Quality Assesment Profiles of Selected Marketed Classical Ayurvedic Preparations by Chromatographic Techniques

Thesis submitted to

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH

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(Accredited 'A^{+}' Grade by NAAC) (3rd cycle)

[Placed in Category 'A' by MoE (GoI)]

For the award of the degree of

Doctor of Philosophy

In the Faculty of Pharmacy

By

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April 2024

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DECLARATION

I hereby declare that the thesis entitled "Quality Assessment Profiles of Selected Marketed Classical Ayurvedic Preparations by Chromatographic Techniques" is a bonafide and original research carried out by me under the guidance of Dr. Pramod J. Hurkadale, Professor Department of Pharmacognosy, KLE College of Pharmacy Belagavi, KAHER, Belagavi-590010 and co-guidance of Dr. Harsha V. Hegde, Scientist C, ICMR-National Institute of Traditional Medicine, Belagavi-590010. The thesis or any part of 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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Lastly I would like to appreciate the support of My Parents, Family and Friends who have been the part of my journey and have supported me.

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LIST OF ABBREVATIONS

^{0}C – Degree Celsius

A- Alkaloids

API -Ayurvedic Pharmacopoeia of India

AQbD- Analytical Quality by Design

C- Carbohydrates

DoE - Design of Experiments

DPPH- 2,2-diphenyl-1-picrylhydrazyl

F- Flavonoids

G- Glycoside

g- Grams

HPTLC- High Performance Thin Layer Chromatography

ICH – International Conference on Harmonization

LOD- Limit of detection

LOQ- Limit of Quantification

M -Mass of the powder in gram

MF - Mass of powder in measuring vessel in gram

ml- Milliliter

NCDs -Non-communicable diseases

nm- Nanometer

NMT- Not more than

P-Protein

RSD- Relative standard deviation

S1- In house Sitopaladi Churna

S2- Sitopaladi Churna Marketed sample A

S3- Sitopaladi Churna Marketed sample B

Sap- Saponin

Ste- Steroids

T1- In house Talisadi Churna

T2- Talisadi Churna Marketed sample A

T3- Talisadi Churna Marketed sample B

TCM - Traditional Chinese medicine

Ter- Terpenoids

T- Tannins

USP- United States Pharmacopeia

UV- Ultraviolet

V_0 - Unsettled apparent volume in mL

V_F - Final tapped volume in mL

WHO- World Health Organization

µ- Micro

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ABSTRACT

This study presents a comprehensive investigation into churna formulations, employing a rigorous and systematic approach to evaluate their quality and composition. Through multifaceted analyses, including determination of foreign matter, powder microscopy, physicochemical characterization, microbial studies, physical powder characterization, organoleptic assessment, macroscopic studies, fluorescence studies, qualitative and quantitative phytochemical investigations and HPTLC analysis, a nuanced understanding of the formulations is achieved. Results indicate consistently low levels of foreign matter, highlighting meticulous quality control. Microscopic examination unveils distinct characteristics aiding in identification. Physicochemical parameters offer insights into solubility and acidity/alkalinity. Microbial studies ensure compliance with safety standards. Physical powder characterization reveals favorable flow properties. Organoleptic characterization indicates standardized sensory profiles. Macroscopic studies facilitate raw material identification. Fluorescence studies depict material behavior under different conditions. Qualitative phytochemical investigations enhance understanding of chemical composition, while quantitative assays provide analytical insight into key parameters. HPTLC analyses showcase method reliability, sensitivity, and eco-friendliness, with quantification of Piperine contributing to formulation characterization. The eco-friendliness evaluation emphasizes sustainable analytical practices. In conclusion, this study employs a holistic methodology to comprehensively characterize churna formulations, offering valuable insights into their quality, composition, and potential therapeutic efficacy, thus contributing to the advancement of herbal medicine.

Keywords: Churna; Pharmacopeial evaluations; HP-TLC; Piperine; ICH guidelines

1. INTRODUCTION:

1.1 Background:

Around four-fifth of the global population is estimated to engage with traditional medicine. Among the 194 Member States of the World Health Organization (WHO), 170 have officially acknowledged the utilization of traditional medicine. These nations have actively sought WHO's support in systematically compiling substantial evidence and data regarding the practices and products associated with traditional medicine. The term "traditional medicine" encompasses the accumulated knowledge, skills, and methodologies employed by various indigenous and cultural communities throughout history to safeguard health. It addresses physical and mental ailments through preventative, diagnostic, and therapeutic measures. The scope of traditional medicine extends to encompass both contemporary pharmaceuticals and time-honored practices, such as acupuncture, Ayurvedic medicine and herbal remedies. [1]

Traditional medicine and medicinal herbs are often utilized as substitutes for routine healthcare and for self-administration in cases of minor and chronic illnesses. This practice is especially favored in economically disadvantaged rural regions and during economic downturns. The incorporation of a holistic approach to human health is a common characteristic found in the majority of "traditional" or regional medical systems. [2] Historically, the focus of medical practice has predominantly centered on the treatment of individual patients, with comparatively less emphasis on preventative measures and the socioeconomic and behavioral determinants influencing overall health and well-being. While this model has proven effective in delivering exemplary patient care over the years, contemporary health challenges, such as non-communicable diseases (NCDs), the global aging population, and intricate structural

barriers to health equity, pose formidable obstacles. Addressing these complex issues necessitates a departure from the conventional individual patient-centric approach. The prevailing bench-to-bedside academic medicine model, foundational to contemporary medical education, research, and practice, is deeply rooted in traditional medicine. [3]

The history of Traditional Chinese Medicine (TCM) spans more than three thousand years and is based on ancient Chinese philosophy. It has developed over time and continues to serve a large population of people in various regions by offering patients dependable, efficient, and cheap health care options. It is an essential component of Chinese cultural history. TCM had a significant impact on Hanja and Kampo medicine in Korea and Japan in a variety of ways. Ayurveda, Siddha, Amchi, Unani, yoga, and naturopathy are "classical" Indian systems. They have a global nature and do not depend on a particular place or language. These methods are all backed up by comprehensive written treatises. Indian traditional medicine is primarily oral in nature, location- and community-specific, and frequently solely used by specific ethnic groups, families, or individuals. African traditional medicine is a rich and complex body of knowledge that is primarily passed down orally from generation to generation. Traditional medical knowledge contains a variety of international and regional components, and Africa is a vast continent that unites extremely diverse cultures and ethnic groupings. [4]

Traditional medical practices constitute an integral aspect of India's rich historical heritage. Ayurveda, the traditional Indian medical system, transcends the mere treatment of ailments by prioritizing preventive measures. Ayurveda encompasses a vast reservoir of knowledge concerning therapeutically significant medications and cultural practices handed down through

generations. The global acceptance of Ayurveda can be attributed to its comprehensive therapeutic approach, profound intellectual foundation, and the enduring efficacy of remedies dating back to prehistoric times.Amidst changes in the environment, lifestyle, culture, and disease patterns, the enduring relevance of ancient medicinal formulations persists. The Ayurvedic concept of health and illness incorporates the Tridosha theory, which considers the balanced state of biological entities. Doshas, comprising Sharirika (somatic) - Vata, Pitta, and Kapha, and Manasika (psychic) - Satva, Rajas, and Tamas, serve to conceptualize the structural, functional, and behavioral aspects of living organisms. The psychosomatic coupling of Manasika and Sharirika doshas is integral to maintaining physiological equilibrium.The three doshas—Vata, Pitta, and Kapha—within Ayurveda elucidate the psychobiological processes inherent in living organisms. Designated as "doshas," these well-organized elements are susceptible to imbalance and derangement. Each dosha possesses distinct attributes and functions, correlating with the kinetic, metabolic, and structural stability mechanisms of a system. For example, Vata contributes to shape expression, cell division, signaling, motility, and waste excretion. This intricate framework underscores the depth and sophistication of Ayurvedic principles in understanding and addressing the complexities of health and well-being. [5]

Ayurveda has undergone a dramatic paradigm shift in recent decades, and researchers perspectives on its uses have also changed significantly. [6] Globally mutual efforts to control the booming traditional medicine and herbal medicines industries and to check product quality is made. The provision of standardized herbal medicines has attracted the active interest of numerous governments and health organizations. The administration of medicinal drugs safely

and effectively depends on their biological activity and composition being consistent. Botanical medicines often fall short of meeting the standards of quality, which encompass methodologies and criteria for assessing and verifying the potency of botanical raw materials, extracts, or formulations. The assurance of quality plays a pivotal role in establishing the safety and effectiveness of botanical medicines. [7]

The Ayurvedic Pharmacopoeia of India (API) stands as a distinct set of standards outlining the quality, purity, and potency parameters for a specific range of medications. These medications are manufactured, distributed, and procured exclusively by authorized producers throughout India. The API is structured into two segments: the first comprises mono-monographs detailing drugs with natural sources, while the second incorporates selected compound formulations sourced from Schedule I books under the 1940 Drugs and Cosmetics Act. These formulations are drawn from renowned Ayurvedic classics spanning various eras. Following the publication of Ayurvedic Pharmacopoeia of India (mono-monograph) Part-I, Vol. I in 1989, subsequent volumes were released, each validated under the legal framework of the Drugs and Cosmetics Act. The inaugural section of the Ayurvedic Formulary of India was initially published in 1978. [8]

Ensuring the quality of natural herbs and products is paramount, and effective quality monitoring of herbal substances becomes imperative in this context. Pharmacopeial elements of quality control encompass the identification of substances, detection of adulterants and substitutes, assessment of material purity, and the quantification of the active chemical constituent specific to the herb in question. Standardization, a critical process, involves the

evaluation of qualitative and quantitative properties of herbs against established or prescribed criteria and parameters. In pursuit of these objectives, the World Health Organization (WHO) has established guidelines for standardization methods and procedures. These guidelines are based on a comprehensive set of significant evaluation parameters, encompassing organoleptic properties, ash values, moisture content, microbial contamination, as well as chromatographic and spectroscopic evaluations. Adherence to these standards facilitates a robust framework for ensuring the quality and integrity of natural herbs and related products. [9]

In accordance with Ayurvedic Pharmacopeia of India the powder of drug/s is termed as- "Churna". The raw materials are sieved and separately pulverised. Each one (of the powders) is weighed individually and well combined, as certain raw materials include more fibrous material than others, the classical literature recommends powdering and weighing each medication individually before combining them. The powder is extremely fine, at least 80 mesh or better. It shouldn't stick together or get wet. The medicinal value of the powder increases with increasing fineness. They should be kept in airtight containers and preserve their potency for one year.

Numerous traditional Churna formulas have been employed in numerous therapies for ages. Sitopaladi Churna (which is referenced in Sarangadharasamhita Madhyamakhanda, Adhyaya 6:134-137) and Talisadi Churna (which is referenced in Sarangadharasamhita Madhyamakhanda, Adhyaya 6: 130-133 1/2) commonly employed formulations integral to Ayurvedic Pharmacopeia of India. The Sitopaladi formula consists of *Piper longum* (48 grams), *Cinnamamoum zeylanicum* (12 grams), *Elettaria cardamomum* (24 grams),

Vamsalochana (96 grams) and Sugar (192 grams). The Shloka that describes Sitopaladi churna composition of raw materials along with ratios,

Sitopalā ̣soḍaśasyādaṣṭausyādvaṃśarocanā ||*134*||

pippalī syāccatuṣkarṣā syādelā cadvikaarsiki|

ekaḥ karṣastvacaḥ kāryaścūrṇayetsaevamekataḥ ||*135*||

sitopalādikaṃ cūrṇaṃ madhusarpirtaṃ lihet|

śvāsakāsakṣayaharaṃ hastapādāṅgadāhajit||*136*||

mandāgniṃ suptajihvatvaṃ pārśvaśūlamarocakam|

jvaramūdhvagataṃ rakttaṃ pittamāśuvyapohati||*137*||

The other classical preparation Talisadi Churna formula consists of *Abies webbina* (12 grams), *Piper longum* (48 grams), *Cinnamoum zeylanicum* (12 grams), *Zingiber officinale* (36 grams), *Piper nigrum* (24 grams), *Elettaria cardamom* (6 grams), *Bambusa bambos* (60 grams) and Sugar (384 grams). The Shloka that describes Talisadi churna composition of raw materials along with ratios,

Tālīsaṅ maricanīsunthī pippalī vaṅśárocanā ||***130***||

ekadvitrichatuḥpaṅcakaṣaṛbhārgānpṛakalpayet|

elātvacostukaṣārdharpṛtyekbhagamāvahet||***131***||

dvatṛiśatkaṣṛtulitā pṛadeyā śakaṛrā budhaiaḥ |

tālīsādhyamidaṅ cūrṇarocanaṅ pācanaṅ smuṛtam|***132***|||

kāsashrvāsajvaraharaṅ chadhyṛtīsāranāśanam|

śoṣādhmānaharaṅ plīhagrahaṇīpaṇḍurogajit|***133***|||

paktvā vā śakaṛramcūrṇakshipetsyad̄ gutikā tataḥ |[10-12].

As research endeavors progress, High-Performance Thin-Layer Chromatography (HPTLC) is gaining prominence as an optimal solution for assuring the quality of herbal plants. Beyond its application in identification, the HPTLC technique serves as a valuable tool for quality control, allowing for a direct comparison of samples with reference standards. HPTLC fingerprint images reveal peak profiles and their intensities, facilitating not only qualitative analysis but also providing quantitative results that can be systematically benchmarked. Essential information regarding the marker compound's identity, purity percentage, and minimal content is systematically obtained. The High-Performance Thin-Layer Chromatography (HP-TLC) technique is commonly employed for the quantitative measurement of phyto-constituents due to its precision and simplicity. Samples, reflecting variations in growth environments and climatic conditions, undergo comprehensive analysis for diverse characteristics and phytochemical components. Beyond chromatograms and digital fingerprint images, the HP-TLC technique allows for visual detection, even when dealing with samples and standards at microliter concentrations. This capability facilitates the simultaneous application of samples and standards on the same HP-TLC plate, streamlining the comparative research of herbal medications and formulations. [13]

The foundational principles of Analytical Quality by Design (AQbD) draw directly from the Quality by Design (QbD) framework, a quality paradigm introduced to the pharmaceutical industry by the US Food and Drug Administration and documented in the ICH Q8(R2) guideline. Employing statistical tools and methodologies such as Design of Experiments (DoE), QbD facilitates the delineation of the design space. Starting with predetermined objectives, the Quality by Design (QbD) approach integrates scientific and risk management

techniques to gain a comprehensive understanding of both the product and the process, ultimately leading to effective process control. Through the implementation of a control plan, risks can be managed, ensuring that the method operates as intended once validated and deployed. This approach allows for a nuanced comprehension of method performance and facilitates necessary adjustments as required. [14-16]

1.2 Review of Literature:

- S. Kumar,*et al*, (2021), In this study the review was carried for understanding traditional use like diarrhoea, flu, leprosy, malaria, UTI and HIV/AIDS. The objective of the review was to gather precise and authenticated information related to uses, phyto-chemicals, pharmacological use and QC/QA of the selected medicinal plant. The search was carried out on different search engines like Google search, Pubchem, Chemspider, PubMed, Elsevier, Wiley etc. The review showed a positive inclination towards the traditional use of the plant material. [17]

- Farag RS, et al, (2020), In the present study medicinal plants are selected (fig, guava, olive and pomegranate) and their crude juice is extracted by mechanical pressing techniques. The crude juice that is obtained is subjected to the phyto-chemical estimations that are flavonoids, tannins, total phenolic content and anthocyanin by HPLC technique. Later DPPH assay, metal chelating assay and reducing power assays are carried out for detecting of the level of anti-oxidant property of the crude juices. After carrying out all the above mentioned parameters and techniques the results reveal the existance of gallic acid, ellagic acid, naringenin, ferulic acid and methygallate. Future scope is towards carrying out more focused parameters for anticancer anti-microbial studies after fractionation of the crude juices. [18]

- Amina M, et al, (2020), In the study, the author meticulously selected five distinct medicinal plants and conducted a comprehensive analysis encompassing preliminary phytochemicals, total phenolic content, in-vitro antioxidant activity, and molluscicidal activity. Following the phytochemical analysis, the results unveiled the presence of tannins, flavonoids,

saponins, terpenoids, steroids, anthraquinones, alkaloids, carbohydrates, and coumarins. Notably, the total phenolic content exhibited higher levels in N. oleander, M. oleifera, and C. papaya. Furthermore, the antioxidant activity was prominently demonstrated by C. papaya, M. oleifera, and T. stans. [19]

- HadadiZ,et al, (2020),In this study the selected six medicinal plants were extracted with methanol and chloroform solvent. The extracts were subjected to anti-microbial study that was carried out against 3 fungal stains and 5 bacterial strains. The essential oils present in the medicinal plants were detected by GC/MS. The plants were screened for anti-oxidant, guaiacolperoxidas and catalase enzymes activity were carried out. The reports presented that the medicinal plants selected have promising antimicrobial agent. [20]

- P. Kaur,et al, (2020), In this research, the polyherbal medicine Mahasudarshanchurna, employed for the treatment of fever, cold, malaria, and various bacterial infections, underwent a comprehensive HP-TLC method development and validation. The targeted compounds included oleanolic acid (OA), ursolic acid (UA), mangiferin (M), gallic acid (GA), quercetin (Q), and curcumin (C), with experimentation involving different mobile phases. The process encompassed visualization and scanning procedures, complemented by HPLC-PDA analysis to validate the obtained results. The developed HP-TLC method proved to be cost-effective, expeditious, as well as precise and accurate in its application. [21]

- Q. Huang,et al, (2020), In this study the quality consistency evaluation by developing a method which relays on the chromatographic fingerprinting method as we cannot completely

depend on chromogenic reaction for quality and safety of the biochemical composition. The HP-TLC fingerprinting is established for evaluation of the quality of the products sold commercially. The developed fingerprinting method for the combination of the chemical potentials and the method was efficient, and reliable. [22]

- X. Liu,et al, (2019), In this study the review is conducted on the quality control of traditional Chinese medicines. The traditional medicines usage have expanded but the information related to the safety, quality, efficacy and authentications is not sufficient. So in this review attempt was made towards the quality aspects by reviewing the chemo-metric technique and the evaluation of quality by fingerprinting by chromatographic method, spectroscopic method and capillary electrophoresis.The application of the fingerprinting is done for multi-component analysis, quantifications, quality control, screening of chemical components. So the study concludes with the challenges which are faced during the quality control along with the future prospects towards the usage of modern techniques in quality control of the traditional Chinese medicines.[23]

- Maria R, et al, (2018), In this study 13 native species were selected from Guayas province for carrying out different parameters for the detection of properties. In the first stage phyto chemical screening was carried out after preparing the ethanolic extracts. Additionally, the quantification of total phenolic content was conducted through the utilization of the Folin-Ciocalteu technique. Simultaneously, a microbial assay was executed targeting S. aureus, E. coli, and V. parahaemolyticus. The study's findings indicate that the predominant active principles across all selected species were notably present in the ethanolic extract. [24]

- Meena AK, et al, (2021), The authors endeavored to establish a comprehensive quality control protocol for Anutailam, an Ayurvedic medicated oil. Employing HP-TLC techniques, they identified key bioactive compounds, including Berberine chloride, Marmelosin, Negundoside, glycyrrhizin, and para-hydroxybenzoic acid (PHBA), Lupeol, and Embelin. Additionally, HPLC was utilized for the precise estimation of Negundoside, Berberine chloride, and Marmelosin, identified as major constituents in Anutailam. The findings affirm adherence to quality parameters throughout the formulation process. Physicochemical analysis was carried out in accordance with the Ayurvedic Pharmacopeia of India. The generated HPLC and HPTLC profiles serve as valuable tools for authenticating the quality and integrity of Anutailam. [25]

- Neethu S, et al, (2021), The authors in the present study have worked on the developing the standardization protocol based on biomarker compounds which are present in the formulation. The formulations were evaluated for pharmacognostical, physicochemical and organoleptic parameters in accordance with the standard procedures for testing the quality of the formulations. HPLC quantification was carried out for compounds luteolin, 6-gingerol, ß-sitosterol and ecdysterone which were used as the biomarkers for theNayopayamKwatha. This study can be used as the reference for carrying out the standardization of the Ayurvedic formulation NayopayamKwatha and the developed HPLC method can be used during the quantifications of the formulation. [26]

- Devipriya S, et al, (2021), In this current study, the authors have conducted a thorough review concerning the correlation between Ayurveda and analytical chemistry. To carryout

this review meta-analysis was carried out of 37 articles. The Analytical techniques like HPLC, LC-MS, UFLC-MS/MS, GC–MS, QTOF-MS, ESI-MS and HRMS are used in identification, determination and estimation of chemical components of the herbal/Ayurveda formulations. Many other analytical techniques are used for carrying out assay, testing stability, purity and potency are also connecting the Ayurveda with the analytical chemistry. The standardization of the Ayurvedic formulations can be carried out by using the analytical techniques which confirms the inevitable relation between Ayurveda and analytical chemistry. [27]

- Meena AK, et al, (2021), In the current study, the standardization of the ashwagandhadileyam formulations was done by using the biomarker Withaferin-A. Organoleptic characters, Physico-chemical along with fingerprinting and by using HP-TLC technique and estimation of standard Witheaferin A by HPLC method. The Rf value was found to be 0.35 for Witheaferin A and 0.092% was estimated. The above method can be used as protocol for standardizing the ashwagandhadileyam. [28]

- Abraham A, et al, (2021), In the present study PathyashadangamKwath which is present in classical Ayurveda, used in treating various diseases like Migraine, head ache, upper respiratory tract infections is studied and standardization protocol is developed for the formulation. The HPLC and HP-TLC was used for standardization of PathyashadangamKwath which is the classical ayurvedic formulation. The kwath underwent a comprehensive evaluation encompassing physical, phytochemical, physicochemical, and chromatographic parameters, following established procedures. This study can serve as a standardized protocol for the standardization of Pathyashadangamkwath.[29]

- Maurya N, et al, (2020), In this study the authors have formulated an Ayurvedic formulation and further the evaluation is done for same. The polyherbal formulation Hingwastakachurna is done as per Ayurvedic formulary which is used in the treatment of gastro-intestinal disease. The formulation is prepared and evaluated for various parameters such as organoleptic characters, Microscopic evaluation, Phytochemical, Physico-chemical, UV spectroscopy, TLC and HP-TLC was done for Piperine standard. These parameters can be employed as quality control benchmarks to ensure the quality of Ayurvedic Hingwastakachurna.[30]

- Sharma, V. et al, (2020),The authors have carried out comparison study of the formulated Triphalachurna and the marketed churna. Various parameters are tested for both the raw materials and formulation which helped in assessing the quality of the formulations. The parameters assessed encompassed Phytochemicals, Physico-chemical, and Pharmacognostical aspects, functioning as integral tools for the quality control of Ayurvedic formulations. The established protocol of procedures can be effectively employed as a tool for the standardization of Triphala churna. [31]

- Vaidya SA, et al, (2022), The HP-TLC method employed simultaneous estimation of berberine, gallic acid, piperine, and quercetin. The mobile phase utilized for separation consisted of toluene: ethyl acetate: methanol: formic acid (6:6:2:1). The calculated rf values were 0.37, 0.51, 0.72, and 0.86, respectively. Demonstrating high precision, the linearity coefficient surpassed 0.99. In the precision study, the %RSD was below 2%, affirming the method's reliability, accuracy, and suitability for simultaneous estimation. [32]

- Chaudhary SK, et al, (2022), Yongchak which is found in Northeast India with botanical name Parkiaroxburghii, this is been used from traditional time in treatment of piles, diabetes, urinary tract infections and blood pressure. The activity are due to the virtue of phytochemicals like flavonoids, saponins, alkaloids, tannins and terpenoids. The development of the HP-TLC method involved utilizing a stationary phase of TLC aluminum plates pre-coated with silica gel 60F254. The selected mobile phase comprised ethyl acetate: acetic acid: formic acid: water (10:1:0.75:1). A well-defined peak corresponding to the standard marker compound catechin was observed at an rf value of 0.61. The method underwent rigorous validation and standardization to establish its efficacy for quality control of P. roxburghii. [33]

- Gamre M, et al, (2022), Simultaneous HP-TLC method was developed for curcumin and cineole. The stationary phase used was pre-coated with silica gel 60F254 with aluminium backing are used. The mobile phase employed consisted of n-hexane: ethyl acetate in the ratio of 6:3.5. The rf values were found to be 0.31 and 0.71 and the R2 value was 0.995 and 0.997 for curcumin and cineol respectively. Following the ICH guidelines, the developed method underwent thorough validation, establishing its attributes of simplicity, accuracy, and rapidity. [34]

- Arage A, et al, (2022) A High-Performance Thin-Layer Chromatography (HP-TLC) method was formulated to simultaneously analyze paracetamol, caffeine, chlorpheniramine, and phenylephrine. The stationary phase employed was a glass plate pre-coated with silica gel 60F254, while the mobile phase comprised methanol: n-butanol: toluene: acetic acid in the ratio of 8:6:4:0.2. The method was executed at 212nm. Validation of the developed method adhered to the guidelines outlined by the International Council for Harmonisation (ICH). This

method demonstrated suitability for the simultaneous estimation of the compounds, applicable to both bulk pharmaceuticals and tablet formulations. [35]

- Prajapati P, et al, (2022), In this current investigation, the author and their research team endeavored to design and develop a method for the estimation of Carvedilol and Ivabradine in a combined formulation. This was achieved through Reverse Phase-High-Pressure Liquid Chromatography (RP-HPLC) and High-Performance Thin-Layer Chromatography (HP-TLC). The quality-based design adopted principles such as Analytical Failure Modes Critical Effect Analysis (AFMCEA) and Design of Experiments (DoE), aligning with the guidelines established by the International Council for Harmonisation (ICH) in Q14.The results obtained from the developed method were found to be within the compliance. The developed method is the better alternative to the available HPLC methods. [36]

- Foudah AI, et al, (2022), The objective of this study is to establish and evaluate an environmentally friendly analytical method for the estimation of ascorbic acid, a parameter not previously addressed. The mobile phase utilized comprises a combination of water and ethanol in a ratio of 7:3 v/v. In this analytical approach, the estimation of ascorbic acid is conducted in P. emblica, P. guajava, and C. annuum. Additionally, the environmental sustainability of the method is assessed using the GREEnness (AGREE) method. The greenness index was found to be 0.88 which shows that the developed method has a very excellent green profile. As the solvents used in this method are green in nature hence the method can be considered as safe to the environment. [37]

- El-Waey AA, et al, (2022),In the present study a novel and green HP-TLC method is developed and validated for quantification of sofosbuvir and ledipasvir simultaneously. The developed HP-TLC method is assessed for the greenness and the eco scale score was found to be 93. On the other part of the study stress degradation is done by the HP-TLC and further HP-TLC-ESI- MS was carried out. The fragments and their identification is done. The method was also validated for various parameters is done. This analytical method developed is more environmental friendly which is the contribution to the increasing interest of the mankind. [38]

- PP Bang et al (2023), In this investigation, the focus is on the utilization of lenvatinib mesylate (LNB), a potent anticancer medication, for the treatment of thyroid cancer. Notably, there exists a gap in the literature concerning the application of green analytical chemistry (GAC) in High-Performance Thin-Layer Chromatography (HPTLC) for LNB. This study is dedicated to the implementation of GAC principles in the development and validation of a green HPTLC method for LNB. For the Reverse Phase High-Performance Thin-Layer Chromatography (RP-HPTLC), the mobile phase consisted of ethanol and water (60:40, v/v), while the green Normal Phase High-Performance Thin-Layer Chromatography (NP-HPTLC) method utilized ethanol and ethyl acetate (50:50, v/v). Notably, RP-HPTLC demonstrated superior sensitivity compared to NP-HPTLC, with the green RP-HPTLC method proving to be faster, more accurate, and more precise. The evaluation of Analytical Greenness (AGREE) scores resulted in values of 0.88 for RP and 0.82 for NP. These findings underscore the potential of the green RP-HPTLC method as a more environmentally friendly and sustainable option. As pharmaceutical enterprises strive to mitigate the potential negative impact of organic solvents on the environment, these ecologically friendly green procedures present

promising alternatives. Both developed methods exhibit exceptional greenness when compared to other reported methods in the literature. [39]

- Gupta M*et al* (2023), This study focused on Dawaul Kurkum, a well-known Unani remedy comprising seven plant materials. Despite its popularity, there has been a notable absence of research characterizing this formulation. Hence, the current study aimed to elucidate the pharmacognostic and phytochemical properties of Duk.The production of Duk adhered to The National Formulary of Unani Medicine Part-I and Bayaz e Kabeer's instructions. The comprehensive characterization encompassed organoleptic characteristics, fluorescence analysis, preliminary phytochemical screening, antioxidant activity assessment, and active ingredient profiling through High-Performance Thin-Layer Chromatography (HPTLC).Analysis revealed the presence of carbohydrates, flavonoids, quinones, glycosides, cardiac glycosides, terpenoids, phenols, coumarin, steroids, and phytosterols in Duk. The total phenolic content was determined to be 5.75 ± 0.23 mg GAE/g, and the total flavonoid content was 10 ± 0.18 mg QUE/g. HPTLC identified P-coumaric acid, cinnamaldehyde, citral, crocin, isovaleric acid, guggulsterone, and dehydrocostus lactone in Duk.In conclusion, this research substantiates the efficacy of Duk as a traditional treatment, offering valuable insights for the standardization of Duk in the future. [40]

- Alam P, *et al,* (2023), The aim of this study was to establish and validate a stability-indicating High-Performance Thin-Layer Chromatography (HPTLC) method with an environmentally friendly approach for the determination of Cordycepin in a laboratory-developed formulation. The selected greener eluent system for the detection of Cordycepin

consisted of ethanol-water (75:25 v/v), and the quantification was performed at a wavelength of 262 nm. To assess the environmental impact, the analytical GREENness (AGREE) approach was employed, providing a comprehensive evaluation of the proposed analytical technology's eco-friendliness. [41]

1.3 Justification:

- **Highlight the growing global interest in Ayurveda:** Ayurveda is a traditional system of medicine with a rich history and growing interest worldwide. Standardizing classical Ayurvedic preparations can help ensure their quality, safety, and efficacy for a global audience.
- **Focus on the scientific approach:** This study uses a scientific approach to standardize classical Ayurvedic preparations. This can help build trust in these traditional medicines and pave the way for their wider acceptance in regulated markets.
- **Address the needs of regulatory bodies:** By standardizing Ayurvedic preparations, this study can provide valuable data to regulatory bodies around the world. This data can be used to establish clear guidelines for the manufacturing and marketing of Ayurvedic medicines.

1.4 Objectives: Primary objectives:

- To carry out the chemo-profiling and identification of bio-active constituents in the selected classical Ayurvedic preparations.
- To develop the analytical methods and their validation by suitable chromatographic technique.(HP-TLC)

Secondary objectives:

- To carryout comparative study of Marketed classical Ayurvedic preparations (Sitopaladi and Talisadi churna) using chromatographic techniques.

2. MATERIALS AND METHODS:

2.1 MATERIALS:

Plant materials: Raw materials used in churna preparations

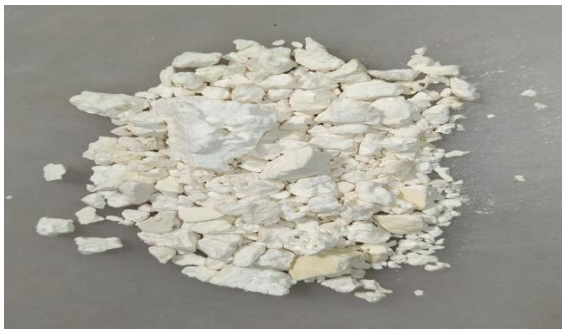
Figure 1: Images of raw materials in the formulations

Abies webbina **(Talisapatra)**

Piper nigrum **(Maricha)**

Elettaria cardamomum **(Elaichi)**

Cinnamomum zeylanicum **(Dalchini)**



Bambusa bamboo **(Vamshalochana)**

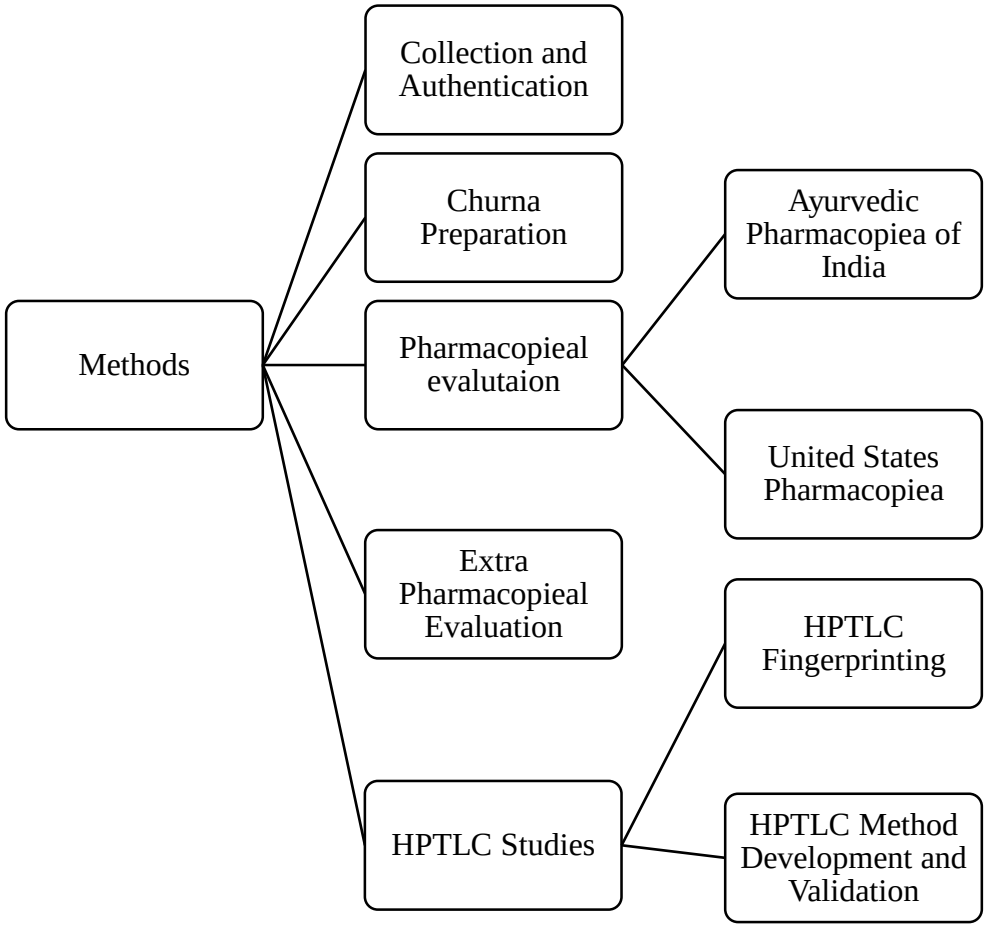
Piper longum **(Pipalli)**

Zingiber officinalis **(Sunthi)**

Marketed products:

Sitopaladi churna (labelled as S2 and S3) and Talisadi Churna (labelled as T2 and T3) are procured from the local market of Belagavi, Karnataka.

