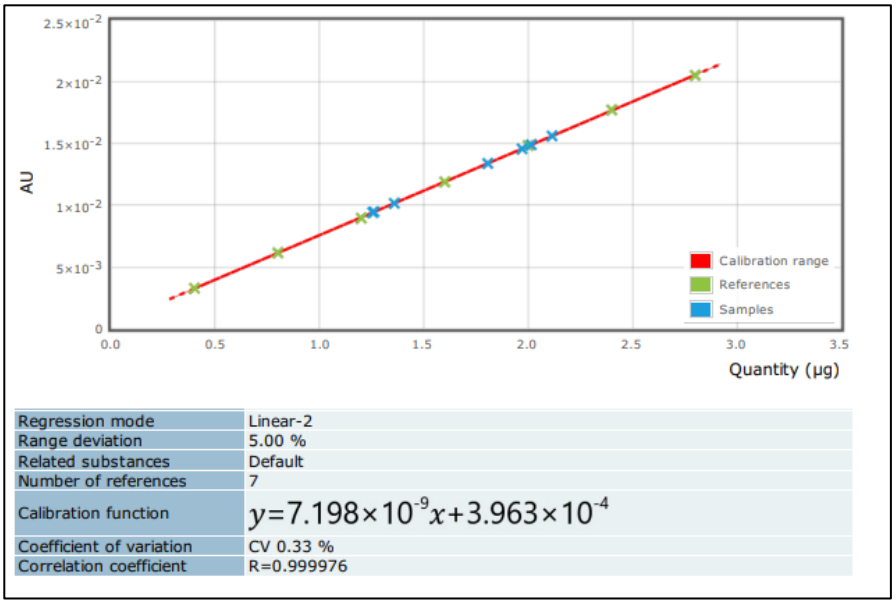


Fig.70: 3D Chromatogram view of Gallic acid present in *T. arjuna* and Ayurvedic formulation

The linearity range for gallic acid was 400 µg/mL to 2000 µg/mL with LOD (350 ppm) and LOQ (570 ppm) values, respectively. The research input achieved a good regression coefficient, r2 = 0.9998 (Gallic acid), as shown in Fig. 71. Spectral data of gallic acid, *T. arjuna*, and Arjunarishta are depicted in Fig. 72. Reproducibility was assessed with 6 levels of standard (Gallic acid) using 3 µl each. The class of compound presence was confirmed using derivatizing reagents for phenols (Anisaldehyde) and terpenoids (Vanillin), as depicted in Fig. 73 and 74.



Regression mode	Linear-2
Range deviation	5.00 %
Related substances	Default
Number of references	7
Calibration function	$y=7.198\times10^{-9}x+3.963\times10^{-4}$
Coefficient of variation	CV 0.33 %
Correlation coefficient	R=0.999976

Fig.71: Linearity, LOD and LOQ of Gallic acid, *T. arjuna* and Ayurvedic formulation

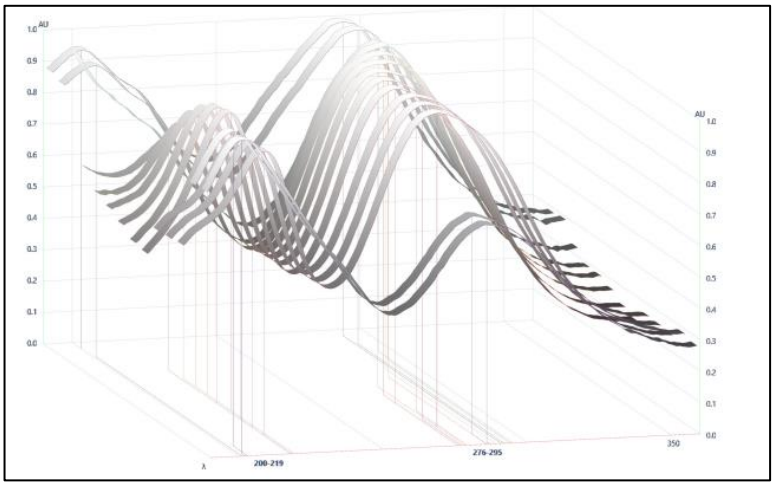


Fig.72: 3D overlaying spectral view of Gallic acid @ 271 nm present in *T. arjuna* and Ayurvedic formulation

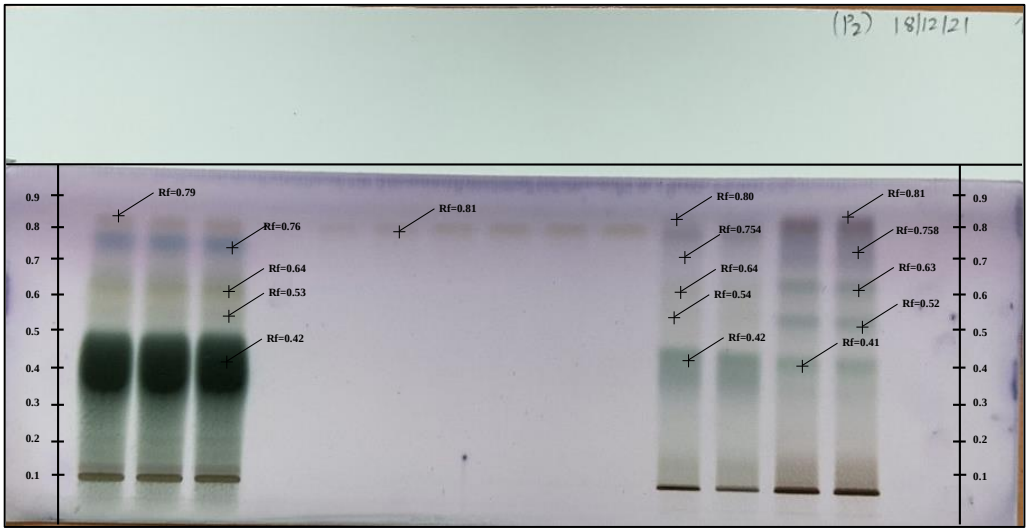


Fig.73: Gallic acid, *T. arjuna* and Ayurvedic formulation derivatized with Anisaldehyde reagent(Plate Image @540nm plate)

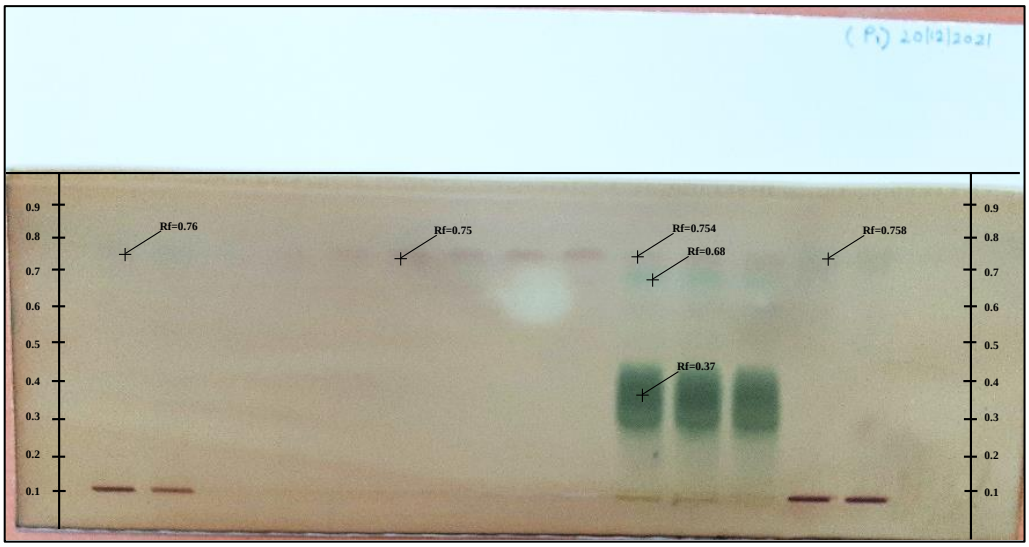


Fig.74: Gallic acid, *T. arjuna* and Ayurvedic formulation derivatized with Vanillin reagent (Plate Image @540 nm)

The recovery data of gallic acid and samples were compared separately at 80%, 100% and 120%. The recovery values are represented in Table 55.The quantification values of coefficient of variance for Gallic acid was found to be in (Table 56).

Table 55: Percentage recovery (n=3) of Gallic acid in *T. arjuna* and Ayurvedic formulation

	%Recovery levels	Conc. of Std spiked on sample (ng/band) (n=3)	Theoretical Conc. (ng)	Observed Conc. (ng)	%Recovery	Average Percentage Recovery
Gallic acid in *T. arjuna (H. A extract)*	80 %	640	0.01340	0.01407	79.58	79.82 %
	100 %	800	0.01343	0.01412	79.86	
	120 %	960	0.01353	0.01415	80.03	
Gallic acid in *T. arjuna (Water extract)*	80 %	640	0.09047	0.01192	93.78	94.77 %
	100 %	800	0.01017	0.01207	94.96	
	120 %	960	0.01024	0.01215	95.59	
Gallic acid in Arjunarishta	80 %	640	0.01550	0.01782	91.52	91.81%
	100 %	800	0.01558	0.01789	91.88	
	120 %	960	0.01561	0.01792	92.03	

Table 56: Quantified values and %CV values of Gallic acid in Ayurvedic formulation

Amount of Gallic acid detected in sample			%CV
A	*Terminalia arjuna* (H_2O Extract)	65.48 µg/ml	5.25 %
B	Arjunarishta	406.6 µg/ml	3.62 %
C	*Terminalia arjuna* (HA Extract)	153.0 µg/ml	25.50 %

12. Detection of Standards present in *Terminalia arjuna, Terminalia bellirica, Terminalia chebula* and *Terminalia catappa* by LC-MS/MS :

The LCMS/MS fingerprinting analysis is processed using the QSight® 220 application, enabling the determination and prediction of the molecular weights for each compound. The molecular weights of the standards are provided in Table 57.

- **Molecular weight of standard compound (polyphenolic compounds):**

Table 57: Molecular weight of phytoconstituents present in *Terminalia* species

Standards	Compound Molecular Weight (g/mol)
Arjunic acid	488.7
Chebulinic acid	956.67
Chebulagic acid	954.70
Ellagic acid	302.197
Gallic acid	170.12
Rutin	610.517

The analysis of the metabolite profile of the standards (arjunic acid, chebulinic acid, chebulagic acid, ellagic acid, gallic acid, and rutin) Fig. 75-80 and the hydro-alcoholic extract of *Terminalia* (*arjuna, bellirica, chebula,* and *catappa*) is depicted in Fig. 81-84. Each peak in the chromatogram corresponds to a specific compound. By measuring the mass values and comparing them with calculated mass values in the spectra, the molecular weights of each compound can be predicted. The LCMS/MS spectra of Arjunic acid (Fig. 75) peak at 487.8 m/z, Chebulinic acid (Fig. 76) peak at 955.7 m/z, Chebulagic acid (Fig.

77) peak at 954.6 m/z, Ellagic acid (Fig. 78) peak at 303.1 m/z, Gallic acid (Fig. 79) peak at 171.0 m/z, and Rutin (Fig. 80) peak at 610.7 m/z respectively.