2.2 METHODS:



2.2.1 Collection and authentication: The raw materials required for the churna were collected from KLE Ayurveda Pharmacy, Belagavi. The authentication was carried out by Dr. Harsha Hegde, ICMR-NITM. The marketed formulations of Sitopaladi and Talisadi Churna were collected from the local market.

2.2.2 Churna Preparation: The Churna were prepared by grounding the raw materials separately and then mixing them in accordance with ratios mentioned in classical references. The churnas are labelled as S1 (Sitopaladi Churna) and T1 (Talisadi Churna). [11]

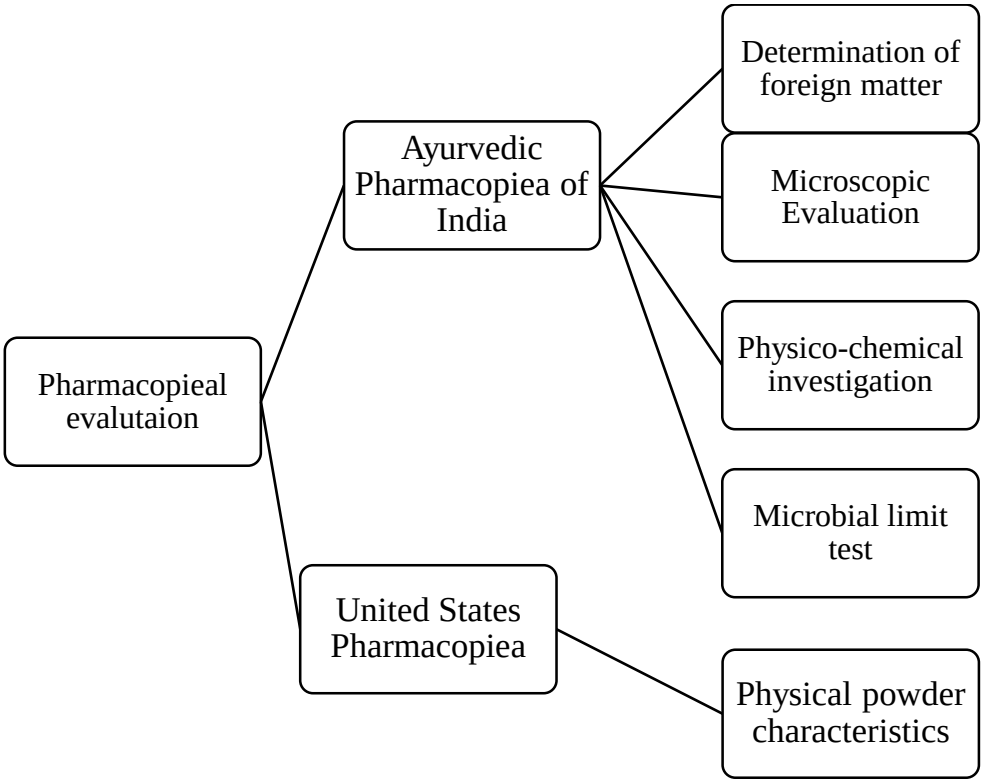
Table 1: Formula of Sitopaladi Churna

Sl. No	Raw materials	Quantity (g)
1	Sitopala	192
2	Vamsalochana	96
3	Pippali	48
4	Ela	24
5	Tvak	12

Table 2: Formula of Talisadi Churna

Sl. No	Raw materials	Quantity (g)
1	Talisa	12
2	Marica	24
3	Sunthi	36
4	Pippali	48
5	Vamsalochana	60
6	Ela	6
7	Tvak	12
8	Sharkara	384

2.2.3 Pharmacopeial Evaluation:



- **Determination of Foreign Matter:**

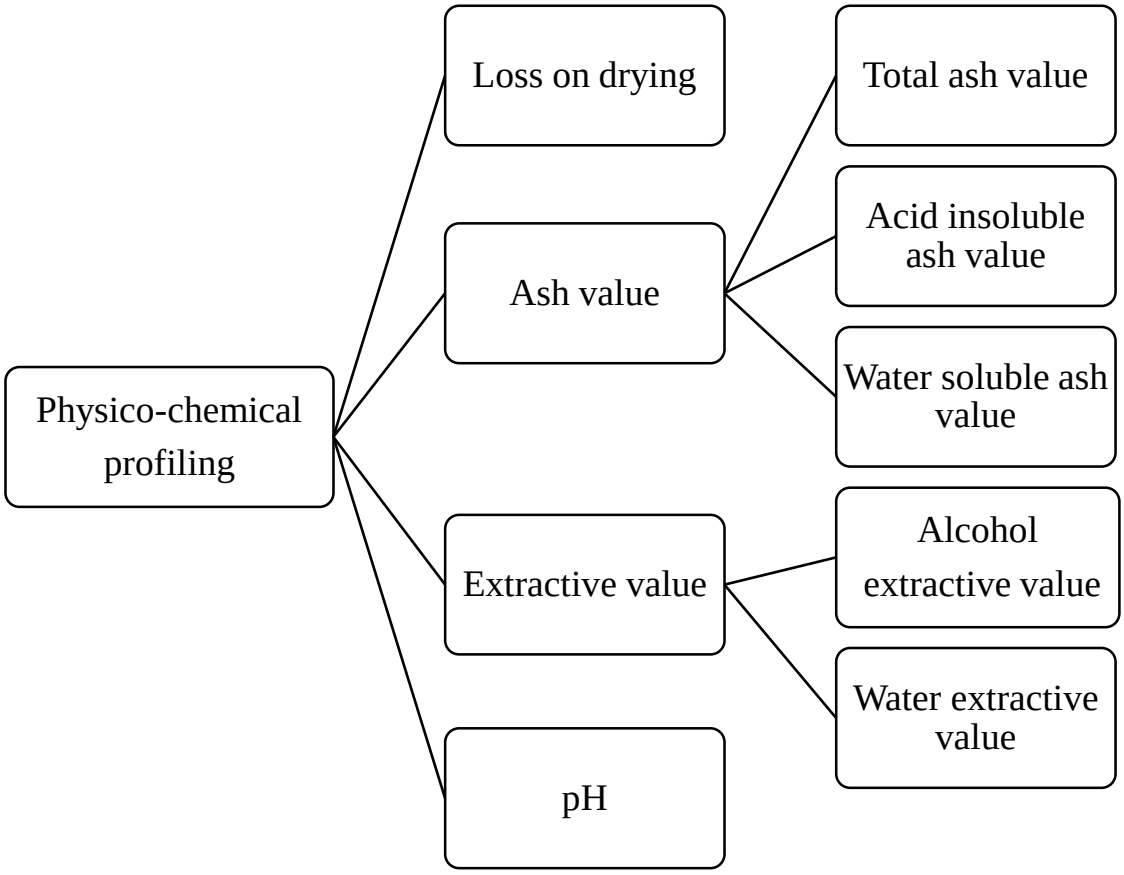
In this parameter the ingredients for detection are spread on a plane surface and detected for the foreign materials with naked eyes and 60X microscope. The weight of the ingredient before and after the detection is noted and substituted in the formula:

Determination of the foreign material (%) = weight after the detection/ weight before the detection *100

- **Microscopic Evaluation:**

Powder microscopy was carried out for all the ingredients. The powdered and sieved ingredients are taken on a glass slide and treated with phloroglucinol and HCL or Potassium iodide and finally glycerin is added before mounting. The instrument used is Trinocular microscope connected to the software output Capture pro.

➢ **Physico-Chemical Profiling:**



- **Loss on Drying:**

  Place an accurately weighed 5-10 g of powder or drug into a tarred evaporating dish. Ensure an even distribution of the test specimen by gently shaking it sideways to a depth of approximately 5-10mm. Proceed to dry the test specimen at 105°C for a duration of 3 hours, followed by weighing. Subsequently, continue the drying and weighing process at half-hour intervals until the observed difference does not exceed 0.25%.

- **Ash Values:**

- Total Ash Values: Combust precisely 2-3g of the finely ground drug in a tarred silica dish at a temperature not surpassing 600°C until devoid of carbon. Subsequently, position it in a desiccator for 30 minutes and promptly record the weight.
- Water Soluble Ash Values: Water-Soluble Ash Values: Boil the obtained ash for 5 minutes in 25ml of water. Gather the insoluble residue in a crucible on ash-less filter paper, rinse it with hot water, and ignite it for 15 minutes at a temperature not exceeding 450°C. Subtract the weight of the insoluble residue from the ash weight; the resulting difference in weight represents the water-soluble ash. Calculate the percentage of water-soluble ash relative to the air-dried drug.
- Acid Insoluble Ash Values: Add dilute hydrochloric acid drop-wise (25ml) to the crucible containing total ash. Collect the insoluble matter using ash-less filter paper, and wash with hot water until the filtrate attains neutrality. Transfer both the filter paper and insoluble matter back to the initial crucible, dry it on a hot plate, and ignite until a consistent weight is attained. Allow the residue to cool in a suitable desiccator for 30 minutes and promptly record the weight. Calculate the acid-insoluble ash content relative to the air-dried drug.

- **Extractive Values:**

(Alcohol Soluble and Water Soluble): Macerate 5g of the air-dried drug, coarsely powdered, with 100ml of alcohol or water in a sealed flask for 24 hours. Vigorously shake during the initial 6 hours and allow to stand for an additional 18 hours. Filter promptly, taking precautions against solvent loss, and evaporate 25ml of the filtrate to dryness in a tarred flat-bottomed

shallow dish. Dry at 105°C until a consistent weight is achieved, and measure. Calculate the percentage of alcohol or water-soluble extractive value in reference to the air-dried drug.

Extractive value = Weight after extraction/Weight of sample *100z

- **pH:**

The electrical pH measuring device is used. The pH meter is standardized using the standard buffer solutions. The sample is prepared 5 %w/v of the extracts; Incase the sample is not completely soluble it is filtered and the filtrate is been used.

- **Microbial Limit Test:**

The bacterial count of the Churna formulations is done by carrying out the microbial study. The pre-treatment of the samples is carried out by treating with buffer sodium chloride peptone solution which has pH value of 7. After the pre-treatment approximately 15mL of liquefied casein soybean digest agar is added. The medium is solidified in the petri-plates (9-10cm) and are incubated at 30-35 ^{0}C for the bacterial growth. The bacterial colony growth is calculated and the criteria are that not more than 300 colonies must be grown and expressed in terms of CFU/mL. [12]

- **Physical Powder Characteristics:**

In this parameter physical powder character of Churna (Sitopaladi and Talisadi) were carried out. The physical characterization determinations include Bulk density, Tapped density, Angle of Repose, and Ratio calculation were calculated for Hausner's ratio and Carr's index. The

above mentioned parameters are carried out in accordance with the USP standard procedures in triplicates and the average value is considered.

The calculations rely on the following formulas:

Bulk Density= Mass of powder (grams)/ Unsettled apparent volume (mL)

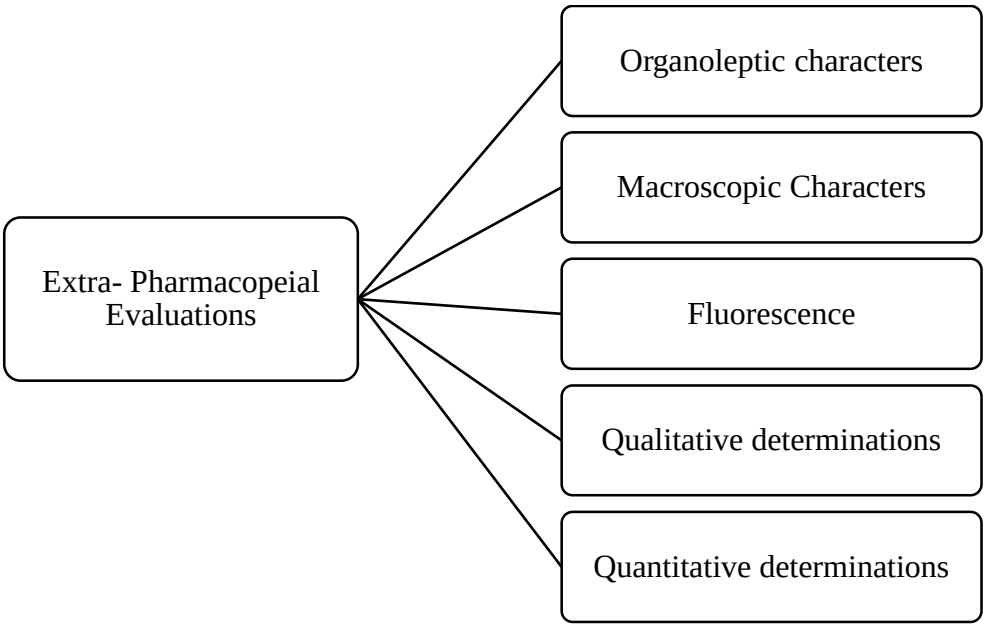
Tapped Density= Mass of powder (grams)/ Final tapped volume (mL)

Angle of Repose (tanα) = height of the cone formed/ 0.5* base of the cone

Hausner's Ratio: Unsettled apparent volume (mL)/Final tapped volume (mL)

Carr's Index: [100* (Unsettled apparent volume (mL)/Final tapped volume (mL)] /[Unsettled apparent Volume (mL)][42-43]

2.2.4 Extra-Pharmacopeial Evaluations:



- **Organoleptic:** In this parameter the color of the ingredient is detected by the eyes against the white background, the texture is felt and the taste and odour are also tested.
- **Macroscopic:** In Macroscopic study the character that are visible to the naked eyes are noted like the shape and size of the raw materials, so these characters were noted.
- **Fluorescence:** The raw materials are powdered separately and passed through the #80 sieve. Different solvents and standard reagents are added to the powdered raw materials and checked for color variation in three different wavelengths at visible range, 254nm and 366nm. The observed colors are reported.

- **Qualitative Phyto-Chemical Investigation:**

For carrying out the phytochemical investigations the raw materials were dissolved in various solvents and this solutions were further used for phyto-chemical investigations. The solvents used are water, methanol, ethyl acetate, chloroform and petroleum ether. The phyto-chemical investigation was carried out for all the raw materials. The phyto-chemical tests were performed were Alkaloids (Dragendroff's reagent), Carbohydrates (Molisch's test), Flavonoids (Alkaline reagent test), Tannins (Ferric Chloride acid), Steroids (Lieberman's test), Protein (Biuret test), Glycoside (Keller killani), Terpenoids (Salkowaski test), Saponin (Foam test) [12, 44]

- **Quantitative Determinations:**
  - **Total Phenolic Content:**

The method namely Folin-Ciocalteau was employed in carrying out the total phenolic content determination of the Churna samples. The concentration range of the samples prepare was 125-

1000µg/mL. Gallic acid was used as the standard in the procedure and the concentration is expressed in equivalents of Gallic acid. To the prepared dilutions 2.5 mL of Folin-Ciocalteau reagent was added along with 2ml of Sodium carbonate (7.5%) and kept for duration of 30 minutes for the reaction process. The results are reported after observations of the absorbance at 765nm using the UV-Visible Spectrophotometer [45].

- **Total Flavonoid Content:**

The quantification of total flavonoid content was conducted using the Aluminium chloride colorimetric method. The concentration range was chosen from 2-10 µg/mL and Quercetin was considered as the standard. The aluminium chloride solution was prepared with concentration of 2%. Subsequently, equal amounts of the samples and aluminium chloride solution were combined, and the mixture was allowed to stand for 1 hour at room temperature. Absorbance readings were recorded using a UV-Visible Spectrophotometer at a wavelength of 420nm. [46]

- **Total Alkaloid Content:**

To ascertain the total alkaloid content, samples were prepared within a concentration range of 20-100 µg/mL, with Atropine serving as the standard. Each sample was supplemented with 5ml of bromo-cresol solution and 5ml of Phosphate buffer solution. Subsequent absorbance measurements were taken using a UV-Visible Spectrophotometer at a wavelength of 470 nm, and the results were meticulously recorded. [47]

- **Total Tannin Content:**

The samples with concentration range 100-500 µg/ mL were prepared for determination of total tannin content. Tannic acid is considered as standard. To the solutions of the samples, 1ml of 8mM potassium ferric cyanide and 1ml of 20mM ferric chloride were introduced, and the volume was adjusted with distilled water. Subsequently, the absorbance of the samples was

measured using a UV-Visible Spectrophotometer at a wavelength of 700 nm, with the results meticulously recorded. [48]

- **Anti-oxidant:**

DPPH-Scavenging method was employed to determine the anti-oxidant potential of the Churna formulation with Ascorbic acid as standard. Samples were meticulously prepared within a concentration range of 20-100 µg/mL. Equal quantities of DPPH reagent (0.1 Mm) were added to the samples, initiating a reaction in dark conditions for 30 minutes. Subsequent absorbance readings of the samples were recorded using a UV-Visible Spectrophotometer at a wavelength of 517 nm, with the results diligently noted. [49]

2.2.5 HPTLC Studies:

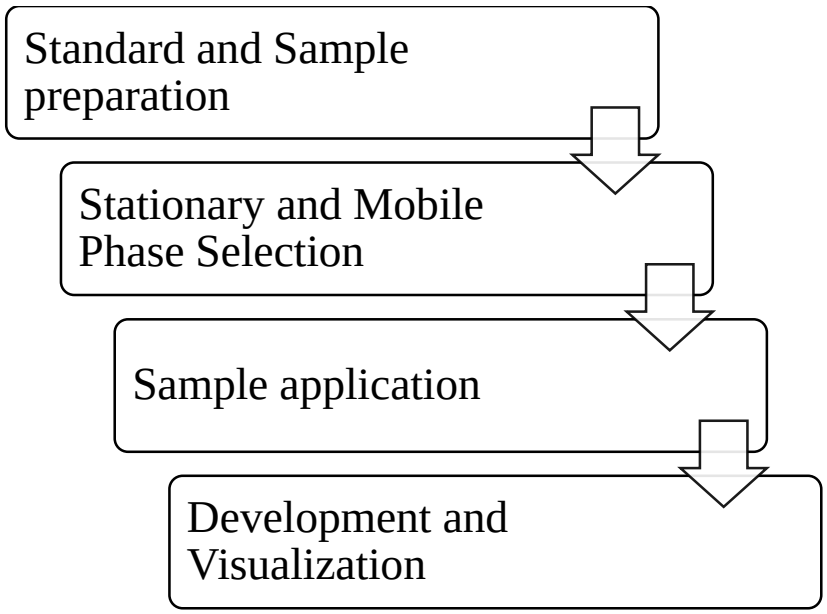
Instrumentation:

The HP-TLC study is carried out utilizing the Camag instrument with Linomat 5 (semi-automatic sampler), Camag Hamilton glass syringe -100 µL (sample application), TLC Visualizer (Photo-documentation), Camag twin trough chamber (for the tlc plate development), Tlc Scanner 4 (Scanning and Chromatograms) and the software visionCATS ver.3 is utilized for co-ordinating the HPTLC modules for developing and validating the method.

Steps to carryout HPTLC method:

- **Sample Preparation:** The samples of the marketed formulation were prepared by dissolving 10mg is dissolved in 1ml of methanol and sonicated. The sonicated solution is then centrifuged for 10 minutes at 2500rpm. After centrifugation the supernatant is collected and used as the sample.

- **Standard Preparation:** The Standard is prepared by dissolving in 1mg/mL in methanol.
- **Stationary Phase and Mobile phase selection:** Stationary phase used was TLC plate with pre coated aluminium backing with silica gel 60F. The Mobile phase for the plate development is chosen based on the literature and trials.
- **Sample Application:** The semi-automatic sampler Linomat 5 is employed for the sample application.
- **Development and Visualization:** For the development twin trough chamber was used. Saturation of about 15minutes was given and after that the plate was placed in the chamber, the development was done till 70mm. The developed plate was allowed to dry at room temperature and visualized at 254nm and 366nm wavelength.



HPTLC Method Development and Validation:

Method Development:

The method development for the standard is carried out using HPTLC technique. Different mobile phase and ratios are tried to develop an optimized method along with Quality by design and DoE principles. The Box-Behneken design is employed with factors Volume of solvent, distance travelled by the solvent front and chamber saturation time. The responses selected are rf value and area under the curve. Design Expert (Stat Ease Inc. Minneapolis, MN, USA) ver 13 is employed for designing of the analytical quality by design method. The design employed give seventeen runs (trails) after the input ranges are given.

Method Validation:

The developed method after optimization is validate for different parameters in accordance with ICH Q2 (R1) guidelines.

Linearity and Range: To obtain a linear dynamic in range different concentrations of the standards were applied, further the slope, co-relation coefficient and intercept were also studied.

LOD and LOQ: These parameters LOD and LOQ are necessary in understanding the method sensitivity and they are obtained by applying statistical formulas

LOD= 3.3 * Standard deviation / Slope

LOQ= 10 * Standard deviation /Slope

Specificity: The sample and standards are applied along with solvent used for sample/standard preparation and the mobile phase. The plate is developed and the chromatograms are obtained.

Precision: In Precision the study is carried out under inter-day and intra-day precision where in triplicates are applied at three different intervals the average value is consider and the %RSD is calculated which has a criteria of not more than 2%.

Robustness: In this parameter intentionally minor changes are made in the mobile phase system and the plate is developed and the % RSD value is calculated. The criteria here is not more than 2%.

Quantification: For quantification the samples with 5µL were applied on the plates and the further calculations were done to quantify the Churna samples. [50- 56]

Greenness Assessment:

The AGREE technique has been employed as an evaluative measure for the developed methodology. The AGREE scale, with a range spanning from 0.0 to 1.00, serves as a quantitative indicator of the method's alignment with environmentally sustainable practices. The analytical tool utilized for this purpose is the Analytical Greenness Calculator, specifically version 0.5 (2020) from Gdansk University of Technology, Gdansk, Poland. The use of this tool ensures a standardized and objective assessment of green analytical chemistry principles.

HPTLC Fingerprinting and determination of Class of Compounds:

The HPTLC Fingerprinting and class of compounds determination of the Churna formulation is carried out. The photo-documentation of the TLC developed plates is done for the results interpretations.

Table 3: HPTLC Fingerprinting attributes:

Class of compounds	Mobile Phase	Derivatisation	Observation
Alkaloids	Toluene: Ethyl acetate: Methanol 3:3:1.5	Dragendroff's Reagent	Orange spots (R White)
Flavonoids	Ethyl acetate: Formic acid: Glacial acetic acid: Water 10: 0.5: 0.5: 1.3	10% Methanolic sulphuric acid	Fluorescent colors to compounds
Tannins	Toluene : Ethyl acetate: Formic acid 6:4:0.3	Ferric chloride	Dark blue color bands
Phenolic	Cyclohexane : Ethyl acetate: Formic acid 4:6:1	Alcoholic Ferric chloride	Dark blue color bands
Anti-oxidant	Toluene: Ethyl acetate: Formic acid 6:4:0.3	DPPH reagent	White bands

3. RESULTS:

3.1 Pharmacopeial Evaluations:

3.1.1 Determination of Foreign Matter:

The pharmacopeial limit of acceptance is NMT 2% and hence all the raw materials pass the criteria of the foreign matter determination. (Table 4)

Table 4: Results of foreign matter determination

Sl.no	Ingredients	Percentage of foreign matter
1	*Abies webbina* (Talisapatra)	0.32%
2	*Piper nigrum* (Maricha)	0.26%
3	*Elettaria cardamomum* (Elaichi)	0.28%
4	*Cinnamomum zeylanicum* (Dalchini)	0.36%
5	*Bambusa bamboo* (Vamshalochana)	0.22%
6	*Piper longum* (Pipalli)	0.28%
7	*Zingiber officinalis*(Sunthi)	0.31%

3.1.2 Microscopic Results:

The results of powder microscopy are depicted in figures (Figure 2a-f)