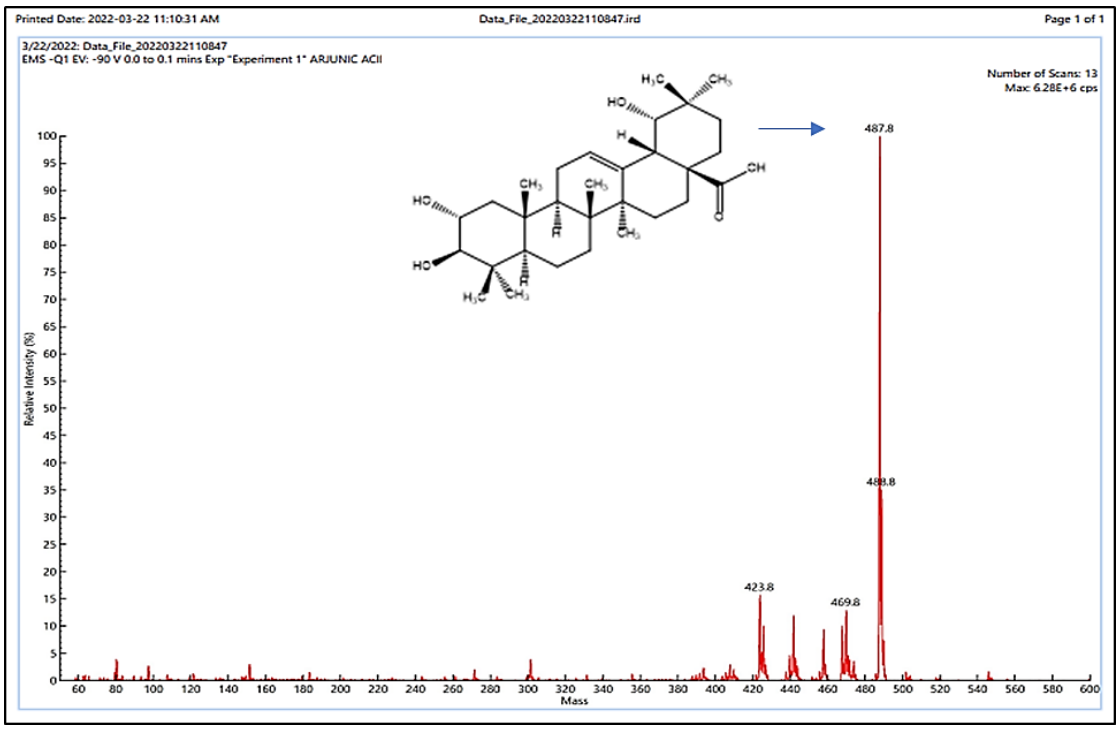


Fig.75: Arjunic acid standard peak at 487.8 m/z

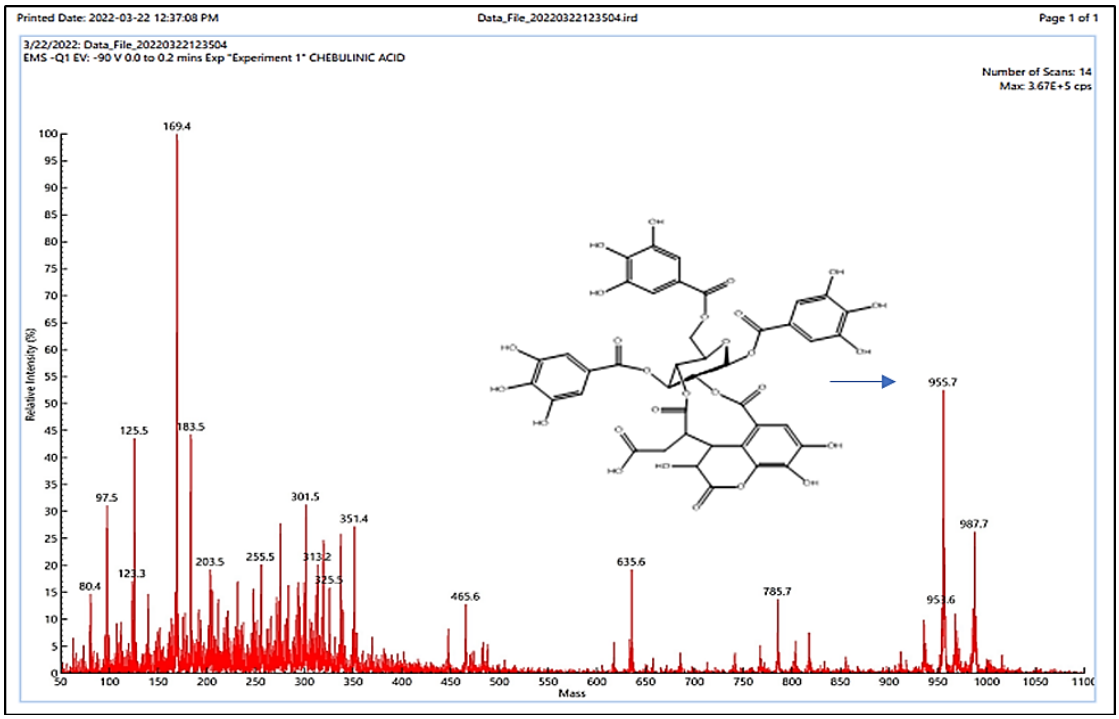


Fig.76: Chebulinic acid standard peak at 955.7 m/z

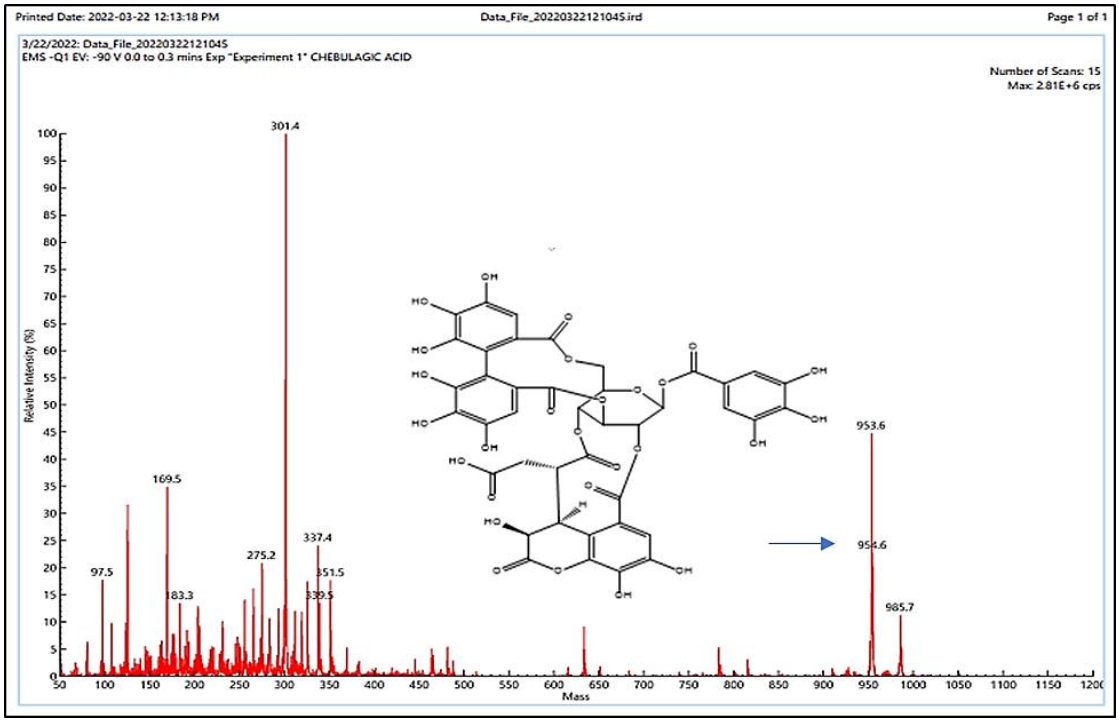


Fig.77: Chebulagic acid standard peak at 954.6 m/z

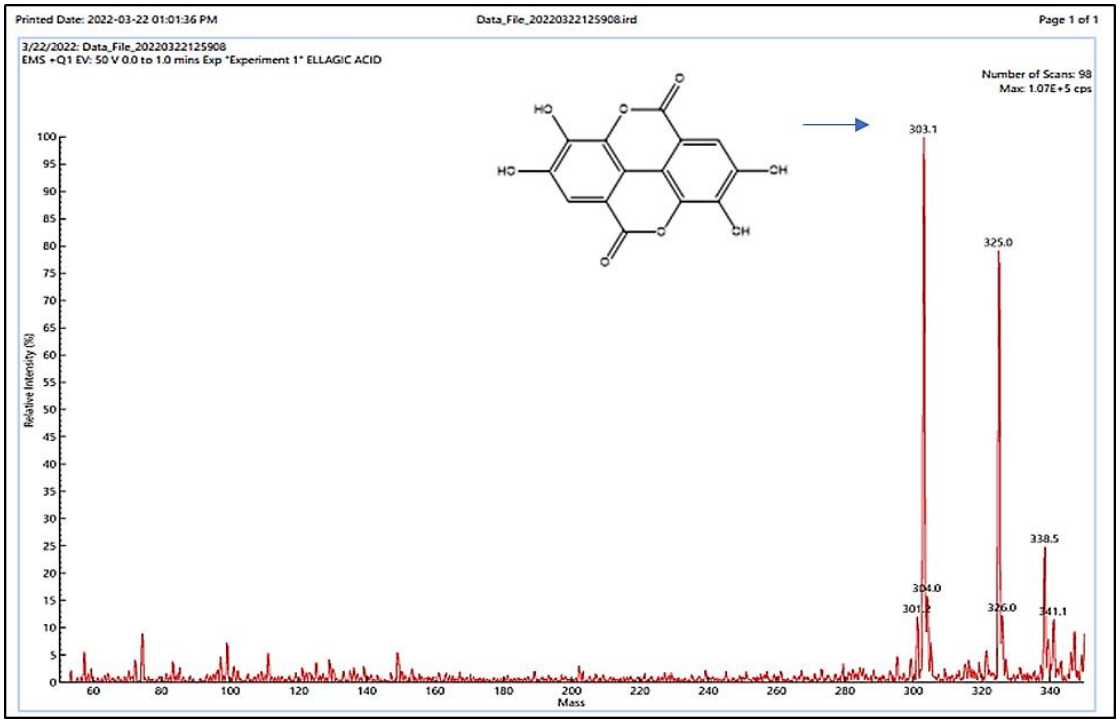


Fig.78: Ellagic acid standard peak at 303.1 m/z

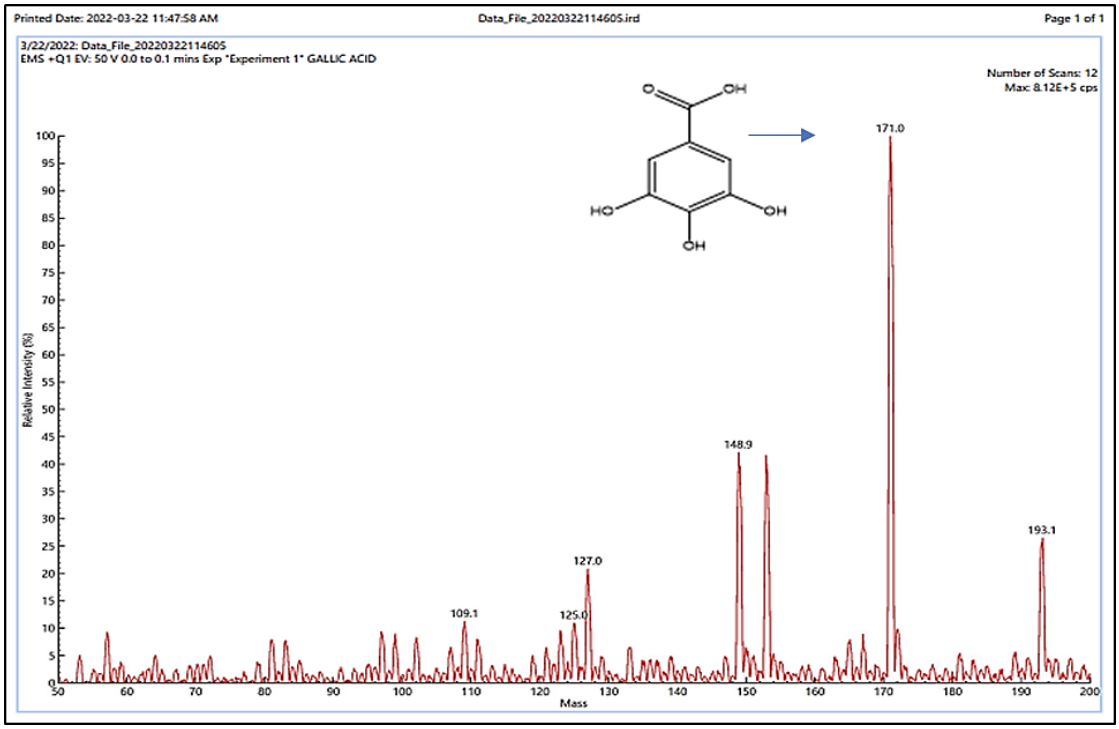


Fig.79: Gallic acid standard peak at 171.0 m/z

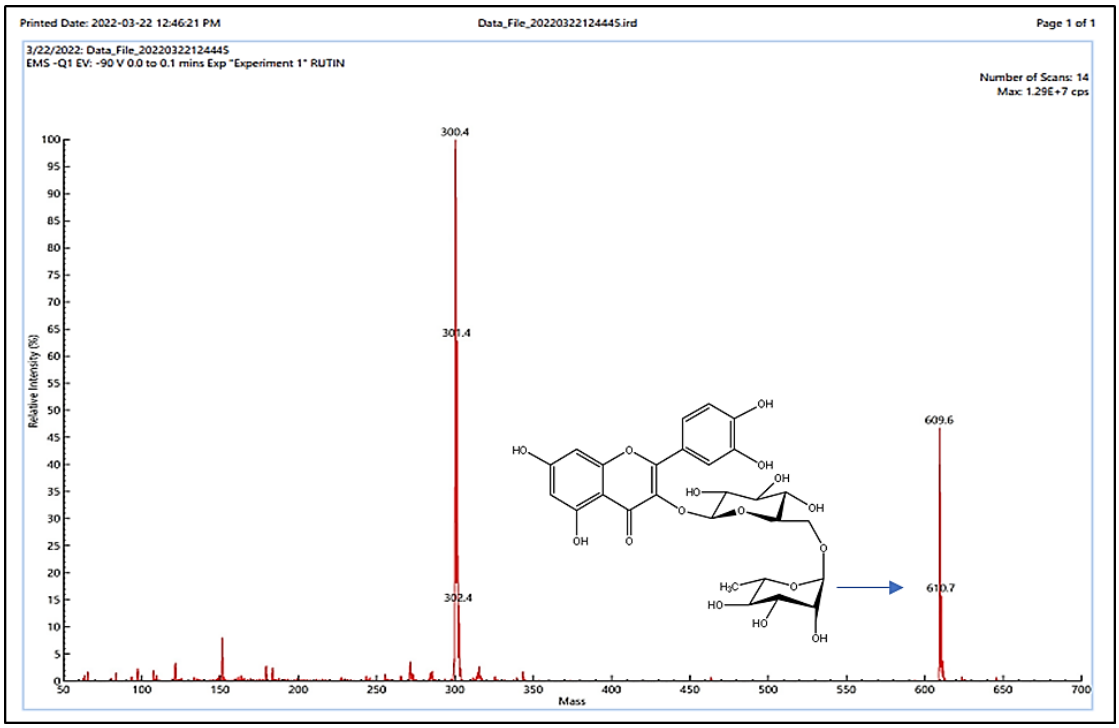


Fig.80: Rutin standard peak at 610.7 m/z

Hence, notably, major compounds with higher area percentages can be identified. In *T. arjuna* (Fig. 81), peaks at 169.4 m/z (GA), 483.5 m/z (AA), and 301.3 m/z (EA) were observed. *T. bellirica* (Fig. 82) exhibited peaks at

169.4 m/z (GA), 301.3 m/z (EA), 355.4 m/z (Chebulinic), and 483.5 m/z (AA). *T. chebula* (Fig. 83) displayed molecular weights at 171.4 m/z (GA), 301.3 m/z (EA), 355.4 m/z (Chebulinic acid), and 955.6 m/z (Chebulagic acid). *T. catappa* (Fig. 84) showcased peaks at 173.3 m/z (GA), 301.3 m/z (EA), and 353.4 m/z (Chebulinic acid). The detected m/z ratios obtained matched exactly with those phytomarkers estimated from selected *Terminalia* extracts.

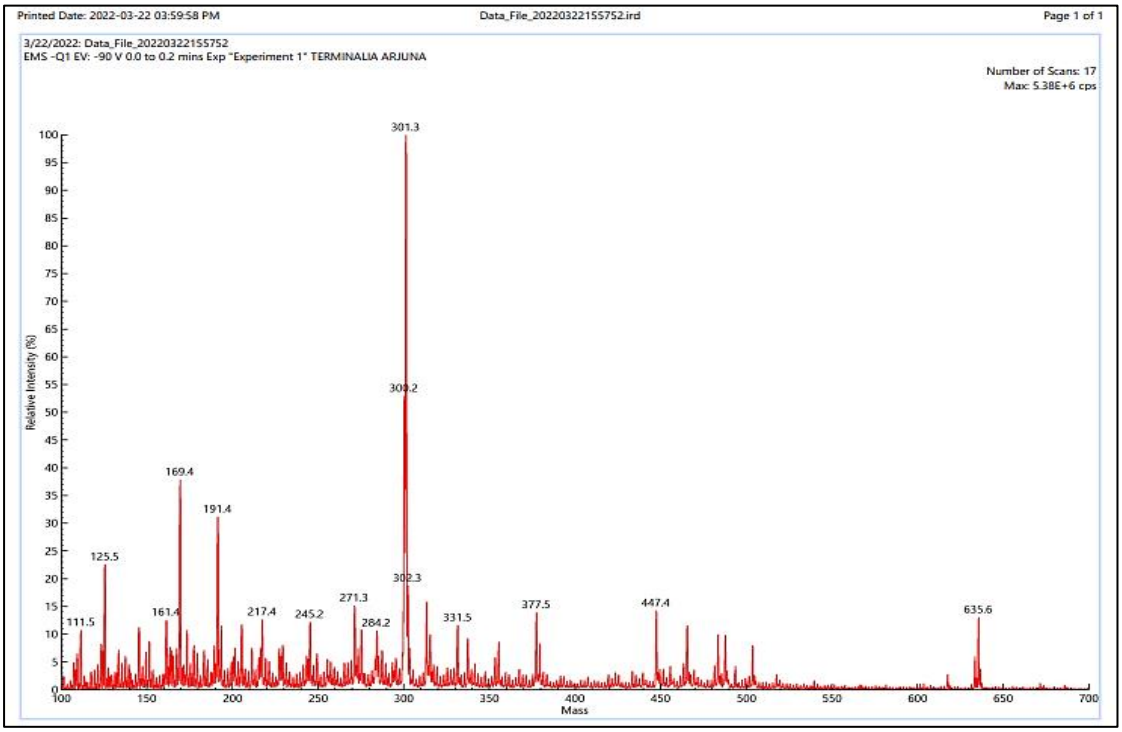


Fig.81: m/z pattern of Hydro-alcoholic extract of *Terminalia arjuna* extract

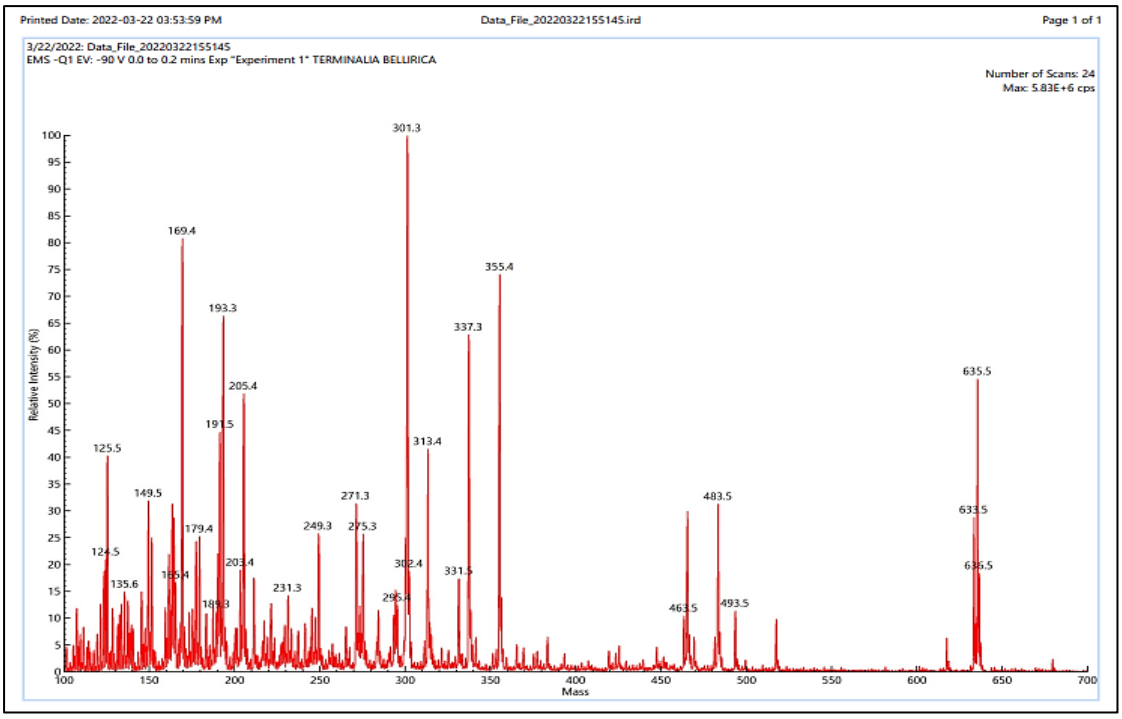


Fig.82: m/z pattern of Hydro-alcoholic extract of *Terminalia bellirica* extract

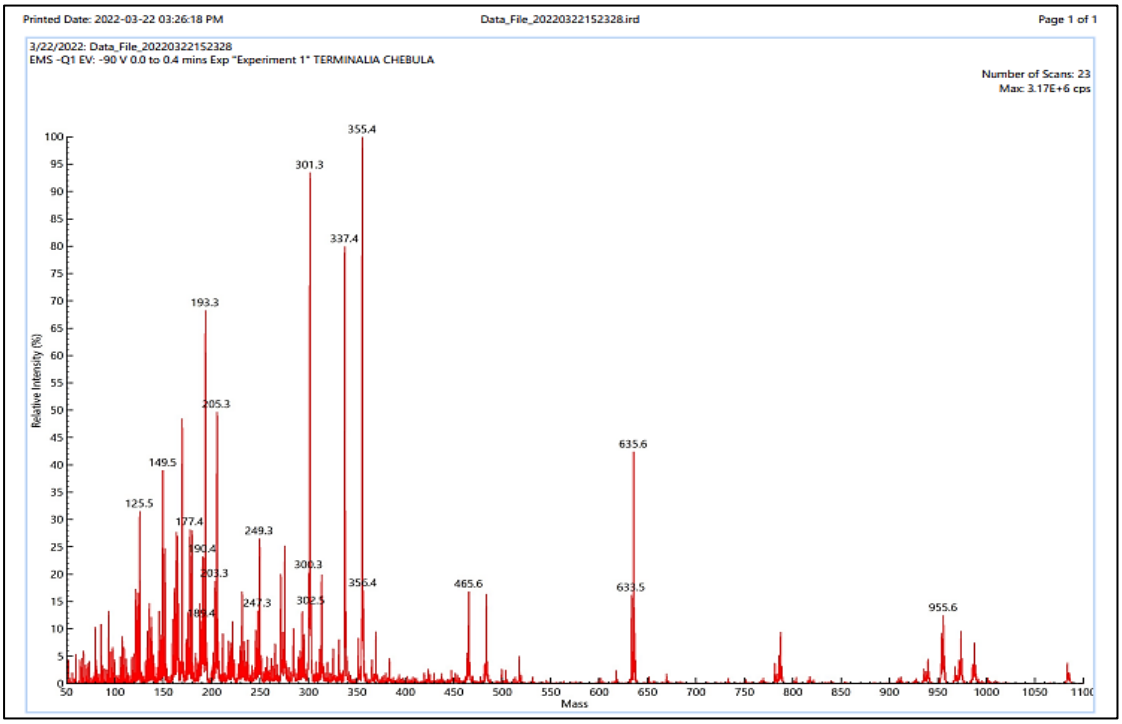


Fig.83: m/z pattern of Hydro-alcoholic extract of *Terminalia chebula* extract

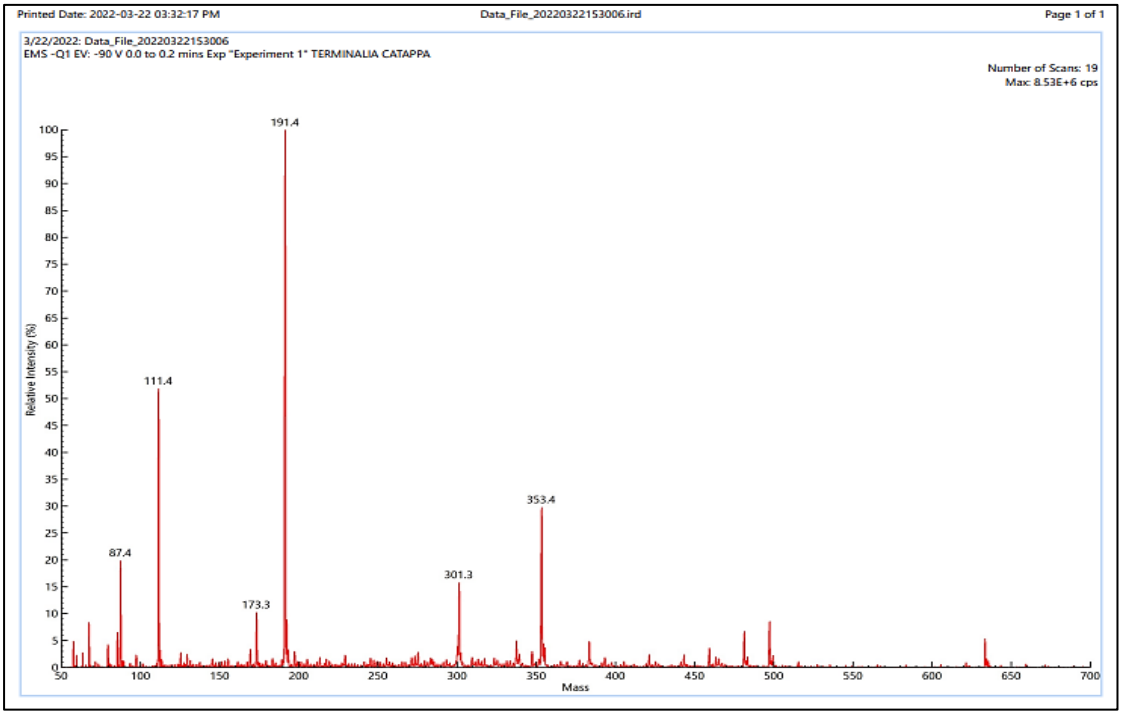


Fig.84: m/z pattern of Hydro-alcoholic extract of *Terminalia catappa* extract

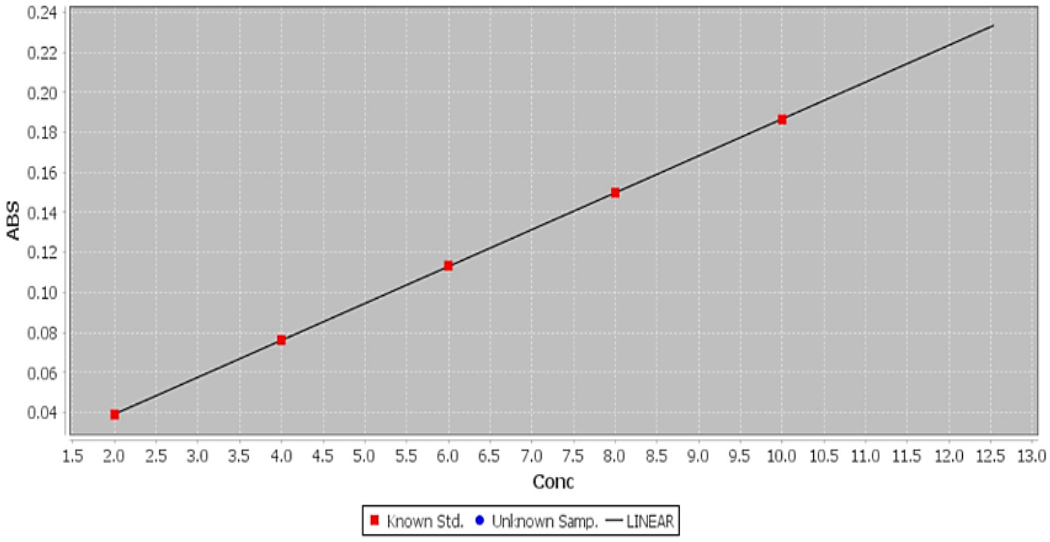
13. Metal Estimation in *Terminalia arjuna, Terminalia bellirica, Terminalia chebula* and *Terminalia catappa* by AAS :

This research focuses on elemental estimation in selected *Terminalia* species using AAS with detection using Calcium (Ca), Iron (Fe), and Zinc (Zn) lamps. Fig. 85 shows the standard calibration curve of Ca with an R=0.999, along with the representation of the sample. The amount of Ca present in *Terminalia* is shown in Table 58, ranked as *Ta* > *Tb* > *Tc* > *Tct*.

Report Type	Application User	Time Stamp
	BSRC	2022-04-19 13:12:54.289

Parameters

Test Name	Element	Integration	Replicates	Rsquare	Spectral Line	Slit	Serial No.
	Ca	3 Sec.	3	1.000	422.7 nm	0.5NM	1011921126



Known Standards

Serial.	Name	Standard Conc.	ABS	T%	RSD
1	STD_1	2.000 ppm	0.0389	091.432	0.0020
2	STD_2	4.000 ppm	0.0761	083.927	0.0060
3	STD_3	6.000 ppm	0.1134	077.019	0.0030
4	STD_4	8.000 ppm	0.1498	070.827	0.0060
5	STD_5	10.000 ppm	0.1865	065.088	0.0040

Fig 85: Standard Calibration Graph of Ca on AAS

Table 58: Detection of Calcium in Selected four *Terminalia* species

Serial	Name	Sample Conc.	ABS	T%	RSD
1	*Terminalia arjuna*	25.925 ppm	1.0441	009.034	0.0490
2	*Terminalia bellirica*	2.818 ppm	0.1172	076.348	0.0030
3	*Terminalia chebula*	1.465 ppm	0.0629	086.517	0.0010
4	*Terminalia catappa*	1.440 ppm	0.0619	086.716	0.0010

In, Fig. 86 presents the standard calibration curve of Fe with an R= 0.995, and the corresponding sample representation. The amount of Fe in *Terminalia* is shown in Table 59, ranked as *Ta* > *Tct* > *Tb* > *Tc*.

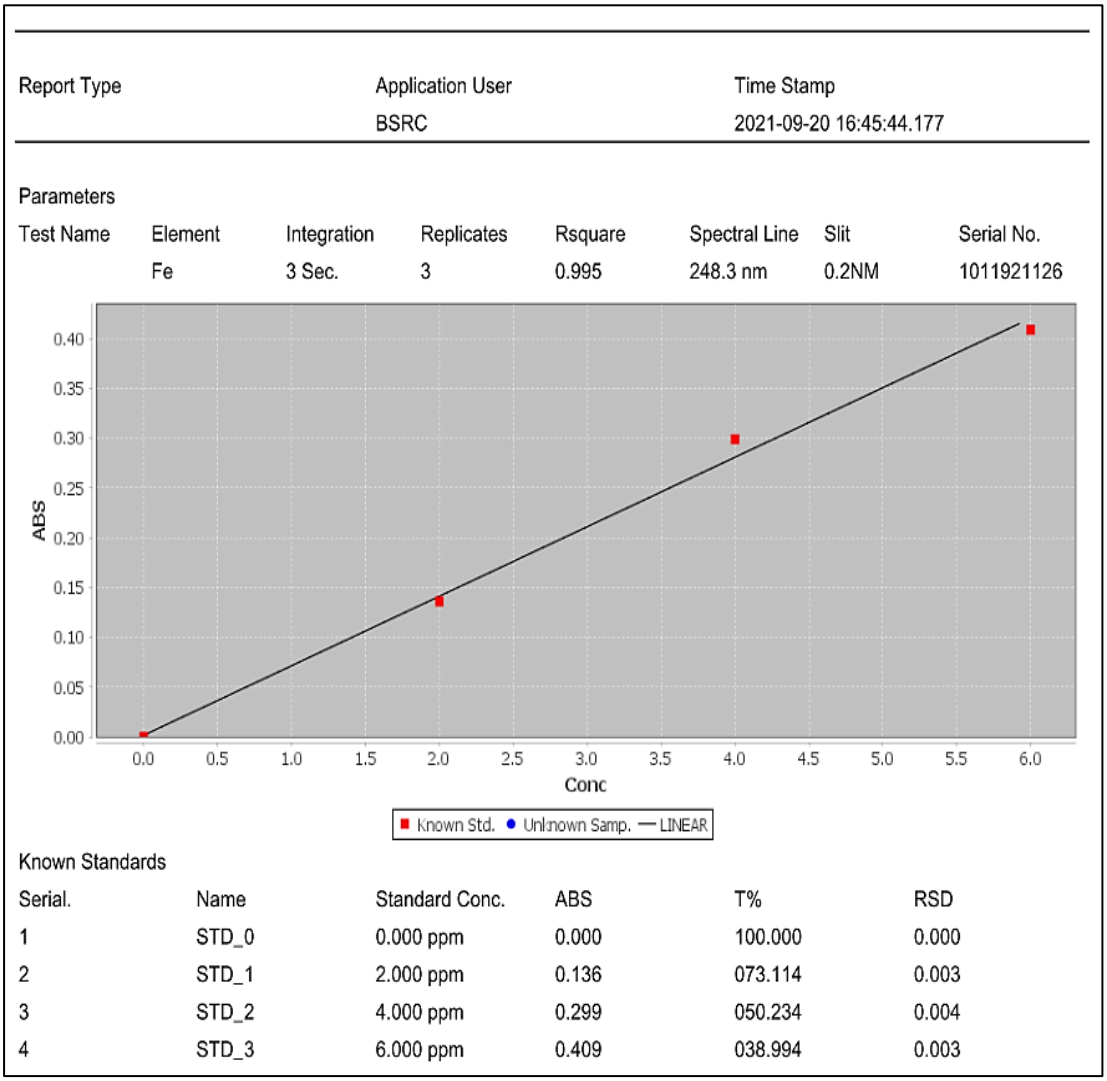


Fig 86: Standard Calibration Graph of Fe on AAS

Table 59: Detection of Iron in Selected four *Terminalia* species

Serial	Name	Sample Conc.	ABS	T%	RSD
1	*Terminalia arjuna*	3.980 ppm	0.021	095.280	0.001
2	*Terminalia bellirica*	1.742 ppm	0.009	097.949	0.000
3	*Terminalia chebula*	0.809 ppm	0.004	099.083	0.000
4	*Terminalia catappa*	2.115 ppm	0.011	097.499	0.000

Similarly, Fig. 87 exhibits the standard calibration curve of Zn with an R= 0.997, along with the sample representation. The amount of Zn in *Terminalia* is shown in Table 60, ranked as *Tc* > *Tb* > *Ta*.

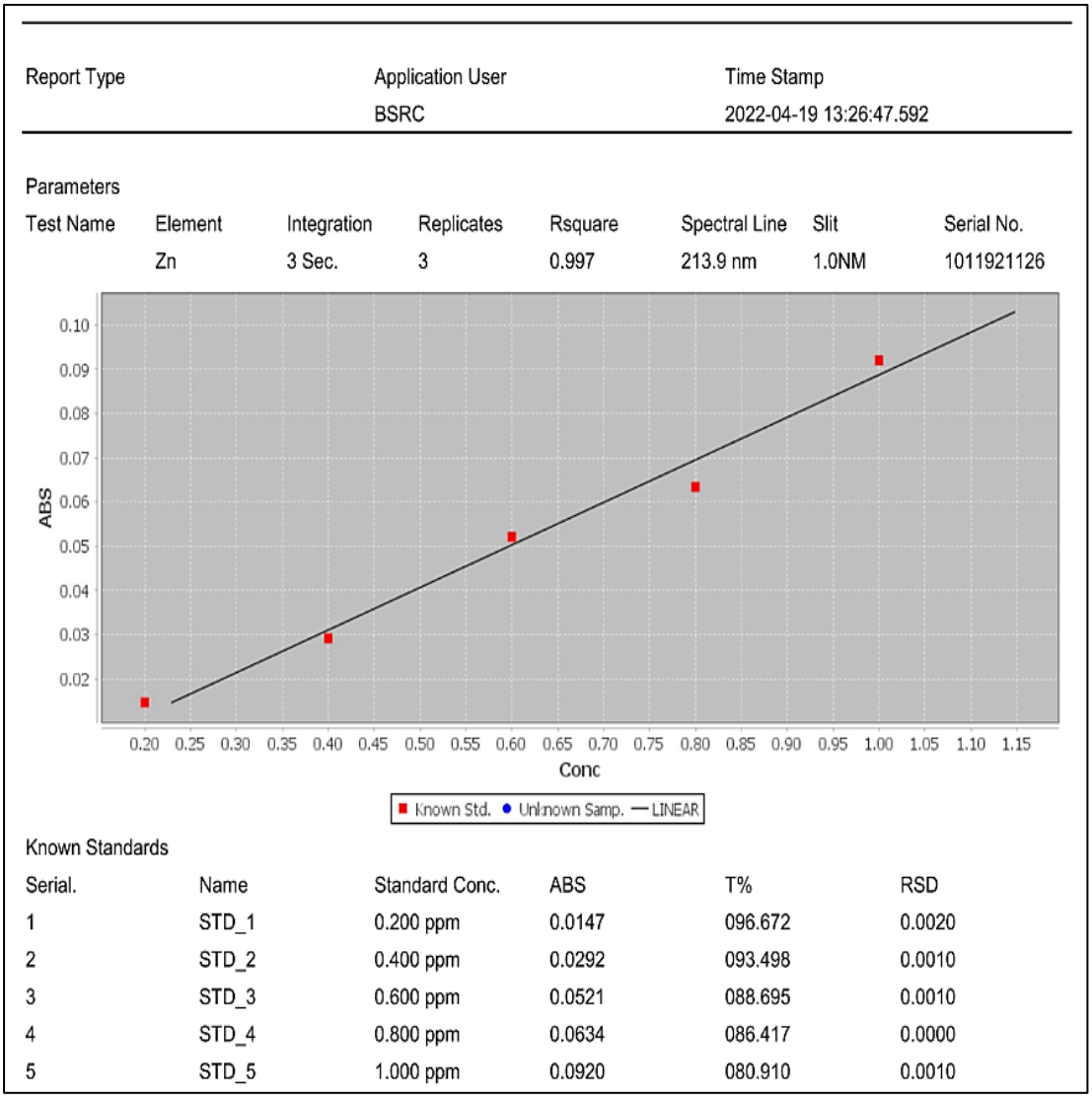


Fig. 87: Standard Calibration Graph of Zn on AAS

Table 60: Detection of Zinc in Selected four *Terminalia* species

Serial	Name	Sample Conc.	ABS	T%	RSD
1	*Terminalia arjuna*	0.423 ppm	0.004	099.083	0.000
2	*Terminalia bellirica*	0.477 ppm	0.005	098.855	0.000
3	*Terminalia chebula*	0.803 ppm	0.011	097.499	0.000
4	*Terminalia catappa*	0.000 ppm	0.001	099.770	0.000

14. Detection of metals in *Terminalia* species by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) :

The developed protocol was validated by performing calibration using calibrated solution of metal reference standards heavy metal reference standards, such as Cadmium (Cd), Lead (Pb), Chromium (Cr), Nickel (Ni), Cobalt (Co), Barium (Ba), Copper (Cu), Aluminium (Al), Zinc (Zn) and Iron (Fe) (Fig. 88). The amounts determined for heavy metals were within the 0.5 ppm (0.00005) limits as specified by FSSAI. Besides, the Terminalia species were found to be enriched with essential phyto-minerals like Iron (Fe), Copper (Cu), and Zinc (Zn) (Table 61). *T. arjuna* reported Iron (Fe) content of 195.2 ppm, Zinc (Zn) 7.863 ppm, and Copper (Cu) 0.714 ppm; *T. bellirica* reported Iron (Fe) content of 156.2 ppm, Zinc (Zn) 11.87 ppm, and Copper (Cu) 0.583 ppm; *T. chebula* reported Iron (Fe) content of 188.8 ppm, Zinc (Zn) 9.681 ppm, and Copper (Cu) 1.737 ppm, and *T. catappa* reported the highest Iron (Fe) content of 199.8 ppm, Zinc (Zn) 12.92 ppm, and Copper (Cu) 3.428 ppm among all. In *T. bellirica*, the Chromium content of 0.562 ppm and in *T. catappa*, the Nickel content of 0.504 ppm were detected, but both values are less than the acceptable limit of 1 ppm (Table 62) and hence, species are found to be safe for human consumption.

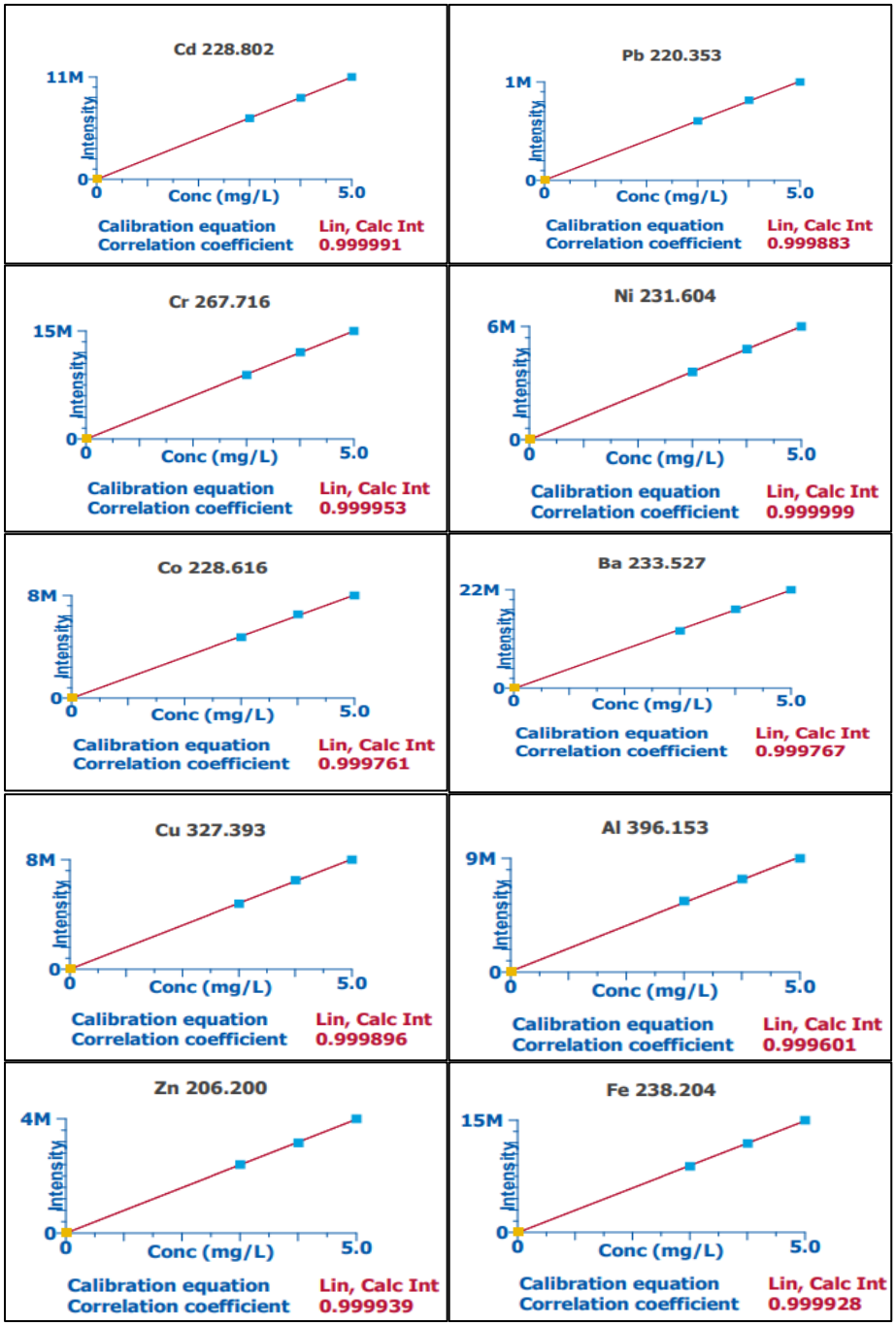


Fig. 88: Calibration graphs of heavy metals Certified Reference Material concentrations

Table 61: Four *Terminalia* species with their Respective Micro / Macro Nutrient Profile with Elemental Quantification by ICP-OES

Terminalia species	Element and Wavelength	Quantification limit (ppm)
T. arjuna	Fe 238.204	195.2
	Cu 327.393	0.714
	Zn 206.200	7.863
T. bellirica	Fe 238.204	156.2
	Cu 327.393	0.583
	Zn 206.200	11.87
T. chebula	Fe 238.204	188.8
	Cu 327.393	1.737
	Zn 206.200	9.681
T. catappa	Fe 238.204	199.8
	Cu 327.393	3.428
	Zn 206.200	12.92

Table 62: Estimated amounts of heavy metals in *Terminalia* species by ICP-OES (ND- Not Detectable)

	Heavy metals		
Sr. No	***Terminalia* species**	**Chromium (ppm)≤ 1**	**Nickel (ppm)≤ 1**
1	***T. bellirica***	0.562	ND
2	***T. catappa***	ND	0.504

15. Metal Estimation in *Terminalia arjuna, Terminalia bellirica, Terminalia chebula* and *Terminalia catappa* by ICP-MS :

The plant samples, comprising fruits from *Terminalia* species (*T.bellirica*, *T. chebula*, and *T.catappa*) and bark from*T. arjuna*, underwent analysis using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) in accordance with established guidelines. The macro nutrients present in *T. arjuna* were found to contain Calcium (Ca) 2520386.02 ppm, Copper (Cu) 4.06 ppm, Magnesium (Mg) 3069.00 ppm, and Iron (Fe) 3766.13 ppm. For *T. bellirica*, the contents were Calcium (Ca) 339682.68 ppm, Zinc (Zn) 2.25ppm, Copper (Cu) 33.42 ppm, Magnesium (Mg) 10254.73 ppm, and Iron (Fe) 1085.02 ppm. In the case of *T. chebula*, the contents were Calcium (Ca) 215979.95ppm, Zinc (Zn) 36.35 ppm, Copper (Cu) 83.91 ppm, Magnesium (Mg) 8323.95 ppm, and Iron (Fe) 4153.78 ppm. Lastly, *T. catappa* contained Calcium (Ca) 141869.06 ppm, Zinc (Zn) 27.23 ppm, Copper (Cu) 55.35 ppm, Magnesium (Mg) 8197.28 ppm, and Iron (Fe) 3794.63 ppm, as represented in Table 63. The consumption of these medicinal plants is believed to be beneficial for human health and is subject to various phyto-pharmaceutical agent and bio-therapeutic studies. However, they also contain heavy metals such as Cadmium, Mercury, and Chromium, whose contents exceed the specified limits, whereas Arsenic and Lead contents are within the acceptable limits (Table 64).

Table 63: Four *Terminalia* species with their Respective Micro / Macro Nutrient Profile with Elemental Quantification by ICP-MS (ND- Not Detectable)

Metals (ppm)	*Terminalia arjuna*	*Terminalia bellirica*	*Terminalia chebula*	*Terminalia catappa*
Calcium (Ca)	2520386.02	339682.68	215979.95	141869.06
Zinc (Zn)	ND	2.25	36.35	27.23
Copper (Cu)	4.06	33.42	83.91	55.35
Magnesium (Mg)	3069.00	10254.73	8323.95	8197.28
Iron (Fe)	3766.13	1085.02	4153.78	3794.63

Table 64: Heavy metals Quantification in four *Terminalia* species by ICP-MS (ND- Not Detectable)

Heavy metal (ppm)	*Terminalia arjuna*	*Terminalia bellirica*	*Terminalia chebula*	*Terminalia catappa*
Cadmium (Cd) ≤ 0.3	1.44	6.73	3.13	2.80
Mercury (Hg) ≤ 1	19.12	6.12	17.37	10.50
Arsenic (As) ≤ 10	0.13	0.43	0.58	1.12
Chromium (Cr) ≤ 2	ND	3.29	4.88	7.85
Lead (Pb) ≤ 10	ND	ND	ND	4.53

Chapter 7 – Discussion

India is rich/ known for its traditional medicinal systems, namely Ayurveda, Siddha, Naturopathy, and Traditional healing etc. and there can be found in the ancient Vedas and scriptures. The concept of Ayurveda emerged and evolved in India between 2500 and 500 BC [199]. Over the past decade, there has been a notable increase in the utilization of herbs and their formulations. The World Health Organization's (WHO) advocacy for traditional medicine has prompted countries to seek WHO's aid in identifying safe and effective herbal medicines for integration into their national healthcare systems. As early as 1989, the World Health Assembly adopted numerous resolutions supporting national traditional medicine programs, emphasizing the significance of herbal medicines in promoting the well-being of individuals and communities.

The developed and developing countries, has a crucial role to provide consumers and healthcare providers with current and authoritative information about the beneficial properties and potential harmful effects of these herbal medicines and their formulations. The primary objectives of these guidelines are to establish fundamental criteria for evaluating the quality, safety, and efficacy of herbal medicines. This, in turn, aims to support national regulatory authorities, scientific organizations, and manufacturers in conducting assessments of documentation, submissions, and dossiers concerning such herbal products and their formulations [200].

The importance of quality is emphasized in the World Health Organization Strategy on Traditional Medicines and their formulations during 2014–2023 for detecting the adulteration and contamination in articles of botanical extracts and blends poses particular challenges were as supplements can be affected by biological and

chemical adulterants and contaminants, adding to the complexity of the issue [199]. Considering this a reliable pharmacopeial standard was considered an essential benchmark for all stakeholders involved for assessing the quality, adherence identity (authenticity of the drug), purity (positive/negative contaminants), including heavy metals and strength (quantifying the active constituents), the necessity for monographs of the standards were emphasized. These monographs served as valuable tools in evaluating the overall quality of Ayurvedic products [201]. Nevertheless, the implementation of enhanced analytical methods, and contemporary assessments of purity various analytical techniques like photometric tools, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) are routinely employed as sophisticated and globally accepted protocols to determine the consistent composition of herbal preparations and their formulations [202].

The contaminant adulteration by metal toxicity in herbal remedies a routine issue can occur either accidentally or intentionally. Hence, heavy metals such as mercury, lead, copper, cadmium, and arsenic can find their way into herbal products due to various factors, majorly via environmental pollution. These contaminants can pose significant health risks to users and should be thus under stringent quality control norms specified as regulatory guidelines for utilization of articles of botanical origin in day to day living. To assess the potential exposure to these toxic metals, their levels can be compared with the Provisional Tolerable Weekly Intake values (PTWI) established by the Food and Agriculture Organization of the World Health Organization (FAO-WHO).When dealing with trace amounts of metals, an imperative need is felt for qualitative and quantitative analysis at same time, Instrumentation becomes pre-eminent and indispensable. Among the primary

methods used for this purpose are atomic absorption spectrophotometry (AAS)[146,147], inductively coupled plasma (ICP-MS) [201] and ICP-OES [202]. Therefore, the most critical requirement at this time is the development of a new, cost-effective, highly sensitive, accurate, and consistent analytical protocol to detect and identify plant database. These protocols should also adhere to regulatory standards and gain global acceptance. The factual overview aforementioned, the parameters that have been examined for proposed research work are outlined below:

1. **Quality assessment of *Terminalia* species :**

   In the current study four selected *Terminalia* species (*T. arjuna, T. bellirica*, *T. chebula* and *T. catappa*) raw materials were evaluated for their quality through botanical, physicochemical, and chemical parameters. The identification of herbal crude drugs begins with the examination of macroscopic characteristics [202]. The macroscopic analysis was conducted to identify the selected plant parts based on their morphological features, including shape, size, color, odor, and taste. This comprehensive assessment aids in establishing the authenticity and quality of the raw materials.

   The physicochemical evaluation plays a crucial role in quality control and identification of crude drugs [202]. Moisture content, extractive values, total ash value, acid insoluble ash value, and water-soluble ash value were assessed for each herbal raw material. Excessive moisture content can lead to microbial growth and hydrolysis, impacting the quality of medicinal plant materials. Extractive value determines the quantity of active constituents extracted from a specific amount of plant material, making it an important parameter in physicochemical analysis. Ash value, on the other hand, helps detect contamination and adulteration with inorganic matter, ensuring the quality and

purity of drugs [202, 204]. The evaluated physicochemical parameters were found to be within specified limits, ensuring the quality of the raw materials as per WHO guidelines.

The phytochemical analysis provides valuable insights into the presence of different secondary metabolites in medicinal plant materials. The preliminary phytochemical analysis indicated the presence of flavonoids, tannins, phenols, alkaloids, triterpenoids, phytosterols, glycosides, saponins, and carbohydrates in all four species (*T. arjuna*, *T. bellirica*, *T. chebula* and *T. catappa*) raw materials of *Terminalia* species. These compounds contribute to the therapeutic potential and bioactivity of the plants, highlighting their significance in traditional medicine and potential applications in various health-related industries

2. Extraction :

The extraction of each herbal raw material was carried out using the Soxhlet extraction method, with ethanol and water selected as solvents due to their non-toxic nature, wide availability and preferability. As per the literature review, ethanol and water solvents are preferred for *Terminalia* extracts due to their ability to effectively extract a broad spectrum of bioactive compounds from the plant raw material, offering a balanced extraction of both polar and non-polar constituents. The specific ratios (70:30) of ethanol and water were chosen for each crude drug to ensure the extraction of desired phytochemical compounds and maximize the extract yield [181]. The resulting percentage yields of *T. arjuna*, *T. bellirica*, *T. chebula*, and *T. catappa* extracts were 26.75%, 25.43%, 22.07%, and 27.54% respectively. These extraction yields provide

important information about the efficiency of the extraction process and the potential concentration of bioactive compounds present in the extracts.