Figure 2a: Powder Microscopy of S1

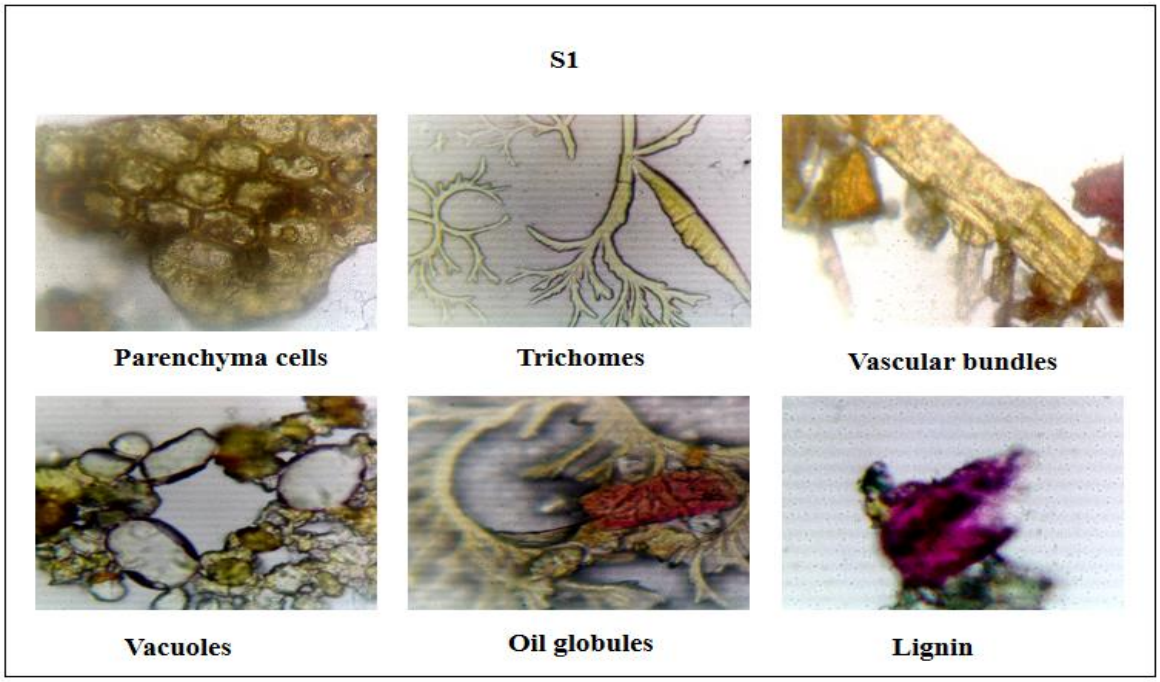


Figure 2b: Powder Microscopy of S2

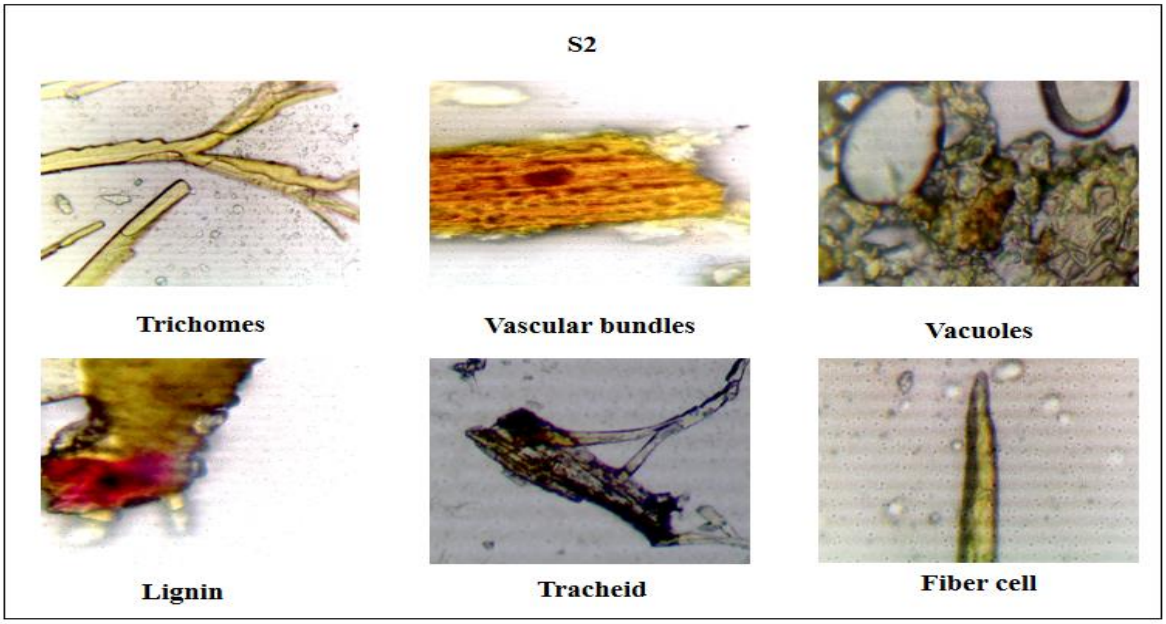


Figure 2c: Powder Microscopy of S3

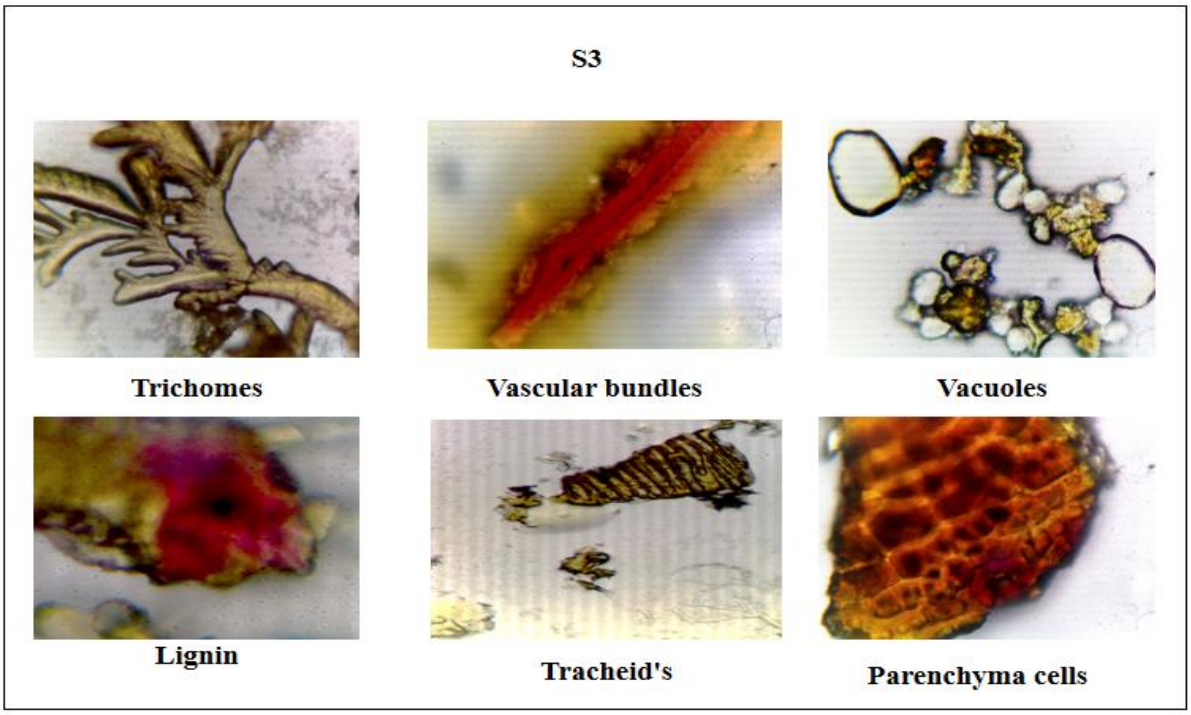


Figure 2d: Powder Microscopy of T1

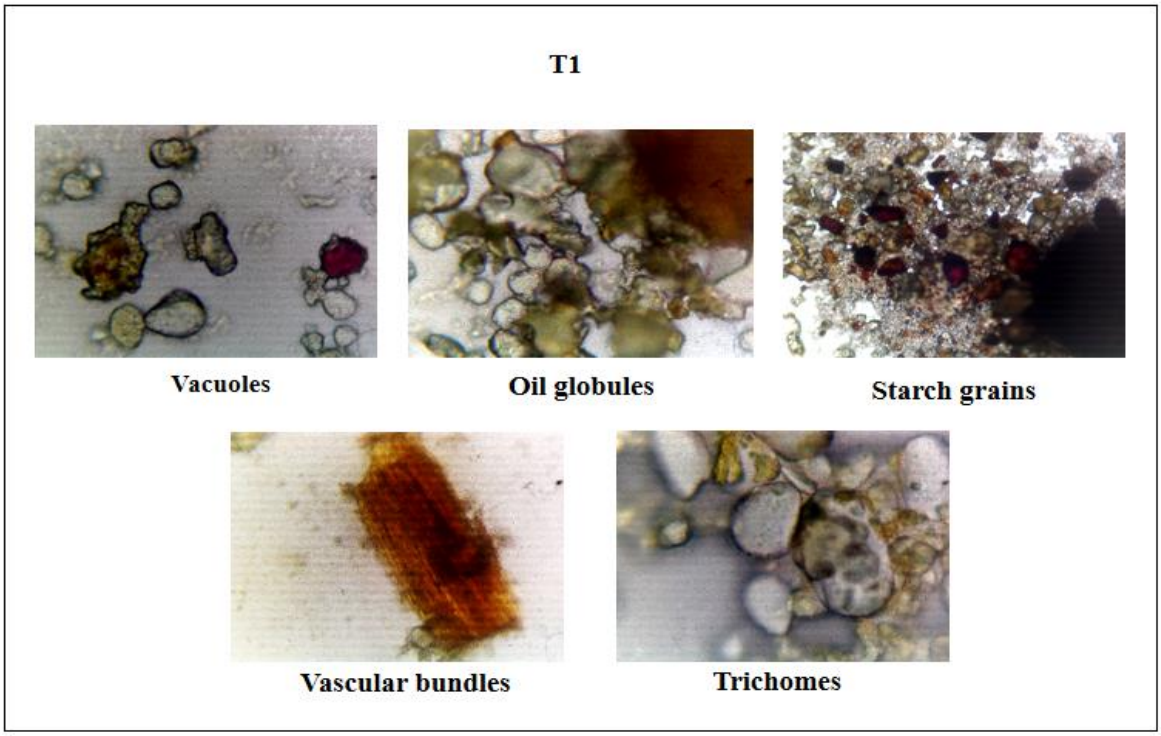


Figure 2e: Powder Microscopy of T2

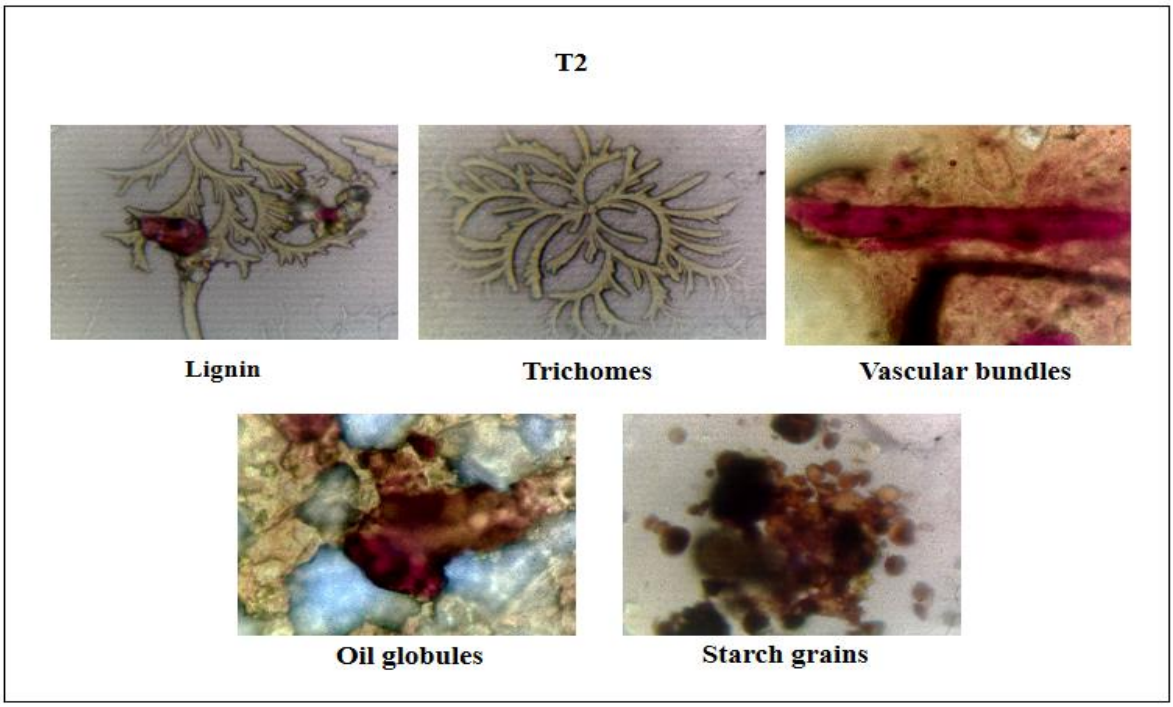
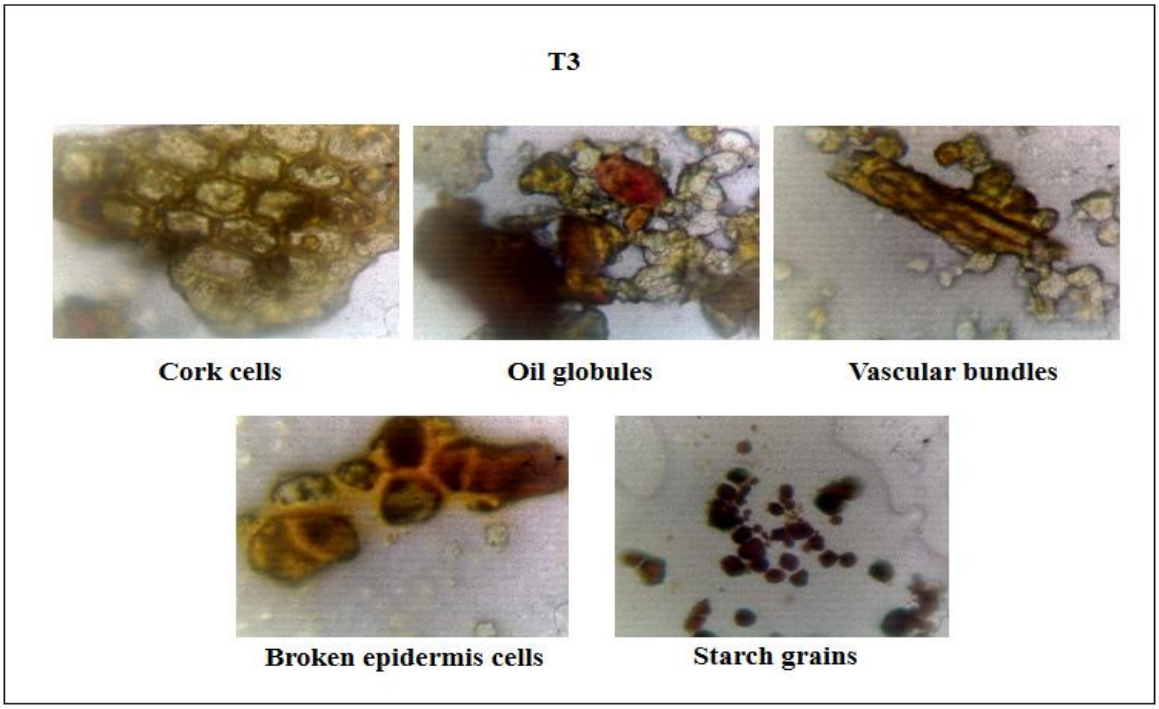


Figure 2f: Powder Microscopy of T3



3.1.3 Physico-Chemical Investigations Results:

The investigations were done and the calculations were carried out. (Table 5)

Table 5: Physico-chemical investigations results of raw materials and formulations:

Sl. no	Raw materials	Extractive Values		Ash Values			Loss on Drying	pH
		Alcohol	Water	Water soluble	Acid insoluble	Total		
1	***Abies webbina*** **(Talisapatra)**	8.40%	4.20%	3.05%	1.98%	5.16%	8.20%	5.33
2	***Piper nigrum*** **(Maricha)**	5.68%	2.20%	6.58%	2.30%	11.05%	8.80%	6.31
3	***Elettaria cardamomum*** **(Elaichi)**	5.64%	4.50%	4.21%	3.32%	6.09%	8.93%	5.3
4	***Cinnamomum zeylanicum*** **(Dalchini)**	11.60%	2.70%	6.09%	4.11%	8.40%	9.70%	5.59
5	***Bambusa bamboo*** **(Vamshalochana)**	1.32%	8.32%	0.63%	0.56%	1.25%	10.50%	6.59
6	***Piper longum*** **(Pipalli)**	15%	11.75%	3.12%	1.95%	8.45%	10.90%	6.01
7	***Zingiber officinalis*** **(Sunthi)**	5.60%	4%	2.13%	1.86%	5.58%	9.80%	5.36
8	**S1**	5.80%	13.40%	10.15%	15.18%	22.05%	5%	7.1
9	**S2**	6%	14.30%	11.03%	16.02%	23.15%	6.30%	7.16
10	**S3**	5.67%	15%	10.56%	15.99%	22.68%	5.60%	7.2
11	**T1**	3.60%	5.38%	7.60%	19%	1.55%	4%	6.05
12	**T2**	3.30%	5.98%	8.10%	18.50%	1.43%	4.70%	6.1
13	**T3**	3.80%	5.66%	8.26%	18.80%	1.49%	5%	6.16

3.1.4 Results of Microbial Study:

The microbial results were also found within the acceptance range in accordance with the API.

Figure 3: Microbial Study of Sitopaladi Churna and Talisadi Churna formulations

S1	S2	S3
		
T1	**T2**	**T3**
		

3.1.5 Results of Physical Powder Characteristics:

The physical powder characteristics were carried out in accordance with USP and the formulation samples were found to be within the acceptance criteria.

Table 6: Physical powder characterization of churna:

Sl. no	Formulations	Bulk density (gm/ml)	Tap density (gm/ml)	Angle of repose (^{0})	Hausner's ratio (gm/ml)	Carr's Index (%)
1	**S1**	0.5555	0.6666	25.28	1.31	16.66
2	**S2**	0.5882	0.6578	23.3	1.24	10.58
3	**S3**	0.5263	0.6622	27.85	1.19	20.52
4	**T1**	0.5952	0.7042	25.22	1.1831	10.9
5	**T2**	0.5882	0.7142	24.14	1.2142	17.64
6	**T3**	0.5813	0.6896	24.67	1.1863	15.7

3.2 Extra-Pharmacopeial Evaluations:

3.2.1 Results of Organoleptic Characters:

The organoleptic characters of both raw materials as well as the formulations were analysed and are reported (Table 7a and 7b)

Table 7a: Organoleptic Characterization Results of Raw Materials

Sl. no	Raw materials	Organoleptic characters		
		Color	**Odor**	**Taste**
1	***Abies webbina* (Talisapatra)**	Green color leaves Brown when dried	Aromatic	Astringent
2	***Piper nigrum* (Maricha)**	Black	Aromatic	Very Pungent
3	***Elettaria cardamomum* (Elaichi)**	Capsule – Greenish color Seeds- brown color	Aromatic, Characteristics	Menthol-like, warm and pungent characteristic taste
4	***Cinnamomum zeylanicum* (Dalchini)**	Tan brown or warm shade of brown	Sweet, spicy and warm fragrance	Sweet and woody flavor with slightly spicy characteristic taste
5	***Bambusa Bamboo* (Vamshalochana)**	White to off white in color	Slightly like the bamboo shoots	Tasteless
6	***Piper longum* (Pipalli)**	Brownish black in color	Peculiar and characteristic odor	In dry form strongly pungent in taste
7	***Zingiber officinalis* (Sunthi)**	Dull cream	Characteristic aromatic and pungent	Strongly pungent in taste

Table 7b: Organoleptic Characterization Results of Formulations:

Sl. no	Raw materials	Organoleptic characters				
		Color	**Odor**	**Taste**	**Appearance**	**Texture**
1	S1	Dutch white	Characteristic	Sweet with slight pungent taste	Powder	Very smooth
2	S2	Dutch white	Characteristic	Sweet with slight pungent taste	Powder	Very smooth
3	S3	Dull white	Characteristic	Sweet with strong pungent taste	Powder	Smooth
4	T1	Dull white	Characteristic	Slightly sweet	Powder	Smooth
5	T2	Dull white	Characteristic	Slightly sweet	Powder	Smooth
6	T3	Dull white	Characteristic	Slightly sweet	Powder	Smooth

3.2.2 Macroscopic Characters: The macroscopic study was carried for all the raw materials and the analysed results are reported (Table 8)

Table 8: Macroscopic Study Results of Raw Materials

Sl. no	Raw materials	Macroscopic characters		
		Shape	**Size**	**Surface and texture description**
1	***Abies webbina*** **(Talisapatra)**	The leaves have needle shape as they belong to conifer category.	1.3-2.2 cm in length	Single, thin leaves with spiral arrangement
2	***Piper nigrum*** **(Maricha)**	Globular berries	3.2-6 mm in diameter	Coarse, rough and wrinkled
3	***Elettaria cardamomum*** **(Elaichi)**	Oblongated in shape	1-2 cm in length	Surface has furrows and ridges and rough in nature
4	***Cinnamomum zeylanicum*** **(Dalchini)**	Longitudinal and wavy	2.5-8 cm in length	Thin with curls on one side and slightly smooth surface
5	***Bambusa bamboo*** **(Vamshalochana)**	Uneven crystals	-	Smooth powder
6	***Piper longum*** **(Pipalli)**	Oblong or long rod like shape	6-8 cm in length	Patterned crevices on the surface, rough surface due to crevices.
7	***Zingiber officinalis*** **((Sunthi)**	Compressed flat and ovate shape	5-9cm in length; 1-2cm in width	Smooth with striations

3.2.3 Fluorescence results: The fluorescence studies were carried out for all the raw materials (Table 9a-g) and also the formulations (table 10a-f).

Table 9a: Fluorescence Analysis of *Abies webbina* (Talisapatra)

Sl. No	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	*Abies webbina* (Talisapatra)-powder	Brown	Brown	Brown
2	Powder + Conc. HCl	Light Brown	Light Brown	Brown
3	Powder + 10% $K_2Cr_2O_7$	Yellowish Brown	Light Brown	Brown
4	Powder + 1M NaOH	Dark Brown	Brown	Dark Brown
5	Powder + Conc.HNO_3	Orange	Dark orange	Brown
6	Powder + Conc.H_2SO_4	Dark Brown	Brown	Brown
7	Powder + Methanol	Light Brown	Light Brown	Brown
8	Powder + CH_3COOH	Reddish Brown	Pale Brown	Brown
9	Powder + NH_3	Brown	Brown	Brown
10	Powder + I_2	Brown	Brown	Brown

Table 9b: Fluorescence Analysis of *Piper nigrum* (Maricha)

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	*Piper nigrum* (Maricha)-powder	Dark brown	Dark brown	Dark brown
2	Powder + Conc. HCl	Pale yellow	Orangish yellow	Orange
3	Powder + 10% $K_2Cr_2O_7$	Yellow	Dark yellow	Light brown
4	Powder + 1M NaOH	Light yellow	Dark yellow	Brown
5	Powder + Conc.HNO_3	Orange	Orange	Brown
6	Powder + Conc.H_2SO_4	Brown	Brown	Brown
7	Powder + Methanol	Dark cream	Yellow	Brown
8	Powder + CH_3COOH	Cream	Light Yellow	Brown
9	Powder + NH_3	Cream	Yellow	Brown
10	Powder + I_2	Black	Black	Black

Table 9c: Fluorescence Analysis of *Elettaria cardamomum* (Elaichi)

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	*Elettaria cardamomum* (Elaichi)- powder	Olive green	Light green	Light green
2	Powder + Conc. HCl	Yellowish orange	Orange	Brown
3	Powder + 10% $K_2Cr_2O_7$	Orangish yellow	Orange	Dark brown
4	Powder + 1M NaOH	Olive green	Green	Brown
5	Powder + Conc.HNO_3	Orange	Light brown	Dark brown
6	Powder + Conc.H_2SO_4	Brown	Brown	Dark brown
7	Powder + Methanol	Pale green	Orange	Brown
8	Powder + CH_3COOH	Creamish green	Orange	Dark brown
9	Powder + NH_3	Olive green	Green	Dark green
10	Powder + I_2	Green	Dark green	Black

Table 9d: Fluorescence Analysis of *Cinnamomum zeylanicum* (Dalchini)

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	*Cinnamomum zeylanicum* (Dalchini)- powder	Orangish brown	Brown	Brown
2	Powder + Conc. HCl	Maroon	Dark maroon	Brown
3	Powder + 10% $K_2Cr_2O_7$	Yellow	Light brown	Brown
4	Powder + 1M NaOH	Dark brown	Dark brown	Dark brown
5	Powder + Conc.HNO_3	Yellowish orange	Orange	Brown
6	Powder + Conc.H_2SO_4	Brown	Dark brown	Darker brown
7	Powder + Methanol	Orange	Dark orange	Dark orange
8	Powder + CH_3COOH	Reddish brown	Brown	Dark brown
9	Powder + NH_3	Brown	Dark brown	Dark brown
10	Powder + I_2	Light brown	Brown	Brown

Table 9e: Fluorescence Analysis of *Bambusa bamboo* (Vamshalochana)

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	*Bambusa bamboo* (Vamshalochana)-powder	White	Pale white	Pale white
2	Powder + Conc. HCl	White	White	White
3	Powder + 10% $K_2Cr_2O_7$	Yellow	Yellow	Light brown
4	Powder + 1M NaOH	Pale White	Pale White	White
5	Powder + Conc.HNO_3	Cream	Pale cream	Dark cream
6	Powder + Conc.H_2SO_4	Light orange	Orange	Light brown
7	Powder + Methanol	Snow white	White	Dull white
8	Powder + CH_3COOH	White	White	Dull White
9	Powder + NH_3	Pale white	White	Dull white
10	Powder + I_2	Black	Black	Black

Table 9f: Fluorescence Analysis of *Piper longum* (Pipalli)

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	*Piper longum* (Pipalli) - powder	Brown	Brown	Brown
2	Powder + Conc. HCl	Orangish brown	Brown	Light brown
3	Powder + 10% $K_2Cr_2O_7$	Yellowish brown	Dark yellow	Brown
4	Powder + 1M NaOH	Orangish brown	Light brown	Brown
5	Powder + Conc.HNO_3	Brown	Brown	Brown
6	Powder + Conc.H_2SO_4	Orange	Brown	Brown
7	Powder + Methanol	Brown	Light brown	Brown
8	Powder + CH_3COOH	Brown	Brown	Brown
9	Powder + NH_3	Yellowish brown	Dark yellow	Brown
10	Powder + I_2	Dark brown	Black	Black

Table 9g: Fluorescence Analysis of *Zingiber officinalis* (Sunthi)

Sl. no	**Drug**	**Visible/Day light**	**UV light**	
			254 nm	**366nm**
1	*Zingiber officinalis* (Sunthi)- powder	Dull cream	Dull cream	Creamish
2	Powder + Conc. HCl	Orange	Brown	Brown
3	Powder + 10% $K_2Cr_2O_7$	Orange	Light orange	Dark brown
4	Powder + 1M NaOH	Yellow	Yellow	Dark brown
5	Powder + Conc.HNO_3	Light orange	Dark brown	Brown
6	Powder + Conc.H_2SO_4	Dark brown	Dark brown	Dark brown
7	Powder + Methanol	Light cream	Cream	Light brown
8	Powder + CH_3COOH	Light cream	Light cream	Light brown
9	Powder + NH_3	Pale yellow	Pale yellow	Pale brown
10	Powder + I_2	Black	Black	Black

Table 10a: Fluorescence Analysis of S1

Sl. no	**Drug**	**Visible/Day light**	**UV light**	
			254 nm	**366nm**
1	S1	Dutch white	Dutch white	Dutch white
2	S1+ Conc. HCl	Creamish brown	Yellow	Light yellow
3	S1 + 10% K2Cr2O7	Yellow	Dark yellow	Orangish yellow
4	S1+ 1M NaOH	Brown	Dark brown	Light brown
5	S1+ Conc.HNO3	Yellowish orange	Yellow	Yellow
6	S1 + Conc.H2SO4	Reddish brown	Brown	Brown
7	S1 + Methanol	Cream	Creamish yellow	Light brown
8	S1 + CH3COOH	Yellow	Pale Yellow	Light brown
9	S1 + NH3	Light brown	Cream brown	Brown
10	S1 + I2	Black	Black	Black

Table 10b: Fluorescence Analysis of S2

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	S2	Off white	Off white	Off white
2	S2+ Conc. HCl	Orange brown	Dull yellow	Light brown
3	S2 + 10% K2Cr2O7	Yellow	Dull yellow	Brown
4	S2+ 1M NaOH	Light brown	Light brown	Brown
5	S2+ Conc.HNO3	Orange	Light brown	Dark brown
6	S2 + Conc.H2SO4	Light brown	Light brown	Brown
7	S2 + Methanol	Cream	Cream	Light brown
8	S2 + CH3COOH	Pale cream	Pale cream	Cream
9	S2 + NH3	Light brown	brown	Dull brown
10	S2 + I2	Black	Black	Black

Table 10c: Fluorescence Analysis of S3

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	S3	Pale white	Pale white	Pale white
2	S3+ Conc. HCl	Brown	Brown	Brown
3	S3 + 10% K2Cr2O7	Brown	Brown	Dark brown
4	S3+ 1M NaOH	Brown	Light brown	Brown
5	S3+ Conc.HNO3	Orange	Dark orange	Light brown
6	S3 + Conc.H2SO4	Maroon brown	Dark brown	Dark brown
7	S3 + Methanol	Cream	Cream	Light brown
8	S3 + CH3COOH	Cream	Pale cream	Yellow
9	S3 + NH3	Brown	Light brown	Brown
10	S3 + I2	Black	Black	Black

Table 10d: Fluorescence Analysis of T1

Sl. no	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	T1	Dull white	Dull white	Dull white
2	T1+ Conc. HCl	Light brown	Yellowish brown	Brown
3	T1 + 10% $K_2Cr_2O_7$	Yellowish brown	Brown	Dark Brown
4	T1+ 1M NaOH	Brown	Creamish brown	Dark brown
5	T1+ Conc.HNO_3	Orange	Dark brown	Dark Brown
6	T1 + Conc.H_2SO_4	Chocolate brown	Dark Brown	Dark Brown
7	T1 + Methanol	Pale cream	Yellowish brown	Brown
8	T1 + CH_3COOH	Pale Brown	Pale Brown	Brown
9	T1 + NH_3	Brown	Light Brown	Light Brown
10	T1 + I_2	Black	Black	Black

Table 10e: Fluorescence Analysis of T2

Sl. No	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	T2	Dull white	Dull white	Dull white
2	T2+ Conc. HCl	Light Brown	Yellowish Brown	Brown
3	T2 + 10% $K_2Cr_2O_7$	Brown	Brown	Dark Brown
4	T2+ 1M NaOH	Light Brown	Light Brown	Brown
5	T2+ Conc.HNO_3	Orange	Pale Brown	Brown
6	T2 + Conc.H_2SO_4	Maroon-brown	Dark Brown	Dark Brown
7	T2 + Methanol	Dull orange	Pale yellow	Brown
8	T2 + CH_3COOH	Pale Brown	Light Brown	Light Brown
9	T2 + NH_3	Light Brown	Light Brown	Light Brown
10	T2 + I_2	Black	Black	Black

Table 10f: Fluorescence Analysis of T3

Sl. No	Drug	Visible/Day light	UV light	
			254 nm	366nm
1	T3	Dull white	Dull white	Dull white
2	T3+ Conc. HCl	Light Brown	Light Brown	Brown
3	T3 + 10% $K_2Cr_2O_7$	Creamish Brown	Creamish Brown	Brown
4	T3+ 1M NaOH	Dull Brown	Dull Brown	Light Brown
5	T3+ Conc.HNO_3	Orange	Brown	Brown
6	T3 + Conc.H_2SO_4	Reddish Brown	Dark Brown	Dark Brown
7	T3 + Methanol	Creamish Brown	Creamish Brown	Brown
8	T3 + CH_3COOH	Dull Brown	Dull Brown	Light Brown
9	T3 + NH_3	Light Brown	Light Brown	Light Brown
10	T3 + I_2	Black	Black	Black

3.2.4 Results of Qualitative Phyto-Chemical Investigations:

Table 11a: Phyto-Chemical Investigations of Raw Material:

Sl. no	Raw Materials	Extracts	Phyto-constituents
1	*Abies Webbina* (Talisapatra)	Aqueous extract	A, Ste, G, C, P
		Methanolic extract	A, Ste, G, F, C, P
		Ethyl acetate extract	A, Ste, G, C,
		Chloroform extract	A, Ste, G, C,
		Petroleum ether extract	A, Ste, G,
2	*Piper nigrum* (Maricha)	Aqueous extract	A, T, Ste, G, C, P
		Methanolic extract	A, Ste, G, C, P
		Ethyl acetate extract	A, Ste, G, C, P, Ter
		Chloroform extract	A, Ste, G, C, P
		Petroleum ether extract	A, Ste, G,
3	*Elettaria cardamomum* (Elaichi)	Aqueous extract	A, C, P
		Methanolic extract	A, C, P
		Ethyl acetate extract	A, G, F, C, P
		Chloroform extract	A, C, P
		Petroleum ether extract	-
4	*Cinnamomum zeylanicum* (Dalchini)	Aqueous extract	A, G, F, C, P
		Methanolic extract	A, G, F, C, P
		Ethyl acetate extract	A, Ste, G, F, C, P
		Chloroform extract	-
		Petroleum ether extract	-
5	*Piper longum* (Pipalli)	Aqueous extract	Sap, G, C, P,
		Methanolic extract	A, Sap, G, C, P,
		Ethyl acetate extract	Ste, G, C,
		Chloroform extract	Ste, G, C, P,
		Petroleum ether extract	Ste,
6	*Zingiber Officinalis* (Sunthi)	Aqueous extract	A, G, Sap, C, P
		Methanolic extract	A, G, F, C, P
		Ethyl acetate extract	A, G, C, P
		Chloroform extract	A, Ste, G, Sap, C, P
		Petroleum ether extract	-

Table 11b: Phyto-Chemical Investigations of Formulations:

Sl. no	Churna Formulations	Extracts	Phyto-constituents
1	S1	Aqueous extract	A, Ste, F, C, P
		Methanolic extract	A, Ste, F,C, P
		Ethyl acetate extract	A, Ste, C, P
		Chloroform extract	A, Ste, C, P
		Petroleum ether extract	A, Ste, C, P
2	S2	Aqueous extract	A, F,S, C, P
		Methanolic extract	A, F,S, C, P
		Ethyl acetate extract	A, F, C, P
		Chloroform extract	A, F, C, P
		Petroleum ether extract	A, C
3	S3	Aqueous extract	A, F,S, C, P
		Methanolic extract	A, F,S, C, P
		Ethyl acetate extract	A, F, C, P
		Chloroform extract	A, F, C, P
		Petroleum ether extract	A, C
4	T1	Aqueous extract	A, F, S, C, P
		Methanolic extract	A, F, C, P
		Ethyl acetate extract	A, F, S, C, P
		Chloroform extract	A, F, C, P
		Petroleum ether extract	A, F, S, C, P
5	T2	Aqueous extract	A, F, C, P, G
		Methanolic extract	A, F, C, P, G
		Ethyl acetate extract	A, F, C, P, G
		Chloroform extract	A, C, P, G
		Petroleum ether extract	A, F, C, P
6	T3	Aqueous extract	A, F, C, P, G
		Methanolic extract	A, F, C, P, G
		Ethyl acetate extract	A, F, C, P
		Chloroform extract	A, F, C, P
		Petroleum ether extract	A, F, C, G

3.2.5 Quantitative Phyto-Chemical Investigations:

The Quantitative phyto-chemical investigations of both the churnas (Sitopaladi and Talisadi Churna) were done for determining Total content of phenolics, flavanoids, alkaloids, tannins. The antioxidant potential of the samples was also carried out by DPPH Scavenging assay.