3. UV data for Qualitative analysis / Confirmation :

The development of the UV-Spectrophotometric method involves two main steps: selecting the appropriate solvent system and determining the detection wavelength for the quantification of arjunic acid, chebulagic acid, chebulinic acid, gallic acid, ellagic acid, and quercetin. The solubility profile of these standards in various solvents was obtained through a literature review and practical analysis; via multiple trials. Methanol was identified as a suitable solvent as per the literature survey since the standards were found to be soluble in it [187].

The wavelength optimization was performed using a Shimadzu 1800 UV-Vis spectrophotometer and further baseline and subsequent blank measurements were taken followed by UV spectrum of the analyte was obtained by scanning the solution containing these standards in the solvent within the range of 400-200 nm. The maximum absorption values (λ max) for each active compound were found to be for Arjunic acid at 205 nm, chebulagic acid at 222.5 nm, chebulinic acid at 281 nm, Gallic acid at 271 nm, Ellagic acid at 254.5 nm, and Quercetin at 374 nm respectively. The process of wavelength determination provided accurate values of maximum absorption, successfully overcoming the limitations mentioned in previous reviews. These λ max values were then used as references for High Performance Thin Layer Chromatographic (HP-TLC) studies. The calibration graphs showed distinct and unique spectral behavior for each compound.

4. Fingerprint Profile by HP-TLC :

HP-TLC fingerprinting, supported by literature review and surveillance data, plays a crucial role in herbal product/ formulations analysis due to its cost-effectiveness, simplicity, and ability to detect multiple compounds simultaneously [131-133]. Compared to GC and HPLC, HP-TLC offers faster analysis, minimal sample preparation, and better resolution for herbal products and their preparations for their quality assessment [135-138]. In this present research work, HP-TLC proves to be a valuable method as it can detect multiple classes of compounds simultaneously, with the aid of derivatizing agents for compound detection. The study focuses on testing, evaluation and inspection of four *Terminalia* species collected from the North Central Corridor of Western Ghats to identify the different classes of compounds present in each of them.

Further, in this study HP-TLC Fingerprinting analysis was employed to detect different (classes of compounds) using various derivatizing agents on TLC F_{254} plates. To assess the effect-directed scavenging activity and identify antioxidants present in *Terminalia* species, an HP-TLC-DPPH assay was conducted. The development of a stable and straightforward USP-HP-TLC densitometric fingerprinting routine was performed which was able to attain good resolution and repeatable findings by combining solvent systems. Nonetheless, the optimal solvent system and resolution found for flavonoids was obtained by- Ethyl acetate: Water: Formic Acid: Acetic Acid (100: 26: 11: 11) v/v/v/v, for Tannins- Ethyl acetate: Water: Formic Acid: Acetic Acid (100: 26: 11: 11) v/v/v/v, for Phenols- Toluene: Ethyl Acetate: Formic Acid (6:4:0.3)

v/v/v and for DPPH, cyclohexane: ethyl acetate: formic acid (4:6:1) v/v/v; n-butanol: glacial acetic acid: water (4:4:1), v/v/v.

Eventually for Spectro densitometric evaluation, the developed plate was then derivatized using the reagents and the final plate image is documented using photo documentation which are represented for flavonoids, phenols, tannins, and antioxidants respectively [189].

- **HP-TLC method development and validation :**

HP-TLC method development and validation, as per literature review and surveillance data, is gaining more prominence in herbal medicinal analysis due to its advantages over other chromatographic tools like GC and HPLC. HP-TLC offers cost-effective, robust, simple, and simultaneous detection of multiple compounds, making it a valuable option for herbal product quality assessment. Its quick analysis, minimal sample preparation, and ability to analyze complex mixtures set it apart. Moreover, HP-TLC allows for higher sample throughput and requires less solvent consumption, aligning with green chemistry principles. The methods accuracy, precision, and robustness are demonstrated through validation studies, making it a reliable choice for herbs and their formulation as well as analysis in both research and industrial applications [135-138].

5. Quantification of Gallic acid and Ellagic acid in four *Terminalia* species :

Using RP-HP-TLC plates for analysis offers advantages over TLC F254 plates due to their reversed-phase characteristics, enabling better separation and quantification of hydrophobic compounds like gallic acid and ellagic acid. RP-HP-TLC allows for improved sensitivity and resolution, making it highly suitable for herbal product analysis [205]. The calibration curve was constructed

within the range of 100–700 ng/mL for both gallic acid and ellagic acid which showed polynomial regression. The standard curve was employed for calculating the correlation coefficient, regression equation, intercepts of the curves, and slopes. Recovery studies involved spiking known amounts of biomarkers equivalent to 80%, 100%, and 120% of gallic acid and ellagic acid onto the hydro-alcoholic extracts. Each level was analyzed in triplicates, and the recovery of gallic acid and ellagic acid at different levels in the hydro-alcoholic extracts of *Terminalia* species was subsequently determined.

6. **Phytochemical screening of *T. chebula, T. bellirica* and *T. catappa* containing Chebulagic acid and Chebulinic acid as biomarker :**

   HP-TLC method development and validation for *Terminalia* analysis, specifically focusing on chebulagic acid and chebulinic acid, is crucial in ensuring the quality and efficacy of herbal products. Literature review and available data support the use of HP-TLC due to the ability to detect multiple compounds simultaneously. Chebulagic acid and chebulinic acid are important bioactive compounds in *Terminalia* species, and the accurate quantification is essential for their medicinal properties. In the earlier research work the detection of chebulagic acid in *T. chebula*, *T. bellirica* and *T. catappa* (fruits) is carried out but by HPLC method. HP-TLC method development considering one plant and samples are available in literature search. But simultaneous estimation of both the standards on HP-TLC analysis or TLC analysis is not reported.

   In the study, the calibration curves were constructed over a concentration range of 50 to 350 ng/spot and determined polynomial regression curve. The standard curve was then utilized to calculate the correlation coefficient, regression

equation, intercepts of the curves, and slopes. The reproducibility study of the standards was also performed. Recovery studies were conducted by spiking known amounts of biomarkers, corresponding to 80%, 100%, and 120% of chebulagic acid and chebulinic acid, onto the hydro-alcoholic extracts. Each level was analyzed in triplicates. The recovery of chebulagic acid and chebulinic acid at different levels in the hydro-alcoholic extracts of *T. bellirica*, *T. chebula*, and *T. catappa* was subsequently determined. These comprehensive analyses contribute to the accurate quantification and assessment of these bioactive compounds in the plant extracts.

7. Quantification of Arjunic acid in *T. arjuna* and *T. chebula* species :

As per the literature review performed and available data, HP-TLC method development and validation were carried out for *T. arjuna* and *T. chebula*, focusing on arjunic acid [205]. In a separate study, HP-TLC analysis was established marker-based standardization for detecting potential adulterants in *T. arjuna* bark. These studies highlight the significance of HP-TLC in accurately identifying and quantifying arjunic acid for quality assessment and adulteration detection in *Terminalia* species [162]. However, the simultaneous quantification of Arjunic acid in both *T. arjuna* and *T. chebula* is not currently available, even though it is a key phytoconstituent in these plants.

The recent study demonstrated the calibration curve for arjunic acid was established in the range of 100–700 ng/mL and obtained linear regression curve. Recovery studies were conducted by spiking known amounts of biomarkers, corresponding to 80%, 100%, and 120% of arjunic acid, onto the hydro-alcoholic extracts in triplicate. The reproducibility study and quantification values along with %CV are reported. These meticulous analyses

contribute to the accurate quantification and assessment of arjunic acid in the plant extracts.

8. **Phytochemical screening of Ayurvedic formulations containing *Terminalia bellirica* and *Terminalia chebula* with respect to Gallic acid as biomarker :**

The earlier research highlights the significance of HP-TLC in quality control analysis of herbal formulations in the various Indian systems of medicine. Hence, the demonstration of application of HP-TLC and UPLC-MS/MS method demonstrates for quality assessment of Itrifal formulations in the Unani system [167]. Similarly, HP-TLC alongside HPLC was utilized to assess the markers present in Triphala formulations and its ingredients from various geographical locations in India [207]. These studies showcase the efficiency and reliability of HP-TLC as an analytical tool for assessing the quality and authenticity of herbal products in traditional medicinal systems. By conducting HP-TLC analysis on these *T. bellirica, T. chebula* and Triphala formulations is more essential due to its numerous advantages highlighted in the literature review.

In the present study, the linearity range for gallic acid was established within the span of 100–700 ng/mL along with 3D spectral detection. The results showed linear calibration curve. Recovery studies were conducted in the range of 80%, 100%, and 120% of gallic acid into both the hydro-alcoholic extracts and Triphala. This comprehensive approach facilitated the analysis of each level in triplicates, enabling the determination of the recovery of gallic acid at different concentrations in the hydro-alcoholic extracts of *Terminalia* species as well as in Triphala. These meticulous procedures contribute to the accurate

quantification and assessment of gallic acid in the plant extracts and Triphala formulation.

9. **Simultaneous Method development and Validation of Gallic acid and Quercetin as Bio-markers in Marketed formulations containing *Terminalia bellirica* and *Terminalia chebula***

   By conducting a study on *T. bellirica* and *T. chebula in* traditional Ayurvedic formulations is crucial, as indicated in the literature review. These plants hold significant importance in Ayurvedic medicinal system and have been used for centuries in various formulations for their therapeutic properties. Hence, investigating their phytoconstituents and bioactive compounds through modern analytical techniques like HP-TLC can validate their traditional uses and provide insights into their potential health benefits. This research work under taken may contribute to the scientific understanding of these plants and aid in developing evidence-based ayurvedic formulations for improved healthcare and well-being. In the present research work, the gallic acid and quercetin were applied over a concentration range of 5 µg/mL to 10 µg/mL and 1 µg/mL to 6 µg/mL, respectively with a polynomial regression. Plate development was conducted using an optimized solvent system. Furthermore, the developed plate underwent derivatization with Anisaldehyde reagent, Vanillin reagent, and DPPH for the detection of various classes of compounds present in selected plant species, as well as in commercially available polyherbal formulations. This multi-step analysis, incorporating varied concentrations and detection methods, provides a comprehensive insight into the composition and characteristics of the target compounds within the selected *Terminalia* species and polyherbal formulations.

10. Phytochemical screening of Ayurvedic formulations containing *Terminalia arjuna* with respect to Gallic acid as biomarker :

The research undertaken for *T. arjuna* and Arjunarishta through traditional Ayurvedic formulations holds immense significance, as highlighted in the literature review. These medicinal herbs have been extensively used in Ayurvedic formulations for their cardioprotective and cardiovascular health benefits. Investigating their active compounds and phytoconstituents through modern analytical techniques, such as HP-TLC, can validate their traditional therapeutic claims. This research will bridge the gap between traditional knowledge and scientific evidence, leading to the development of evidence-based Ayurvedic formulations that can effectively support cardiac health and contribute to improved well-being for individuals seeking natural remedies for cardiovascular conditions [168].

This research undertaken highlights on the detection of gallic acid in *T. arjuna* and Arjunarishta Ayurvedic formulations. The standard stock solution of gallic acid 40 µg/ml was applied over a concentration range of 1 µg/mL to 6 µg/ml and achieved a linear regression curve. The plate was developed using an optimized solvent system. Subsequently, the developed plate underwent derivatization with Anisaldehyde reagent and Vanillin reagent for the detection of various classes of compounds present in the selected plant, as well as in commercially available polyherbal formulations. This approach allows for a detailed analysis of the presence and characteristics of gallic acid, providing valuable information on the composition of the target compounds within both the *Terminalia arjuna* and Ayurvedic formulation.

11. Detection of Standards present in *Terminalia arjuna, Terminalia bellirica, Terminalia chebula* and *Terminalia catappa* by LC-MS/MS :

In earlier research work, on the fingerprinting of *Terminalia* (*arjuna*, *bellirica*, *chebula*, and *catappa*) through LC-MS/MS is crucial, as underlined in the literature review. The recent research, has focused on understanding the pharmacokinetics and metabolite profiling of these plants using advanced analytical techniques like UPLC-MS/MS. Investigating the active compounds and metabolites in these *Terminalia* species will provide valuable insights into their pharmacological properties and potential therapeutic benefits. This research will contribute to a better understanding of their medicinal values and aid in the development of evidence-based herbal formulations for healthcare and wellness purposes [154, 172].

Firstly, these plants have a long history of Ethano-medicinal use and are known for their diverse bioactive compounds. Hence, by conducting fingerprinting analysis, we can identify and quantify these compounds, providing valuable insights into their chemical composition and potential health benefits. Secondly, LC-MS/MS offers high sensitivity and specificity, enabling the detection of a wide range of compounds in complex mixtures. Thus, this advanced analytical technique will enhance our understanding of the phytochemical profiles of these plants and contribute to the development of standardized herbal formulations with proven quality efficacy and safety. In the recent study, the LCMS/MS fingerprinting analysis of four selected *Terminalia* species was conducted, including the use of standards. The objective was to detect and confirm the presence of standards (arjunic acid, chebulinic acid, chebulagic acid, ellagic acid, gallic acid, and rutin) within the hydroalcoholic

extract of four selected *Terminalia* species by comparing the m/z (mass-to-charge ratio) of the standards with the peaks obtained in the plant extracts. This analytical method allows for precise identification and verification of specific compounds based on their mass spectral characteristics, providing valuable insights into the chemical composition of the *Terminalia* species and facilitating the establishment of a comprehensive fingerprint for each species.

12. Metal Estimation in *Terminalia arjuna, Terminalia bellirica, Terminalia chebula* and *Terminalia catappa* by AAS:

The literature frameworks indicate that AAS plays a crucial role in elemental analysis. AAS provides valuable insights into plant mineral nutrients and their compositions [146]. Additionally, this emphasis was on the significance of AAS in elemental analysis, underscoring its relevance in various fields, including environmental monitoring and pharmaceutical research [147]. AAS allows us to quantitatively determine the presence of various trace metals and elements in these plant species. By analyzing their elemental composition, we can gain insights into their nutritional value, potential toxic elements, and overall quality. AAS can provide valuable information about the presence of essential minerals and heavy metals, which can impact their safety and therapeutic properties. Exploring the elements of *Terminalia* using atomic absorption is of paramount importance for various reasons. The use of AAS in studying the selected *Terminalia species* (*arjuna*, *bellirica*, *chebula*, and *catappa*) will contribute to a deeper understanding of their elemental profiles and aid in developing safer and more effective herbal formulations.

In the present study, atomic absorption spectrophotometry was employed to determine the concentrations of zinc (*Tc > Tb > Ta),* calcium (*Ta > Tb > Tc >*

Tct), and iron (*Ta > Tct > Tb > Tc)* in *Terminalia species.* The results obtained from this analysis provide valuable information about the levels of these essential metals present in the plant material. Given that these metals act as micronutrients with significant importance in human nutrition, understanding their concentrations in *Terminalia* species becomes crucial. The data obtained from this study can contribute to assessing the potential nutritional benefits of *Terminalia* species consumption and aid in understanding their role as a source of essential micronutrients for human health. The presence of Ca, Fe, and Zn in these species makes them valuable nutritional supplements and energy boosters, commonly utilized in various ayurvedic and herbal formulations.

13. Detection of metals in *Terminalia* species by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) :

The earlier research based on a plant study using ICP-OES allows for the simultaneous and precise quantification of multiple elements in plant samples, providing valuable insights into their elemental composition. This information is crucial for assessing the nutritional value, potential toxic elements, area of collection and overall quality of the plants. Additionally, ICP-OES is a sensitive and reliable analytical technique, enabling the detection of trace elements at low concentrations. Thus, utilizing ICP-OES in plant studies ensures accurate data and supports evidence-based approaches in various fields such as agriculture, environmental monitoring, and herbal medicinal formulations [148, 149].

In this research study, plant samples from fruits of *Terminalia* (*bellirica*, *chebula*, and *catappa*) and bark (*arjuna*) were analyzed using ICP-OES following WHO guidelines. This method allowed for accurate determination of

trace elements like Arsenic (As), Lead (Pb), Cadmium (Cd), and Mercury (Hg), as well as essential elements such as Iron (Fe), Calcium (Ca), and Magnesium (Mg), providing valuable micro-macro nutrient profiles. The selected four *Terminalia* species were subjected to elemental estimation using Perkin Elmer TITAN MPS digestor model ICP-OES AVIO 200 equipped with a nebulizer and dual axial plasma for fast elemental analysis, in accordance with USP 233 and USP 231 pharmacopeial dossiers [162]. The heavy metal content was found to be less than 1 ppm, which was within the limits specified by FSSAI [145]. The ICP-OES tool thus proved to be a novel and effective tool.

14. Metal Estimation in *Terminalia arjuna*, *Terminalia bellirica*, *Terminalia chebula* and *Terminalia catappa* by ICP-MS :

The literature evidences for the plant study should be carried out by ICP-MS due to its high sensitivity and traceability of elements in plants. ICP-MS provides accurate and precise quantification of various elements, enabling to assess plant nutritional content, potential toxic elements, and their impact on human health. This analytical technique is essential for understanding the elemental composition of plants and their medicinal properties, supporting the development of safe and effective herbal formulations. Additionally, ICP-MS is widely recognized as a reliable and robust analytical technique, making it a preferred choice for plant elemental analysis in research and quality control settings. The measurements were performed on a PerkinElmer NexION 2000 ICP-MS/MS instrument (PerkinElmer, USA) which is according to the international guidelines reported in 2020, specific limits were set for the heavy metal content in medicinal plants [208]. The heavy metal limits as per WHO and

FDA are Cadmium (Cd) ≤ 0.3 ppm, Mercury (Hg) ≤ 1 ppm, Arsenic (As) ≤ 10 ppm, Chromium (Cr) ≤ 2 ppm, and Lead (Pb) ≤ 10 ppm respectively [209].

In the present study, this analytical technique enabled precise determination of essential elements such as Calcium (Ca), Zinc (Zn), Copper (Cu), Iron (Fe), and Magnesium (Mg), alongside heavy metals including Cadmium (Cd), Mercury (Hg), Arsenic (As), Chromium (Cr), and Lead (Pb). The results obtained provide a comprehensive understanding of the elemental composition of these plant samples, contributing valuable information to assess their nutritional content and potential health implications. This analysis aligns with standards for quality control and safety considerations, particularly in the context of human consumption and medicinal applications of these selected *Terminalia* species.

Chapter 8 – Summary

The literature review encompasses a comprehensive analysis of various research studies and case reports that manifests modern applications of a novel, 21CFR approved, USP-HP-TLC for phytochemical testing and inspection analysis while adhering to the guidelines set forth by International regulatory bodies across globe specified for herbal and related articles of botanical origin. The focus of proposed research highlights the significance of quality assurance and safety as well as efficacyof herbal and traditional medicinal systems in addition to compliance with regulatory guidelines eventually ensuring their efficacy and authenticity. The methods developed were validated with 21 CFR guidelines in all aspects issued by the US Food and Drug Administration (FDA). By employing State- of- Art ultra-modern tools like HP-TLC and LCMS/MS techniques we have made meticulous efforts to accurately identify and quantify the active components and potential contaminants in herbal formulations which would enable future researchers to utilisealmost all type of proposed diverse analytical techniques to the fullest. The ICH guidelines are globally recognised standards that aim to harmonise the development and registration of pharmaceutical products, including herbal and traditional medicinal products.

The proposed research demonstrates the effectiveness of these techniques in characterizing and evaluating the quality and authenticity of herbal formulations. HP-TLC has been remarkably instrumental in identifying marker compounds and assessing the presence of adulterants, ensuring consumer safety and promoting the rational use of traditional medicinal plants from centuries. The research studyexplores LC-MS/MS, as another confirmatory technique in herbal drug

analysis, highlighting its potential for identifying bioactive compounds in highest accuracy and sensitivity at same time.

The study also entails and investigates how beneficial it is or toxic is the presence of minerals or metals can impact overall utilisation of herbal components in herbal and herbaceutical as well as pharmaceutical industries. For the mineral metal estimation, tools like Atomic Absorption Spectroscopy (AAS),Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Inductively coupled plasma mass spectrometry (ICP-MS) have been utilised as acutting-edge technique. The results of elemental estimation highlight notable presence of micronutrients quint essential for human biology like zinc, iron, and calcium in *Terminalia* species. Employing through rigorous analysis, the study reveals significant levels of these essential minerals in *Terminalia species*, making it a valuable natural source for nutrition and potential therapeutic applications. The elemental tools used, are scientifically proven to be an effective analytical tool for being most robust and precise quantification of trace elements in herbal medicinal products, further contributing to the understanding of *Terminalia*species bioactive constituents and its potential health benefits.

The study utilized the State-of-Art ICP-OES Avio 200 technique to determine the elemental composition, with a specific focus on trace heavy metals viz. (Mercury, Arsenic, Cadmium, and Lead) in accordance with FSSAI norms. Further, the analysis included screening for micro-macro essential mineral nutrient profiles, following the recommended guidelines as stated in accordance with USP- 232-chapter elemental impurity limits. The screening results identified *Terminalia* species as non-toxic with abundant essential nutrient rich compounds. These compounds show potential for treating blood disorders like sleep sickness

and anaemia, and also exhibit antibacterial, antifungal, and wound healing properties, making them valuable candidates for potent biotherapeutic phytomedicines. Hence, through meticulous sample preparation and calibration using standard reference materials, both ICP-OES and ICP-MS demonstrated their suitability for elemental quantification in *Terminalia*. ICP-OES offered a robust and cost-effective solution for identifying major elements in the samples, while ICP-MS provided exceptional sensitivity and allowed the detection of trace metals at ultra-low as ppb or ppt levels.

The findings of the study revealed substantial concentrations of zinc, iron, and calcium in various *Terminalia* species, indicating their potential significance is potential lead in treatment of malnutrition and other related nutritional applications of their precise quantification of these essential minerals opens avenues for further research into the health benefits and bioactive properties of *Terminalia*and their selected species.

The research study aimed to assess the quality of four selected *Terminalia* species: *T. arjuna, T. bellirica*, *T. chebula*, and *T. catappa*. The assessment involved botanical, physicochemical, and chemical parameters, as well as the quantification of bioactive compounds present in these plant species. This research utilize various analytical techniques such as macroscopic analysis, soxhlet extraction, UV-spectrophotometry, High Performance Thin Layer Chromatography (HP-TLC), Reverse Phase High Performance Thin Layer Chromatography (RP-HP-TLC), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), and Inductively coupled plasma mass spectrometry (ICP-MS) to achieve its objectives.

The macroscopic analysis involved examining morphological features to identify the selected plant parts and physicochemical parameters, includes moisture content,

extractive values, total ash value, and ash insoluble in acid and water soluble ash values, were assessed to ensure the quality of the raw materials. The phytochemical analysis revealed the presence of various bioactive compounds, such as flavonoids, tannins, phenols, alkaloids, and glycosides. Soxhlet extraction with ethanol and water(70:30) as solvents was used to extract bioactive compounds from the plants. The optimized extraction process yielded extract percentages for each species, providing information about the efficiency and potential concentration of bioactive compounds. UV-sspectrophotometric methods were developed to quantify specific compounds like arjunic acid, chebulagic acid, chebulinic acid, gallic acid, ellagic acid, and quercetin.

The developed HP-TLC fingerprinting analysis effectively detected multiple classes of compounds simultaneously indicating impactful cost effective method developed for selected *Terminalia* species and further quantification of gallic acid, ellagic acid, chebulagic acid, chebulinic acid, and arjunic acid was carried out using TLC and RP-HPTLC plates, allowing for better separation and quantification of hydrophobic compounds. The research study also allowed exploring the presence of essential elemental profiles in selected *Terminalia* species using AAS which were found to be enriched with essential minerals like calcium, iron, zinc, and copper. The ICP-OES objective to assess the elemental composition of four *Terminalia* species (*arjuna*, *bellirica*, *chebula*, and *catappa*) as per WHO guidelines. The results indicated low levels of heavy metals within acceptable limits and high levels of essential elements, making them beneficial and safe for human consumption. The ICP-MS technique utilized to measure the macro and trace elements with highest sensitivity confirmed ICP-OES observations in the same *Terminalia* species, revealing their potential benefits for human health. Nevertheless, the heavy metal composition particularly

(As, Pb, Cd and Hg) were also estimated from findings in which these findings emphasize the importance of metal estimation for further ensuring the safety and efficacy of herbal medicinal formulations and their applicability use in various fields like agriculture and environmental monitoring, foodonomics etc.Overall, we are sure and confident that the efforts taken in this comprehensive research study would provide valuable insights into the quality and bioactive composition of ecologically important species of medicinal plants, supporting their traditional medicinal use and potential applications in phyto-pharmaceuticals and Nutraceutical industries as well as paving further paths and scopes to future researchers in this field.

Thus, to conclude, the analytical techniques employed in proposed research demonstrated their effectiveness in assessing the quality and authenticity of herbal products, contributing to the development of evidence based Ayurvedic formulations for healthcare and well-being of human mankind for years to come.

Chapter 9– Conclusion

Medicinal plants and their formulations in various forms have a rich therapeutic history that enables them to furnish the health needs of a significant global population which consist of multiple intrinsic chemical constituent matrices, making it difficult to attribute efficacy to extract/ isolate a single active constituent However, ensuring Quality Control / Quality Assurance, poses challenges due to the vast variability of geographical sources and complex chemical components involved.. hence, for establishing quality control standards for raw materials and standardizing finished herbal drugs and their formulations remains an unmet challenge. Therefore, chemical fingerprinting analysis methods like TLC, HP-TLC, and LCMS/MS have emerged to address these challenges that impact the quality of herbal drugs which are able to resolve the intricate interactions among multiple ingredients that contribute to the therapeutic effects, featuring herbal medicines apart from conventional chemical drugs.

HP-TLC monographs USP<203>and USP<1064> serve as benchmark regulations for standardizing and ensuring the quality control and identification of articles of botanical origin, including drug substances, drug products, dietary supplements, and herbal medicines. The World Health Organization (WHO) acknowledges the significance of TLC, HP-TLC, HPLC, and AAS methodologies found in national or regional pharmacopoeias in addition to WHO documents. However, it emphasizes the need for proper validation of these methods, including trace detection limits, which may vary based on the instrument and sample type which also advocates for improved and harmonized validated methods for their inclusion in international and regional pharmacopoeias to ensure accurate assessment and authentication of herbal materials.

The proper utilization of various analytical instrumental techniques, including AAS, ICP-OES, and ICP-MS, assumes a vital role in safeguarding the quality, safety and efficacy of medicinal products. These methods offer comprehensive insights into chemical composition, specific compound identification, and trace element estimation in addition to heavy metal quantification. The adherence to regulatory guidelines such as 21 CFR, ICH, and USP enhances not only the quality assurance of Ayurvedic and traditional formulations, but quality control as well ensuring consistency and reliability across diverse studies and laboratories. The focus on quality and safety not only establishes the authenticity and standardization of these products but also facilitates their integration into modern healthcare systems, supporting potential applications in healthcare, pharmaceuticals, and nutraceuticals. Hence, such measures instill confidence among consumers and healthcare practitioners alike, promoting responsible use and appreciation of these ancient medicinal practices in contemporary contexts.

The findings of the present research study remarkably exemplify that USP-HP-TLC analytical tool adheres to international guidelines, making it widely accepted and the preferred technical for herbal drug analysis worldwide. The designed HP-TLC method proves to be novel, robust and cost-effective, surpassing the present time consuming and expensive methods, as confirmed by regression results and validation criteria. This study findings showcases the successful screening and identification of essential phytotherapeutic markers, such as Arjunic acid, Chebulagic acid, Chebulinic acid, Gallic acid, Ellagic acid and Quercetin, in selected *Terminalia* species (*T. arjuna*, *T. bellirica*, *T. chebula*, and *T. catappa*) using the HP-TLC technique. HP-TLC offers advantages of simple visual evaluation, flexibility in use, reproducible analysis, reliable quantification, and the ability to

detect multiple analytes without cross-contamination. The detection of HP-TLC fingerprinting for compound classes was achieved using derivatizing reagents such as, the Natural product reagents, Ferric chloride, and the HP-TLC-DPPH assay confirmed the presence of antioxidants in selected*Terminalia*species. The parameters for the methodare aligned well with the guidelines of ICH and USP protocols, which satisfies the regulatory requirements, which are outlined in 21CFR. The developed protocol serves as an excellent alternative for herbal drug analysis, routine quality control, and testing of marketed Polyherbal formulations, surpassing the costs associated with other chromatographic and spectroscopic tools frequently used in medicinal plant and phytochemical research. This advancement paves the way for wider global acceptance of HP-TLC as the official ultra-modern chromatographic tool.

The application of LC-MS/MS facilitates the determination and prediction of molecular weights for each compound, using the available spectrum library for molecular weights. By comparing mass values with calculated spectral patterns, the molecular weights of each compound can be predicted and major compounds with higher area percentages are identified. Prominently, the detected compounds in *T. arjuna*, *T. bellirica*, *T. chebula*, and *T. catappa* matches exactly with m/z of those of the standard phyto-markers utilized in the research study.

In this research utilization of AAS with Ca, Fe, and Zn lamps to estimate Micro and macro nutrient profile in selected *Terminalia* species was undertaken and the standard calibration curves for Ca, Fe, and Zn are with high Rvalues (0.999, 0.995, and 0.997, respectively) and quantification results reveal the abundance of Ca, Fe, and Zn in selected *Terminalia*species which highlights the nutritional beneficence of

these elements in *Terminalia* species, reinforcing their value as essential supplements and energy enhancers in various ayurvedic and herbal formulations.

As per compliance of USP and WHO guidelines, the ICP-OES, a State -of-Art tool was utilized for confirmation of micro-macro nutrient profile along with safety profile concerns for consumption of human mankind. In this parameter, fruits (*T. bellirica*, *T. chebula*, and *T. catappa*) and bark (*T. arjuna*) samples from selected *Terminalia* species were subjected for elemental estimation by ICP-OES technique. Essential elements like Fe, Ca, and Mg were accurately determined, with heavy metal content (As, Pb, Cd, Hg) found to be below the 1 ppm the limit set by FSSAI . The protocol was validated using heavy metal reference mix solution from Perkin Elmer to ensure accurate sensitive and reliable results. These *Terminalia* species exhibited enrichments in essential phyto minerals like Zn, Fe and Cu while Arsenic and Lead remained within acceptable ranges (≤ 1 ug/ml).

ICP-MS method accurately determines the heavy metals trace elements like Arsenic, Lead, Cadmium, and Mercury, as well as essential elements such as Iron, Calcium, and Magnesium, providing valuable micro-macro nutrient profiles. The protocol developed was validated using heavy metal reference standards. Additionally, the selected *Terminalia* species were found to be enriched with essential phyto-minerals, making them potentially beneficial for human health and this ICP-OES and ICP-MS tools proved to be a novel and effective means to assess the safety and nutritional value of these medicinal plants for human consumption, justifying further with phyto-pharmaceutical and bio-therapeutic investigations.

Chapter 10- Future Scope

The research findings presented in this study have significant future scope and implications in the varied areas of phyto pharmaceuticals and nutraceuticals. The study employed by various analytical techniques, such as UV-Vis spectrophotometry, HP-TLC, DPPH assay, AAS, ICP-OES and ICP-MS, to analyze the phytochemical composition of different *Terminalia* species. The findings presented are below:

1. **HP-TLC Bio-autography:** Future studies could focus on isolating and identifying novel bioactive compounds from *Terminalia* species and these medicinal plants are already known to contain numerous beneficial compounds, that indicates including flavonoids, tannins, and terpenoids which exhibits antibacterial, anti-fungal and anti-microbial activities. HP-TLC bio-autography is an analytical technique that combines HP-TLC with biological assays. Then, the plate is exposed to live microorganisms or biological targets to assess the bioactivity of separated compounds which results in streamlining the process of bioassay-guided fractionation, leading to the isolation of potential lead compounds from complex plant extracts efficiently.
2. **Identification of Novel Compounds**: The impactful point of research would be to identify and isolate specific compounds from *Terminalia* species that exhibit potent anticancer activity which can be explored from different parts of *Terminalia* plants, such as leaves, bark, fruits, or roots, to identify unique bioactive compounds that effectively inhibit cancer cell growth.
3. **Plant Protein and *Terminalia*species:** The potential of *Terminalia* species as sources of plant protein could also be explored through Proteomic techniques like two-dimensional gel electrophoresis and mass spectrometry that can be

used to analyze the protein content of these plants which could lead to the development of new plant-based protein sources, which are in high demand due to the growing popularity of vegetarian and vegan diets.

4. **Food Quality and Safety:** Foodonomics could also contribute to improving the quality and safety of foods derived from *Terminalia* species through, omics technologies that are used to detect contaminants or adulterants in *Terminalia* products which further improve food safety andestablish consumer confidence in these products.
5. **Toxicological Considerations:** The determination of heavy metal content within acceptable limits in *Terminalia* species ensures their safety for human consumption which is crucial for regulatory purposes and assuring the safety of herbal products and their formulations indicating export trades of *Terminalia* species for medicinal and cosmetic purposes.
6. **Drug Development and Ayurvedic Formulations:** The phytochemical profiling and quantification of active compounds in *Terminalia* species lay the foundation for further research indeveloping novel drug molecules and improving existing Ayurvedic formulations.

Overall, the findings proposed research, open up avenues for further research in the domains of new bioactive compounds, lead molecule, plant protein, food quality, herbal formulations, heavy metal and drug development and the development of new analytical techniques could further enhance our understanding of these plants and facilitate their commercial utilization.

Chapter 11- Bibliography

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Annexure

I: Authentication letter

राष्ट्रीय पारम्परिक चिकित्साविज्ञान संस्थान
ICMR-NATIONAL INSTITUTE OF TRADITIONAL MEDICINE
(भूतपूर्वक्षेत्रीयआयुर्विज्ञानअनुसंधानकेन्द्रFormerly Regional Medical Research Centre)
Nehru Nagar, Belagavi-590 090

भारतीयआयुर्विज्ञानअनुसंधानपरिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
स्वास्थ्यअनुसंधानविभाग, स्वास्थ्यऔरपरिवारकल्याणमंत्रालय, भारतसरकार
Department of Health Research,
Ministry of Health & Family Welfare, Govt. of India

Dr. Harsha Hegde
Scientist-E
harshah@icmr.gov.in

Date: 20-05-2021

AUTHENTICATION

This is to authenticate that the plant materials brought by Ms. Chaitrali Bidikar, Ph. D Scholar, Dept. of Pharmacognosy, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi, are identified as ***Terminalia chebula*** **Retz.,** ***Terminalia bellirica*** **(Gaertn.) Roxb.,** ***Terminalia arjuna*** **(Roxb.) Wight & Arn. and** ***Terminalia catappa*** **L.,** all belonging to family **Combretaceae.** The herbarium specimens of the same havebeen deposited in our herbaria with accession numbers RMRC-1632, RMRC-1633, RMRC-1634 and RMRC-1635 respectively.

Harsha Hegde
Scientist-E

II: Industrial Training Certificates

Chika
Overseas Pvt. Ltd.

Marketing. Off. : C-1/401,G.I.D.C. Phase II, Vatva, Ahmedabad:382 445. INDIA
Corp.Off. : Plot No.68,Sector No.1, Dronagiri Node, Navi Mumbai-400 707.INDIA

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E-mail : chika@chikaoverseas.com
Website: www.chikaoverseas.com

Date: 14-12-2021

Certificate Of Training

This is to certify that, ***Ms. Chaitrali Bidikar*** *Ph.D. Research Scholar student from college of Pharmacy, KLE University Belgavi Has successfully undergone the method development of -*

1. *Terminalla Arjuna and its Bio-Markers (Gallic and Ellagic Acid)*
2. *Terminalia Catappa.*
3. *Additionally carried work on four selected species of Terminalia. Using same solvent system (Mobile Phase).*

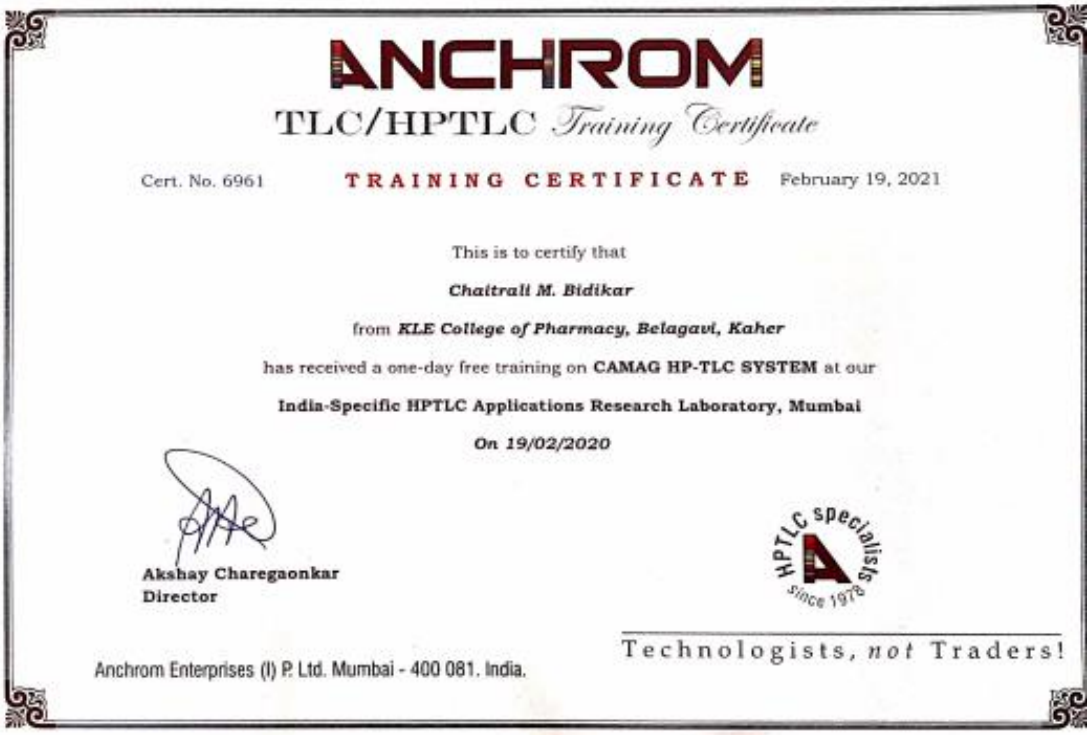
Training conducted from, 29.11.2021 to 06.12. 2021.during this period of training she has completed her training on operational techniques of most advance recently launched UV-VIS spectrophotometer and completed her project work as well, on the same instrument.

She has completed her desired work on High Performance Thin Layer Chromatography (HPTLC) – Advance version; vision Cats- from CAMAG, Switzerland.

Importantly she is lucky to have hands on training experience with our most advance recently launched, ***inductively coupled plasma instrument****- ICP-OES. trace metal detection analysis system. Under the guidance of undersigned. During the training in above mention period* ***Ms. Chaitrali Bidikar*** *has shown keen interest in knowing new operational techniques of different type of analytical instruments.*

For Chika Overseas Pvt Ltd.

M.G.NANDANWADKAR
GENERAL MANAGER – Q.A
REGULATORY COMPLIANCES



ANCHROM

TLC/HPTLC *Training Certificate*

Cert. No. 6961 **TRAINING CERTIFICATE** February 19, 2021

This is to certify that

Chaitrali M. Bidikar

from ***KLE College of Pharmacy, Belagavi, Kaher***

has received a one-day free training on **CAMAG HP-TLC SYSTEM** at our

India-Specific HPTLC Applications Research Laboratory, Mumbai

On 19/02/2020

Akshay Charegaonkar
Director

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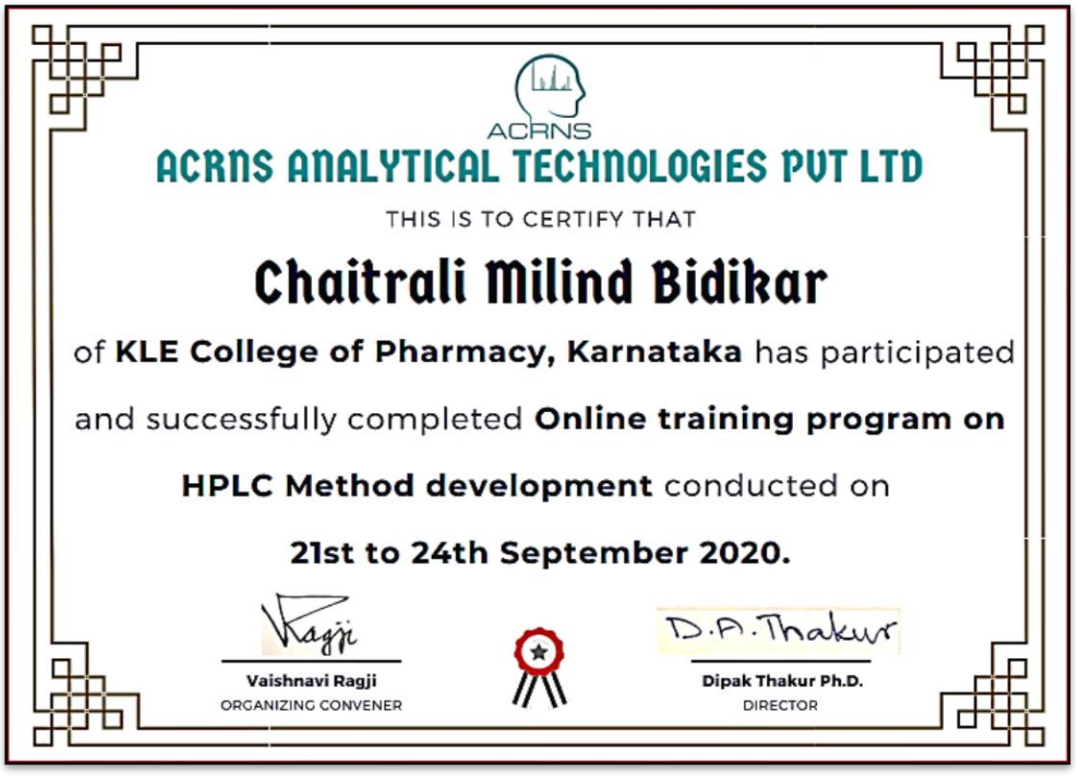

ACRNS

ACRNS ANALYTICAL TECHNOLOGIES PVT LTD

THIS IS TO CERTIFY THAT

Chaitrali Milind Bidikar

of **KLE College of Pharmacy, Karnataka** has participated and successfully completed **Online training program on HPLC Method development** conducted on **21st to 24th September 2020.**

Ragji

Vaishnavi Ragji
ORGANIZING CONVENER

D.A.Thakur

Dipak Thakur Ph.D.
DIRECTOR

III: List of Publications

1. Bidikar CM, Hurkadale PJ, Nandanwadkar SM, Hegde HV. A validated spectro densitometric regulatory compliant USP-HP-TLC protocol for quantification of polyphenols and antioxidants from polyherbal formulations containing Terminalia species. Journal of Chromatography B. 2022 Sep 1;1207:123379.https://doi.org/10.1016/j.jchromb.2022.123379 (I.F= 3)

2. Bidikar CM, Hurkadale PJ, Nandanwadkar SM, Hegde HV, Singh S, Khale A, Phanse M. High-performance thin-layer chromatography fingerprint profile analysis and spectro-densitometric evaluation of antiproliferative antioxidants such as ellagic acid and gallic acid from four widely used Terminalia species. JPC–Journal of Planar Chromatography–Modern TLC. 2023 Aug;36(2-3):169-78.https://doi.org/10.1007/s00764-023-00238-z(I.F = 1.6)

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A validated spectro densitometric regulatory compliant USP-HP-TLC protocol for quantification of polyphenols and antioxidants from polyherbal formulations containing *Terminalia* species

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[b] *Food and Micronutrient Analysis Laboratory, KLE University, KLE Academy of Higher Education and Research (KAHER), Belagavi 590010, Karnataka, India*
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Terminalia chebula
Triphala
HP-TLC
ICH

ABSTRACT

The phytochemical profiles of ethno-medicinal plants from Southern Asia have been extensively studied, due to their wide utilization in various traditional systems of India, Bhutan, Maldives, Nepal and China. *Terminalia bellirica* (Gaertn.) Roxb. and *Terminalia chebula* Retz. are the two most important and widely utilized medicinal plants across the traditional system in India. The herbal products comprising the fruits of these two plants, example Triphala, Vyoshadi-Gulgulu Gulika and also marketed Ayurvedic products like Pilonil Tablet are proven to have high medicinal value and biotherapeutic efficacy. The current study is an effort to develop highly precise, sensitive and reproducible HP-TLC protocol for the standardization herbal preparations comprising of hydro-alcoholic extract of selected *Terminalia* species as their major ingredients. The selected herbal products were assessed through HP-TLC for quantifying gallic acid and quercetin, followed by their visualization using DPPH*, Anisaldehyde and Vanillin as derivatizing reagent. The USP official protocol was followed for the method development using digitally optimized HP-TLC system. The results demonstrated good sensitivity and regression value of 99.999% for proposed method with optimized chromatographic analysis. The developed protocol was validated in accordance with ICH guidelines and all the parameters were found to be within the specified limits. Thus, the proposed HP-TLC method would surely serve as a classical tool for analysis and standardization of *Terminalia* species and their traditional products.