Figure 4: Quantitative Phyto-Chemical Investigations of Sitopaladi Churna

Figure 4a: Total Phenolic Content of Sitopaladi Churna

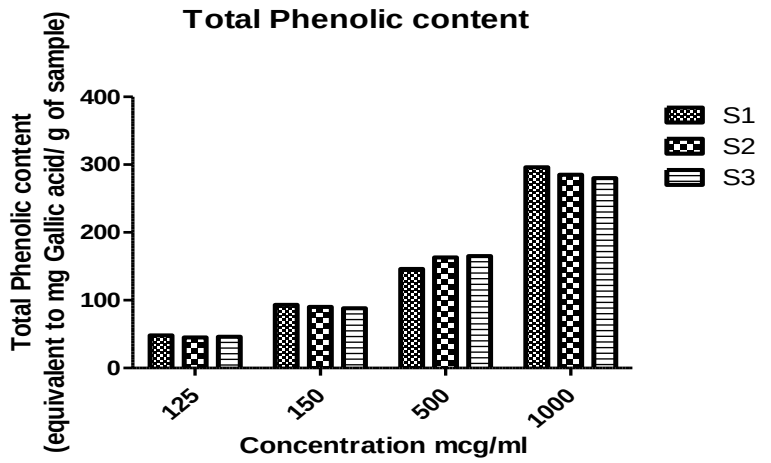


Figure 4b: Total Flavonoid Content of Sitopaladi Churna

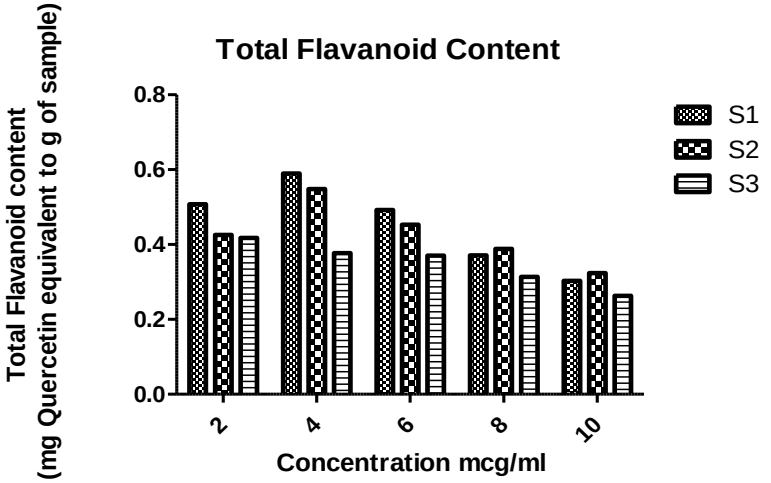


Figure 4c: Total Alkaloid Content of Sitopaladi Churna

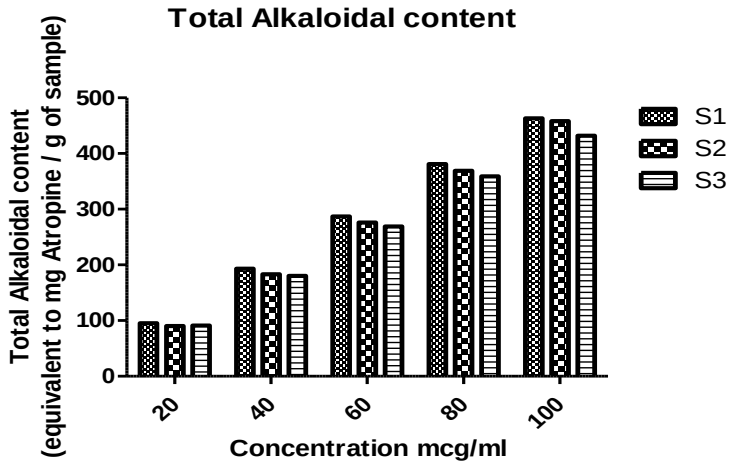


Figure 4d: Total Tannin Content of Sitopaladi Churna

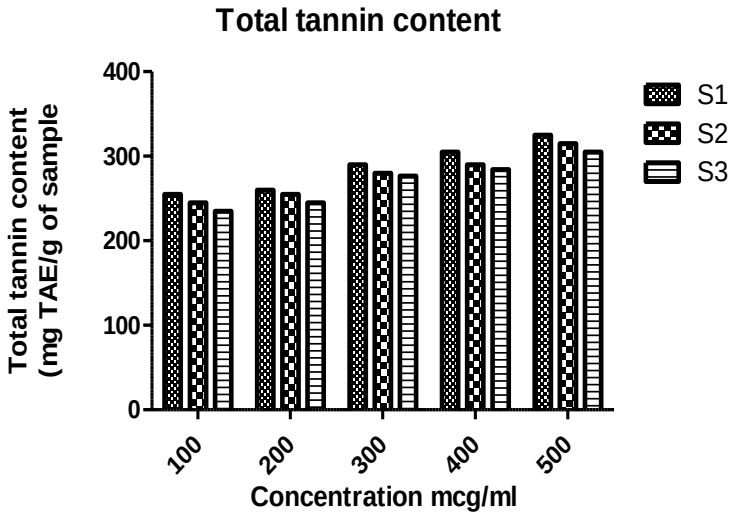


Figure 4e: Anti-oxidant Potential of Sitopaladi Churna

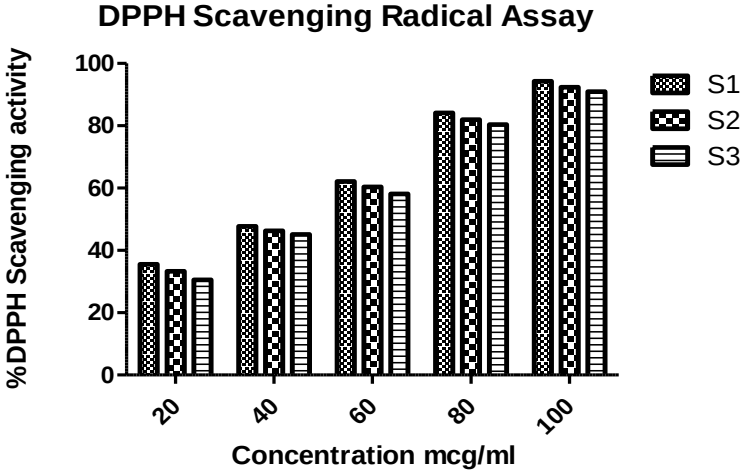


Figure 5: Quantitative Phyto-Chemical Investigations of Talisadi Churna

Figure 5a: Total Phenolic Content of Talisadi Churna

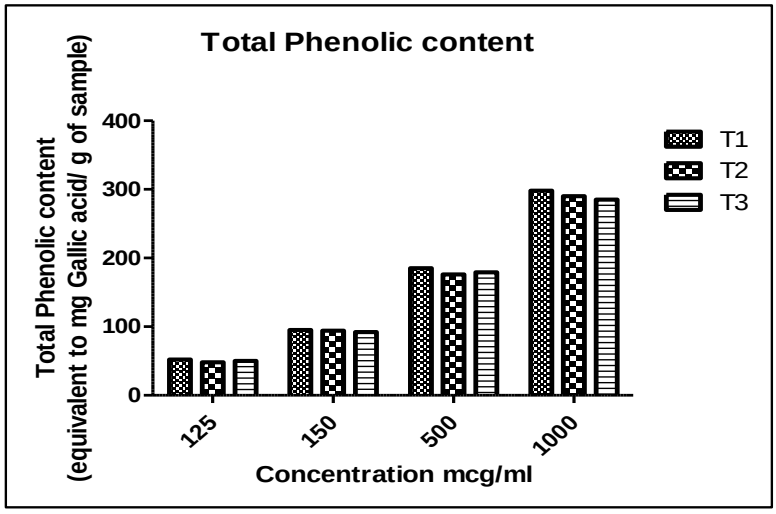


Figure 5b: Total Flavonoid Content of Talisadi Churna

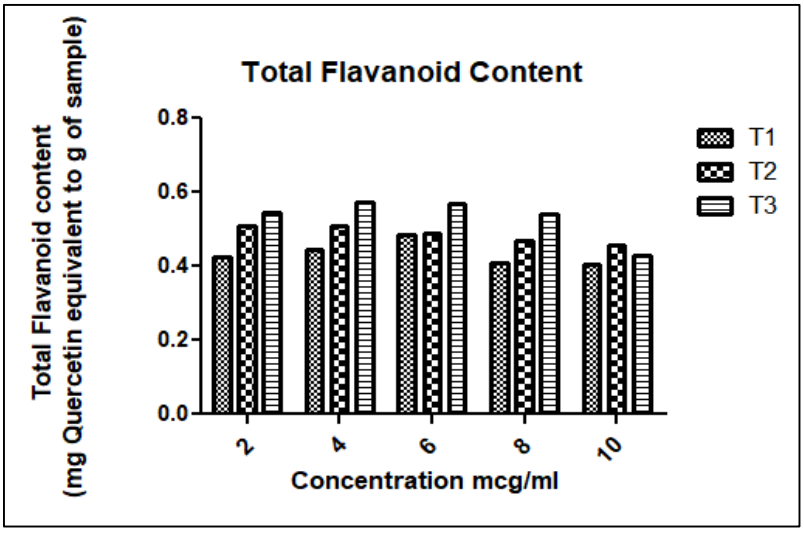


Figure 5c: Total Alkaloid Content of Talisadi Churna

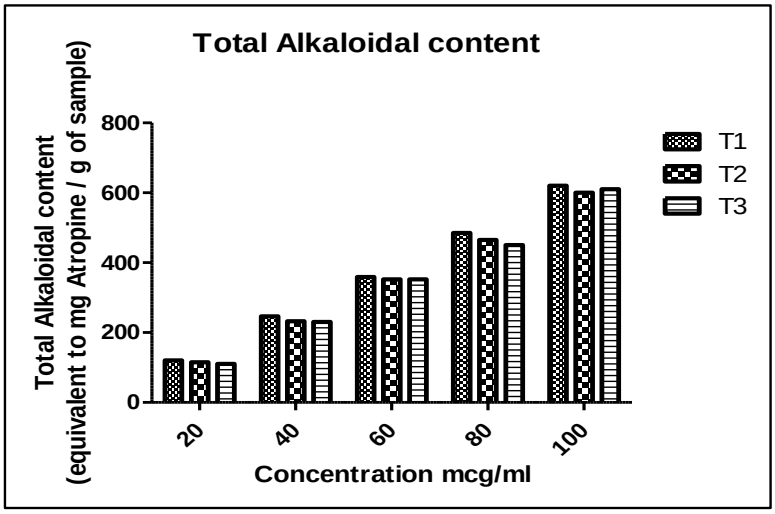


Figure 5d: Total Tannin Content of Talisadi Churna

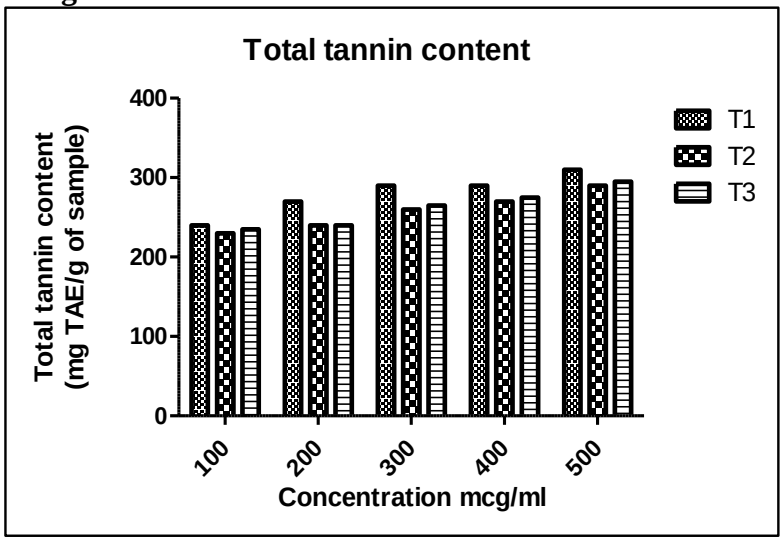
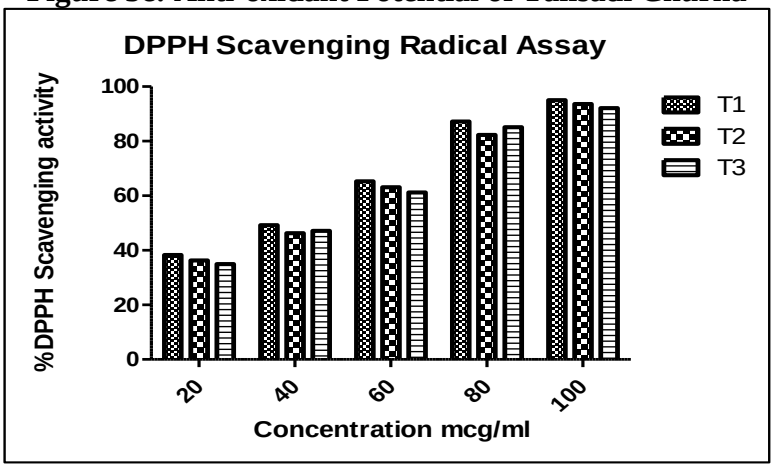


Figure 5e: Anti-oxidant Potential of Talisadi Churna



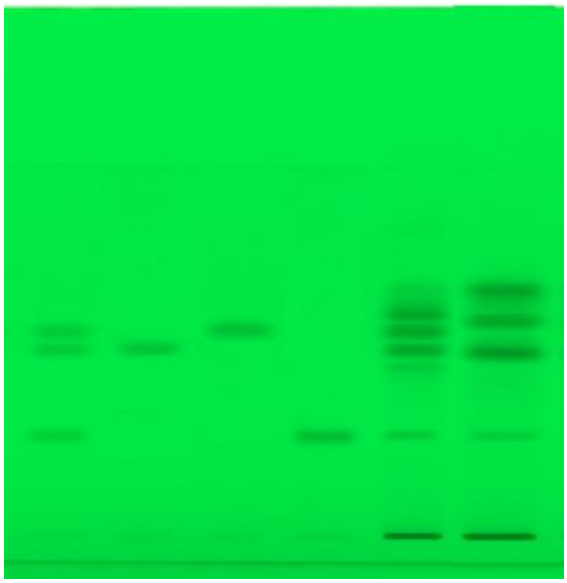
3.3 HPTLC results:

3.3.1 HPTLC Method Development and Validation:

Table 12: Chromatographic conditions of the developed simultaneous method

Parameters	**Chromatographic conditions**
Sample applicator	CAMAG Linomat V
Sample application rate	0.1µL/s
Stationary Phase	TLC plate silica gel G 60 F_{254} pre-coated
Mobile Phase (v/v/v)	Ethyl acetate : Hexane : Formic acid 5 : 5: 1
Plate development distance	70 millimeter
Chamber saturation time	5 minutes
Scanner	CAMAG tlc Scanner 4
Software	visionCATS

Figure 6: Plate image of the developed simultaneous method



Track 1: Mixture; Track 2: Piperine; Track 3: Cinnamic acid;
Track 4: Gallic acid; Track 5: Sample 1; Track 6: Sample

Figure 7: Pictorial Representation of the AGREE scale for the simultaneous developed method

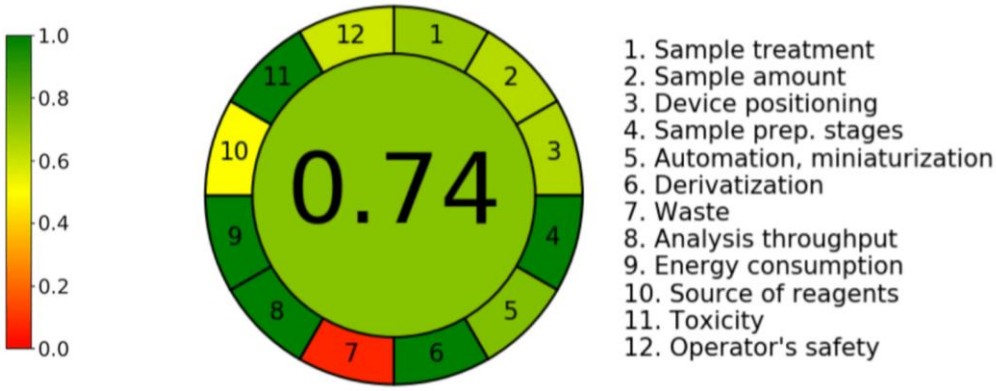
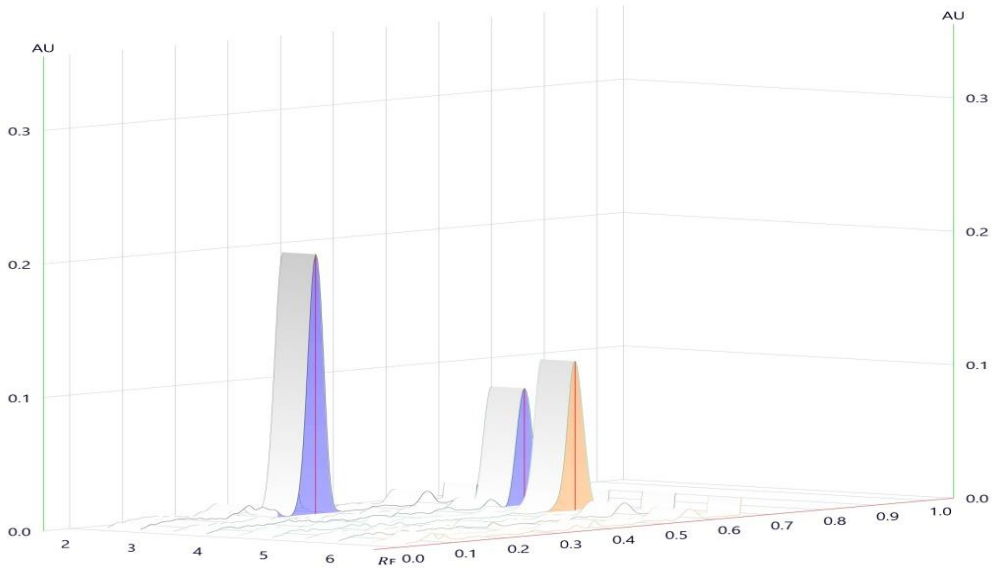


Figure 8: Specificity Parameter Chromatograms



Track 2-4: Standards; Track 5:Mobile Phase ;Track 6:Methanol;

Table 13: Validation parameter results of simultaneous method

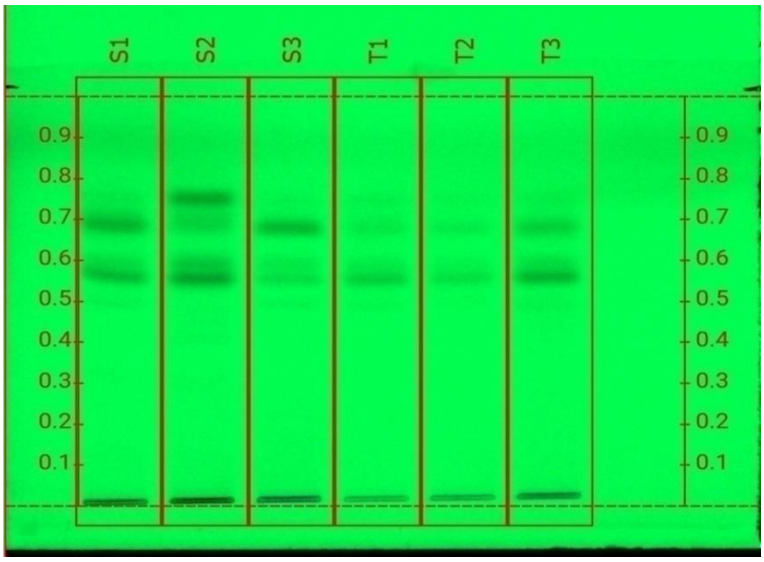
Parameters	Cinnamic acid	Gallic acid	Piperine
Linearity	y= 0.0009x+0.0005	y= 0.0016x-0.0005	y= 0.0009x-0.0002
Range (µL/band)	1-5	1-5	1-5
R^2	0.989	0.998	0.993
LOD(µg/mL)	0.525056	0.524666	0.53896
LOQ(µg/mL)	1.59108	1.5898	1.6332
Precision %RSD			
Intra-day Precision (%RSD)			
Rf value	1.018922026	0.56765	1.4769
Area under curve	0.009501	0.63145	0.5208
Inter-day Precision (%RSD)			
Rf value	0.2513	0.9211	0.1002
Area under curve	0.8424	1.1226	1.6284
Robustness (Rf value) %RSD			
Mobile phase composition	0.0950	0.6314	0.1002
Mobile phase volume	0.2513	0.4638	0.5208
Duration of chamber saturation	0.1900	1.0663	0.17421

3.3.2 Fingerprinting:

Figure 9: HPTLC fingerprinting images

Figure 9a: HPTLC fingerprinting

At 254nm



At 366nm

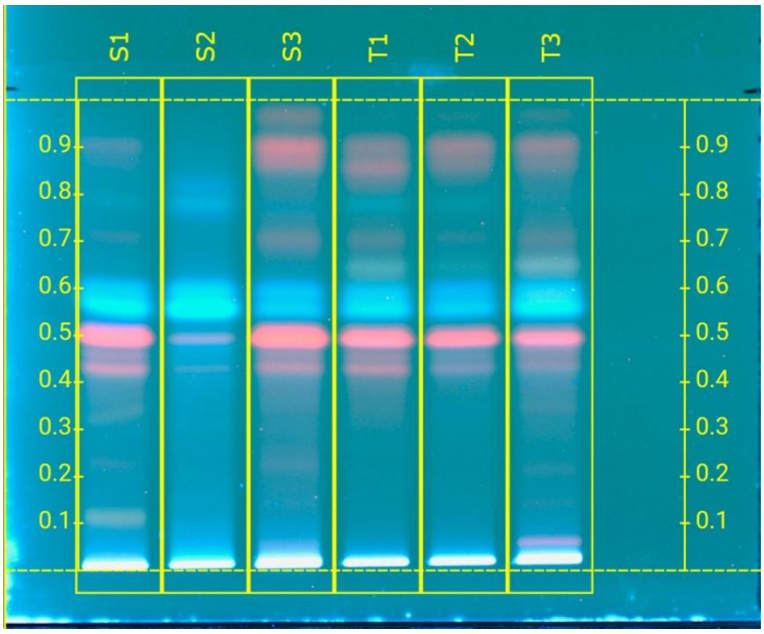
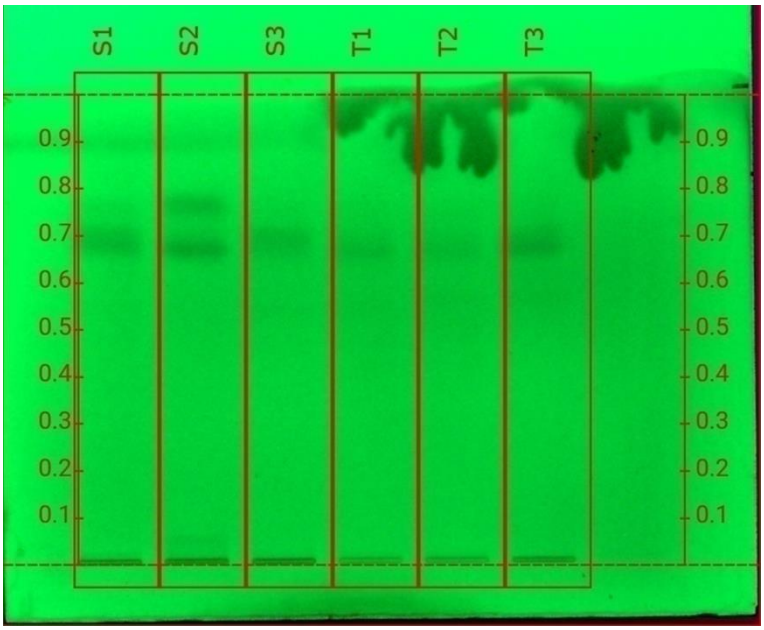


Figure 9b: HPTLC fingerprinting images of Alkaloids

At 254nm



At 366 nm

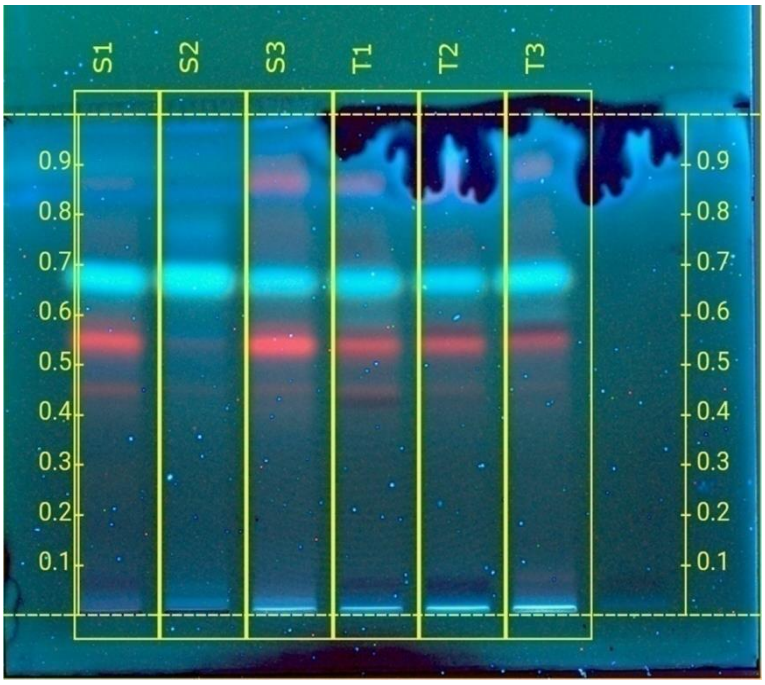
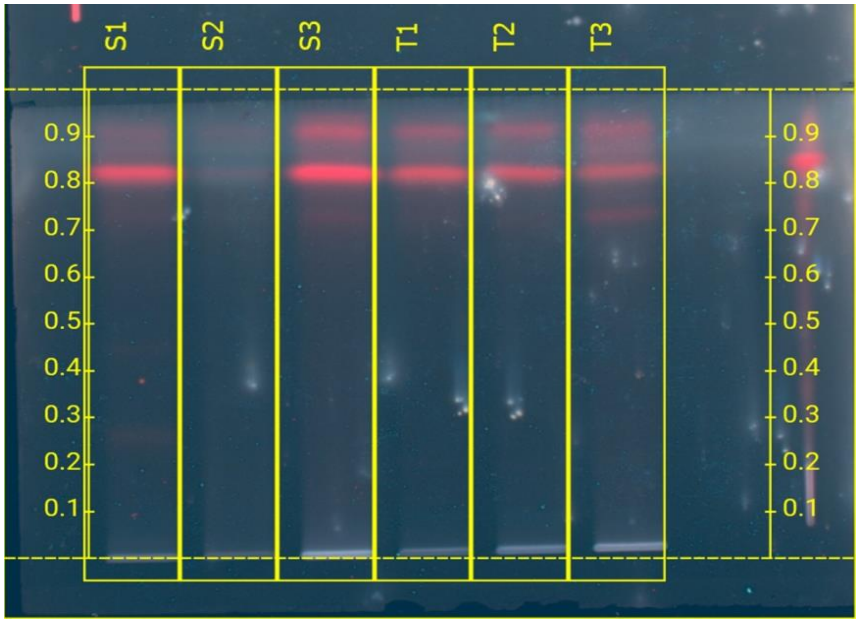


Figure 9c: HPTLC fingerprinting images of Flavonoids

Before Derivatization



After Derivatization

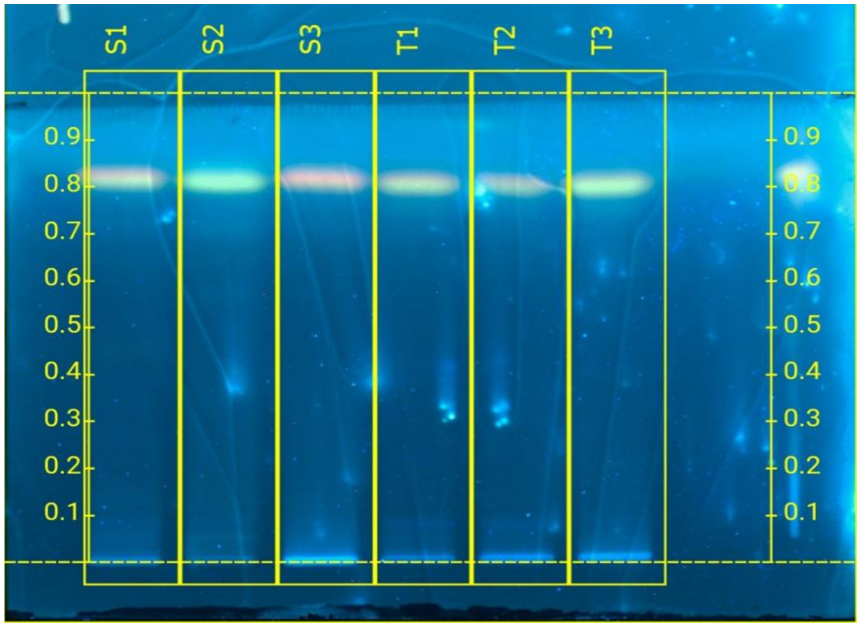
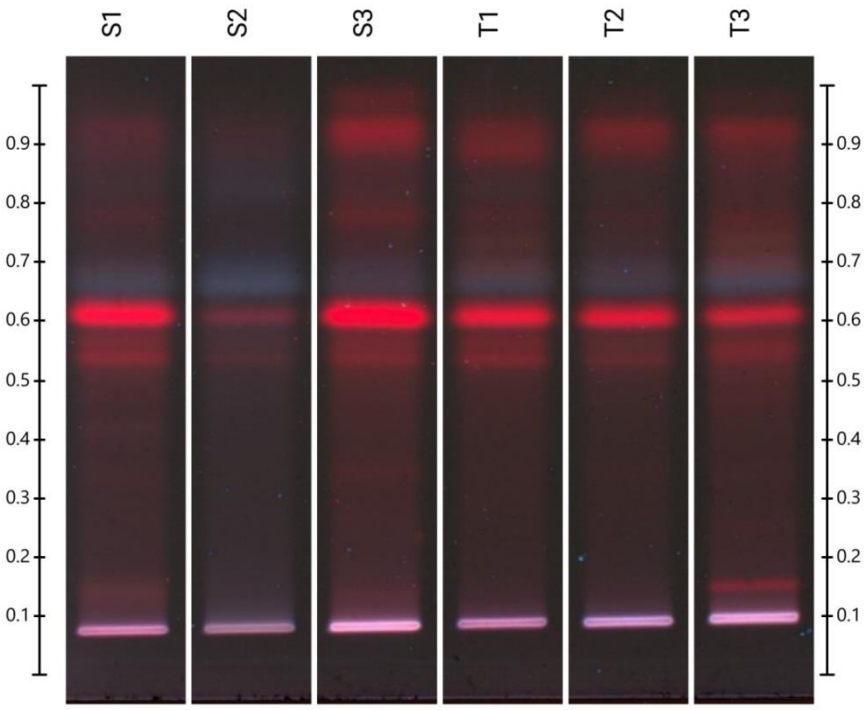


Figure 9d: HPTLC fingerprinting images of Tannins

Before Derivatization



After Derivatization

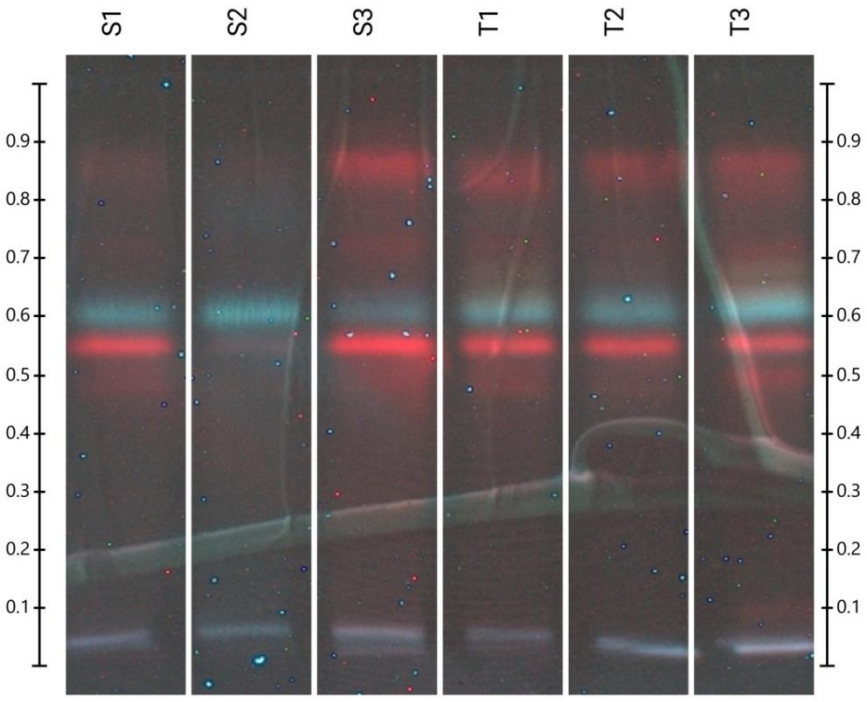
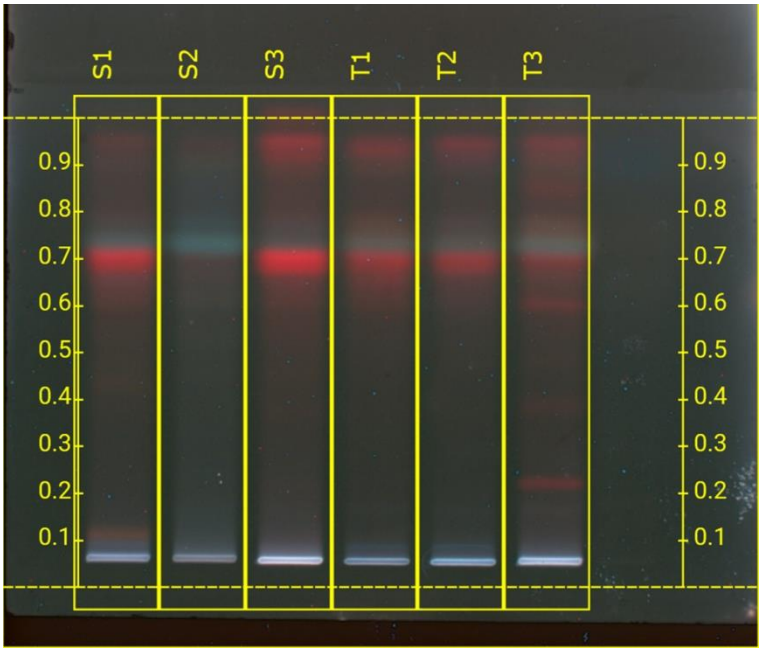


Figure 9e: HPTLC fingerprinting images of Phenolics

Before Derivatization



After Derivatization

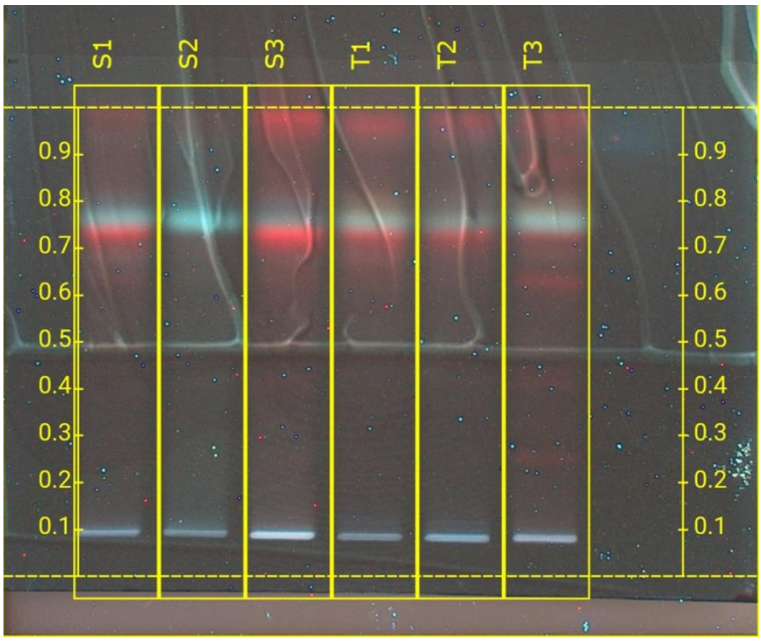
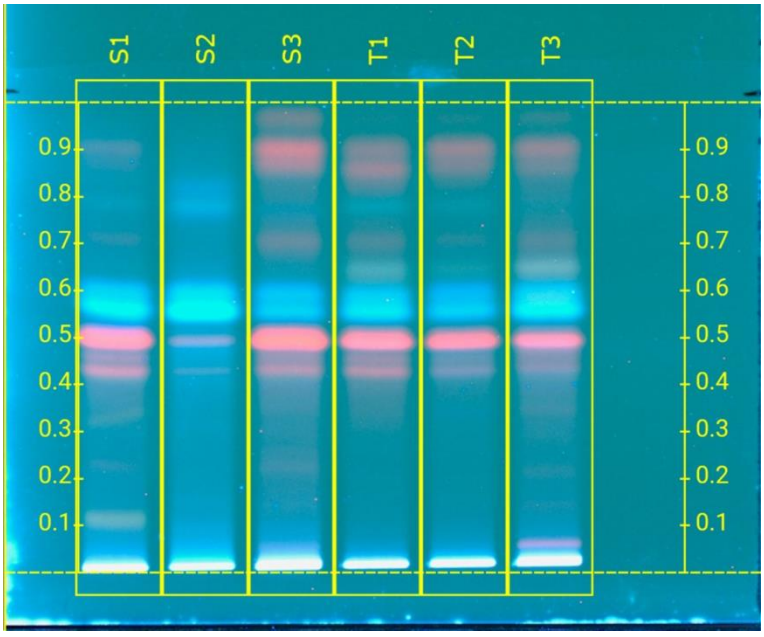
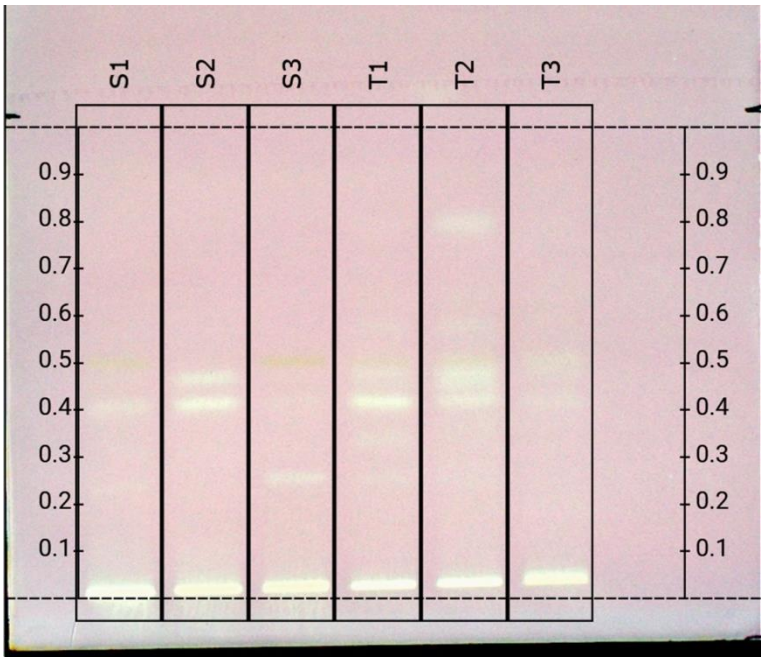


Figure 9f: HPTLC fingerprinting images of DPPH assay

Before Derivatization



After Derivatization



3.3.3 Quantification of Piperine: The Piperine sample is applied on the plates along with the churna formulations.

Figure 10: Chemical structure of Piperine

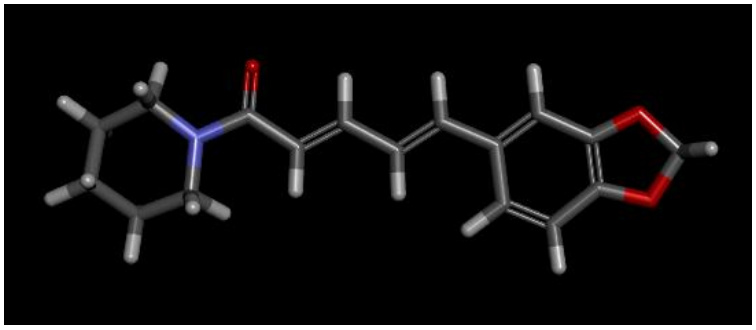


Figure 11: Chromatogram of Standard Piperine

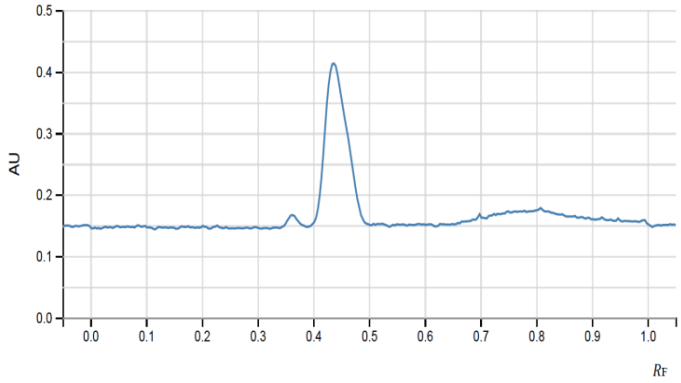


Figure 12: Spectra of Piperine:

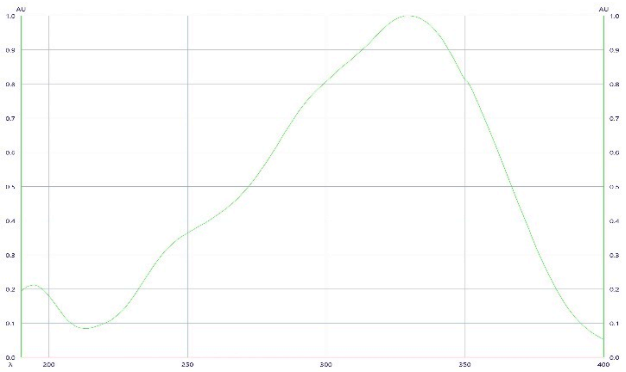


Table 14: Box-Behneken design

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
		A: Volume of Solvent (mL)	B: Solvent front (cm)	C: Saturation time (minutes)	Rf value	Area
14	1	8	8	10	0.561	0.00516
16	2	8	8	10	0.569	0.00523
8	3	9	8	15	0.325	0.00625
1	4	7	7	10	0.621	0.00500
5	5	7	8	5	0.666	0.00567
9	6	8	7	5	0.437	0.00468
12	7	8	9	15	0.512	0.00408
2	8	9	7	10	0.368	0.00685
13	9	8	8	10	0.459	0.00597
7	10	7	8	15	0.682	0.005002
11	11	8	7	15	0.492	0.005998
15	12	8	8	10	0.563	0.00518
3	13	7	9	10	0.684	0.00564
10	14	8	9	5	0.456	0.00569
17	15	8	8	10	0.562	0.0052
6	16	9	8	5	0.386	0.006
4	17	9	9	10	0.398	0.00685

Table 15: Statistical analysis of the Box-Behneken design

Response	Equation	Model	p-value	PRESS	CV (%)	S.D
Rf value	+0.5142-0.1470A +0.0165B+0.0083C	Linear	0.0310	0.0414	1.507	0.0445
Area	+0.0053+0.0006A-0.001C-0.0002AB+ 0.0002AC-0.0007A^2+0.0001B^2-0.0003C^2	Linear	0.0179	0.0977	1.721	0.0400

Table 16a: ANOVA analysis for Response 1- Rf value

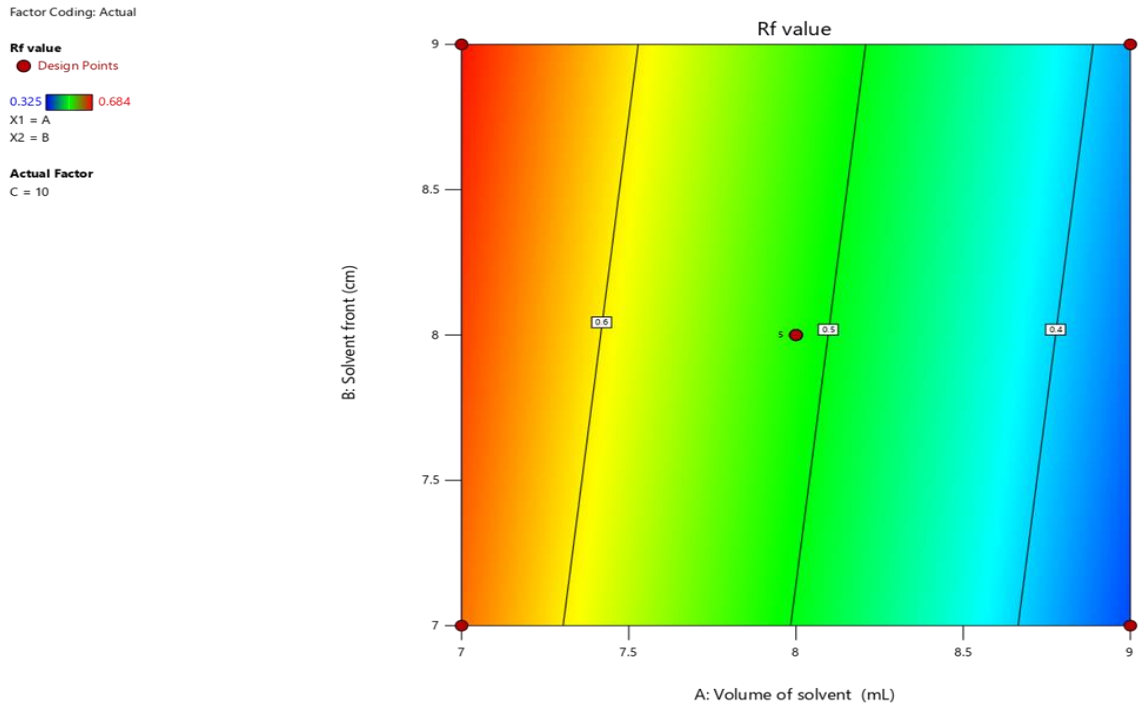
Source	Sum of squares	df	Mean square	F-value	p-value	
Model	0.1756	3	0.0585	29.60	0.0310	**Significant**
A-Volume of solvent	0.1729	1	0.1729	87.43	< 0.0001	
B-Solvent front	0.0022	1	0.0022	1.10	0.3130	
C-Saturation time	0.0005	1	0.0005	0.2754	0.6086	
Residual	0.0257	13	0.0020			
Lack of Fit	0.0169	9	0.0019	0.8513	0.6159	**Not significant**
Pure Error	0.0088	4	0.0022			
Cor Total	0.2013	16				

Table 16b: ANOVA analysis for Response 2- Area

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	7.463E-06	9	8.293E-07	5.46	0.0179	**Significant**
A-Volume of solvent	2.689E-06	1	2.689E-06	17.72	0.0040	
B-Solvent front	8.978E-09	1	8.978E-09	0.0592	0.8148	
C-Saturation time	6.301E-08	1	6.301E-08	0.4152	0.5399	
AB	1.024E-07	1	1.024E-07	0.6747	0.4385	
AC	2.107E-07	1	2.107E-07	1.39	0.2772	
BC	2.143E-06	1	2.143E-06	14.12	0.0071	
A^2	1.934E-06	1	1.934E-06	12.74	0.0091	
B^2	1.478E-08	1	1.478E-08	0.0974	0.7641	
C^2	3.670E-07	1	3.670E-07	2.42	0.1639	
Residual	1.062E-06	7	1.518E-07			
Lack of Fit	5.761E-07	3	1.920E-07	1.58	0.3265	**Not significant**
Pure Error	4.863E-07	4	1.216E-07			
Cor Total	8.526E-06	16	8.293E-07			

Figure 13 : Surface response curves 2D and 3D images of rf value

Figure 13a: Surface response curves 2D and 3D images of rf AB



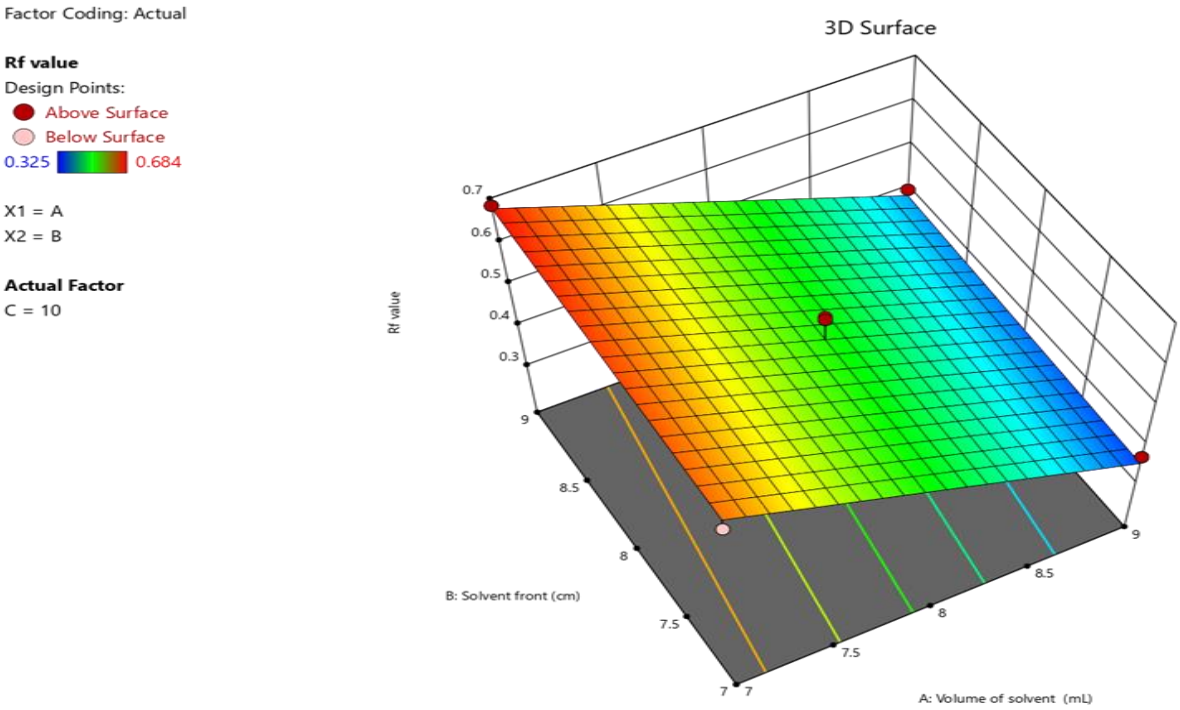
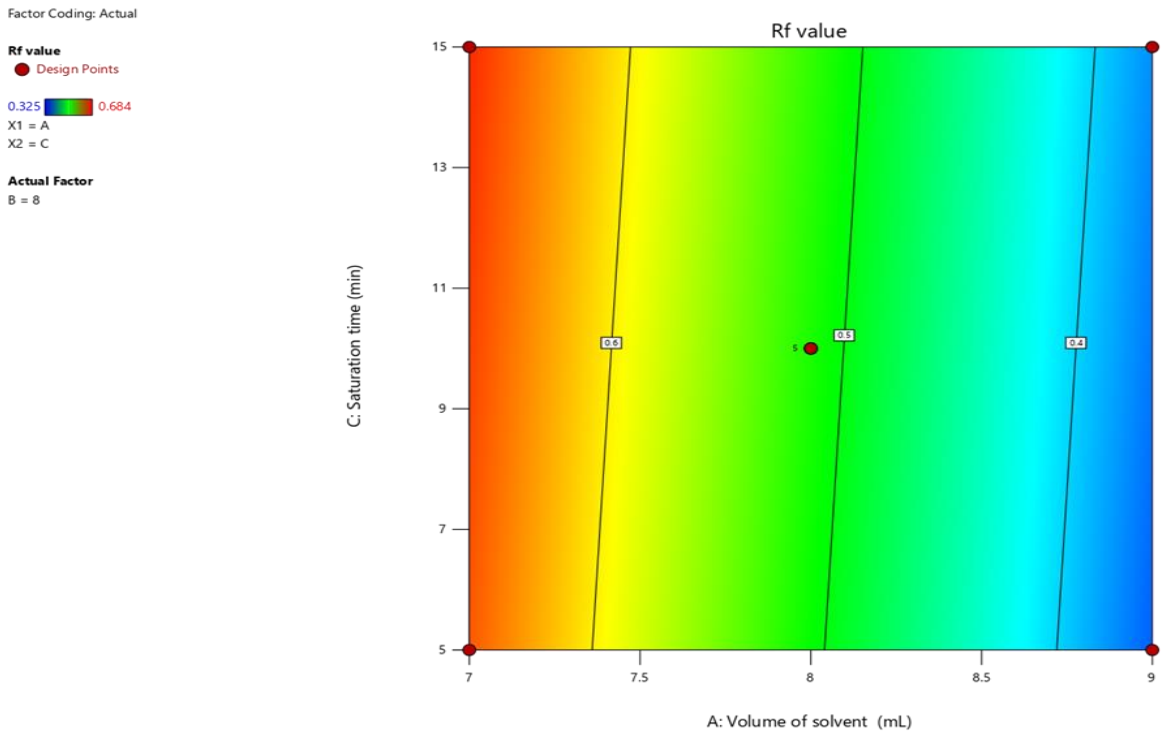


Figure 13b: Surface response curves 2D and 3D images of rf AC



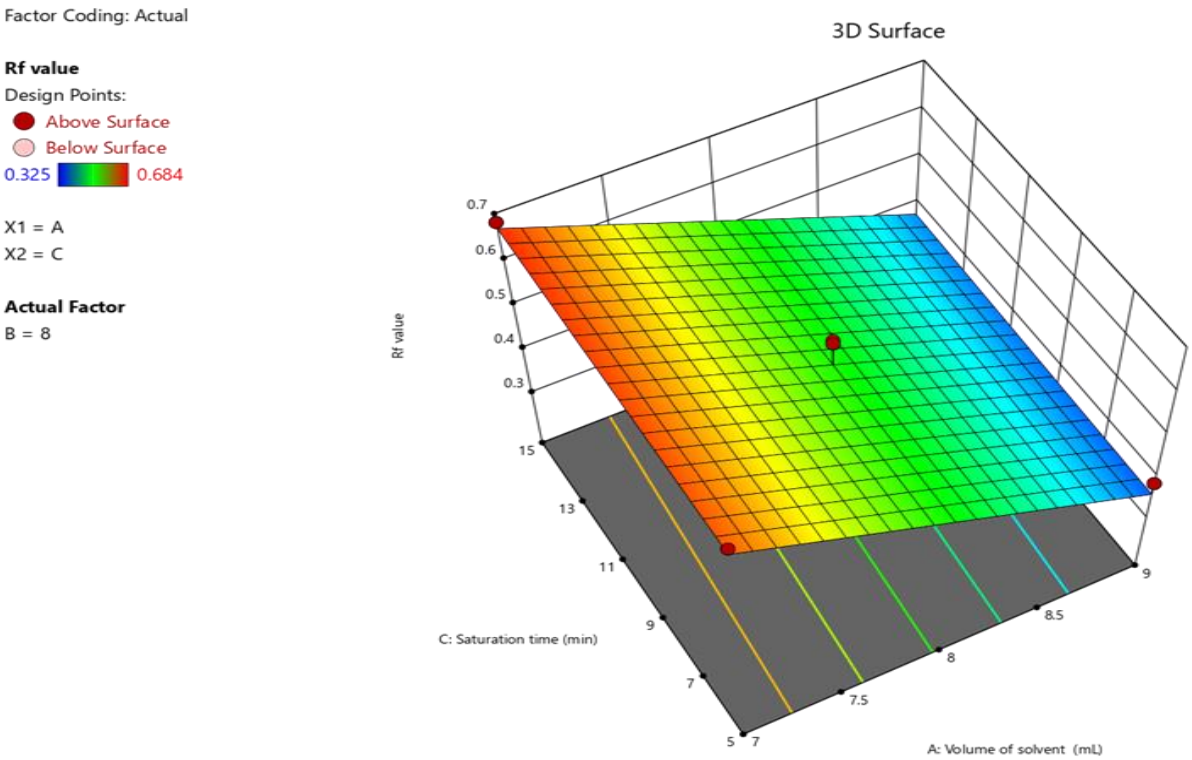
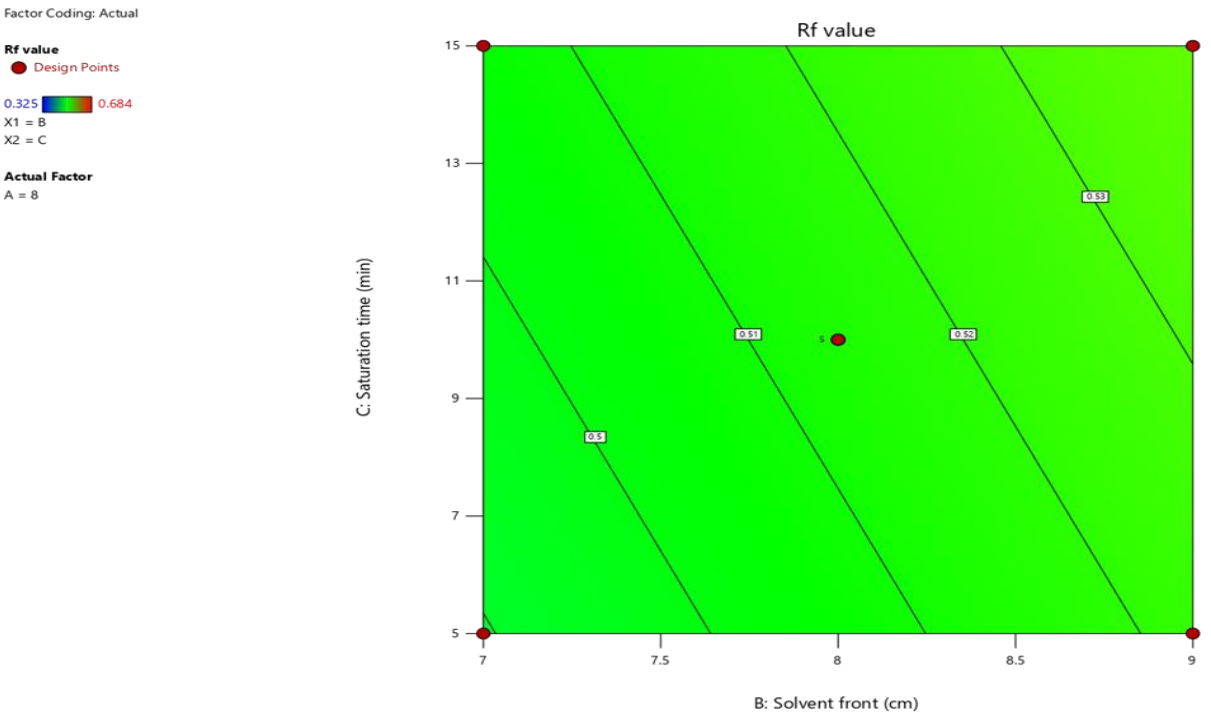


Figure 13c: Surface response curves 2D and 3D images of rf BC



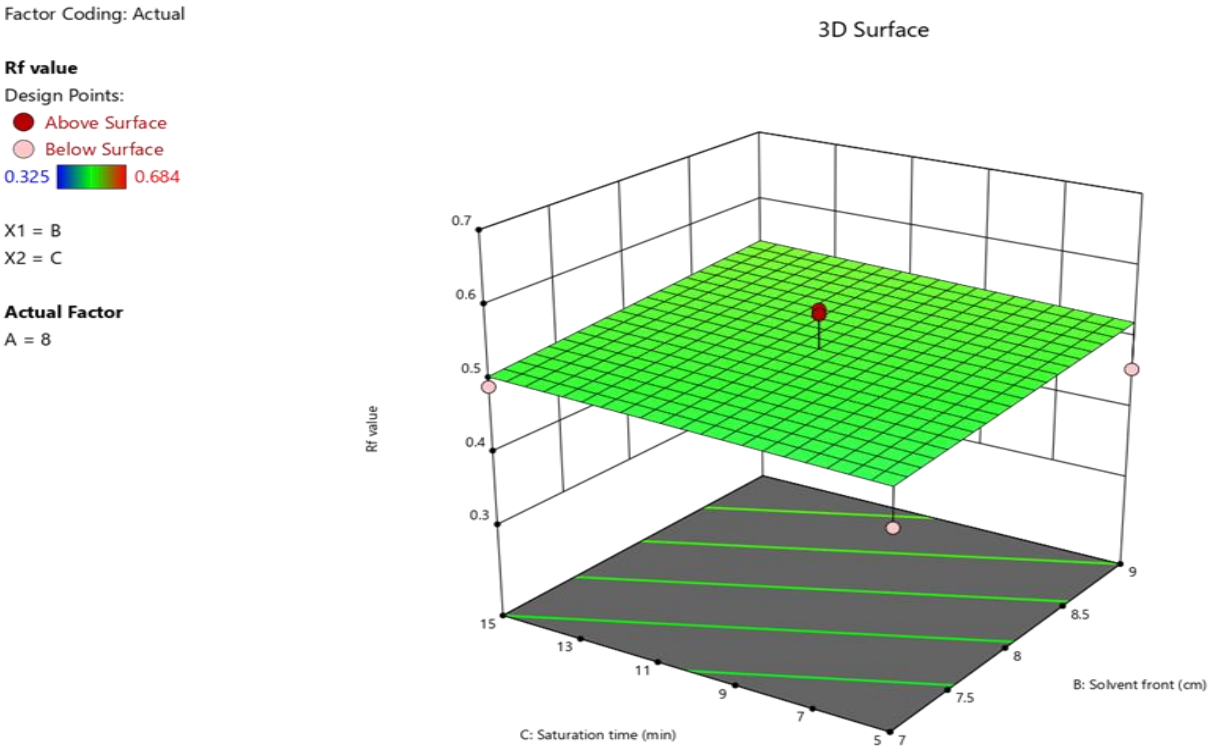


Figure 14: Surface response curves 2D and 3D images of area:

Figure 14a: Surface response curves 2D and 3D images of area AB

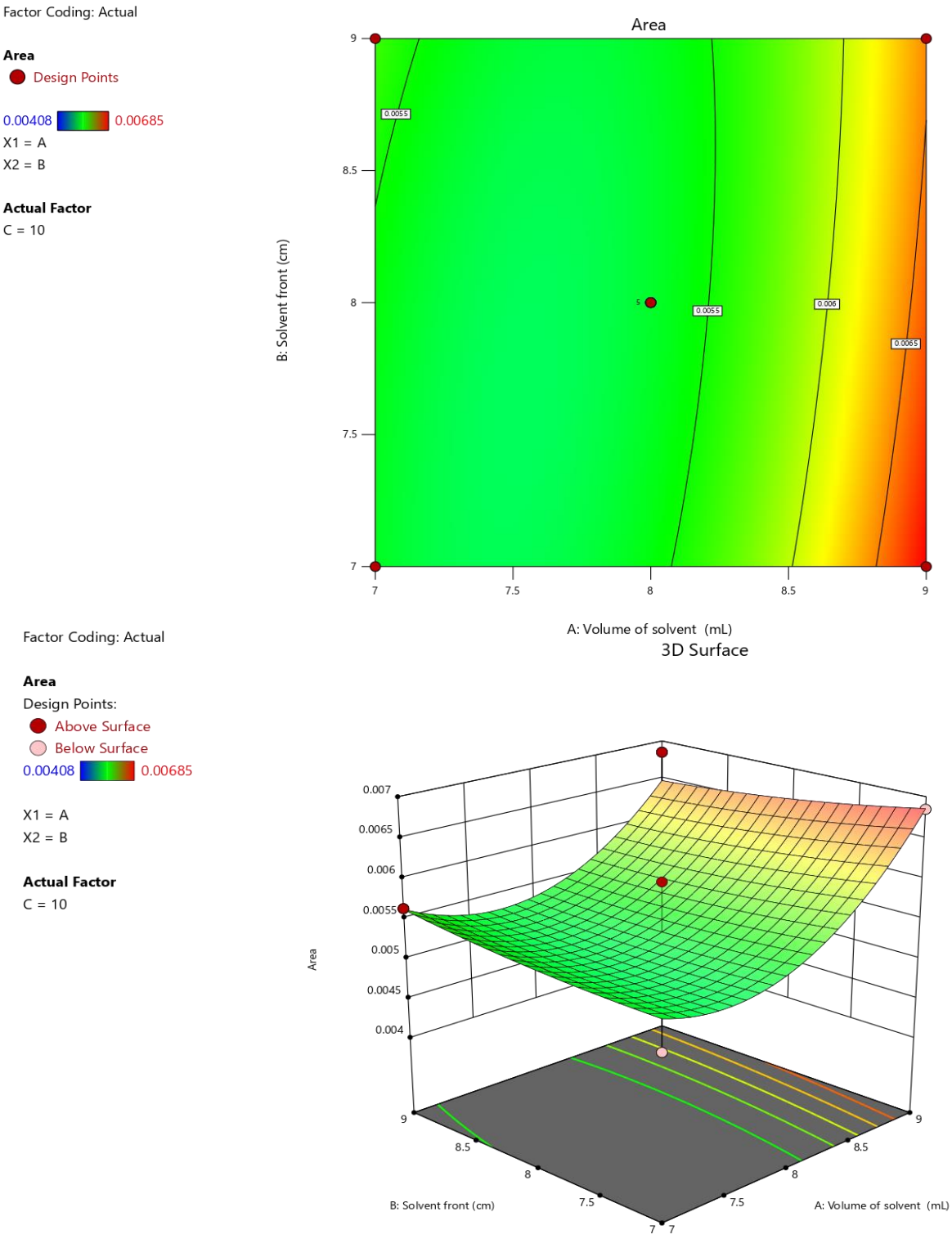
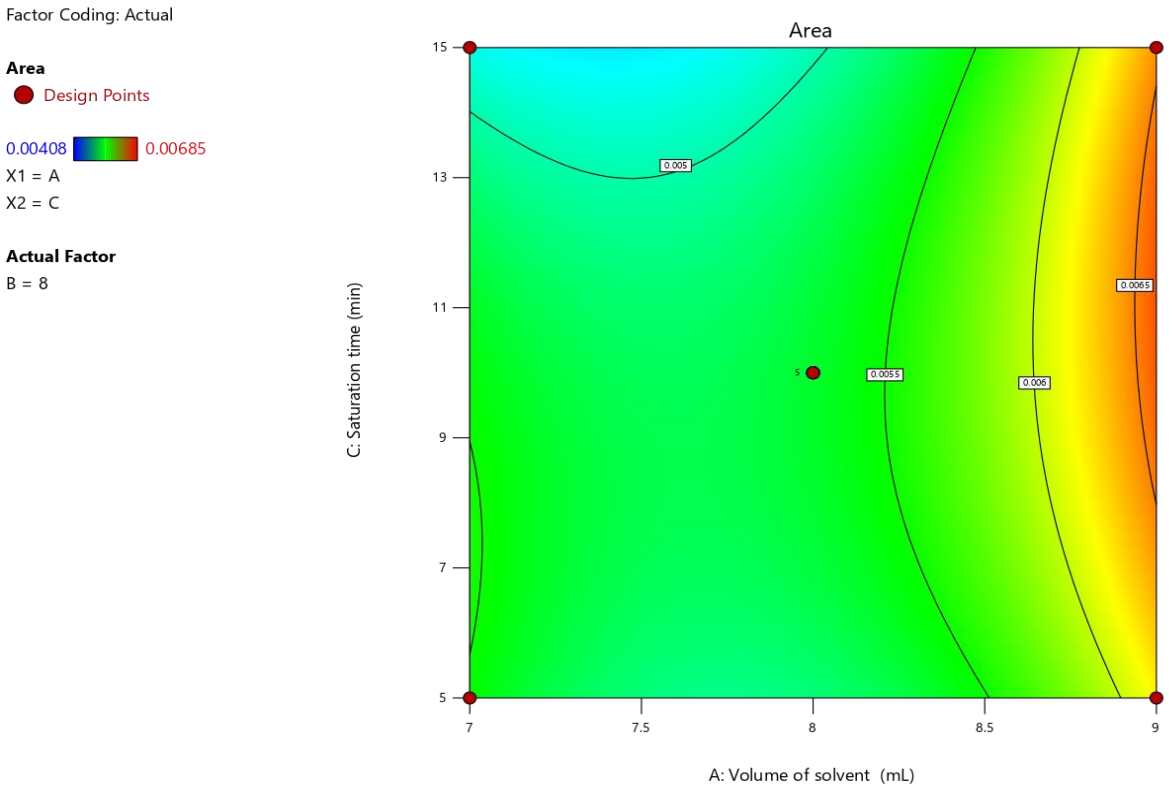


Figure 14b: Surface response curves 2D and 3D images of area AC



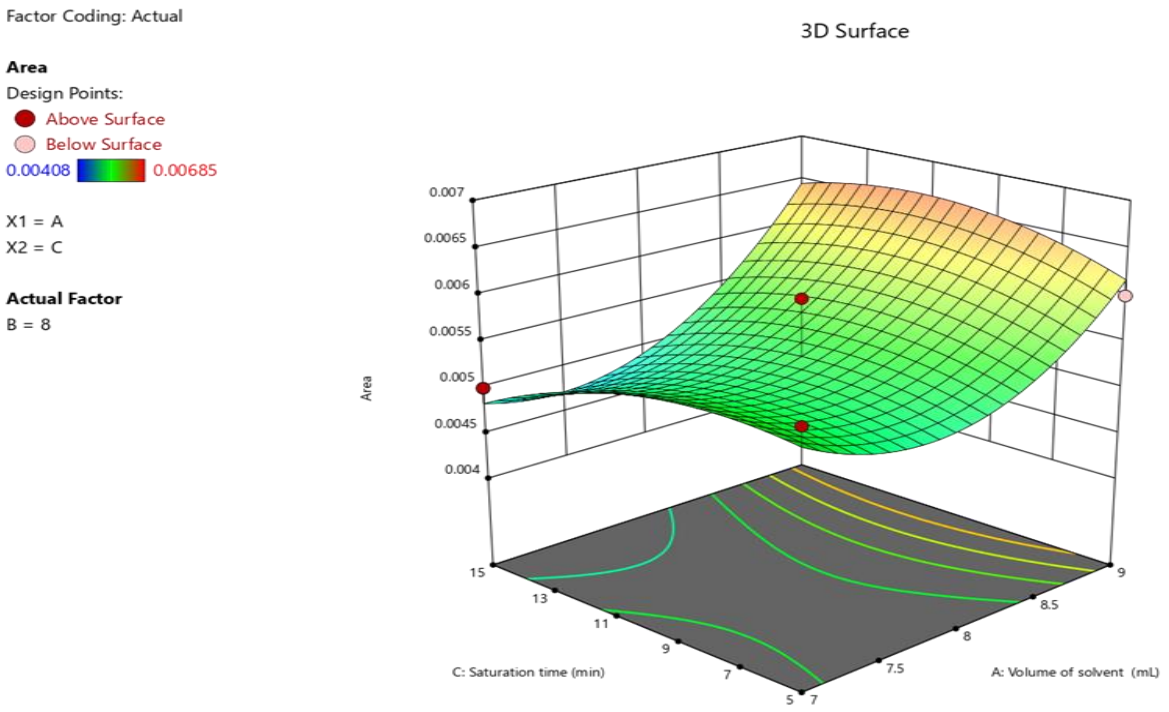


Figure 14c: Surface response curves 2D and 3D images of area BC

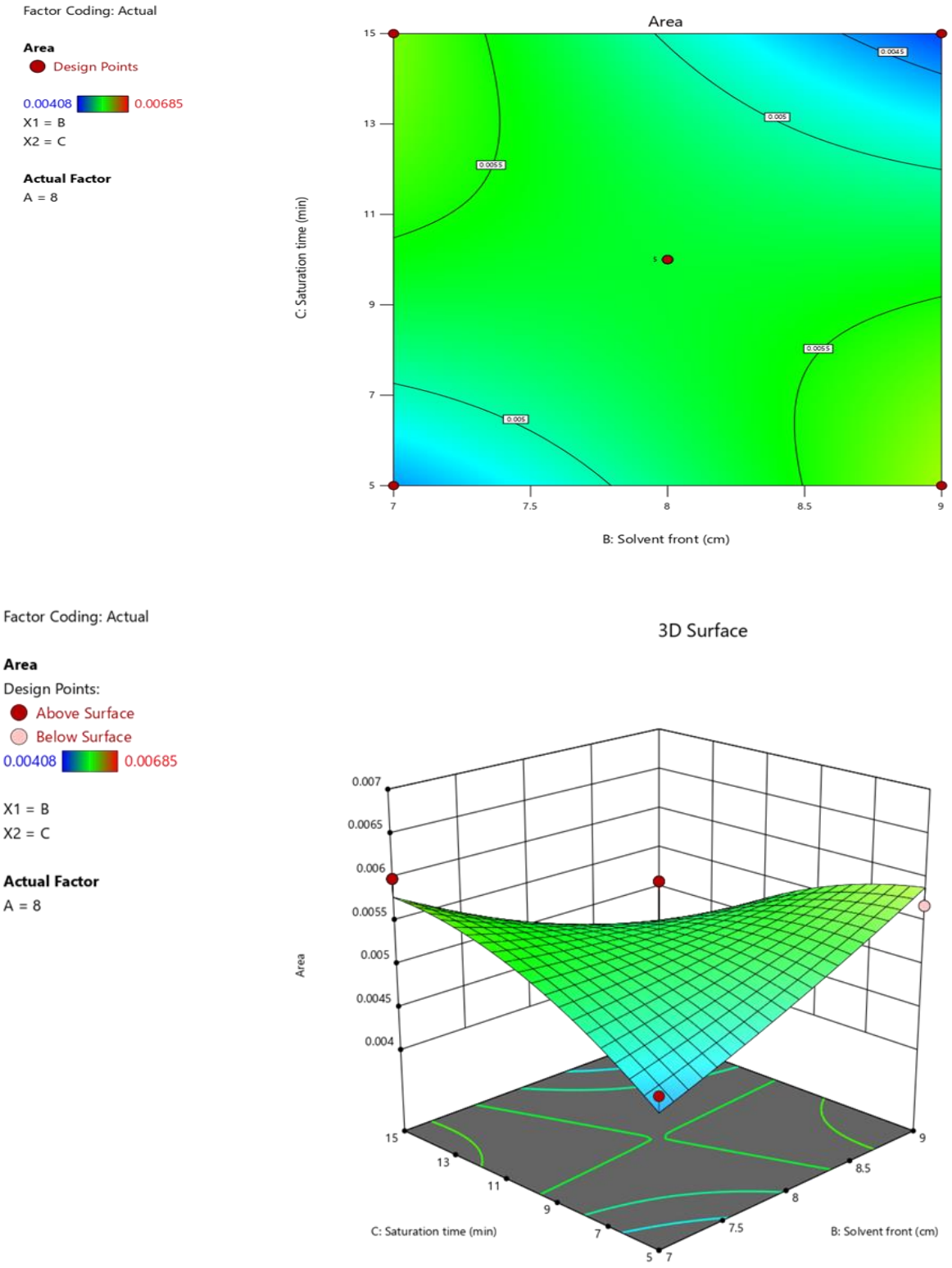


Figure 15: Overlay Plot for Graphical Optimization

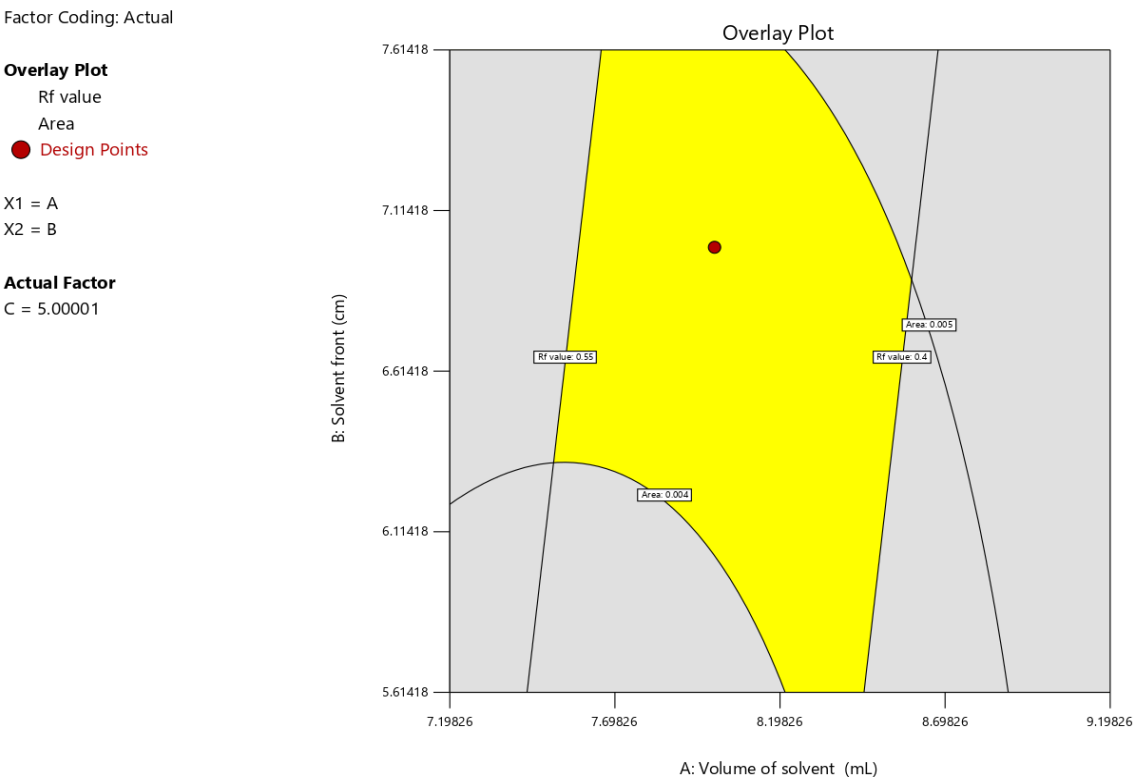
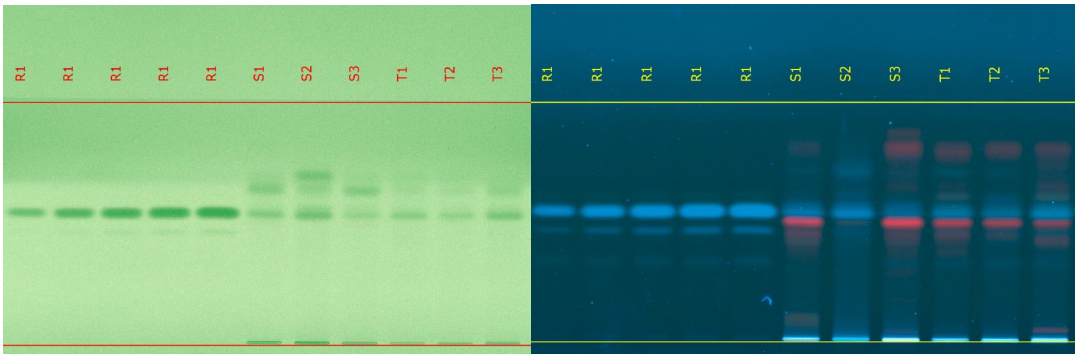