1. Introduction

Traditional medication framework is favorable to humankind; however there is yet absence of suitable normalization strategies for deciding its consistency, amount/ quantity and quality. Altogether, to normalize and admeasure the principal biomarker through medication and formulations from poly-herbal, chromatographic techniques are highly preferred, tools according to WHO guidelines [1]. The primary prerequisites for their adequacy in western medication/ treatment are quality control and Standardization of unrefined components and traditional medications. These ensure the right of the client to get essential, healthy and potent solution. Bioactive concentrates can be normalized utilizing chromatographic and spectroscopic methods as indicated by the dynamic hypothesis or primary compound(s) [2].

The quality of traditional medicinal plants are evaluated based on Current Good Manufacturing Practice (cGMP) and thus, manufacturers are required to assess the identity, integrity, and content of the ingredients of the raw drug sources and final finished products, for which developing scientifically validated methods is a pre-requisite [1,3–5]. Pharmacopoeias and other official dossiers ready-to-execute methods and pre-requisites for controlling quality of several traditional medicinal plants, preparations and products, to meet the necessities of cGMP [6,7]. Nevertheless, the availability of such information is limited to only few plant drug materials and even lesser herbal formulations and products.

The plants belonging to genus *Terminalia* from Combretaceae are widely used in the traditional systems of medicine across Asian countries, including Ayurveda e.g. Triphala, Pilonil, etc. The most important

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Annexure

Abbreviations

HP-TLC	High Performance Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
DPPH	2,2-diphenyl-1-picrylhydrazyl or α, αdiphenyl-β-picrylhydrazyl
ICH	International Council for Harmonization
CFR	Code of Federal Regulations
cGMP	Current Good Manufacturing Practice
TLC	Thin Layer Chromatography
USP	United states of Pharmacopeia
v	Volume
µg/mL	Microgram per Milli Litre
ng/mL	Nanogram per Milli Litre
mins	Minutes
H_2SO_4	Sulphuric acid
Rf	Retardation Factor
%	Percentage
ICMR	Indian Council of Medical Research

Table 1
Derivatization reagent preparation.

Derivatization reagents	Used technique for preparation
Anisaldehyde reagent (ASR)[35]	Anisaldehyde reagent (ASR) was obtained dissolving 1 mL of p-anisaldehyde in 200 mL of Methanol in glass bottle and cool it down in ice water bath then to the mixture, slowly add acetic acid and sulphuric acid (20:10 V/V).
DPPH (2, 2-Diphenyl-1 picrylhydrazyl) reagent	DPPH (2, 2-Diphenyl-1 picrylhydrazyl) reagent was obtained dissolving 8 mg of DPPH in 200 mL ethanol in dark environment as DPPH is light sensitive and prepares it in ambered color bottle.
Vanillin reagent	The acidified Vanillin reagent was prepared by weighing 3 g of vanillin, 1.5 mL conc. H_2SO_4 and absolute Ethanol (100 mL).

of them are *T. bellirica* and *T. chebula* [8]. *T. bellirica* is well known with various names commonly as Bibhitaki [9] in Sanskrit, Baheda in Marathi [10] and Hindi. It is anthelmintic [11], anodyne, styptic, aperients, sweet and antipyretic, antiemetic in nature, while seeds are enriched with analgesic properties [12]. It is likewise acceptable in persistent chronic diarrhea, dysentery, and increases appetite and also effective in colds-cough and acts well when taken with honey during sore throats. Potent pharmacological properties like anti-oxidant, anti-mutagenic, anti-proliferative effects [13] have also been often represented. The fruit of *T. chebula* or Myrobalan (Haritaki) is known as "King of Ayurveda" because of it's to immune modulatory efficacy and wide utility. These dried fruits are rich in tannins, gallic acid [14], ellagic acid, and chebulinic acid [15], phenols, ethyl gallate, chebulaginic acid, bellericanin, gallo-tannic acid, gallic acid and lignans resins and anthraquinone [16–18].

In the modern times, advancement in chromatography techniques and fingerprint profiles play a quint essential role. HP-TLC became the official approved protocol globally. The technique has advanced from an offline, quick, qualitative technique online to an analytical technique of high reproducibility, enabling retaining the lower running costs. HP-TLC is the most preferred quality control tool [19] that analyses multiple samples of diverse nature in parallel (around 14 samples of diverse nature on single stationary phase i.e., TLC plate), USP-HP-TLC emphasis heavily on quality as well as quantity when herbal analysis is the target of research as it complies with guidelines mentioned in official methods of plant analysis and is also approved with 21 CFR compliance [20–22].

While the quality control protocols for synthetic drug substances are simple and straightforward, this process is complex for traditional herbal drugs and formulations. Traditional remedy and their formulations are often enriched and encapsulated with complex composition of multiple phyto-components. Therefore, quality testing of traditional medicines has become more challenging and laborious especially with the improvised regulatory approved protocols for such testing [23].

In this regards, focusing on the aspect of the traditional medicine, USP-HP-TLC chapters USP 203 and USP 1064 for articles of botanical origins [24,25] have been mentioned as official protocols in form of USP monographs highlighting various assays targeting quality. Comprehensive HP-TLC [26] fingerprint can be reproducibly developed on HP-TLC plate with maximum separation. Evaluation of quality of the constituents based on qualitative-quantitative data simultaneously can be identified. The novel free radical scavenging activity by DPPH assay can be applied to exhibit the antioxidant activity of complex mixtures like plant extracts and inherent biomarkers [27–29]. The DPPH* (2,2-diphenyl-1-picrylhydrazyl or α, αdiphenyl-β-picrylhydrazyl) assay [30] is simple, fast to perform, cost-effective, efficient and thus one of the most usually utilized tools for antioxidant screening of constituents, extracts or biological matrices, mixtures of constituents [31,32].

Therefore, the present work is an effort to determine the existence of bio-active phytochemical markers in selected medicinal plants, viz. fruits of *T. bellirica*, *T. chebula* and their herbal products [33,34].

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals, solvents and reagents were of analytical grade. Gallic acid was provided as gift sample by Himalaya, Pvt. Ltd. Bangalore, India. Quercetin was procured from Sigma Aldrich, Mumbai, India. Methanol, Toluene, Isopropyl alcohol, Acetic acid were purchased from Merck, Germany.

2.2. Sample collection

The marketed formulations containing selected *Terminalia* species; Triphala from zandu, while vyoshadi gulgulu gulika (tablet) and pilonil tablet were procured from Vaidyaratnam, Kerala. Fruits of *T. bellirica* and *T. chebula* were purchased from the local Market. The materials were authenticated at ICMR-National Institute of Traditional Medicine, Belagavi.

2.3. Sample preparation/ extraction

The dried fruits were grind to obtain coarse powder (mesh size #40) and then it was together extracted through hydro-alcoholic media (30:70 v/v); through soxhlate process at temperature 80 °C [35]. These extracts were evaporated using rotary evaporator under vacuum to obtain concentrated extract. In case of marketed formulation Triphala powder was weighed as per the daily dose. Vyoshadi gulgulu gulika and pilonil in form of tablets, each tablet was weighed and the average weight of 10 tablets was considered for analysis. The tablets were crushed, and final weight was dissolved in 10 mL of methanol which was further sonicated following filtration to obtain clear supernatant used for analytical studies as per USP ⟨203⟩ chapter [20].

2.4. Instrumentation

A CAMAG HPTLC system controlled by Linomat V applicator, Twin trough Developing Chamber, TLC Plate Heater III, Scanner IV, vision CATS software version 3.0.2 and Hamilton HPTLC syringe was used.

2.5. High-performance thin layer chromatography

Analysis was carried out in accordance with USP Chapter ⟨203⟩ (The

Annexure

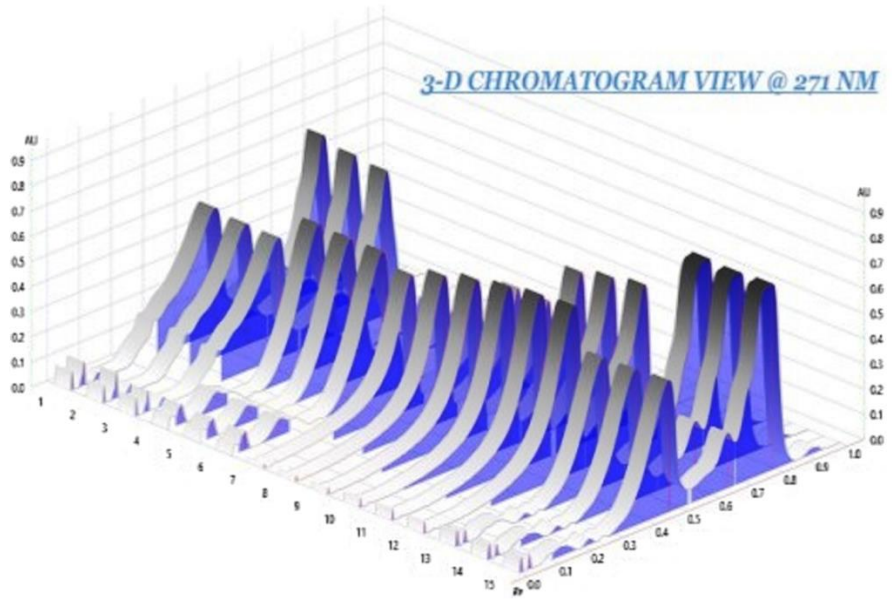

Fig. 1. 3D Chromatogram of Gallic acid and Quercetin.

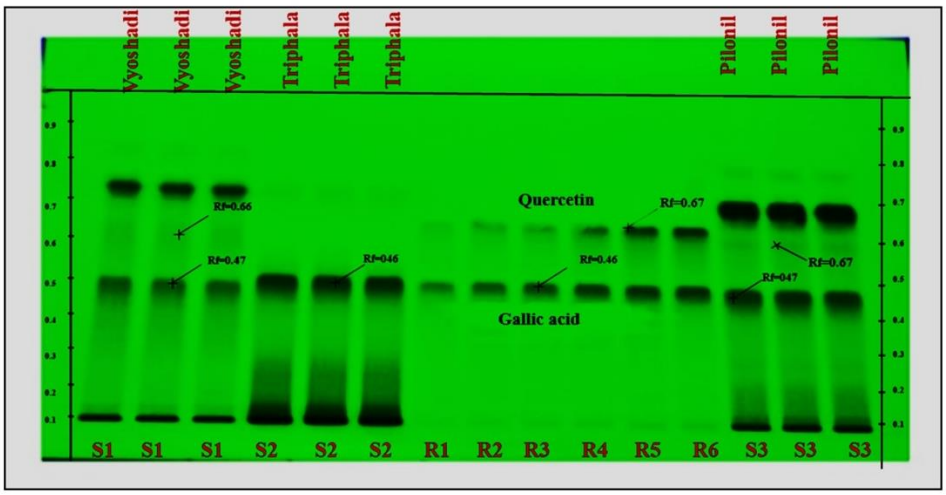

Fig. 2. Plate Image @254 nm.

United States Pharmacopoeia (USP), 2017). Plates backed with aluminium coating silica gel F_{254} Merck Darmstadt, Germany were activated by using methanol by procedure as per USP. Band application was done using Linomat V sample applicator. Ascending development of plate was done with optimized solvent system of **toluene: isopropyl alcohol: acetic acid** (7:2.5:0.5) **v/v/v** in CAMAG Twin-trough chamber without saturation. Densitometric scanning was performed using CAMAG Scanner IV system controlled with vision Cats version 3.0.2 software.

2.5.1. Standard/stock solution

A mixture of Gallic acid and Quercetin was made ready by dissolving it in Methanol to obtained 800 µg/mL and 1000 µg/mL respectively and further aliquots of same were prepared as required in standard.

2.5.2. Sample/test solution preparation

Sample of Triphala in form of dried powder of the extract was dissolved in Methanol to obtain **10,000 µg/mL or (10 mg/mL).** The average powdered tablets of Vyoshadi and Pilonil were dissolved in Methanol, sonicated for 2–3 mins at ambient temperature and centrifuged for 5 mins. The supernatant was taken for final analysis.

2.5.3. Derivatization reagents

Table 1.

Annexure

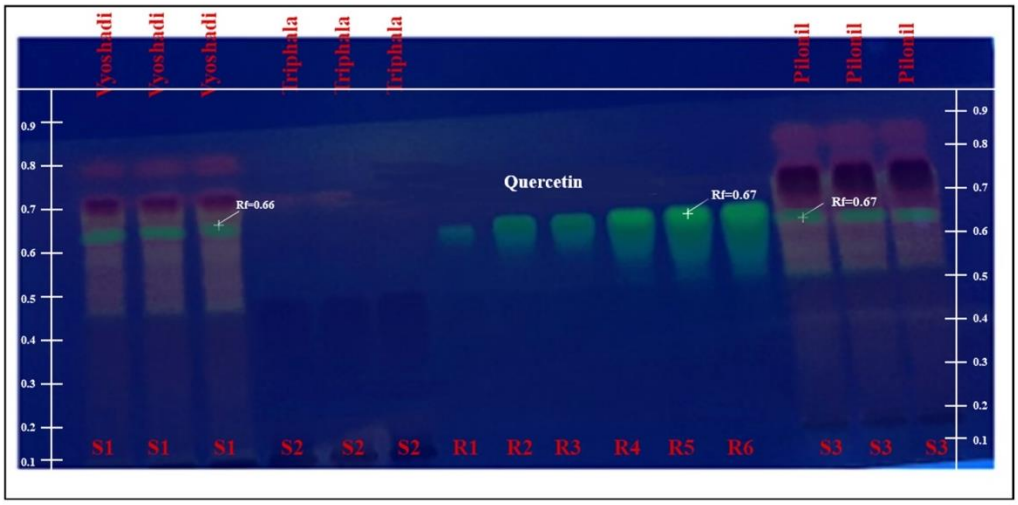

Fig. 3. Plate Image @366 nm.

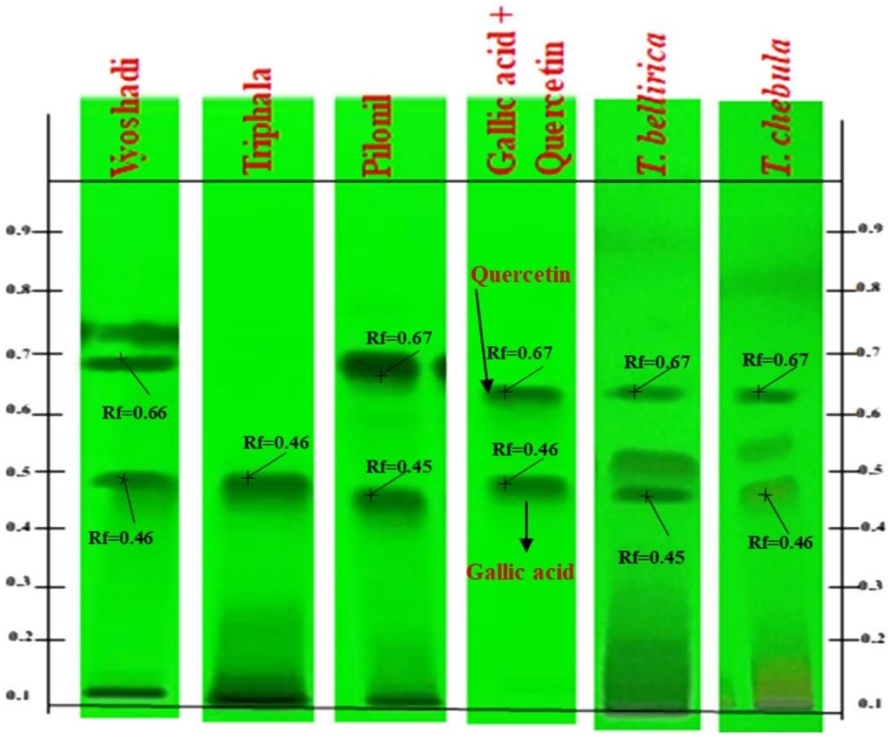

Fig. 4. Plate Image @ 254 nm with 3 Polyherbal formulations, *T. bellirica* & *T. chebula*.

3. Results and discussion

3.1. Optimized mobile phase

The optimized solvent system, as mobile phase was confirmed after trial and error with best Rf toluene**: isopropyl alcohol: glacial acetic acid (7:2.5:0.5) v/v/v** obtained in maximum separation of bands with compact spots for Gallic acid (Rf 0.53) and Quercetin (Rf 0.67) respectively (Figs. 1, 2, 3 and 4).

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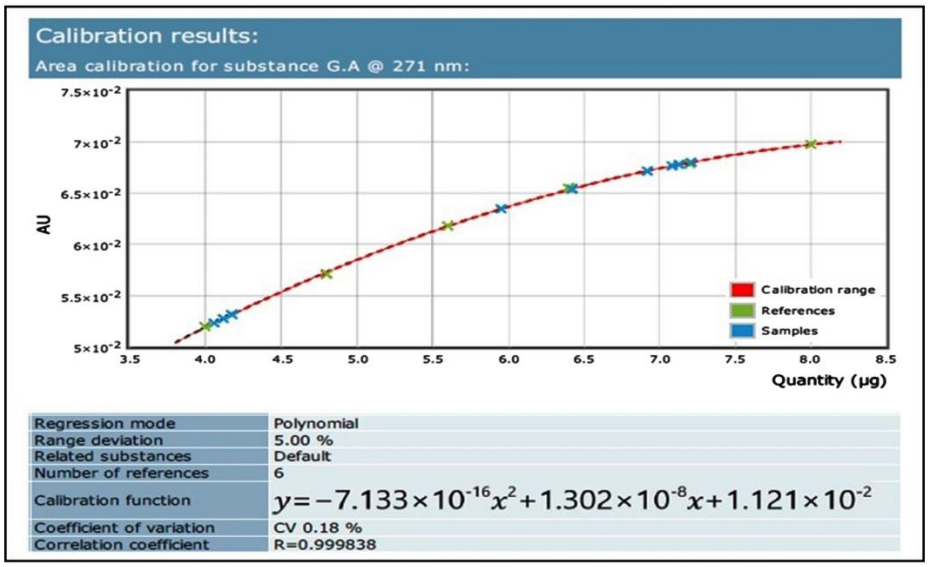

Fig. 5. Linearity, L.O.D and L.O.Q of Gallic acid along with samples.

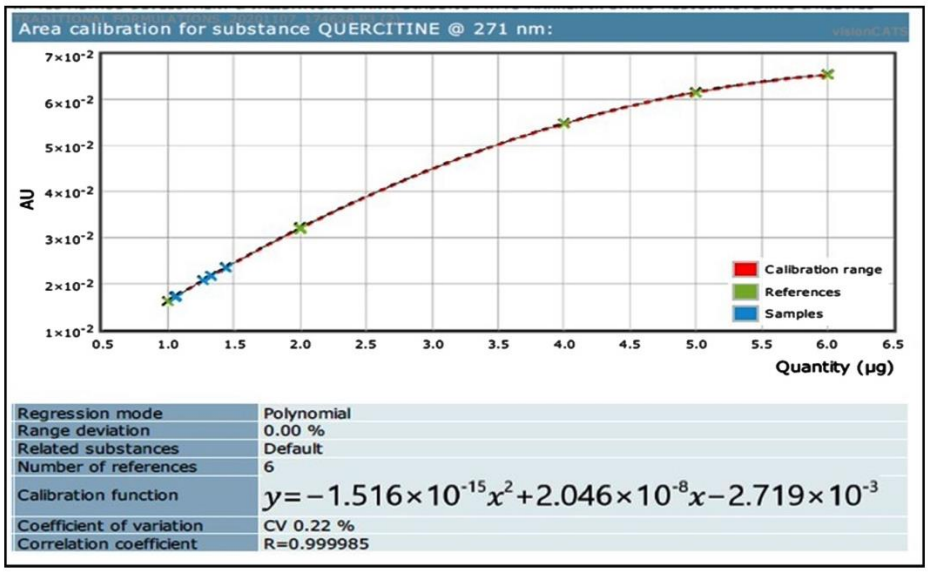

Fig. 6. Linearity, L.O.D and L.O.Q of Quercetin along with samples.

3.2. Wavelength determination

The standard solutions of gallic acid and quercetin were scanned from 190 to 400 nm keeping solvent system as blank. Gallic acid and Quercetin maximum absorbance was confirmed at 271 nm and 366 nm respectively and same was cross checked with C.O.A provided by supplier of biomarker.

3.3. Validation of Gallic acid and Quercetin in traditional formulation

3.3.1. Linearity

Merck TLC plates F254 were activated using methanol and dried with the help of heater III. Gallic acid and Quercetin from the stock solution of 800 µg/mL and 1000 µg/mL respectively, was applied in the range of (5 µg/mL-10 µg/mL) and (1 µg/mL-6 µg/mL). The good regression coefficient was obtained as $r^2 = 0.9998$ (Gallic acid) and $r^2 = 0.9999$ (Quercetin) respectively as shown in the (see Fig. 5).