Table 17: Chromatographic criteria's of the optimized method for Piperine quantification

Parameters	Conditions
Stationary Phase	TLC silica gel F254 plate with aluminium backing
Mobile Phase	Ethanol: Water: Formic acid (8:2:0.1) v/v/v
Saturation Time	5mins
Distance Travelled by Solvent Front	7 cm

Figure 16: Plate image of piperine quantification:



R1: Piperine; ; S(1,2,3): Sitopaldi churna sample; T(1, 2, 3): Talisadi Churna sample;

Figure 17: Pictorial representation of the AGREE scale for quantification method

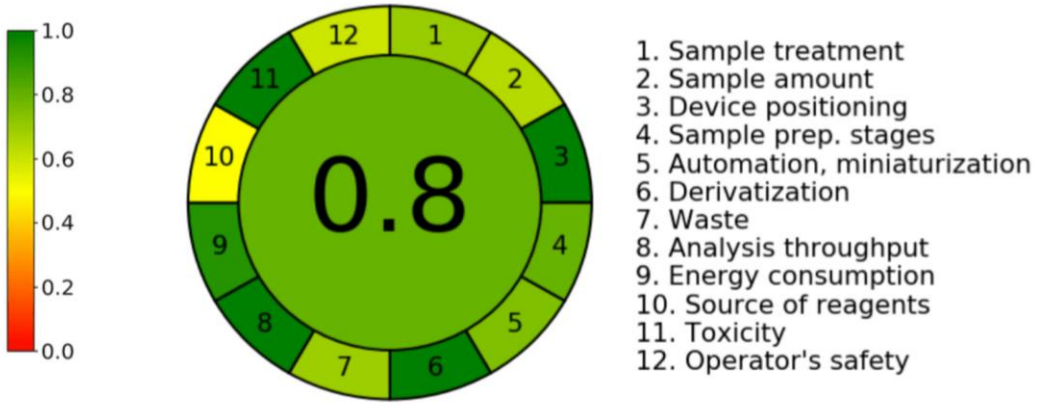


Table 18: Method validation results for piperine quantification method:

Parameters	**Piperine**
Linearity	y= 0.0021x+ 0.0199
Range (µg/mL)	2-10
R^2	0.992
LOD(µg/mL)	1.033293
LOQ(µg/mL)	3.131192
Rf value	0.437
Inter-day Precision	
Rf value	0.437± 0.00473
Area under curve	0.03281±0.00018
Intra-day Precision	
Rf value	0.436± 0.00379
Area under curve	0.03285±0.00024
Robustness (Rf value) %RSD	
Mobile phase composition	0.5697%
Mobile phase volume	0.3591%
Duration of chamber saturation	0.4789%

4. DISSCUSION:

4.1 Discussion of Pharmacopeial evaluation:

4.1.1 Determination of foreign matter: Upon examination of the results, it is evident that the levels of foreign matter are relatively low across the board (Table 4). Abies webbiana (Talisapatra) exhibits a foreign matter percentage of 0.32%, while Piper nigrum (Maricha) and Elettaria cardamomum (Elaichi) show values of 0.26% and 0.28%, respectively. Cinnamomum zeylanicum (Dalchini) has a slightly higher foreign matter content at 0.36%. Notably, Bambusa bamboo (Vamshalochana) registers a particularly low percentage of 0.22%, indicating a high level of purity. Similarly, Piper longum (Pipalli) and Zingiber officinalis (Sunthi) exhibit foreign matter percentages of 0.28% and 0.31%, respectively. The consistent low levels of foreign matter across the ingredients suggest a meticulous quality control process in place.

4.1.2 Microscopic studies: Powder microscopy serves as a crucial investigative tool in the detailed analysis of powdered samples. In this methodology, the powdered samples undergo microscopic examination, often following treatment with specific reagents to unveil distinct characteristics. The figure 2a- 2f depict the microscopic details of the formulations.

4.1.3 Physicochemical results: The provided table offers a detailed insight into the physico-chemical characteristics of various raw materials used in pharmaceutical or herbal formulations (Table 5). Starting with the water extractive values, Piper longum (Pipalli) stands out with the highest percentage at 11.75%, indicating a substantial solubility of compounds in water. Moving to the alcoholic extractive values, Piper longum (Pipalli) again leads with a notable 15%, suggesting efficient solubility in alcohol. Total ash values, reflecting the inorganic

content, show Piper nigrum (Maricha) with the highest at 11.05%. In contrast, Bambusa bamboo (Vamshalochana) demonstrates the lowest acid insoluble ash value, signifying potential higher purity. Water-soluble ash values highlight Piper nigrum (Maricha) with the highest solubility at 6.58%, emphasizing its potential for water-based formulations. Loss on drying percentages, indicating moisture content, vary from 8.20% to 10.90%, with Piper longum (Pipalli) having the highest loss on drying. This emphasizes the need for careful drying processes during production. The pH values, ranging from 5.30 to 6.59, offer insights into the acidity or alkalinity of the raw materials. Bambusa bamboo (Vamshalochana) stands out with the highest pH, while Piper nigrum (Maricha) has the highest total ash value at 11.05%.

The same studies were carried out in case of formulations (Table 5), Examining the water extractive values, S3 has the highest at 15.00%, indicating a robust solubility of compounds in water. Alcoholic extractive values show a variation among formulations, with S2 displaying the highest at 6.00%, suggesting its efficient solubility in alcohol. Moving to the ash values, total ash values range from 1.43% to 23.15%, with S2 having the highest total ash content. Acid insoluble ash values vary between 15.18% and 18.80%, indicating the portion of ash insoluble in acid. Water-soluble ash values show a range from 7.60% to 11.03%, emphasizing the diversity in solubility characteristics across formulations. Loss on drying percentages, representing moisture content, range from 4.00% to 6.30%, with S2 displaying the highest loss on drying. This highlights the importance of controlling moisture levels during formulation processes. The pH values, ranging from 6.05 to 7.20, provide insights into the acidity or alkalinity of the formulations. Formulation S3 has the highest pH, indicating a slightly alkaline nature.

4.1.4 Microbial studies: In compliance with the procedures outlined in the Ayurvedic Pharmacopeia of India, the microbial limit test was conducted for churna formulations (Figures 3). Notably, formulation T3 exhibited superior results, while the other two formulations also fell within the acceptable limit of not exceeding 300 colonies. The microbial limit test, conducted as per Ayurvedic Pharmacopoeia guidelines, revealed colony counts below the specified threshold for all three formulations. Specifically, S2 demonstrated a higher colony count in comparison to S1 and S3. These findings are indicative of the microbial load in the respective formulations, with S2 exhibiting a comparatively higher microbial presence. Such microbial limit assessments are pivotal in ensuring the safety and quality of churna formulations, aligning with regulatory standards and pharmacopeial requirements.

4.1.5 Physical Powder Characterization: The table 6 presents crucial data on the physical powder characterization for different formulations. Examining the bulk and tap densities provides insights into the packing properties of the formulations. S2 exhibits the highest bulk density at 0.5882, indicating a higher mass of powder per unit volume, and T1 has the highest tap density at 0.7042, reflecting the maximum packing efficiency achievable under tapping conditions. The angle of repose values, ranging from 23.3 to 27.85 degrees, signify the flow properties of the formulations. A lower angle of repose, as seen in S2, T1, T2, and T3, suggests better flow characteristics. Hausner's ratio, a measure of powder flowability, varies between 1.1831 and 1.31. Lower Hausner's ratios in T1 and T3 indicate improved flow properties. Carr's index, representing the compressibility of the powder, ranges from 10.58% to 20.52%. A lower Carr's index, observed in T1 and T2, suggests higher compressibility and potentially better tableting properties. Conversely, higher values in S3 indicate lower compressibility.S2 and T1,

with their favorable bulk and tap densities, low angle of repose, and lower Carr's index show the better results.

4.2 Discussion on Extra-Pharmacopeial Evaluations:

4.2.1 Organoleptic characterization: The table outlines the organoleptic properties of raw materials (Table 7a) employed in formulations, Starting with Abies webbina (Talisapatra), its green leaves take on a brown hue when dried, offering an aromatic odor and an astringent taste. Moving on to Piper nigrum (Maricha), characterized by its black color, it introduces an aromatic odor and an intensely pungent taste. The unique characteristics of Maricha imply its potential to impart both color and a bold flavor to the end product.Elettaria cardamomum (Elaichi) exhibits greenish capsules with brown seeds, boasting an aromatic and characteristic odor. Its taste profile, described as menthol-like, warm, and pungent. Cinnamomum zeylanicum (Dalchini), ranging from tan brown to a warm shade of brown, carries a sweet, spicy, and warm fragrance. Its taste, characterized as sweet and woody with a slightly spicy undertone. Bambusa bamboo (Vamshalochana), identified by its white to off-white appearance and a mild bamboo shoot odor, stands out as tasteless. Piper longum (Pipalli), with a brownish-black hue, has a peculiar and characteristic odor. In its dry form, it presents a strongly pungent taste. Finally, Zingiber officinalis (Sunthi), with a dull cream color, offers a characteristic aromatic and pungent odor. Its strongly pungent taste.

In case of formulations (Table 7b) the first set represented by S1, S2, and S3, shares a consistent Dutch white color, a characteristic odor, and a taste profile noted for its sweetness with a subtle pungent undertone. These formulations are presented in a powdered form, boasting a very smooth texture. This uniformity across S1, S2, and S3 suggests a deliberate effort to maintain

a standardized sensory experience, appealing to a particular flavor and texture profile. On the other hand, the second set, denoted as T1, T2, and T3, deviates slightly in color, featuring a dull white hue. However, like the first set, they exhibit a characteristic odor and a taste that leans towards slight sweetness. The powdered presentation and smooth texture are consistent with the first set, emphasizing a deliberate choice in the formulation process to ensure a cohesive sensory experience.

4.2.2 Macroscopic studies: The table 8 presents a comprehensive overview of the macroscopic characteristics of various raw materials. Abies webbina (Talisapatra) is characterized by needle-shaped leaves, ranging from 1.3 to 2.2 cm in length, single and thin, with a spiral arrangement. Piper nigrum (Maricha) exhibits globular berries measuring 3.2-6 mm in diameter, featuring a coarse, rough, and wrinkled surface. Elettaria cardamomum (Elaichi) appears oblongated, 1-2 cm in length, with a surface marked by furrows, ridges, and rough texture. Cinnamomum zeylanicum (Dalchini) displays a longitudinal and wavy structure, 2.5-8 cm in length, thin with curls on one side, and a slightly smooth surface. Bambusa bamboo (Vamshalochana) is characterized by uneven crystals, resulting in a smooth powder texture. Piper longum (Pipalli) takes an oblong or long rod-like shape, measuring 6-8 cm in length, with patterned crevices on the surface contributing to a rough texture. Zingiber officinalis (Sunthi) presents a compressed flat and ovate shape, 5-9 cm in length, 1-2 cm in width, featuring a smooth surface with distinct striations. This detailed summary offers insights into the unique shapes, sizes, and surface textures of each raw material, facilitating their identification and understanding in various applications.

4.2.3 Fluorescence studies: Abies webbina (Talisapatra) powder exhibits a consistent brown color (Table 9a). Acidic treatments darken the color, while alkaline treatments maintain brown. Oxidizing agents induce yellowish-brown or light brown hues. Methanol and acetic acid produce light brown and reddish-brown colors, respectively. Iodine addition has no effect, keeping the original brown color. Piper nigrum (Maricha) powder is consistently dark brown (Table 9b). Acidic treatments yield yellow to orange hues, alkaline treatments result in light yellow to cream colors, and oxidizing agents induce yellow to dark yellow or light brown. Methanol and acetic acid treatments create dark cream to cream colors. Iodine turns the powder black under all light conditions. Elettaria cardamomum (Elaichi) powder exhibits an olive green color (Table 9c), transitioning to light green under UV light. Acidic treatments produce shades of orange to dark brown, while alkaline treatments induce green to dark green hues. Oxidizing agents yield orangish yellow to dark brown colors, and methanol or acetic acid treatments result in pale green to creamish green tones. Iodine turns the powder green under visible light and dark green or black under UV light. Cinnamomum zeylanicum (Dalchini) powder is orangish-brown in daylight and brown under UV light (Table 9d). Acidic treatments result in maroon to darker brown shades, alkaline treatment maintains a dark brown color, and oxidizing agents yield yellow to light brown. Methanol and acetic acid treatments create orange to reddish-brown hues, while ammonia and iodine maintain various shades of brown. Bambusa bamboo (Vamshalochana) powder is white in daylight and pale white under UV light (Table 9e). Acidic and alkaline treatments maintain a consistent white or pale appearance, while oxidizing agents yield yellow to light brown hues. Methanol and acetic acid treatments create snow white to dull white shades. Ammonia maintains a pale white color, and iodine turns the powder black under both daylight and UV light. Piper longum (Pipalli) powder is brown in

both daylight and UV light (Table 9f). Acidic and alkaline treatments result in orangish brown to orange hues, while oxidizing agents yield yellowish brown to dark yellow. Methanol and acetic acid treatments create various shades of brown. Ammonia induces a yellowish brown color, and iodine turns the powder dark brown under daylight and black under UV light. Zingiber officinalis (Sunthi) powder appears dull cream under daylight and UV light, transitioning to a creamish shade (Table 9g). Acidic and alkaline treatments induce orange to dark brown hues, while oxidizing agents yield various shades of orange. Methanol and acetic acid treatments create light cream to light brown tones. Ammonia maintains a pale yellow to pale brown color, and iodine turns the powder black under all light conditions.

In the S1 samples (Table 10a), the drug maintains a Dutch white color in both daylight and UV light. Acidic treatments induce creamish brown to reddish-brown hues, while alkaline treatment yields brown to dark brown. Oxidizing agents produce yellow to orangish-yellow shades. Methanol and acetic acid treatments create cream to pale yellow tones. Ammonia maintains a light brown to cream-brown color, and iodine turns the powder black under all light conditions. In the S2 samples (Table 10b), the drug exhibits an off-white color in both daylight and UV light. Acidic treatments induce orange-brown to dark brown hues, while alkaline treatment yields light brown. Oxidizing agents produce yellow to brown shades. Methanol and acetic acid treatments create cream to pale cream tones. Ammonia maintains a light brown to dull brown color, and iodine turns the powder black under all light conditions. In the S3 samples (Table 10c), the drug starts as pale white in both daylight and UV light. Acidic treatments induce brown to maroon-brown hues, while alkaline treatment yields brown to light brown. Oxidizing agents produce brown to dark brown shades. Methanol and acetic acid treatments create cream to pale cream tones. Ammonia maintains a brown to light brown

color, and iodine turns the powder black under all light conditions. In the T1 samples (Table 10d), the drug maintains a dull white color in both daylight and UV light. Acidic treatments induce light brown to chocolate brown hues, while alkaline treatment yields brown to creamish brown. Oxidizing agents produce yellowish brown to dark brown shades. Methanol and acetic acid treatments create pale cream to pale brown tones. Ammonia maintains a brown to light brown color, and iodine turns the powder black under all light conditions. In the T2 samples (Table 10e), the drug starts as dull white in both daylight and UV light. Acidic treatments induce light brown to maroon-brown hues, while alkaline treatment yields light brown. Oxidizing agents produce brown to dark brown. Methanol and acetic acid treatments create dull orange to pale brown tones. Ammonia maintains a light brown color, and iodine turns the powder black under all light conditions. In the T3 samples (Table 10f), the drug remains dull white in both daylight and UV light. Acidic treatments induce light brown to reddish-brown hues, while alkaline treatment yields dull brown to light brown. Oxidizing agents produce creamish brown. Methanol and acetic acid treatments create creamish brown to dull brown tones. Ammonia maintains a light brown color, and iodine turns the powder black under all light conditions.

4.2.4 Qualitative Phytochemical investigations:

The systematic analysis of raw materials and extracts reveals a comprehensive breakdown of phyto-constituents present in each component. The utilization of various solvents in the extraction process has yielded diverse profiles, enhancing our understanding of the chemical composition. The results are presented in Table 11a and 11b for raw materials and formulations respectively.

4.2.5 Quantitative Phytochemical investigations:

In the pursuit of a thorough understanding of the churna formulations, the study delved into quantitative phyto-chemical assays, offering insights into key parameters such as Total Phenolic Content, Total Tannic Content, Total Flavonoid Content, and Total Alkaloid Content. Rigorous quantitative estimations were meticulously conducted to ascertain the concentration of these essential phyto-chemical constituents, providing a quantitative foundation for the samples under scrutiny. The results for Sitopaladi churna formulations are depicted in (Figure 4a- 4e) and for Talisadi churna formulations are depicted in (Figure 5a- 5e).

4.3 Discussion on HPTLC results:

4.3.1 HPTLC method development and validation: The HPTLC method (Figure 6) was meticulously developed and optimized (table 12). Subsequently, the developed method underwent a comprehensive validation process in strict adherence to the guidelines set forth by the ICH and USP. The developed analytical method underwent rigorous validation following ICH Q2 R1 guidelines (Table 13). Key parameters, including Linearity, Range, LOD, LOQ, Precision, Specificity (Figure 8) and Robustness, were systematically assessed to ensure method reliability. Linearity and Range spanned 1-5 µL/band with high correlation coefficients. LOD and LOQ values were determined, demonstrating sensitivity. Precision, evaluated for intra-day and inter-day variations, exhibited %RSD values below 2%. Specificity tests confirmed the method's selectivity. Robustness, assessed through deliberate variations, yielded %RSD values consistently below 2%. The environmental sustainability of the method, assessed through the AGREE scale for Green Analytical Chemistry (GAC), achieved a score of 0.74. (Figure 7), emphasizing its eco-friendly attributes. In summary, the method proved

reliable, sensitive, specific, and environmentally sustainable, meeting the highest analytical standards.

4.3.2 HPTLC fingerprinting: In the realm of botanical extractions, a meticulous exploration unfolded as the methanolic extracts from diverse samples underwent rigorous scrutiny through various High-Performance Thin-Layer Chromatography (HPTLC) fingerprinting analyses. Employing distinct mobile phases detailed in Table 3, the resulting developed plates were meticulously documented using a TLC visualizer. Figure 9a delve into the fingerprints of samples showcases patterns under 254nm & 366nm wavelength. Noteworthy observations include the Sitopaladi churna S2 sample exhibiting fewer separated bands at 366nm compared to S1 and S3. In Talisadi churna samples, T2 reveals lighter bands in contrast to T1 and T3. Further scrutiny unveils a distinct band (near 0.1) present in S1, conspicuously absent in S2 and S3.The identification of Alkaloids (Figure 9b) employed Dragendroff's Reagent, with an absence of orange bands under white light in all samples, signifying the absence of alkaloids. Flavonoids(Figure 9c), elucidated through 10% Methanolic sulphuric acid derivatization revealed a transformation in S2 samples post-derivatization, with a fluorescent band emerging at 0.8 rf value, indicating the presence of flavonoid secondary metabolites. The exploration of Tannins (Figure 9d) involved Ferric chloride reagent derivatization, showcasing consistent blue color bands across all samples, with S3 displaying a slightly less intense band at rf 0.6.The analysis of Phenolic compounds (Figure 9e) utilized alcoholic ferric chloride reagent, unveiling vibrant blue bands. Notably, S2 exhibited the most prominent band, S3 displayed a less distinct one, and T2 demonstrated a subdued presence at rf value 0.75. Antioxidant potential, evaluated through HPTLC-DPPH (Figure 9f) method, showcased white bands on the

plate's post-derivatization, indicating the presence of antioxidant compounds in all samples except T3. This underscores the formulations' efficacy in combatting oxidative stress. The comprehensive chromatographic narrative attests to the robustness and distinctive characteristics of the herbal formulations under investigation.

4.3.3 Quantification of Piperine by HPTLC: During the development of the High-Performance Thin-Layer Chromatography (HPTLC) method, a methodical exploration was undertaken through a series of preliminary trials, utilizing varied ratios of the mobile phase. As an integral facet of quality assessment, chromatographic (Figure 11) and spectral (Figure 12) analyses of the standard Piperine (Figure 10) were conducted, with the developed chromatogram captured at 330nm .To methodically elucidate the factors influencing HPTLC method development, the Box-Behnken design, renowned for its flexibility, was judiciously selected. This design, as detailed in Table 14, facilitated the systematic study and optimization of key factors and responses, namely the volume of solvent, chamber saturation, and the distance travelled by the mobile phase. The responses, specifically Rf value and Area, were meticulously recorded. A comprehensive understanding of the study and optimization process was achieved through statistical analysis, as reflected in Table 15 & 16. Surface response plots, both in 2D and 3D, contributed to graphical optimization, supplemented by the overlay plot (Figure 13, 14 & 15). Notably, the interpretation of statistical results and graphical representations identified run std 9 and run 6 as the most optimized conditions. The implementation of QbD fundamentals guaranteed the comprehensive optimization of chosen parameters, resulting in a method of exceptional quality. The chromatographic conditions, a product of this optimized approach, are succinctly summarized in Table 17. This method,

developed through an Analytical Quality by Design (AQbD) paradigm, stands as a testament to the meticulous consideration and refinement of critical parameters in pursuit of a robust and high-quality analytical method. The optimized method underwent validation following the ICH Q2 (R1) guidelines, and the outcomes are succinctly presented in Table 18. Precision and robustness evaluations yielded %RSD values well within the acceptable criteria of <2%, underscoring the method's reliability and stability. These validation results affirm the suitability of the developed method for accurate and precise analytical applications within the defined parameters.

In the realm of quantitative analysis, the focus narrowed to the quantification of Piperine (Figure 16) —a key constituent within the formulations. The meticulous examination revealed quantitative concentrations of 0.621% w/w, 0.8179% w/w, and 0.4138% w/w for samples S1, S2, and S3, respectively. The quantitative assessment revealed concentrations of 0.591% w/w, 0.4574% w/w, and 0.7468% w/w for T1, T2, and T3 churna samples, respectively. This analytical insight provides a nuanced understanding of the relative abundance of Piperine within each classical formulation, contributing to the comprehensive characterization of these herbal compositions. The evaluation of the environmental impact of the developed method utilized the AGREE scale, yielding a noteworthy score of 0.80. This comprehensive assessment considered all twelve components of Green Analytical Chemistry (GAC).The visual representation of this environmental score is depicted in Figure 17, providing a concise and informative overview of the method's eco-friendly attributes

5. SUMMARY:

The comprehensive study on churna formulations delves into a multifaceted evaluation, employing a rigorous and systematic approach to ensure thorough characterization and quality assessment. The investigation encompasses various analytical techniques and protocols, contributing to a nuanced understanding of the herbal formulations under scrutiny.

One crucial aspect addressed in the study is the determination of foreign matter in the raw materials. The results, presented in Table 4, reveal consistently low levels of foreign matter across the board. Noteworthy is the low percentage of foreign matter in Abies webbiana (Talisapatra) at 0.32%, indicating a high level of purity. Similarly, Piper nigrum (Maricha) and Elettaria cardamomum (Elaichi) exhibit values of 0.26% and 0.28%, respectively, emphasizing meticulous quality control in place. These findings suggest a robust quality assurance process that ensures the purity of the herbal ingredients, vital for maintaining high standards in herbal formulations. Powder microscopy serves as a crucial investigative tool in the detailed analysis of powdered samples. The microscopic examination, as illustrated in Figure 2a-2f, involves treating the powdered samples with reagents like PhuroglucinolHCl and Iodine solution. This process unveils distinct characteristics, aiding in the identification and understanding of the powdered samples. The microscopic details presented in the figures provide valuable insights into the morphology and composition of the herbal ingredients, contributing to the overall characterization of the formulations. Physicochemical results, as detailed in Tables 5a and 5b, offer a comprehensive insight into the physico-chemical characteristics of various raw materials and formulations. The water extractive values, alcoholic extractive values, ash content, and pH levels provide crucial information about the solubility, inorganic content, and

acidity or alkalinity of the materials. For instance, Piper longum (Pipalli) stands out with the highest water extractive value at 11.75%, indicating substantial solubility in water. These physicochemical parameters are essential for understanding the properties of the raw materials and formulations, contributing to their quality assessment. Microbial studies, conducted in compliance with Ayurvedic Pharmacopeia guidelines, involve a microbial limit test for churna formulations. The results, depicted in Figures 3, reveal that formulation T3 exhibited superior microbial results, while the other two formulations also fell within the acceptable limit of not exceeding 300 colonies. This microbial limit assessment is pivotal in ensuring the safety and quality of churna formulations, aligning with regulatory standards and pharmacopeial requirements.

Physical powder characterization, presented in Table 6, provides crucial data Formulations S2 and T1 stand out with favorable bulk and tap densities, low angle of repose, and lower Carr's index, indicating better flow properties and compressibility.

Organoleptic characterization (Tables 7a and 7b) outlines the sensory properties of raw materials and formulations. The uniformity in sensory profiles across formulations suggests a deliberate effort to maintain a standardized sensory experience, appealing to specific flavor and texture preferences. Macroscopic studies (Table 8) offer a comprehensive overview of the unique shapes, sizes, and surface textures of each raw material. This information facilitates the identification and understanding of the raw materials in various applications. Fluorescence studies (Tables 9a-9g and 10a-10f) provide insights into the behavior of the materials under different treatments and wavelengths. The variations in color and fluorescence patterns under

different conditions contribute to the overall characterization of the samples. Qualitative phytochemical investigations (Tables 11a and 11b) break down the phyto-constituents present in each component, enhancing our understanding of the chemical composition. Quantitative phytochemical assays (Figures 4a-4e and 5a-5e) provide a quantitative foundation for key parameters like Total Phenolic Content, Total Tannic Content, Total Flavonoid Content, and Total Alkaloid Content.

HPTLC outcome summarized (Tables 12-17), showcase method development, validation, and distinctive characteristics of herbal formulations. The HPTLC method is meticulously developed and validated following international guidelines, ensuring its reliability, sensitivity, and eco-friendly attributes. The fingerprinting analyses using different mobile phases provide a detailed chromatographic narrative, attesting to the robustness and distinct characteristics of the herbal formulations. The discussion on HPTLC emphasizes the reliability, sensitivity, and eco-friendly attributes of the method. Quantification of Piperine through HPTLC reveals concentrations, contributing to a nuanced understanding of the relative abundance of this key constituent within each classical formulation. In the realm of quantitative analysis, the focus narrows to the quantification of Piperine — a key constituent within the formulations. The meticulous examination reveals quantitative concentrations for each sample, providing analytical insight into the relative abundance of Piperine within each classical formulation and contributing to the comprehensive characterization of these herbal compositions. The eco-friendliness evaluation of the developed method employing the AGREE scale emphasizes its eco-friendly attributes, further highlighting its alignment with sustainable analytical practices.

In conclusion, this comprehensive study employs a holistic and advanced methodology to characterize churna formulations thoroughly. The integration of various analytical techniques and protocols provides valuable insights into the quality, composition, and potential therapeutic efficacy of these herbal formulations.

6. CONCLUSION:

In the rigorous examination of churna formulations, a comprehensive approach was adopted to ensure the highest standards of quality. The assessment encompassed various facets, including Pharmacognostical, Photo-chemical, Physico-chemical, microbial, and HPTLC fingerprinting analyses. These meticulous examinations played a pivotal role in characterizing both Sitopaladi and Talisadi churna formulations. Quality, being a cornerstone in the realm of herbal formulations, demanded the implementation of standardized procedures. The protocols employed in this study can be unequivocally regarded as benchmarks, setting a precedent for future endeavors in the field. The establishment of such standardized procedures not only fortifies the reliability of the study but also contributes to the broader scientific community by providing a robust framework for quality assessment.

A crucial aspect of this investigation involved method development and validation, specifically geared towards the quantification of Piperine. This meticulous attention to detail underscores the commitment to precision and accuracy in the study. Piperine quantification, as an integral component, enhances the depth and comprehensiveness of the research, contributing valuable insights to the herbal sciences. Recognizing the inherent vulnerabilities of herbs and herbal products to external factors such as geographical locations, climatic variations, adulteration, substitutions, and transportation, the study advocates for the necessity of standard protocols. These protocols serve as a bulwark against circumstantial challenges, ensuring the preservation of high-quality standards for herbs and herbal products. By addressing these challenges through rigorous methodologies and standardized procedures, the study endeavors to elevate the reliability and integrity of herbal formulations in the face of diverse environmental influences.

7. LIMITATIONS AND FUTURE SCOPE:

- **Limited Scope:** The study focused on only two specific churna formulations, Sitopaladi Churna and Talisadi Churna. Further research is needed to determine if the developed HPTLC method can be universally applied to a wider range of churna formulations.

- **Long-Term Stability:** The study may not have addressed the long-term stability. Future research could explore how the method performs over extended storage periods.

- **Clinical Correlation:** This study focused on analytical methods for quality control. Future studies could explore how the established quality control parameters correlate with the clinical efficacy of the churna formulations.

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9. ANNEXURES

Sl. N	Topic	Date
1	Authentication letter	10/01/2022
2	Antibiotic Resistance- webinar	20/12/2020
3	Sample size estimation, systemic review and meta-analysis	04/01/2021 to 06/01/2021
4	Use of sterile preparations- Webinar	13/01/2021
5	Intensive Biostatistics course	11/01/2021 to 22/02/2021
6	On-site HP-TLC training	12/03/2021
7	Marketed herbal drug evaluation by HP-TLC	13/03/2021
8	Health research Fundamentals	Jan-Mar 2021
9	Regulatory compliance on Laboratory practices	20/04/2021
10	Regulatory in HP-TLC- chapters of HP-TLC in Pharmacopeia	12/05/2021
11	Application of LC-MS/MS in pharmacopeia	07/06/2021
12	Method development in HP-TLC	16/06/2021
13	**Online Oral Presentation at** 8th International Congress of SFEC India, Theme: Ethanopharmacology& Medicinal Plants- Approach towards Product development.	27/08/2021 to 29/08/2021
14	**Online Oral Presentation at** Graduate School of Pharmacy organised by Society of Pharmacognosy on theme: New Horizons of Natural Products and Ayush Remedies.	27/11/2021 to 28/11/2021
15	**Online Poster Presentation** at National Seminar on "Ethnopharmacology for wellness: Tradition to Translation" Organized by CSIR-Indian Institute of Chemical Biology Kolkata, India.	10/12/2021
16	Webinar on Extractable and leachable study of Phthalates in pharmaceutical products and bio-monitoring of phthalates by HP-TLC vs LC-MS/MS	19/02/2022
17	Webinar series on- Ethnopharmacology: A Perspective of Immunoboosters and Antivirals.	28/02/2022 to 02/03/2022
18	Presented Poster at – 9th International Congress of the society for Ethnopharmacology 2002 at JSS College of Pharmacy, Mysore.	22/04/2022 to 24/04/2022
19	DST –STUTI sponsored and Organized by ICT Mumbai and Bombay College of Pharmacy- Seven day Hands on training Program on -- "Application of Sophisticated Instrumental Techniques for Evaluation of Drugs and Pharmaceuticals during Discovery and Development.	30/05/2022 to 05/06/2022
20	Workshop on Opertaions of Atomic Absorption Spectrophotometer, Total Organic Carbon Analyser, Acoustic Doppler Current Profiler and Epifluorescence Microscope	23/08/2022 to 29/08/2023
21	SERB Sponsored Workshop- "Role of artificial Intelligence and Machine Learning in Drug Discovery	12/12/2022 to 17/12/2022
22	International Bioresource Conclave and Ethnopharmacology Congress (ISESFEC-2023) At Imphal, Manipur- India.	24/02/2023 to 26/02/2023

राष्ट्रीय पारम्परिक चिकित्साविज्ञान संस्थान
ICMR-NATIONAL INSTITUTE OF TRADITIONAL MEDICINE
(भूतपूर्व क्षेत्रीय आयुर्विज्ञान अनुसंधान केन्द्र **Formerly Regional Medical Research Centre**)
Nehru Nagar, Belagavi-590 090

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

भारतीय आयुर्विज्ञान अनुसंधान परिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार कल्याण मंत्रालय, भारत सरकार
Department of Health Research,
Ministry of Health & Family Welfare, Govt. of India

Date: 10-01-2022

AUTHENTICATION

This is to authenticate that the plants submitted by Ms. Amruta Balekundri, Ph.D Scholar, KAHER, Belagavi are identified as ***Bambusa bambos* (L.) Voss**. (Poaceae), ***Piper longum* L.** (Piperaceae), ***Piper nigrum* L.** (Piperaceae), ***Zingiber officinale* Roscoe.** (Zingeberaceae), ***Elettaria cardamomum* (L.) Maton.** (Zingeberaceae) and ***Cinnamomum verum* J. Presl. (Syn. C. zeylanicum Blume.)** (Lauraceae). The voucher specimens of the same have been deposited in our herbaria with accession number RMRC-1668, RMRC-1669, RMRC-1670, RMRC-1671, RMRC-1674 and RMRC-1675 respectively.

Harsha Hegde
Scientist 'E'

डॉ. हर्ष हेगडे वैज्ञानिक-ई
Dr. Harsha Hegde Scientist-E
भा.आ.सं.अ.-राष्ट्रीय पारम्परिक चिकित्साविज्ञान संस्थान
ICMR - National Institute of Traditional Medicine
स्वास्थ्य अनुसंधान विभाग, भारत सरकार
Department of Health Research, Govt. of India
Nehru Nagar, Belagavi-590010

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B. PHARM PROGRAM ACCREDITATED BY NBA, NEW DELHI, FOR A PERIOD OF SIX YEARS UP TO JUNE 2025

Certificate

This certificate is awarded to

AMRUTA RAVI BALEKUNDRI

For attending the webinar on "Antibiotic Resistance"
organized by KLE College of Pharmacy, Belagavi, Karnataka
on 29th December, 2020.

Dr. V.S.Mannur
Coordinator

Dr. Sunil Jalalpure
Principal

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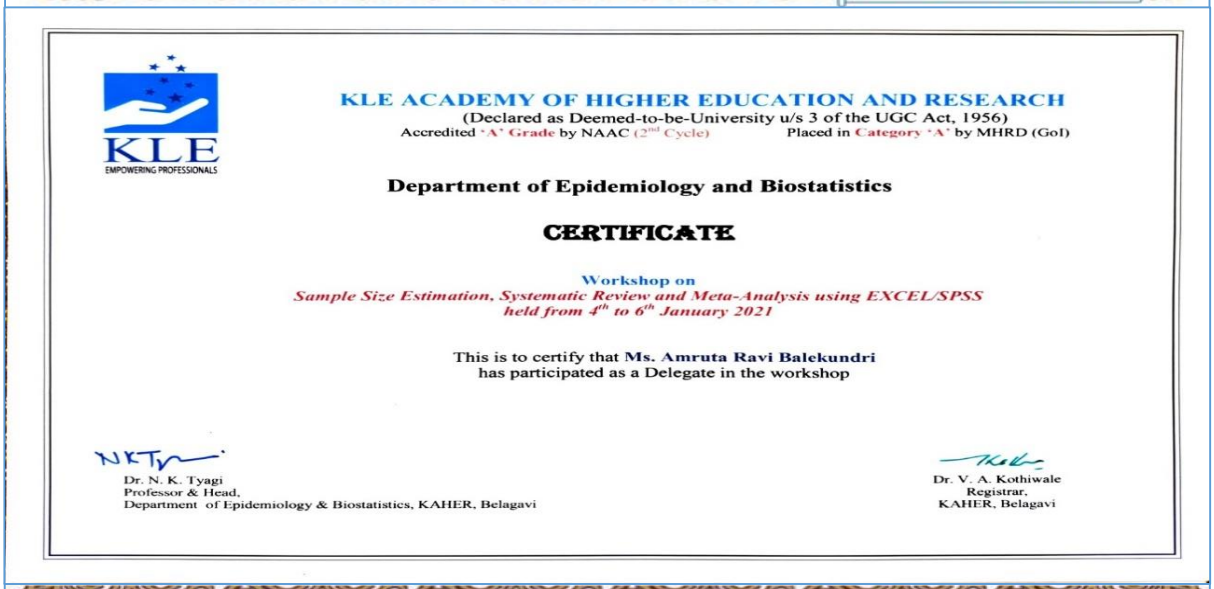

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Department of Epidemiology and Biostatistics

CERTIFICATE

Workshop on
Sample Size Estimation, Systematic Review and Meta-Analysis using EXCEL/SPSS
held from 4th to 6th January 2021

This is to certify that **Ms. Amruta Ravi Balekundri**
has participated as a Delegate in the workshop

Dr. N. K. Tyagi
Professor & Head,
Department of Epidemiology & Biostatistics, KAHER, Belagavi

Dr. V. A. Kothiwale
Registrar,
KAHER, Belagavi

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Certificate

This certificate is awarded to

AMRUTA RAVI BALEKUNDRI

For attending the webinar on "Use of Sterile Preparations"
organized by KLE College of Pharmacy, Belagavi, Karnataka
on 13th January, 2021.

Dr. V.S.Mannur
Coordinator

Dr. Sunil Jalalpure
Principal

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Department of Epidemiology and Biostatistics
KAHER/EBD/IN2021/D-014

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This is to certify that **Ms. Amruta Ravi Balekundri**
has attended the 'Intensive Course in Research Methodology and Biostatistics'
held from 11th January to 22nd February, 2021 at KAHER, Belagavi
and passed with **87.3 Percent (Grade A)**

Dr. N. K. Tyagi
Prof. & Head,
Department of Epidemiology & Biostatistics,
KAHER, Belagavi

Dr. V. A. Kothiwale
Registrar,
KAHER, Belagavi

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Cert. No. 7162 **ON-SITE HPTLC TRAINING CERTIFICATE** March 15, 2021

This is to certify that
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from ***CAMAG HPTLC*** *customer*
KLE Academy of Higher Education and Research, Belagavi
has *received a one-day free training* ***on 12/03/2021***
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BELAGAVI

nirf 37th 2020 Rank

Certificate

This is to certificate is that Dr./Mr./Ms. Amruta Balekundri

has participated as a **Resource Person / Delegate** in the KAHER sponsored One-Day workshop on "**Marketed Herbal Drug Evaluation by HPTLC**" organized by Dept. of Pharmacognosy, KLE College of Pharmacy, Belagavi, on 13th March 2021.

Dr. D. Nithyananda Sastry
Program Coordinator

Prof. (Dr.) Kalpana S Patil
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Prof. (Dr.) Sunil Jalalpure
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Health Research Fundamentals

(ICMR-NIE e-Certificate course: NIeCer 101)

with a score of **77** %

Online Assignments	24.46/25	Proctored Exam	52.5/75

Total number of candidates certified in this course: **852**

Dr. Manoj Murhekar
Scientist 'G' & Director
ICMR-National Institute of Epidemiology, Chennai

Prof. Balram Bhargava
Secretary to Govt. of India, Dept. of Health Research
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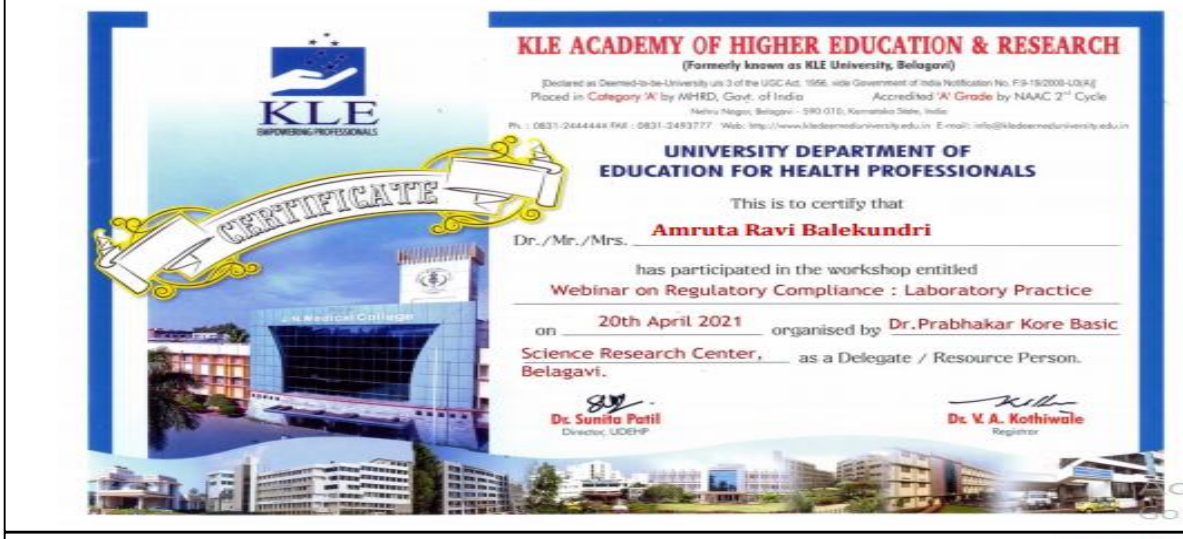

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CERTIFICATE

UNIVERSITY DEPARTMENT OF
EDUCATION FOR HEALTH PROFESSIONALS

This is to certify that

Dr./Mr./Mrs. Amruta Ravi Balekundri

has participated in the workshop entitled

Webinar on Regulatory Compliance : Laboratory Practice

on 20th April 2021 organised by Dr.Prabhakar Kore Basic Science Research Center, Belagavi. as a Delegate / Resource Person.

Dr. Sunita Patil
Director, UDEHP

Dr. V. A. Kothiwale
Registrar

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This is to certify that

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Has attended the workshop on

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Akshay Charegaonkar
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Anchrom Enterprises (I)
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MHRD
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Vidyanagar, Hubballi - 580031.
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Certificate of Appreciation

This certificate is awarded to Dr./Ms./Mrs./ Mr. **Amruta Ravi Balekundri** for attending the webinar on "Application of LC-MS/MS in Pharmaceuticals" organized by Dept. of Pharmaceutical chemistry, KLE College of Pharmacy, Vidyanagar, Hubli, on 07th of June, 2021.

Dr. AHM V Swamy
Principal
KLE College of Pharmacy,
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(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. AMRUTA BALEKUNDRI
from.....for his / her contribution as a Delegate/Volunteer 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.: GSPICON21OPT1R3

CERTIFICATE OF PARTICIPATION

THIS CERTIFICATE IS BEING AWARDED TO

DR./MR./MS. MS. AMRUTA RAVI BALEKUNDRI

HAS SUCCESSFULLY PRESENTED A POSTER / ORAL ENTITLED

Phytochemical, Physicochemical And Preliminary Parameters For Quality Assessment Of Sitopaladi Churna

AND SECURED **THIRD RANK** 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

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. Amruta Ravi Balekundri 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

CERTIFICATE
OF ATTENDENCE

This is to certify that
AMRUTA BALEKUNDRI
Has attended the webinar on
Extractable and Leachable study of Phthalates in pharmaceutical products and biomonitoring of phthalates by HPTLC Versus LC-MS/MS
Conducted on 19th February 2022
Organised by
Anchrom Enterprises (I) Pvt. Ltd

AKSHAY CHAREGAONKAR
Director
Anchrom Enterprises (I) Pvt. Ltd
Mumbai

DR. KASHYAP N. THUMMAR
Asst. Professor
Graduate School of Pharmacy
GTU, Gujarat

Late Shri Laksmanji Motghare
Charitable Trust, Nagpur's
Dr. Arun Motghare College of Pharmacy, Kosra-Kondha, Tah. Pauni, Dist. Bhandara, (MS) India
Dr. Arun Motghare Diploma in Pharmacy, Kosra-Kondha, Tah. Pauni, Dist. Bhandara, (MS) India
Dr. Arun Motghare Mahavidyalaya
Kondha-Kosra, Tah. Pauni, Dist. Bhandara, (MS) India
In Association With Society For Ethnopharmacology (SFE) Nagpur Chapter, Nagpur
"ETHNOPHARMACOLOGY: A PERSPECTIVE OF IMMUNOBOOSTERS AND ANTIVIRALS"
3 DAYS NATIONAL E-SEMINAR

Certficate

Dr./Mr./Mrs./Miss AMRUTA RAVI BALEKUNDRI
of KLE College of Pharmacy Belagavi
in recognition of his/her attendance as a Participant at 3 Days National e – Seminar on
"Ethnopharmacology: A Perspective of Immunoboosters and Antivirals"
from 28th Feb. – 02nd Mar. 2022

Dr. Arun L. Motghare
President
Late Shri Laksmanji Motghare Charitable Trust, Nagpur, (M.S.) India

Dr. Mohammad Tauqeer Sheikh
Principal
Dr. Arun Motghare College of Pharmacy Kosra –Kondha, Tah. Pauni, Dist. Bhandara (M.S.) India

Mr. Adesh Meshram
Principal
Dr. Arun Motghare Diploma in Pharmacy Kosra –Kondha, Tah. Pauni, Dist. Bhandara (M.S.) India

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Bombay College of Pharmacy

NRT National Facility for Research and Training

Certificate of Participation

This is to certify that

Amruta Ravi Balekundri

from KLE College of Pharmacy, Belagavi
has successfully completed
7-Day Hands-on Training Program on

"Application of Sophisticated Instrumental Techniques for Evaluation of Drugs and Pharmaceuticals during Discovery and Development"

held under the initiative

Synergistic Training Program Utilizing the Scientific and Technological Infrastructure (STUTI) of Department of Science and Technology (DST), India,

jointly organized by Bombay College of Pharmacy & National Facility for Research and Training (DST-DPRP supported) with Institute of Chemical Technology as Program Management Unit.

30th May – 5th June 2022

Dr. Ratnesh Jain
DST STUTI ICT Coordinator

Prof. Krishna Iyer
In-charge Principal & Convenor

Prof. Krishnapriya Mohanraj
Coordinator

Dr. Vaishali Shirsat
Co-coordinator

7 Days Workshop on Operation of Atomic Absorption Spectrophotometer, Total Organic Carbon Analyser, Acoustic Doppler Current Profiler and Epifluorescence Microscope
23rd to 29th August 2022

CERTIFICATE OF PARTICIPATION

This is to certify that ~~Mr.~~ / ~~Mrs.~~ / Ms. / ~~Dr.~~ / ~~Prof.~~

AMRUTA RAVI BALEKUNDRI

has participated in the Synergistic Training Program Utilizing the Scientific and Technological Infrastructure (**STUTI**) **Workshop on Operation of Atomic Absorption Spectrophotometer, Total Organic Carbon Analyser, Acoustic Doppler Current Profiler and Epifluorescence Microscope** held during 23rd to 29th August 2022 at School of Earth, Ocean and Atmospheric Sciences (**SEOAS**), Goa University in association with Department of Science & Technology (**DST**) and Indian Institute of Technology (**IIT**) Gandhinagar.

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Assistant Professor
SEOAS
Co-ordinator

Azadi Ka Amrit Mahotsav

Dr. C. U. Rivonker
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Dean, SEOAS
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Indian Society for Ethnopharmacology

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9th International Congress of
SOCIETY FOR ETHNOPHARMACOLOGY, INDIA
SFEC 2022
22nd to 24th April 2022
(Globalizing Local Knowledge and Localizing Global Technologies)
Theme : Redefining Ethnopharmacology for the Global Health and Wellbeing

Certificate of Participation

This is to certify that Prof. / Dr. / Mr. / Ms. Amruta Ravi Balekundri has Presented a PAPER in ORAL / POSTER SESSION in the 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.

Title of presentation: Analytical Quality by design approach for high performance Thin layer Chromatography method development and validation.

Dr. T. M. Pramod Kumar
Organizing Secretary, SFEC - 2022

Dr. K. Shrutisagara
Organizing Secretary, SFEC - 2022

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SHORT COMMUNICATION

Greenness Evaluation and Simultaneous High-Performance Thin-Layer Chromatography Method for Determination of Powder Mixture and Ayurvedic Formulation

Amruta Balekundri[1] · Pramod Hurkadale[1] · Harsha Hegde[2]

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Abstract Quality is the essential aspect of pharmaceuticals for both synthetic and herbal products. The quality assessment of the herbals is the most challenging task. In this study, analytical method is developed for simultaneous estimation of the three different standards. The separation method known as high-performance thin-layer chromatography (HPTLC) is very adaptable and economical. The method developed on HPTLC system where in the stationary phase used is tlc silica gel G F254 with aluminum backing and the mobile phase finalized was ethyl acetate: hexane: formic acid (5:5:1) v/v/v. The developed method was then validated in accordance with International Conference on Harmonization and United State Pharmacopeia guidelines. After validation, the developed method was found to be easy, simple, precise and specific. The greenness of the method was evaluated using the AGREE scale.

Keywords HPTLC · Simultaneous · ICH guidelines · Validation · AGREE scale

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Introduction

The quality of any product is of utmost important to mankind; it becomes more important when it comes to pharmaceuticals and herbal medicine we consume, as the quality is an essential aspect and hence assessment of quality also becomes a crucial part. Herbal product that are marketed also requires standardization. Various modern techniques are applied to standardize the herbal products. Herbal products are prone to adulteration both intentional and unintentional. Determination of adulteration is one of the tedious job; the modern techniques help in identification of adulteration and quality assessment [1].

Sitopaladi churna is one of the classical preparation, which been widely used for ages. It has been consumed for treatment of various disease like cough, cold, respiratory tract infections and also as immune modulator. Sitopaladi churna consists of Cinnamonumzeylanicum (Twak), Piper longum (Pippali), Elettariacardamomum (Ela), Bambusaarundinacea (Vamsalochana) and Saccharumofficinarum (Sugar candy) [2].

The modern analytical techniques have been used in analysis of herbal medicines and products. The different analytical techniques that are utilized in the modern times are high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), gas chromatography mass spectroscopy (GC-MS) and liquid chromatography mass spectroscopy (LC-MS). Any of these techniques combined or single can be used for the analysis purpose. When it comes to the herbal or Ayurvedic formulation analysis, most of the time HPTLC is the technique of choice. HPTLC has advantages like it utilizes less amount of solvent, sensitive, precise and reliable. The HPTLC modules comprise an applicator, stationary phase, mobile phase, developing chamber, scanners and visualizer. The analysis

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is carried out for developing and validating the analytical techniques. The methods developed can help us in identification, determination as well as quantifications. Validation of the developed method is also an important aspect of the quality control. The various international bodies have their guidelines, which are to be followed while validating the analytical methods; the important once are, namely United State Pharmacopeia (USP) <1064>and International Conference on Harmonization (ICH Q2 R1), United State Pharmacopeia (USP) <203>.

Cinnamic acid is majorly available in cinnamon bark. Cinnamic acid is found to be effective in treatment of various diseases and disorders like bacterial infections, diabetes, cancer and neurological [3]. Gallic acid is highly anti-oxidant in nature, which makes it more reliable pharmaceutical agent for categories such as antioxidant, anticancer, antibacterial, antiulcer and antifungal [4]. Piperine is widely found in Piperaceae family; it has been utilized for ages in treating tumor, diabetes, obesity. It also acts as anti-microbial and immune-modulatory agent [5]. The structures of the standards are presented in supplementary figure 1.

Eco-friendly procedures are one of the important goal of green analytical chemistry. In green chemistry concept, 12 principles considering the environmental, safety and health issues are considered. The 12 criteria assessment is converted into the score ranging from 0.0 to 1.0. The assessments are based on significance principles, which are flexible, easy to interpret and easy to perform [6].

Instrumentation and Methods

Materials

The Piperine (Semi-labs), gallic acid (Hi-media) and cinnamic acid (Rankem) were the standards used in the method development; apart from that, other chemicals and solvents utilized were of analytical grade. The tlcsilica gel 60 F254 plates are used as the stationary phase. Two marketed formulations were taken as the samples.

HPTLC Instrumentation

CAMAG HPTLC instrument is used in this method development and validation. The 100-µL CAMAG Hamilton syringe and CAMAG Linomat V are utilized for sample application. The plate development is done in CAMAG twin-trough chamber, and the developed plate is scanned by CAMAG tlc Scanner 4. The chromatograms and spectra are obtained after the scanning. The software that was utilized was vision CATS.

Method Development [7–10].

Preparation of standard and samples: The standard solutions were prepared by accurately weighing and dissolving in methanol solvent, which has a concentration of 1 mg/ml. The samples were also prepared in a similar manner with a concentration of 10 mg/ml; further, they were sonicated and centrifuged for 10 min.

Mobile-phase optimization: For optimizing the method, various trails were carried out with different mobile phases and ratios. The mobile phase that gave a proper separation was considered as the optimized system. The plate was developed up to 70 mm; chamber saturation of 5 min was done.

Method Validation

For the acceptance of the developed method, the method must be validated. The validation of the method was done in accordance with the guidelines like USP chapters (<203> <1064>) and ICH Q2R1 [11–13]. Various parameters like linearity, range, LOD, LOQ, specificity, precision and robustness were validated in this method.

Linearity and Range: Linearity and range of the developed method were finalized after various trail applications. The concentration range in which the linearity was obtained was chosen based on the regression equations obtained.

LOD and LOQ: Based on the principles of Statistics, the limit of detection and limit of quantification of all the standards were calculated, wherein the linearity values were also considered. The following formulas are applied for calculations that is, LOD = 3.3 SD/slope; LOQ = 10 SD/slope; SD is standard deviation.

Specificity: The specificity parameter in this method was validated by applying on plate different bands that is of analytes, mobile phase and solvents. After the application, the plate was dried, developed and scanned to obtain chromatograms.

Precision: The precision parameter is subdivided into inter-day and intra-day precision. In this parameter, the triplicates of the selected concentration are applied and their %RSD is calculated by applying the statistical principles. The value of %RSD if obtained less than 2% then is considered as the method is precise.

Robustness: In this parameter, a deliberate change is being made in the developed method wherein the factors like tank saturation time, change in the volume of mobile phase and slight change in the mobile phase compositions are done.

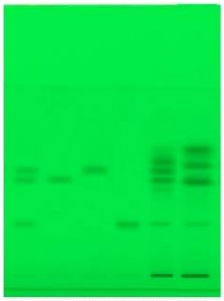
Greenness Assessment

The AGREE technique is utilized to assess the developed method. The AGREE scale ranges from 0.0 to 1.00. The tool used is the Analytical Greenness Calculator with version 0.5 2020 Gdansk University of Technology, Gdansk, Poland) [6, 14–16].

Results and Discussion

Method Development

The HPTLC method developed with the stationary phase was tlc silica gel 60 F254 and mobile phase finalized was ethyl acetate: hexane: formic acid (5:5:1) v/v/v. Figure 1 shows the developed method plate with different bands after development. The supplementary figure 2 shows spectra obtained after scanning of the bands. Table 1 shows the optimized chromatographic condition along with the parameters.



Track 1: Mixture; Track 2: Piperine;
Track 3: Cinnamic acid;
Track 4: Gallic acid;
Track 5: Sample 1; Track 6: Sample

Fig. 1 Image of the developed tlc plate

Method Validation

The developed method was validated as per the guidelines specified in ICH Q2 R1. The method was validated for parameters like linearity, range, LOD, LOQ, and precision.

Linearity and Range: The linearity of the standard was found from 1 to 5 µL/band range. The correlation coefficient was found to be 0.989, 0.998 and 0.993 for cinnamic acid, gallic acid and piperine, respectively. The results and equation found are reported in Table 1. The linearity plots of the standards and the linearity and range chromatograms are shown in supplementary figure 3 and supplementary figure 4, respectively.

LOD and LOQ: The LOD was found to be 0.525056, 0.524666 and 0.53896 µg/band and LOQ was found to be 1.59108, 1.5898, and 1.6332 µg/band for cinnamic acid, gallic acid and piperine, respectively.

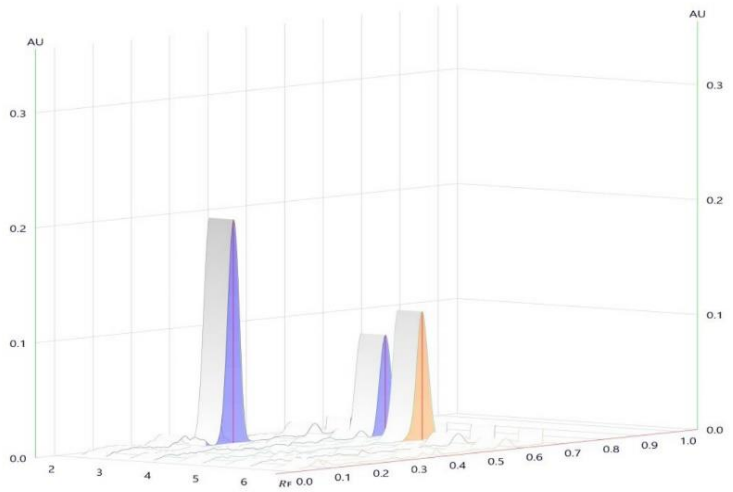
Precision: The precision is divided into two types, namely intra-day and inter-day precision. In this parameter, the replicates of all the standards with concentration 3 µL/band were applied. The results of precision and area both were found to have %Relative Standard Deviation < 2. The results are presented in Table 1.

Specificity: In specificity parameter, the developed chromatograms of the solvent and mobile phase had no specific interference with the chromatograms of standard and the sample; hence, the developed method was considered to be

Table 1 Validation parameters and results

Parameters	Cinnamic acid	Gallic acid	Piperine
Linearity	$y=0.0009x+0.0005$	$y=0.0016x-0.0005$	$y=0.0009x-0.0002$
Range (µL/band)	1–5	1–5	1–5
R^2	0.989	0.998	0.993
LOD (µg/mL)	0.525056	0.524666	0.53896
LOQ (µg/mL)	1.59108	1.5898	1.6332
Precision %RSD			
Intra-day precision (%RSD)			
Rf value	1.018922026	0.56765	1.4769
Area under curve	0.009501	0.63145	0.5208
Inter-day Precision (%RSD)			
Rf value	0.2513	0.9211	0.1002
Area under curve	0.8424	1.1226	1.6284
Robustness (Rf value) %RSD			
Mobile phase composition	0.0950	0.6314	0.1002
Mobile phase volume	0.2513	0.4638	0.5208
Duration of chamber saturation	0.1900	1.0663	0.17421

Fig. 2 Specificity parameter



Track 2-4: Standards; Track 5:Mobile Phase ;Track 6:Methanol;

specific in nature. Figure 2 shows the chromatograms found in specificity parameter.

Robustness: The %RSD (relative standard deviation) for robustness was found to be < 2% after making deliberate changes in the time of chamber saturation, mobile phase composition and mobile phase volume.

Greenness Assessment

In this assessment, the agree scale was utilized to determine the greenness assessment of the developed method. The score was found to be 0.74 for the method developed after carrying out the assessment of the 12 GAC (green analytical chemistry) component. The score pictogram is shown in Fig. 3 along with all the components.

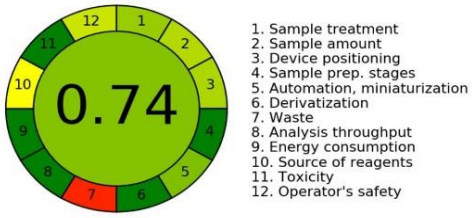


Fig. 3 Pictogram of AGREE scale of the developed method using AGREE calculator

Conclusion

In this study, a simple HPTLC method was developed and validated in accordance with ICH Q2 R1 guidelines. The developed method was rapid, easy, precise, specific and robust. The method can be applied in determination of cinnamic acid, gallic acid and piperine simultaneously, which is helpful in saving time and economic. The greenness score was found to be 0.74, which is a greener score.

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Author Contributions AB has performed the experiment, collected, analyzed the data and prepared the manuscript. PH and HH have designed the experiment, analyzed and finalized the manuscript.

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Declarations

Conflict of interest No competing interests to declare.

Ethics Approval and Consent to Participate Not applicable.

Availability of Data All data and materials are available upon request.

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Quality assessment, HPTLC-DPPH, analytical quality by design based HPTLC method development for estimation of piperine in piper species and marketed formulations

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AGREE scale

ABSTRACT

Background: Quality assessment of herbs is an essential aspect. In this study HPTLC method is developed by employing the quality based optimization approach with the aid of analytical quality by design tools and the quality assessment of the Piper species.
Result: The Quality assessment was carried out for the piper species which was under the acceptance criteria of Ayurvedic Pharmacopeia of India. Eco-friendly and Quality based HPTLC method was developed and validated for Piperine in Extracts as well as marketed formulation. Further the antioxidant potential was checked by the HPTLC-DPPH (2,2-diphenyl-1-picrylhydrazyl) method.
Conclusion: Quality assessed extracts and Analytical quality based HPTLC method which was eco-friendly, simple and reliable was developed and validated.

Abbreviations

HPTLC	High Performance Thin Layer Chromatography
AQbD	Analytical quality by design
WHO	World health Organization
API	Ayurvedic Pharmacopeia of India
ATP	Analytical target profile
CQA	Critical quality attributes
DoE	Design of Experiments
S1	Sitopaladi Churna
T1	Talisadi Churna
ICH	International Conference on Harmonization
LOD	Limit of Detection
LOQ	Limit of quantification
Std.dev	standard deviation
S	slope of the calibration curve
DPPH	2,2-diphenyl-1-picrylhydrazyl
A	Volume of Solvent
B	Distance travelled by the solvent front
C	Chamber saturation
AGREE	Analytical GREEnness
GAC	Green analytical chemistry

Background

Humans prioritise quality above anything else in all facets of life. Since medications are meant to promote human welfare, their quality is crucial when it comes to products that humans ingest. There are severe norms and regulation for the quality control of the synthetically manufactured chemical medicines. Before being marketed and ingested by patients and consumers, they must pass a number of tests and quality controls. This regulatory rigour ensures the safety and effectiveness of the pharmaceutical goods by ensuring that the quality of medications produced synthetically is up to par. Apart from these, a number of quality control measures are utilised to ensure the quality features of the herbals. Standardisation and the phytochemicals investigation are conducted. The quality assurance of them requires both qualitative and quantitative measurements.

As there is an increasing demand for herbal medications, there is a need to assure their quality. The quality of the herbals is influenced by many physical, chemical, and geographical characteristics which contribute to the quality of these materials. Apart from that,

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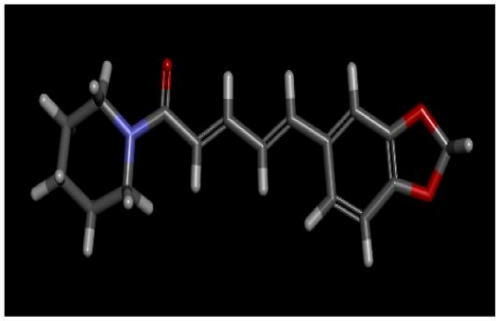


Fig. 1. Chemical structure of Piperine.

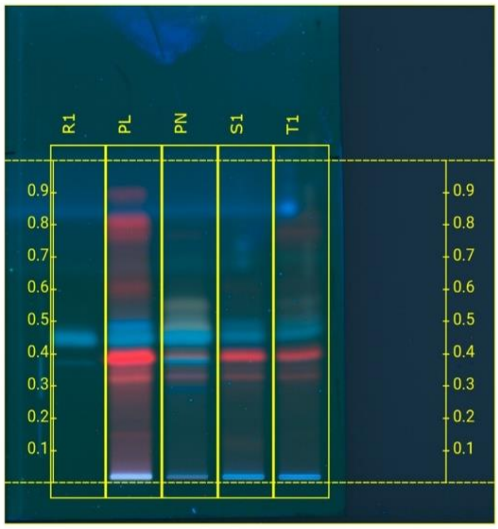


Fig. 2. Plate image of method development
R1: Piperine; PL:*Piper longum*; PN: *Piper nigrum;* S1: Sitopaldi churna marketed sample; T1: Talisadi Churna marketed sample;.