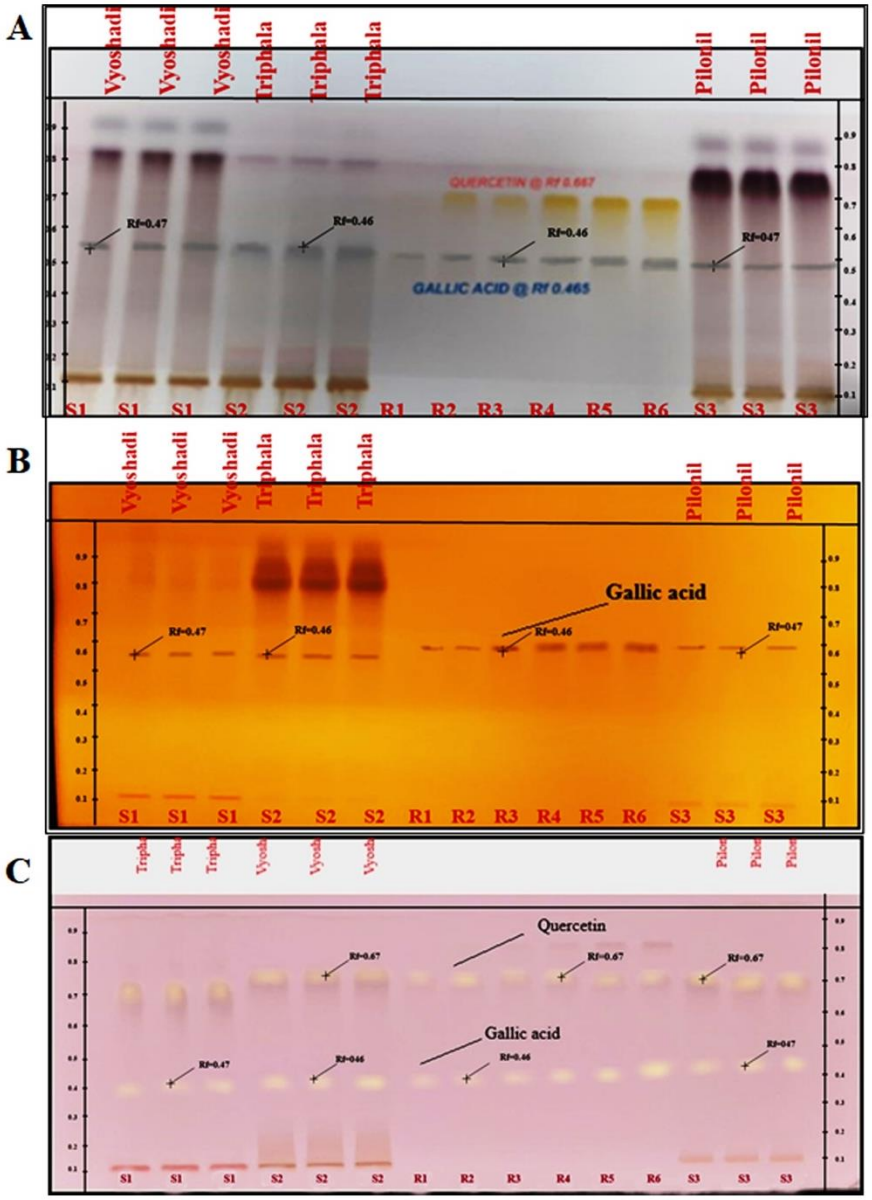

Fig. 7. A) Plate Image @540 nm plate derivatized with Anisaldehyde reagent, **B)** Derivatized with Vanillin reagent (blue color indicates presence of phenolic compound), **C)** Derivatized with DPPH reagent (Yellow color indicates Antioxidant activity).

Quercetin coefficient of variation (CV 0.22%) and correlation coefficient R = 0.999985 (see Fig. 6).

3.3.2. Sensitivity(L.O.D. & L.O.Q)

The sensitivity of Gallic acid and Quercetin was estimated as Limit of detection (L.O.D) and Limit of Quantification (L.O.Q) as per the method developed. The L.O.D and L.O.Q for Gallic acid were 800 ppm and 2400 ppm; while for Quercetin was 5 ppm and 8 ppm respectively.

3.3.3. Specificity

The specificity was analyzed for the ability to differentiate the method based on Rf and changes in the mobile phase with the respective resolutions. The spot of Gallic acid and Quercetin was confirmed with the comparison of Rf and spectra of the standard and sample. Three different levels spectra were calculated to obtained peak purity.

3.3.4. Precision

Precision is expressed with units of standard deviation or relative

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standard deviation. In Intra-day, 4 replicates of standard solution at 6 μg/mL of Gallic acid and 4 μg/mL of Quercetin were carried out twice a day. Inter-day was done by repeating the same application program with same concentration on two different days. The results are calculated as percent relative standard deviation (%RSD).

3.3.5. Reproducibility

The reproducibility was determined by application of 6 levels of standard (Gallic acid & Quercetin) of 5 μl of each and was expressed as % CV. The CV of Gallic acid and Quercetin was found to be (gallic acid in Pilonil is 652.8 μg/mL/ 9.71%, Triphala is 352.2 μg/mL / 1.57%, Vyoshadi is 411.9 μg/mL/ 1.41%) and (quercetin in Pilonil is 134.5 μg/mL / 6.43%, Vyoshadi is 106.0 μg/mL / 0.44%). The (0.44%-2.5%) which is acceptable term for herbal analysis as per USP guidelines.

3.3.6. Derivatization reagents

a) The applied plate was derivatized using Anisaldehyde reagent (ASR) followed by heating at 100–120 °C. The appearance of phenolic compounds was determined by development of red color.
b) 2, 2-Diphenyl-1 picrylhydrazyl reagent (DPPH) in ambered color bottle was used for dipping the applied plate in dark room and the plate was left in dark for around 10–15 mins. The appearance of lemon yellow spots with bluish purple background confirms the presence of antioxidant activity.
c) The plates were dipped in Vanillin reagent and were heated at 100–120 °C. The appearance of blue or red color spots indicates the presence of terpenoids and phenolic compounds (Fig. 7 A), B) & C))

4. Conclusion

The literature review portrays due needs to develop authenticated and validated chromatographic protocol for identification and estimation of phyto-marker which are robust, precise, cost effective and at the same time the method developed needs to be regulatory compliant that to be approved globally. The results obtained from proposed study indicates that HP-TLC method development is in compliance with global guidelines thereby leading to better acceptability of tool as preferred tool for herbal analysis. Also the regression values, the amount of quantification and the parameters of validation demonstrated that HP-TLC method developed is the best alternative to tedious, complex and costly methods like HPLC, LC-MS, GC–MS and must be used as preferred tool for routine Quality control and related analysis.

CRediT authorship contribution statement

Chaitrali M. Bidikar: Conceptualization, Methodology, Visualization, Validation. **Pramod J. Hurkadale:** Investigation, Resources, Supervision, Project administration. **Shrikrishna M. Nandanwadkar:** Supervision, Data curation, Formal analysis, Writing – original draft. **Harsha V. Hegde:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ORIGINAL RESEARCH PAPER

High-performance thin-layer chromatography fingerprint profile analysis and spectro-densitometric evaluation of antiproliferative antioxidants such as ellagic acid and gallic acid from four widely used *Terminalia* species

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Abstract
The emerging need for quality control to assess the chemical composition and stability of herbal raw materials has grown significantly along with their demand. The proposed research focuses on phytochemical analysis of hydro-alcoholic bark and fruit extracts from *Terminalia* (*arjuna*, *bellirica*, *chebula*, and *catappa*) followed by high-performance thin-layer chromatography (HPTLC) technique. HPTLC fingerprinting carried out on precoated silica gel F_{254} TLC revealed the presence of alkaloids, flavonoids, tannins, phenols, and antioxidants. The developed RP-HPTLC method was validated to quantify gallic acid (GA) and ellagic acid (EA). The calibration plot from linear regression analysis demonstrated a good polynomial regression relationship, with R values of 99.99% for the peak areas of GA and EA, respectively. The calibration range for GA and EA is 100–700 ng per band. Spectro-densitometric scanning verified GA and EA by four-way confirmation via R_F, chromatogram, spectra, and visual comparison of chromatograms. The method was developed and validated in accordance with the International Council for Harmonisation guidelines. From the validation results, it can be concluded that this method would prove an excellent alternative to existing costly techniques such as gas chromatography and high-performance liquid chromatography.

Keywords *Terminalia* · Reversed-phase high-performance thin-layer chromatography (RP-HPTLC) · High-performance thin-layer chromatography (HPTLC) · International Council for Harmonisation (ICH) · Fingerprinting · Reproducible

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Abbreviations

HPTLC	High-performance thin-layer chromatography
TLC	Thin-layer chromatography
RP-HPTLC	Reversed-phase high-performance thin-layer chromatography
HPLC	High-performance liquid chromatography
GA	Gallic acid
EA	Ellagic acid
ICH	International Council for Harmonisation
ppm	Parts per million
ppb	Parts per billion
V/V	Volume by volume
AR	Analytical grade
rpm	Rotations per minute
R	Correlation coefficient
r^2	Regression coefficient
ICMR	Indian Council of Medical Research

USP	United States Pharmacopeia
DPPH	2,2-Diphenyl-1-picrylhydrazyl or α,α-diphenyl-β-picrylhydrazyl

1 Introduction

Medicinal plants serve as a rich source of natural resources with abundant therapeutic values. Interest in the utilization of medicinal plants is increasing because of the various implementations documented for them and their byproducts [1, 2]. The rising demand for medicinal plants is due to their ready availability, affordability when raw material is concerned, and being culturally acceptable, holistic, with wider scope of confirmation, and comparatively less expensive [3]. With about 250 species, the genus *Terminalia* ranks second among the Combretaceae family, which is broadly spread in tropical areas of the world exclusively in Southeast Asia, South Africa, and Australia [4, 5].

The popular Ayurvedic drug derived from *Terminalia* species is *Terminalia arjuna* (also known as *arjuna*) W. & A. It is a deciduous tree widely distributed across the country [6]. As a medicinal and biotherapeutic agent, it is utilized in a variety of traditional classical formulations of Ayurvedic medicine including Siddha Ghrita, Kshirpaka, Arista, etc. [7]. It consists of flavonoids, triterpenoids, saponins, tannins, antioxidants, polyphenols, and various phytoconstituents such as ellagic acid, arjunone, arjunic acid, arjunolic acid, gallic acid, arjunolone, oligomeric proanthocyanidins, etc. The stem bark contains the majority of flavonoids and phenols, which are significantly utilized in the treatment of cardiomyopathy, high blood pressure, and hypercholesterolemia, as well as fungicidal and antibacterial, antimicrobial, anti-inflammatory, immunomodulatory, and antinociceptive activity [8, 9]. It is also used in cases of obesity, heart disease, hypertension, hyperglycemia, Kshatashaya (debility), wounds, diabetes, Prameha, and chloasma [7].

In India, *Terminalia bellirica* (Gaertn.) Roxb. is a common plant and exhibits a wide range of pharmacological activities related to its conventional medicines. It contains numerous bioactive secondary metabolites, including alkaloids, flavonoids, lignans, tannins, phenols, coumarin, terpenoids, glycosides, and saponins. *T. bellirica* is a medicinal plant that is used in a variety of herbal formulations and has ethnomedicinal qualities. It has other useful properties such as antipyretic, astringent, laxative, and anthelmintic effects [10, 11]. Fruits can be used as a hair tonic to treat conditions such as asthma, bronchitis, hepatitis, diarrhea, piles, dyspepsia, eye diseases, hoarseness of voice, and scorpion stings. Coughs are treated with the green fruit's decoction, partially ripe fruits have a purgative effect, and the fruit kernel is narcotic. Leprosy, piles, dropsy, and dysenteric diarrhea are all treated with fruit pulp. In Bangladesh and neighboring countries as well as in rural inaccessible parts of India, tribes have been utilizing the fruit of *T. bellirica* for the treatment of menstrual disorders [12–15].

The Indian systems of medicine (Ayurveda, Unani, and homeopathy) have extensively used the valuable medicinal components and elements of *Terminalia chebula* (Haritaki). The fruits of *T. chebula* contain resin, palmitic, stearic, oleic, and linoleic acids, which are anthraquinone derivatives. The active biomarkers are chebulinic acid, chebulagic acid, tannic, ellagic acid, and gallic acid. Among the critical components of Triphala's formulation are *T. chebula* (harda) and *T. bellirica* (baheda) fruits [16–18]. Extensive documented Ayurvedic texts and Ayurvedic pharmacopoeias have reported potent diverse properties, including antipurgatives, antiviral, astringents, antioxidants, antimicrobials, stomach ache relief, tonics, laxatives, and carcinogenic, hypocholesterolemic, radioprotective, and antispasmodic effects [19]. The fruit-bearing Combretaceae family, which includes *Terminalia catappa*, has a wealth of macro- and micronutrients. Documented evidence suggests that root, bark, fruit, and leaf extracts of *T. catappa* have antidiabetic, antimicrobial, antibacterial, anti-inflammatory, antioxidant, and anticancer properties when used with petroleum jelly [20]. It is also commonly known as "tropical almonds" or "Indian almonds" derived from the ripe fruits of *T. catappa*, which can be either raw or roasted, and is also a rich source of fatty acids with high nutritional characteristics and anticardiovascular potential. The phenolic extracts of *T. catappa* contain gallic acid, catechin, chlorogenic acid, kaempferol, ellagic acid, rutin, quercetin, quercitrin, iso-quercitrin, epicatechin, and caffeic acid [21].

As per World Health Organization (WHO) database survey, 80% of the country's population are dependent on herbal medicine for disease treatment [22]. Herbal medicines are economically important because the market has expanded at an impressive rate due to a global resurgence in conventional and alternative healthcare systems. The machinery needed to control manufacturing procedures and quality standards is required to be utilized to the fullest when it comes to quality control and quality assurance aspects of herbal and related phytomedicines [23, 24]. The process of standardization involves assessing the quality, complex nature of the raw materials, chemical composition, compatibility, and solubility in various solvents. Gas chromatography and liquid chromatography are the preferred techniques for the detection and separation of the complex compounds. However, they are time consuming, and sample pretreatment procedures are cumbersome as well as costly [25, 26]. Criteria for quality control of herbal and medicinal plants include physicochemical analysis. Color, odor, appearance, clarity, viscosity, moisture content, pH, disintegration time, friability, hardness, flowability, flocculation, sedimentation, settling rate, and ash values are examples of physical

parameters. Limit tests, chemical tests, chemical assays, and other chemical parameters are examples of chemical parameters. In such cases, standardization and evaluation guarantee the content of one or more active constituents and marker compounds [27, 28].

Chromatography is an effective analytical technique that can separate and quantify many substances. The detection, identification, and estimation of chemical markers within plant extracts are made possible by analytical technologies, including thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC). As per United States Pharmacopeia (USP) chapters, these are standard official methods for analysis. HPTLC is the simplest and most highly reproducible, precise, and low-cost high-throughput tool, in comparison with other chromatography tools such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) [27–30]. TLC and HPTLC both have a wide range of applications in the biotechnology, chemical, phytochemistry, food, and cosmetics industries, among many other sectors [31–34]. HPTLC is a type of planar chromatography in which the sample components are separated on specialized layers of silica gel bonded with fluorescent binders. HPTLC fingerprinting also plays an impactfully pivotal role in quality-control profiles and for documentation/authentication/identification for the class of compounds (alkaloids, tannins, phenols, antioxidants, etc.) present in the plants [35, 36]. Further detection via spectrodensitometric scanning increases the separation's effectiveness, enabling quick, robust, and economic [37] throughput, establishing HPTLC as an apt tool for qualitative, quantitative, and preparative research [38, 39]. In this investigation, we designed a validated reversed-phase high-performance thin-layer chromatography (RP-HPTLC) method for the analysis of gallic acid (GA) and ellagic acid (EA) biomarkers from four *Terminalia* species, utilizing RP18 silica gel plates as per the International Council for Harmonisation (ICH) guidelines.

2 Experimental

2.1 Materials

2.1.1 Chemicals and reagents

All the reagents, solvents, and chemicals were of analytical reagent (AR) grade. EA and GA are among the common biomarkers procured from Sigma-Aldrich (Mumbai, India). Water, methanol, formic acid, acetic acid, ethyl acetate, toluene, cyclohexane, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and ferric chloride were purchased from Merck (Darmstadt, Germany).

2.1.2 Sample collection

The fruits of *T. bellirica* (*Tb*), *T. chebula* (*Tc*), and *T. catappa* (*Tct*) and the bark of *T. arjuna* (*Ta*) were purchased from the local market of Belagavi (Karnataka, India). Authentication of the collected plant material was verified by a taxonomist from ICMR-National Institute of Traditional Medicine, Belagavi.

2.2 Methodology

2.2.1 Standard solutions

Standard solutions of 50 µg/mL GA and 100 µg/mL EA were prepared in methanol at various concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 µg/mL, subjected to ultrasonication for 5 min, and filtered via polytetrafluoroethylene (PTFE) nylon filter 0.45 µm.

2.2.2 Sample extraction

T. arjuna dried stem and the fruits of *T. bellirica*, *T. chebula*, and *T. catappa* were subjected to grinding, and the coarse fine powder was passed through mesh size #40 and extracted with a hydro-alcoholic solution, via soxhlation process at 80 °C [22, 38]. The solvent was evaporated using rotary evaporator that is composed of a vacuum system, a rotating evaporation flask, and a condenser with a collecting flask. Further, the extract was stored in a refrigerator at 4 °C [40].

2.2.3 Sample preparation for RP-HPTLC analysis and HPTLC fingerprinting

(a) *RP-HPTLC analysis*: The hydro-alcoholic extracts of *T. arjuna* and *T. catappa* (100 mg), *T. bellirica* (1 mg), and *T. chebula* (10 mg) were weighed and dissolved in 10 mL volumetric flask with methanol. The applied volumes on the plate were 10 ng/µL (*Ta* and *Tct*), 100 ng/µL (*Tb*), and 1000 ng/µL (*Tc*).
(b) *HPTLC fingerprinting*: The *Terminalia* species (*arjuna*, *bellirica*, *chebula*, and *catappa*) hydro-alcoholic extracts were dissolved in ethanol at a concentration of 100 mg/10 mL, and the applied volumes on the plate for all samples were 10 µg/µL.

In both instances (a and b), the final sample was subjected to centrifugation (5000 rpm, 10 min), ultrasonic processing (15 min), and vortex for 30 s. Each supernatant was transferred to a 2 mL vial, and the required quantity was taken

for the investigation. All extracts and samples were stored in a refrigerator [41].

2.2.4 RP-HPTLC method

Chromatographic analysis was performed on RP-HPTLC plate silica gel 60 RP18 WF_{254}s. A band length of 8 mm was applied on the plate with the help of a CAMAG Linomat 5 sample applicator (Muttenz, Switzerland). Development was performed (20 × 10 cm CAMAG twin-trough chamber) with methanol−water−formic acid−acetic acid (80:100:4:4, *V/V*) up to a developing distance of 80 mm, measured from the lower plate edge; both separations took about 25 min. Photo documentation was done using the CAMAG TLC Visualizer at 254 nm (UV), 366 nm (fluorescence mode), and with white light illumination (Vis). Further the plate was subjected to densitometric scanning at UV 254 nm and 275 nm (absorbance measurement of other compounds; deuterium lamp) with CAMAG Scanner 4.

2.2.5 HPTLC fingerprinting

HPTLC fingerprint was performed on Merck Silica gel $60F_{254}$ TLC precoated plates, and sample application and scanning were done with the above-mentioned CAMAG HPTLC instruments. Alcohol rejuvenated ferric chloride ($FeCl_3$) reagent was used for derivatization; then the samples were heated at 110 °C for 10 min, and densitometric scanning was done at UV 254 nm and 366 nm with CAMAG Scanner 4. The other duplicate developed fingerprint plate was then photodocumented, and the plate was dried by heating at 100 °C by CAMAG TLC Plate Heater 3, and sprayed with different derivatization reagents. The plates were then treated with 1% methanolic natural product reagent and documented in fluorescence mode. After derivatization with the natural product reagent, it was scanned in fluorescence mode at 366/> 400 nm. One more similar plate was developed and derivatized using DPPH reagent in the dark room. The derivatized plate was dried using CAMAG TLC Plate Heater 3 and photodocumented, and densitometric scanning was carried out at 540 nm.

2.3 Validation parameters of HPTLC as per ICH guidelines

2.3.1 Calibration

The method was validated as per ICH guidelines. The calibration curve was obtained in the range of 100–700 ng/mL for both GA and EA. For the calculation of correlation coefficient, regression equation, and intercepts of the curves and slopes, the standard curve was used.

2.3.2 Sensitivity

Limit of detection (LOD) taking into consideration the analyte concentration (*S*/*N* value 3) and limit of quantification (LOQ) taking into consideration the analyte concentration (*S*/*N* value 10) were evaluated.

2.3.3 Specificity

In chromatography, specificity refers to accurately measuring a specific analyte when other components are present (or are expected to be present) in a given sample. The specificity of the method was determined by comparing the standard with sample drugs. The bands for GA and EA in *Terminalia* species were confirmed by comparing the R_F and overlaying peak purity spectra with those of the standard.

2.3.4 Precision

A study on inter-day, intra-day, and repeatable precision of the method was evaluated to determine the precision. The developed method's interpretation precision and repeatability were expressed in terms of percent relative standard deviation (%RSD) of peak area. For three consecutive days, the inter-day study was performed. %RSD was used for calculating the findings of repeatability as well as intra-day and inter-day precisions [42].

2.3.5 Reproducibility

Reproducibility is expressed as the precision between the results obtained. The method was performed by evaluating six aliquots of standard solution containing 600 ng per spot of GA and 300 ng per spot of EA, which were applied on TLC plates, and the developed plates were measured to obtain the final results. Reproducibility was expressed as coefficient of variation (CV%) of the measured concentrations of each calibration level.

2.3.6 Accuracy/recovery

Recovery studies were performed by spiking known amounts of biomarkers corresponding to 80%, 100%, and 120% of GA and EA on the hydro-alcoholic extracts. Each level was analyzed in triplicate. The recovery of GA and EA at different levels in hydro-alcoholic extracts of *Terminalia* species was calculated.

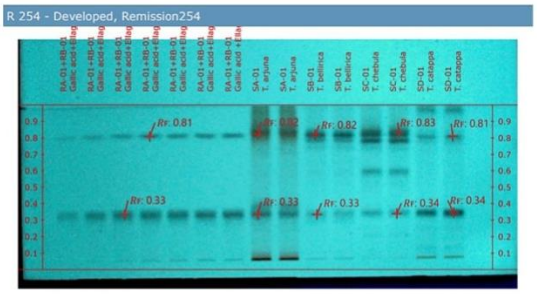

Fig. 1 RP-HPTLC plate image at $\lambda = 254$ nm

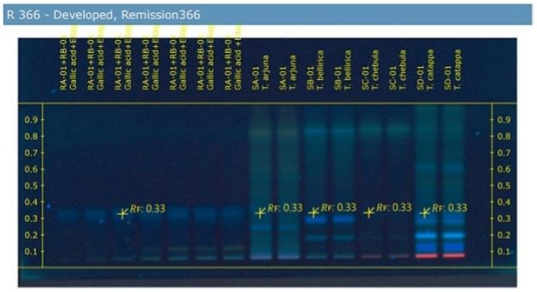

Fig. 2 RP-HPTLC plate image at $\lambda = 366$ nm

3 Results and discussion

3.1 Optimized mobile phase

The maximum band separation was obtained with optimized solvent system of methanol−water−formic acid−acetic acid (80:100:4:4, *V/V*) with the best R_F and compact spots (R_F 0.82) GA and (R_F 0.33) EA, respectively (Figs. 1, 2, 3).

3.2 Wavelength determination

During wavelength determination, mobile phase was taken as blank for background correction and further scanning was performed for analytes of interest from 0 to 8 mm, respectively, of visual bands. The standard solutions of GA and EA were scanned from 190 to 400 nm [43]. The maximum absorbances of GA and EA were verified at 279 nm and 254 nm, respectively, and the results were cross-checked with coenzyme A supplied by Sigma-Aldrich.

3.3 Validation of GA and EA

3.3.1 Calibration

GA and EA were administered at concentrations between 100 ng/mL and 700 ng/mL, respectively, from stock solutions of 50 µg/mL and 100 µg/mL. As demonstrated in Figs. 4 and 5, good polynomial regression values were obtained for GA ($r^2 = 0.9999$) and EA ($r^2 = 0.9999$). Average RSD was found to be 1.17%, and the correlation coefficients were $R = 0.999556$ (GA) and $R = 0.999317$ (EA), respectively [44]. The quantification of GA in *T. arjuna* was 32.05 µg/mL, in *T. bellirica* 46.11 µg/mL, in *T. chebula* 36.72 µg/mL, and in *T. catappa* 32.12 µg/mL and that of EA in *T. arjuna* was 24.15 µg/mL, in *T. bellirica* 15.74 µg/mL, in *T. chebula* 22.10 µg/mL, and in *T. catappa* 106.2 µg/mL.

3.3.2 Sensitivity (LOD and LOQ)

According to the method created, the sensitivity of GA and EA was estimated for their LOD and the LOQ values. The LOD and LOQ values for GA were found to be 75 ng and 225 ng, whereas for EA, LOD and LOQ values were calculated as 80 ng and 240 ng, respectively [42].

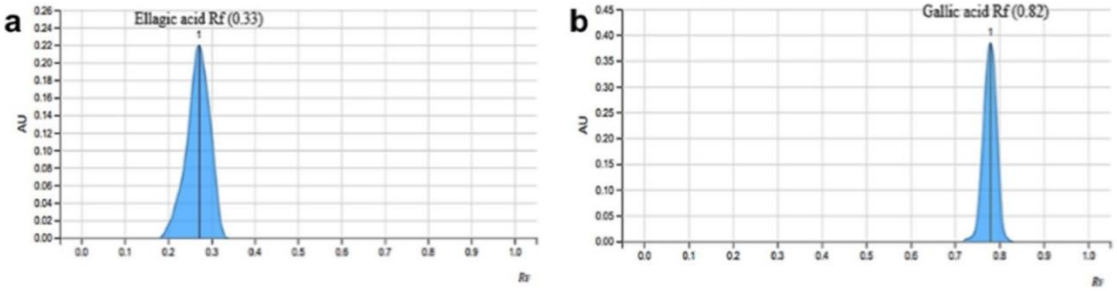

Fig. 3 Chromatogram of **a** EA at R_F 0.33 and **b** GA at R_F 0.82

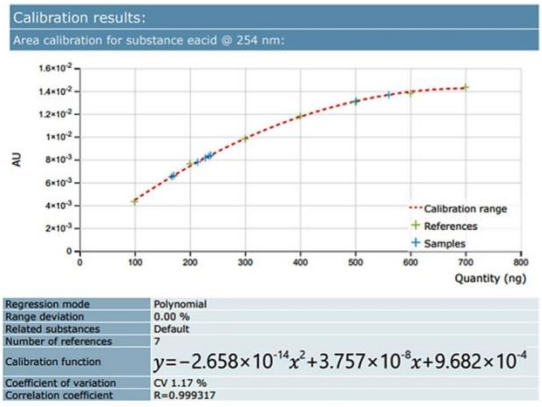

Fig. 4 Polynomial regression, LOD, and LOQ of ellagic acid along with samples

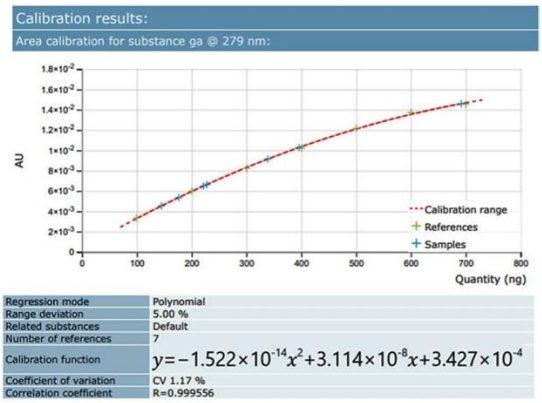

Fig. 5 Polynomial regression, LOD, and LOQ of gallic acid along with samples

3.3.3 Specificity

The specificity of the method was determined by a comparison of the R_F and spectra of the standard and sample established to determine the purity of the peak. The peak purity of both standards and samples was confirmed by comparing the overlaid spectra at the peak start, peak apex, and peak end positions of the band. The R_F value of GA was 0.82, and that of EA was 0.33.

3.3.4 Precision

Standard deviation or relative standard deviation units are used to express repeatability/precision. Four replicates of the standard solution of 600 ng/mL of GA and 200 ng/mL of EA were performed twice for intra-day study. Inter-day study was performed by repeating the same application program with the focus on two different days. The %RSD for (intra-day precision) repeatability was found to be 0.56%, and (inter-day precision) intermediate was found to be 0.78%. The %RSD for (intra-day and inter-day) precisions was less than 2%, indicating the precision of the method [45].

3.3.5 Reproducibility

The reproducibility was assessed by applying six levels of standard GA and EA. GA 6 µL (600 ng) and EA 3 µL (300 ng) were selected as optimized reproducibility levels. The final quantified results for GA and EA were found to be 1.99% CV and 1.21% CV, respectively.

3.3.6 Recovery

Accurately weighed quantities of GA and EA were transferred to individual volumetric flasks and made up to a volume of 25 mL to achieve three different levels (80%, 100%, and 120%) of recovery. The standard and sample were spiked together in triplicate at levels of 80%, 100%, and 120%. The obtained areas of sample and standard were further compared separately at 80%, 100%, and 120%. The results of recovery studies are expressed in terms of % recovery and were within acceptable limits (Table 1).

3.3.7 HPTLC fingerprinting analysis

The utilization of HPTLC fingerprinting analysis for the detection of different classes of compounds was performed using various derivatizing agents on TLC F_{254} plate. The free radical scavenging activity was evaluated by HPTLC–DPPH assay for the detection of antioxidants present in *Terminalia* species. In the present study, a stable and simple RP-HPTLC densitometric fingerprinting method was developed. A combination of solvent systems was used for obtaining good resolution and reproducible results. Nevertheless, the optimized solvent system and resolution observed for flavonoids was obtained by ethyl acetate–water–formic acid–acetic acid (100:26:11:11, *V/V*), for tannins toluene–ethyl acetate–formic acid (6:4:0.3, *V/V*), for phenols cyclohexane–ethyl acetate–formic acid (4:6:1, *V/V*), and for DPPH *n*-butanol–glacial acetic acid–water (4:4:1, *V/V*).

The fingerprinting exhibited five dark-blue bands (*Ta*), six dark-blue bands (*Tb*), six dark-blue bands (*Tc*), and eight dark-blue bands (*Tct*) after derivatization with alcoholic ferric chloride reagent corresponding to phenolic compounds. There were two blue and two green bands (*Ta*); one dark-green, two dark-blue, one green, and two blue bands (*Tb*); six dark-blue and two blue bands (*Tc*); six green, one red, and two blue bands (*Tct*) after derivatization with natural product reagent corresponding to flavonoid compounds.

Annexure

Table 1 Percentage recovery ($n = 3$) of GA and EA

	Percentage recovery levels (%)	Concentration of standard spiked on sample (ng per band) ($n = 3$)	Theoretical concentrations (ng)	Observed concentration spot (ng)	Recovery (%)	Average percentage recovery (%)
Gallic acid in *T. arjuna*	80	240	0.00664	0.00668	98.52	97.22
	100	300	0.00678	0.00690	97.76	
	120	360	0.00712	0.00735	98.40	
Gallic acid in *T. bellirica*	80	240	0.01460	0.01473	96.40	98
	100	300	0.01528	0.01566	98.48	
	120	360	0.01673	0.01698	97.12	
Gallic acid in *T. chebula*	80	240	0.00915	0.00920	89.58	97.96
	100	300	0.01027	0.01075	94.67	
	120	360	0.01112	0.01126	99.63	
Gallic acid in *T. catappa*	80	240	0.00453	0.00458	85.44	95.64
	100	300	0.00536	0.00582	97.58	
	120	360	0.00618	0.00632	98.91	
Ellagic acid in *T. arjuna*	80	240	0.00661	0.006620	96.64	95.46
	100	300	0.00685	0.007495	94.41	
	120	360	0.00707	0.008380	98.33	
Ellagic acid in *T. bellirica*	80	240	0.00824	0.00830	96.28	97.16
	100	300	0.00862	0.00912	95.80	
	120	360	0.00894	0.00995	97.42	
Ellagic acid in *T. chebula*	80	240	0.00779	0.00782	95.95	96.37
	100	300	0.00815	0.00845	98.68	
	120	360	0.00895	0.00925	97.49	
Ellagic acid in *T. catappa*	80	240	0.01256	0.01275	97.17	98.36
	100	300	0.01312	0.01345	96.51	
	120	360	0.01368	0.01383	98.41	

There were three lemon-yellow bands (*Ta*); three lemon-yellow bands (*Tb*); three lemon-yellow bands (*Tc*); and two lemon-yellow bands (*Tct*) after derivatization with DPPH reagent corresponding to antioxidants. There were one dark-blue band (*Ta*); two dark-blue bands (*Tb*); two dark-blue bands (*Tc*); and one dark-blue band (*Tct*) after derivatization with iron chloride ($FeCl_3$) reagent corresponding to tannin compounds. For spectro-densitometric evaluation, the developed plate was then derivatized using the reagents listed in Table 2, and the final plate image was documented using photodocumentation, which is represented in Fig. 6, respectively [46].

Table 2 Detection of different classes of compounds using different derivatizing reagents for HPTLC fingerprinting analysis

Derivatizing reagents	Procedure of derivatizing reagents	Observations
Natural product reagent (Fig. 6b)	Dissolve 1 g of (2-aminoethyl diphenyl borinate) dissolved in 200 mL of ethyl acetate. Preheat the plate before derivatization, and derivatize it with natural product reagents	Fluorescent compounds; red fluorescence confirmed flavonoids, plant pigments, and chlorophyll pigments [47]
Iron chloride ($FeCl_3$) (Fig. 6a and c)	2 g $FeCl_3$ is dissolved in 10 mL of water and diluted up to 200 mL with ethanol	Dark-blue bands confirmed the presence of tannins and phenols [47]
DPPH-antioxidant activity (Fig. 6d)	The applied plate was dipped in 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent prepared in an amber bottle and kept in the dark for 10−15 min	Lemon-yellow dots on a bluish-purple background confirmed the presence of antioxidants [48–50]

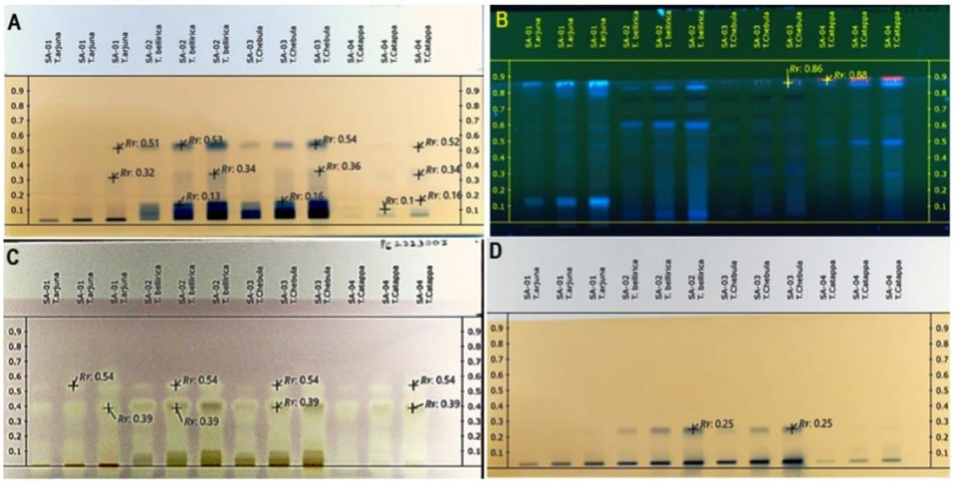

Fig. 6 Fingerprinting analysis. **A** Phenols by $FeCl_3$ reagent; **B** flavonoids by natural product reagent; **C** antioxidants by DPPH reagent; **D** tannins by $FeCl_3$ reagent

4 Conclusion

The ideal instruments for routine quality control and related analysis are exhaustive, requiring higher pretreatment times and sophisticated protocols such as HPLC, liquid chromatography−mass spectrometry (LC−MS), and GC−MS requiring complex and tedious sample pre- as well as post-treatment procedures. A quick and simple, cost-effective method is required to verify the reliability and quality of these raw materials. The proposed study's findings firmly demonstrate that the HPTLC technology complies with international guidelines, improving wider acceptability across the globe, making it the tool of choice for herbal analysis. Additionally, the regression results and the criteria of validation quantification proved that the developed HPTLC method is the best robust and cost-effective replacement over existing time-consuming and costly methods. This work portrays the screening and identification of essential phytotherapeutic markers such as GA and EA effectively in selected *T. arjuna*, *T. bellirica*, *T. chebula*, and *T. catappa* in single application with higher accuracy and precision by HPTLC technique. Ultramodern HPTLC has relatively simple visual evaluation and flexible use, reproducible analysis, and reliable quantification, along with multiple detection of separated analytes. It also enhances sensitivity by analyzing multiple samples in parallel without cross-contamination, which is a costly affair when compared with other chromatographic techniques. The detection of HPTLC fingerprinting for classes of compounds was done using natural product reagent ferric chloride, and the radical HPTLC−DPPH assay was carried out for antioxidants present in *Terminalia*. The research conducted showcased that the results validated are suitable for the analytical use. The parameters of the method proposed were well within accordance with guidelines of ICH and USP protocols. The developed protocol also complies with regulatory requirements of 21CFR (Code of Federal Regulations). This indicates that the present method may be used as the best alternative for analysis of herbals and routine quality control as well as, but not limited to, marketed polyherbal formulation analysis when compared with costlier chromatographic and spectroscopic tools employed in plant/phytochemical research. This would eventually facilitate wider and global acceptability of HPTLC as an official modern chromatographic tool.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval Not applicable.

Research involving human and animal participants Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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IV: International and National Conferences

8th International Congress of
Society for Ethnopharmacology, India
(Globalizing local knowledge and localizing global technologies)

SFEC 2021
27th to 29th, AUGUST 2021

THEME : Ethnopharmacology & Medicinal Plants - Approach towards product development

ORGANISED BY
SFE—INDIA PUNE CHAPTER
&
BHARATI VIDYAPEETH (DEEMED TO BE UNIVERSITY)
POONA COLLEGE OF PHARMACY & ALL PHARMACY COLLEGES OF PUNE REGION

This certificate is awarded to

Professor/Dr. /Ms. /Mrs. /Mr. Chaitrali M. Bidikar

from.....

for presenting Poster/ Oral Presentation on the topic .....

.....

at International Congress of Society for Ethnopharmacology, India (SFEC 2021).

Dr. Sathiyanarayanan L., Organizing Secretary, SFE India
Dr. K. R. Mahadik, LOC Chairman, SFE India
Mr. Birendra K. Sarkar, SFE President

Graduate School of Pharmacy
Gujarat Technological University
&
Society of Pharmacognosy
Formerly Indian Society of Pharmacognosy

CERTIFICATE NO.: GSPICON21PP022

CERTIFICATE OF PARTICIPATION

THIS CERTIFICATE IS BEING AWARDED TO

DR./MR./MS. Ms CHAITRALI MILIND BIDIKAR

HAS SUCCESSFULLY PRESENTED A POSTER / ORAL ENTITLED

ASYSTEMATIC NETWORK PHARMACOLOGY APPROACH OF TERMINALIA CHEBULA RETZ. FOR TYPE II DIABETES MELLITUS, PCOD AND METABOLIC SYNDROME

DURING
25TH NATIONAL CONVENTION OF SOCIETY OF PHARMACOGNOSY & INTERNATIONAL CONFERENCE ON
"NEW HORIZONS OF NATURAL PRODUCTS AND AYUSH REMEDIES" DURING
NOVEMBER 27-28, 2021.

MS. JIGNA VADALIA
Organizing Secretary,
Loc & Asst. Prof., GSP- GTU

DR. SANJAY CHAUHAN
Chairman, Loc & Director
GSP-GTU

DR. UMESH PATIL
General Secretory
Society Of Pharmacognosy

PROF. (DR.) NAVIN SHETH
Vice Chancellor Gtu &
President Society Of
Pharmacognosy

Indira Bahuuddeshiya Shikshan Sanstha, Buldana's

Dr. Rajendra Gode Institute of Pharmacy,

University-Mardi Road, Amravati (444602)

Certificate

This is to be certified that Dr./Prof./Mr./Ms/Mrs Chaitrali Bidikar KLE College of Pharmacy, Belagavi, Karnataka Presented a poster on topic "Identification of anti-Bacterial activity of *Terminalia bellirica* and *Terminalia chebula* by high performance thin layer chromatography coupled with direct bioautography technique" in Two Days (Virtual) International Conference on, RECENT ADVANCES IN DRUG DISCOVERY (QSAR Modeling, Molecular Docking, Molecular Simulation) held at Dr. Rajendra Gode Institute of Pharmacy, Amravati on 15th and 16th Dec. 2021.

Prof. Rahul D. Jawarkar
ORGANIZING SECRETARY

Dr. Snehal S. Manekar
CO-CONVENOR

Dr. Ravindra L. Bakal
CONVENOR

Annexure

(Oral Presentation and Young Scientist Certificate at International Conference)

9th International Congress of
SOCIETY FOR ETHNOPHARMACOLOGY, INDIA
(Globalizing Local Knowledge and Localizing Global Technologies)
Theme : Redefining Ethnopharmacology for the Global Health and Wellbeing

SFEC 2022
22nd to 24th April 2022

Certificate of Participation

This is to certify that Dr./Mr./Ms. Chaitrali M. Bidikar
has Presented a PAPER Titled Fast and Rapid Analysis of anti-oxidant biomarkers in selected Terminalia species by HP-TLC and GC-MS technique
in SFE Young Ethnopharmacologist Award Competition - 2022 "Prof. P. K. Debnath Memorial Award" at 9th International Congress of the Society for Ethnopharmacology, India (SFEC-2022) held at JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India from 22nd to 24th, April 2022.

Annexure

Poster Presentation Certificate of National Conference

International Bioresource Conclave & Ethnopharmacology Congress

22nd International Congress
International Society for Ethnopharmacology (ISE)

&

10th International Congress
Society for Ethnopharmacology (SFE)

Theme: "Reimagine Ethnopharmacology: Globalization of Traditional medicine"

Jointly organized by

Institute of Bioresources and Sustainable Development (IBSD)
Takyelpat, Imphal, Manipur, India

Society for Ethnopharmacology (SFE)
23/3 Shaktigarh, Jadavpur , Kolkata, India

February 24-26, 2023

Venue: City Convention Centre, Imphal 795005, Manipur, India

Certificate for Presentation

This to certify that

Mr./Ms./Prof./Dr. ……Chaitrali M. Bidikar…… participated & presented a paper in the E-presentation session in online mode during the International Bioresource Conclave & Ethnopharmacology Congress (ISESFEC-2023) at City Convention Centre, Imphal 795005, Manipur, India during February 24-26, 2023.

Dr. Subhash C Mandal
Secretary, SFE

Prof. Marco Leonti
Secretary, ISE

Dr. Nanaocha Sharma
Organizing Secretary, ISESFEC 2023

8th Convention

Society for Ethnopharmacology, India – 2021

23/3 Shaktigarh, Kolkata, India

National Seminar on

"Ethnopharmacology for wellness: Tradition to Translation"

Organized by

CSIR-Indian Institute of Chemical Biology

Kolkata, India

December 10, 2021

Certificate

This to certify that

Mr./Ms./Prof./Dr. Chaitrali Milind Bidikar participated & presented a paper in the Poster session in the 8th Convention of SFE-India and National Seminar held at CSIR-IICB, Salt Lake Campus, Kolkata 700 091, India on December 10, 2021.

Mr. Birendra Kumar Sarkar
President
Society for Ethnopharmacology, India

Dr. Arun Bandyopadhyay
Chairman
8th Convention, SFE-India

Dr. Subhash C Mandal
Executive Secretary
Society for Ethnopharmacology, India