Table 1
Quality assessment data.

Parameter	*Piper longum*	*Piper nigrum*
Foreign matter	0.30 %	0.32 %
Color	Brownish black	Black
Odor	Characteristic, Aromatic	Characteristic, Aromatic
Taste	Pungent	Pungent
Shape	Elongated	Round and/or globular
Extractive Values		
Alcoholic	12 %	6.5 %
Aqueous	10 %	3.2 %
Ash Values		
Total Ash	9 %	10 %
Alcohol	2 %	2 %
Water	4 %	5 %
Loss on drying	11 %	8 %
pH	6.0	6.2

adulteration is also an increasing concern when it comes to herbal material quality. The quality characteristics of the herbal materials in the herbal pharmaceuticals are assessed using a variety of chemical and

Table 2
Box-Behnken design with factors and responses.

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
		A:Volume of solvent (mL)	B:Solvent front (cm)	C:Saturation time (minutes)	Rf value	Area
14	1	8	8	10	0.561	0.00516
16	2	8	8	10	0.569	0.00523
8	3	9	8	15	0.325	0.00625
1	4	7	7	10	0.621	0.005
5	5	7	8	5	0.666	0.00567
9	6	8	7	5	0.437	0.00468
12	7	8	9	15	0.512	0.00408
2	8	9	7	10	0.368	0.00685
13	9	8	8	10	0.459	0.00597
7	10	7	8	15	0.682	0.005002
11	11	8	7	15	0.492	0.005998
15	12	8	8	10	0.563	0.00518
3	13	7	9	10	0.684	0.00564
10	14	8	9	5	0.456	0.00569
17	15	8	8	10	0.562	0.0052
6	16	9	8	5	0.386	0.006
4	17	9	9	10	0.398	0.00685

Table 3
Statistical analysis parameters and equations.

Response	Coded equation	Model	PRESS	S.D
Rf value	0.5428- 0.1470*A + 0.0165*B + 0.0083*C −0.0083*AB −0.0193*AC + 0.0003*BC+ 0.0077*A^2–0.0328*B^2–0.0358*C^2	Quadratic	0.0875	0.0438
Area	0.0053+ 0.0006*A −0.0001*B −0000.1*C-0.0002*AB+ 0.0002* AC −0.0007* BC +0.0007A^2 +0.0001B^2 −0.0003C^2	Quadratic	9.977E-06	0.0004

phytochemical tests, analytical techniques, and hyphenated analytical techniques. [1]

Piper longum (Supplementary Fig. 2a) also referred to as "long-pepper" or "Pippali" and belonging to the Piperaceae family, can be cultivated as a perennial shrub or a herbaceous vine. It is extensively dispersed throughout the tropical and subtropical world, including the Indian subcontinent, and is a native of the Indo-Malayan region. The fruits are mostly utilised in food as spices and preservatives, and they are also an effective treatment in a number of conventional medical systems. [2]

Black pepper/*Piper nigrum (*Supplementary Fig. 2b) is a spice used in both contemporary and conventional cuisine. *P. nigrum* is a member of the Piperaceae family. The presence of certain phenolic components including alkaloids, flavonoids, carotenoids, terpenoids, etc. is what gives black pepper its pharmacological properties. Alkaloids (piperine). [3]

The most prevalent plant alkaloid in the Piperaceae family, piperine (PIP), has recently attracted a lot of attention due to its wide range of beneficial biological and pharmacological characteristics. Piperine is a yellow crystalline substance with the chemical formula $C_{17}H_{19}NO_3$. It is a weakly basic, polar molecule. [4] (Fig. 1)

Material and methods

Chemicals and plant materials

Standard Piperine was procured from Hi-media, and all the other chemicals and solvents utilized in the research work belong to analytical grade. The plant material *Piper Longum* and *Piper nigrum* were procured from Shri B. M. Kankanwadi Ayurveda Mahavidyalaya, Karnataka and

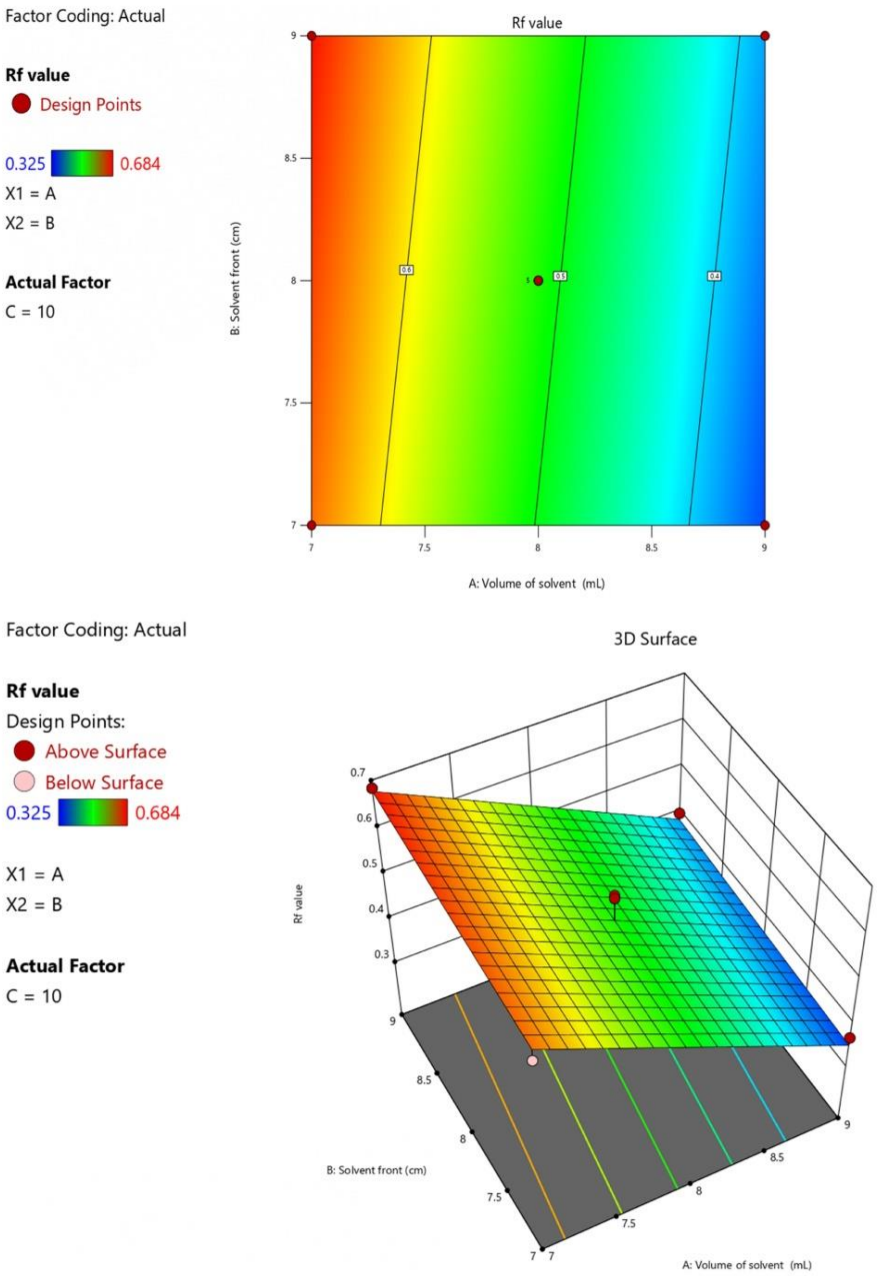


Fig. 3. Surface response curves 2D and 3D images.

authenticated.

Quality assessments

The fruits of both these piper species were allowed to dry at room temperature in shade, further the fruits were ground to prepare powder and sieved. The powder was subjected to cold extraction with methanol solvent and extract was prepared. The quality assessment of the *Piper longum* and *Piper nigrum* were assessed for the parameters namely- Physico-chemical (moisture content, extractive value, and ash value) Phyto-chemical analysis and Pharmacognostic analysis in accordance with WHO guidelines and Ayurvedic Pharmacopeia of India. [5]

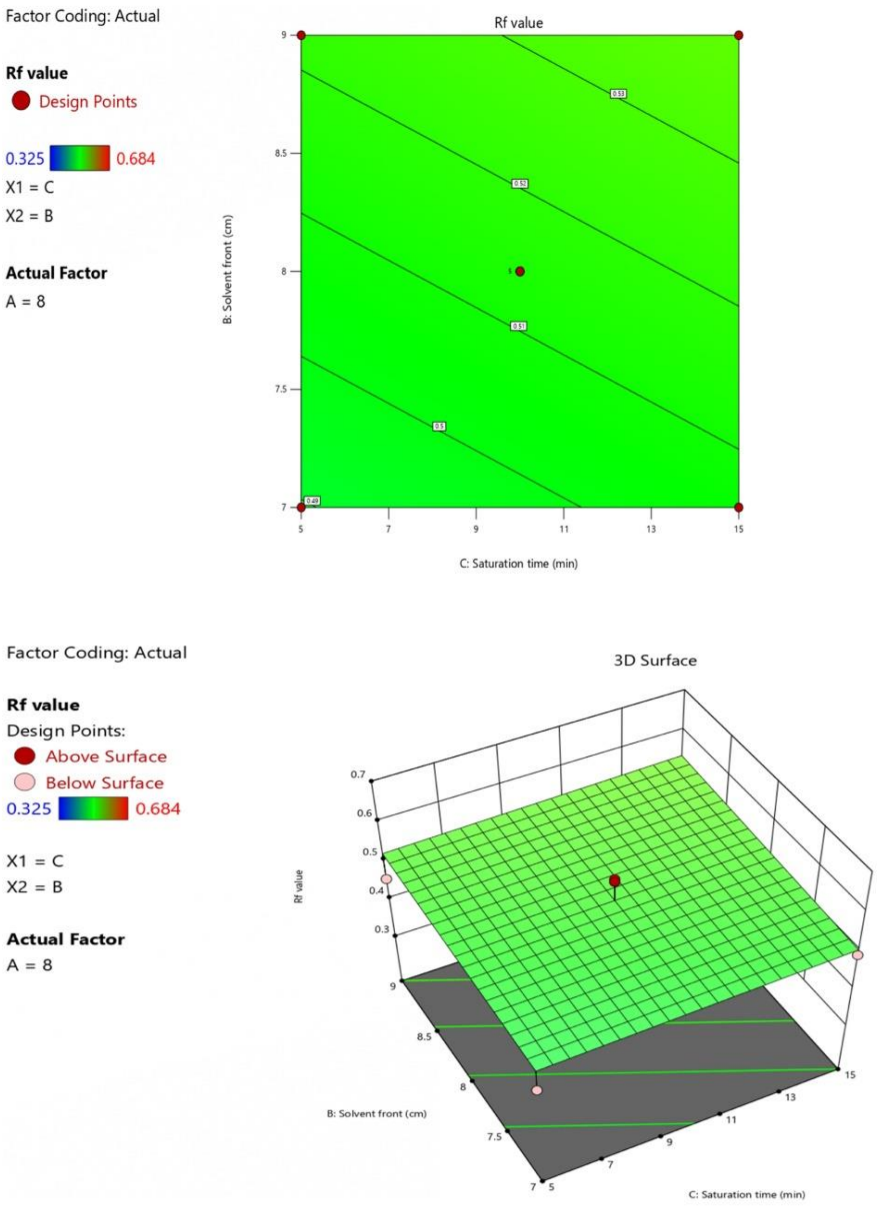


Fig. 3. (*continued*).

HPTLC

Instrumentation: HP-TLC Camag instrument with applicator Linomat 5, TLC Visualizer, TLC Scanner 4, TLC twin trough chamber (Camag Switzerland), TLC pre-coated silica gel aluminum plates with 60 F_{254} and TLC Hamilton glass syringe (100 µL) is utilized to carry out the research work. The software visionCATS version 3 by Camag Switzerland was implied.

Preparation of standard and sample: The standard Piperine with concentration (1 mg/ml) was prepared by dissolving it in methanol solvent. In case of extracts the samples and Marketed samples [Sitopaladi Churna (S1) and Talisadi Churna(T1)] are prepared by dissolving 100 mg/ml in methanol and allowed to sonicate for 10 min, further kept for centrifugation for 10 min and filtered. [6]· [7]

Analytical quality by design

Defining of analytical target profile (ATP) and critical quality attributes (CQA)

Analytical Target Profiles (ATP) must first be established before the

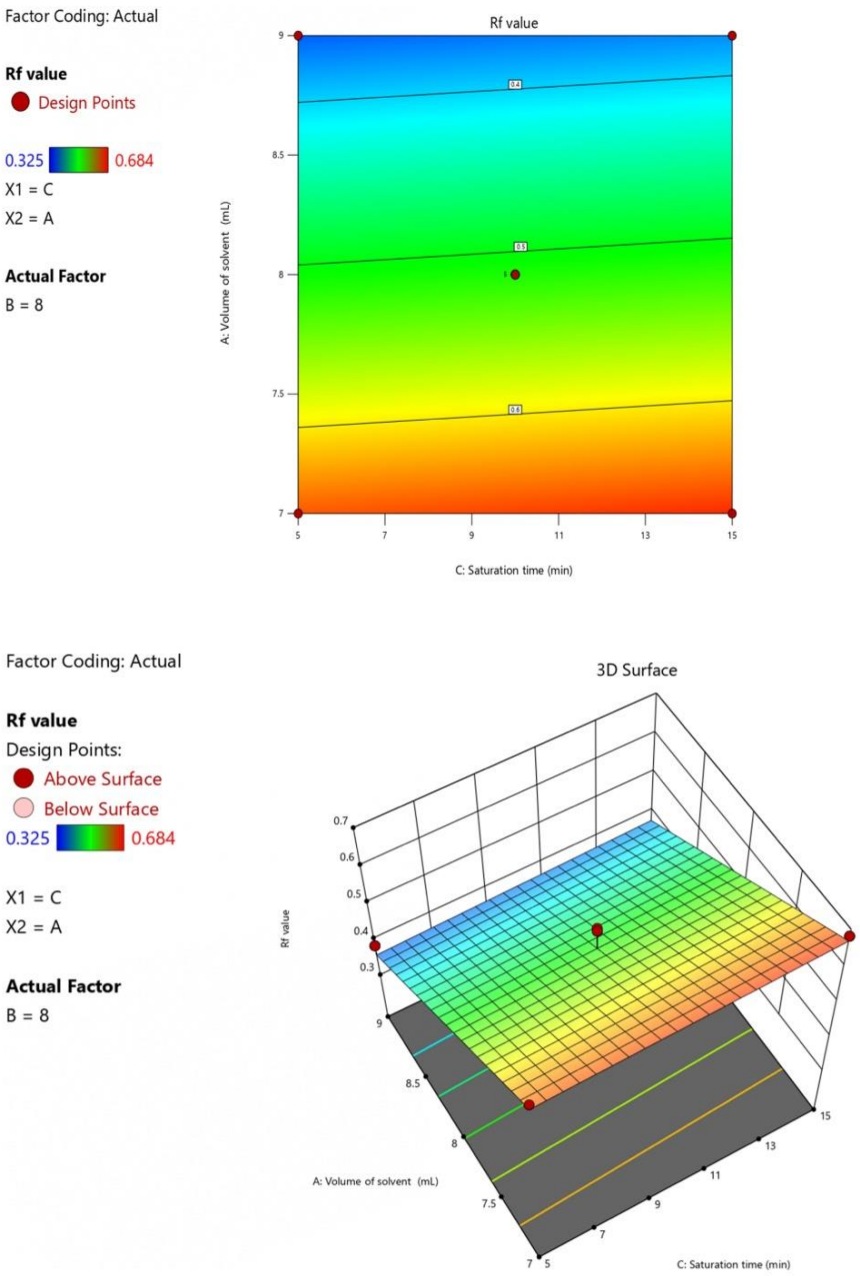


Fig. 3. (*continued*).

Qbd technique can be used to build analytical methods, where in the fundamental qualities that are to be decided upon are indicators of the performance of the method. Critical Quality Attributes (CQAs) from the defined ATP that will be helpful in determining the good performance of the created technique must be determined in order to achieve reliable results.

Analytical Target Profile (ATP) for the HPTLC: The purpose is likely to develop an optimized HPTLC method for a specific analysis. The critical responses (CQAs) identified are the Rf value and Area. These are essential for characterizing and quantifying the components in the sample. The factors considered for method development are the volume of solvent (A), distance travelled by the mobile phase (B), and chamber saturation (C).The Box-Behnken design was chosen for method development and optimization, indicating a systematic and structured

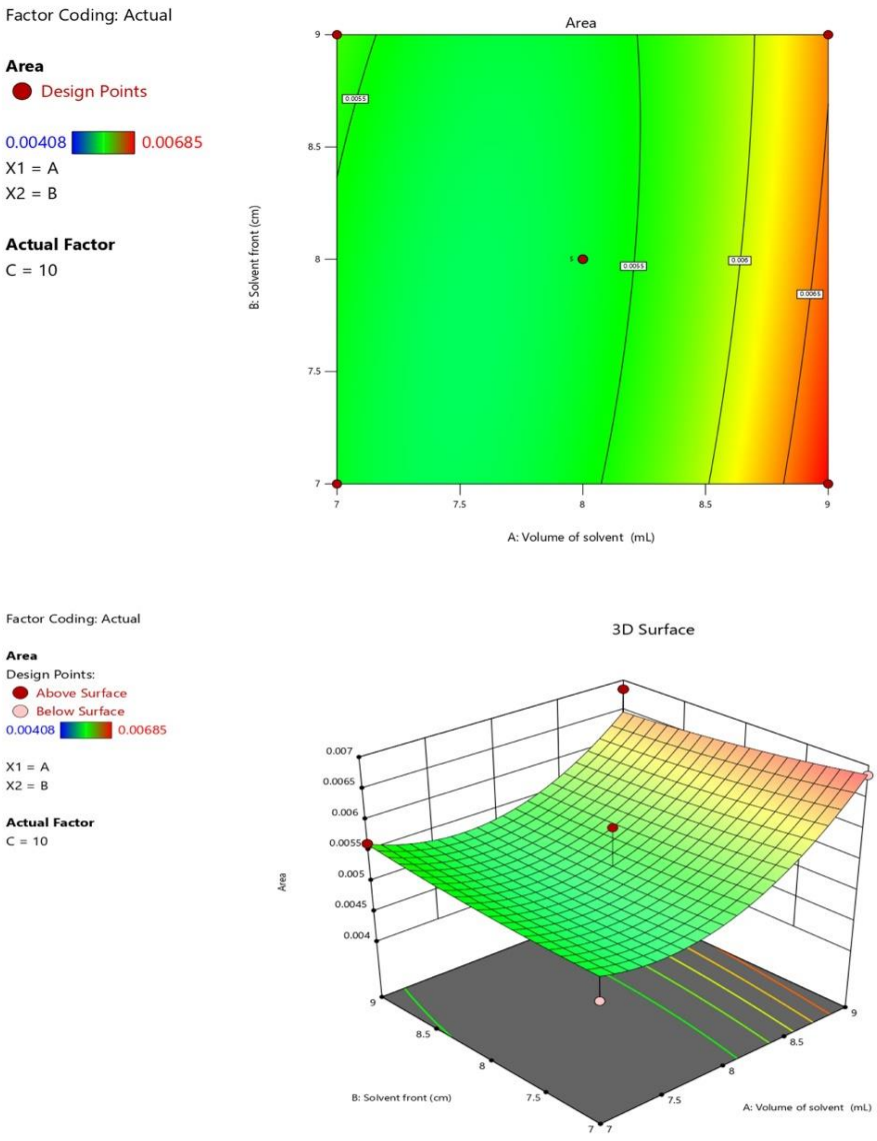


Fig. 3. (*continued*).

approach. The responses, Rf value, and Area are recorded, reflecting the performance of the method. Statistical analysis, surface response plots in 2D and 3D, is conducted to understand the impact of factors on responses. Optimization: The results indicate that the most optimized conditions are associated with specific factor levels: A (Volume of Solvent) - 8 ml; B (Distance travelled by the solvent front) - 7 cm; C (Chamber saturation) - 5 min; Method Development by AQbD: The method is developed using Analytical Quality by Design (AQbD) principles, optimizing all selected parameters to ensure a quality-imbibed method. Chromatographic Conditions: The optimized conditions are summarized in Table 5, providing a clear reference for the developed method.

Method development and optimization by design of experiments (DoE)

In this study the HP-TLC method was optimized by implementing the DoE for understanding the effects of the selected parameters and their responses. The Box-Behnken design is implied for the study which gives us seventeen experimental runs as three factors were selected (Volume of solvent, Distance travelled by solvent front and Saturation time) and the responses noted were the rf value and peak area of the chromatograms. Design Expert v.13 is applied to carry out the DoE which is provided by Stat-Ease Inc., Minneapolis, MN, USA). The software helps in understanding the critical values which will be helpful in achievement of desired response from the independent variables selected.

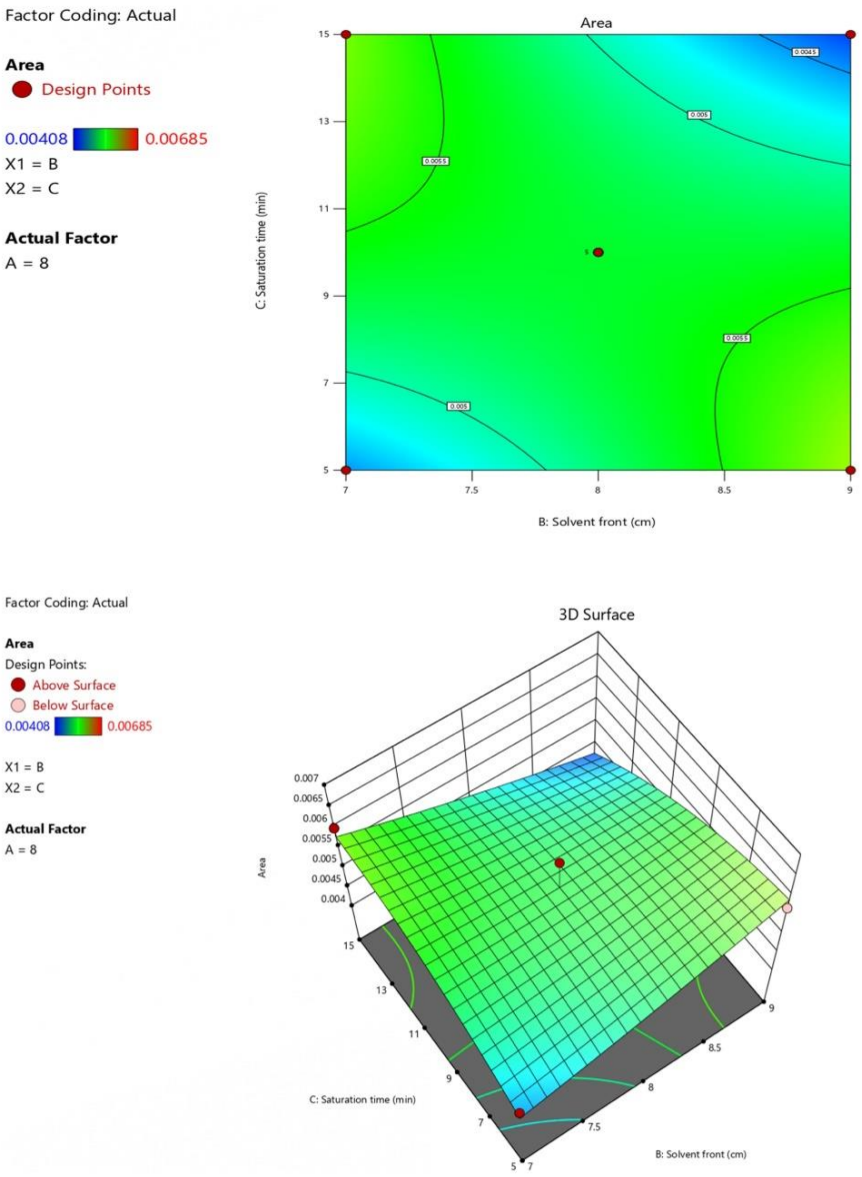


Fig. 3. (*continued*).

Validation of the optimized method

The method that is developed by DoE was validated in compliance with the ICH Q2 (R1) guidelines. The method was validated for Linearity and Range, LOD, LOQ, Precision, Specificity and Robustness.

Linearity and range: The standard Piperine was applied with different concentration to obtain a linear dynamic range. The slope, correlation coefficient (r^2) and y intercept are calculated from the linearity.

LOD and LOQ: The LOD and LOQ are helpful in evaluating the method sensitivity and calculated by applying the statistical formulas

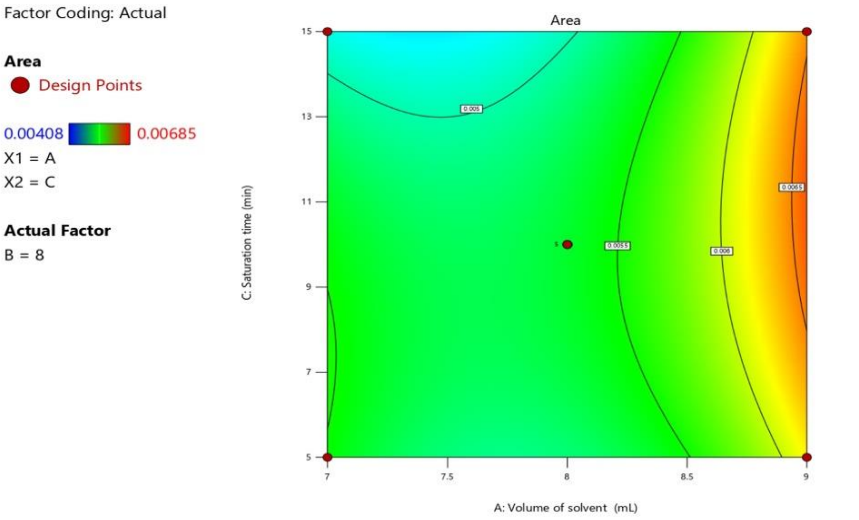
$$LOD = 3.3 * \text{std. dev}/S$$

$$LOQ = 10 * \text{std. dev}/S$$

where, std.dev- standard deviation; S- slope of the calibration curve.

Precision: The precision parameter is carried out in two ways namely inter-day and intra-day precision. In inter-day the parameter is done for three consecutive days and in intra-day the precision parameter is carried out at three different time intervals on the same day.

Specificity: In this parameter the tracks with sample, standard, solvent (used in sample preparation) and mobile phase are applied on the plate to check the specificity of the method.



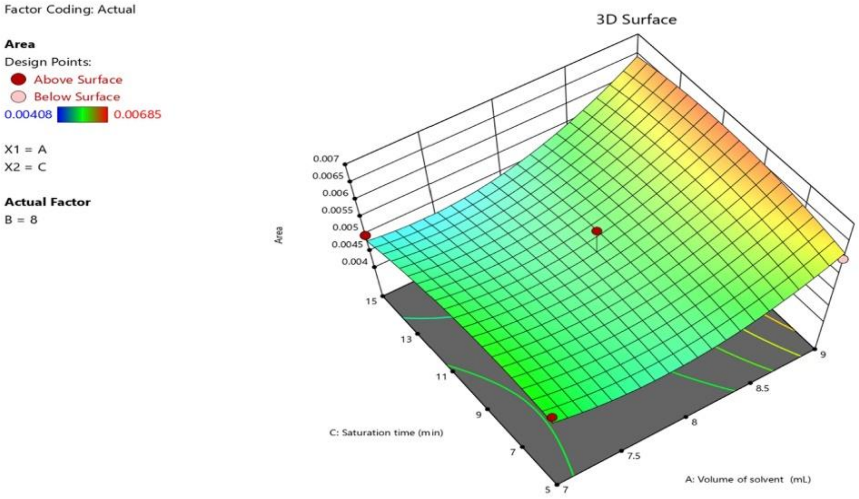


Fig. 3. (*continued*).

Quantification of piperine

The quantification of the Piperine in different samples was carried out by the developed HPTLC method after applying them in triplicates. The wavelength employed was 330 nm (lambda max). The sample applied was 5 µL.

HPTLC-DPPH

The antioxidant study was carried out by the HPTLC-DPPH method. In this method the plate was developed by using the same criteria and conditions as in the method mentioned earlier. The plate after drying was subjected to derivatization by immersing the plate in DPPH (0.5 mM methanolic) solution for duration of 5seconds and then further drying at room temperature for 90 s and then heating the plate at 60 °C temperature for next 30 s. The images are clicked in white light and the plates are scanned at 517 nm tungsten wavelength.

Greenness scale

AGREE refers to a Greenness scale or rating system, it is likely related to assessing the environmental impact or sustainability of a process, product, or system. Greenness scales are often used to evaluate and communicate the ecological and environmental aspects of various

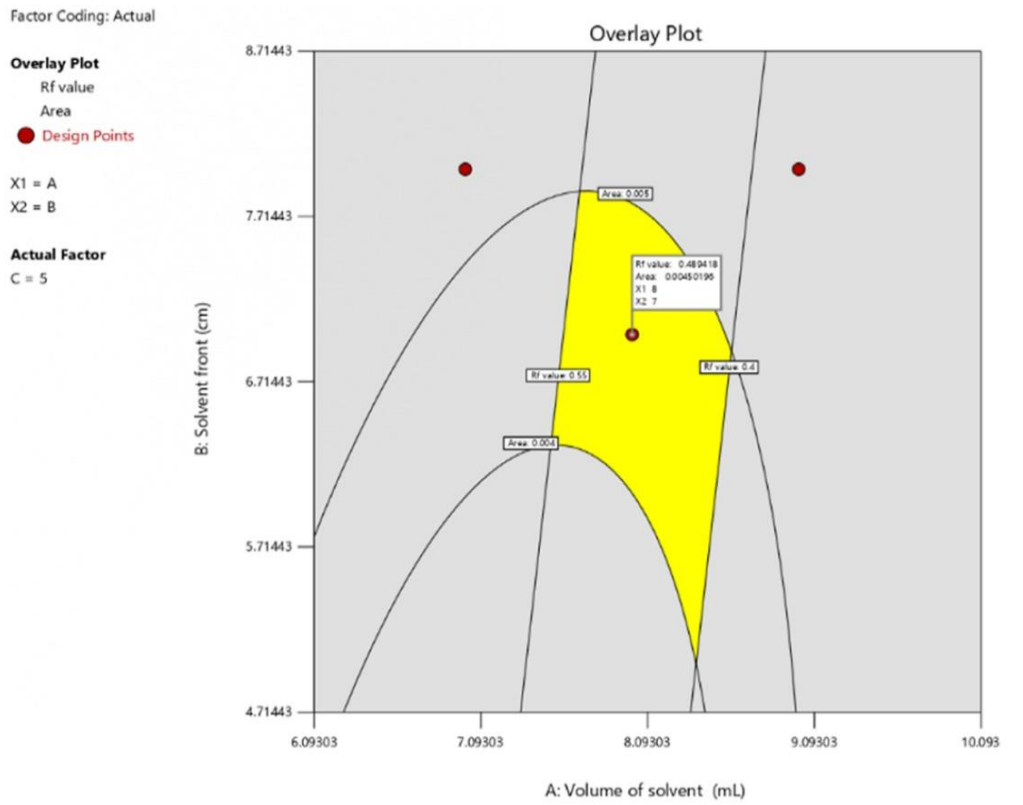


Fig. 4. Overlay plot for Graphical optimization.

practices. Related to evaluating the environmental friendliness of manufacturing processes, products, or technologies.

To assess the method developed by AQbD approach for its greenness index the AGREE scale Technique was employed. The tool namely Analaytical Greenness calculator (with ver 0.5 2020 Gdansk University of Technology, Gdansk, Poland) which has scale ranging from 0.0 to 1.00.

Fig. 2

Results and discussion

Quality assessment

The preliminary phytochemical investigation was carried out and *Piper longum* and *Piper nigrum* showed the presence of alkaloids, flavonoids, tannins, and steroids. The physicochemical and pharmacognostic parameter are carried out for *Piper longum* and *Piper nigrum* and the results are depicted in Table 1.

AQbD based method development and optimizations

For developing the HPTLC method various initial trails were carried out with different ratios of mobile phase. The Box-Behnken design is flexible in nature so it was chosen to study the factors and responses in HPTLC method development. The Box-Behnken design (Table 2) is employed for the method development and optimizations and the responses are recorded. The factors considered are volume of solvent, chamber saturation and the distance travelled by the mobile phase. The responses namely Rf value and Area are recorded. For further understanding of the study and optimization process the statistical analysis of the developed method was carried out and the values are depicted in the Table 3. The graphs showing the prediction vs actual values and perturbation are studied (Supplementary Fig. 2). The surface responses plots both in 2D and 3D are studied for graphical optimization along with the overlay plot (Figs 3, 4 and 5) which shows that the run std 9 and run 6 is found to be the most optimized after interpreting the statistical (Table 4,4b) and graphical results, that is the A (Volume of Solvent) - 8 ml, B (Distance travelled by the solvent front) –7 cm and C (Chamber saturation) - 5 min. The method developed by AQbD has optimized all the selected parameters to develop a quality imbibed method and the chromatographic conditions are summarized in Table 5. [8–10]

Method validation

The validation of the optimized method is carried out in accordance with the ICH Q2 (R1) guidelines and the results are summarized in the Table 6. It was found that the developed method (Fig. 6a, 6b and 7) was linear within the range of 2–10 uL/band, LOD was 1.03 whereas LOQ was 3.13 µg/mL and the%RSD for precision, robustness was found to be within criteria which is <2 %. [11]

Quantitative estimation of piperine

The developed and optimized method was employed for carrying out

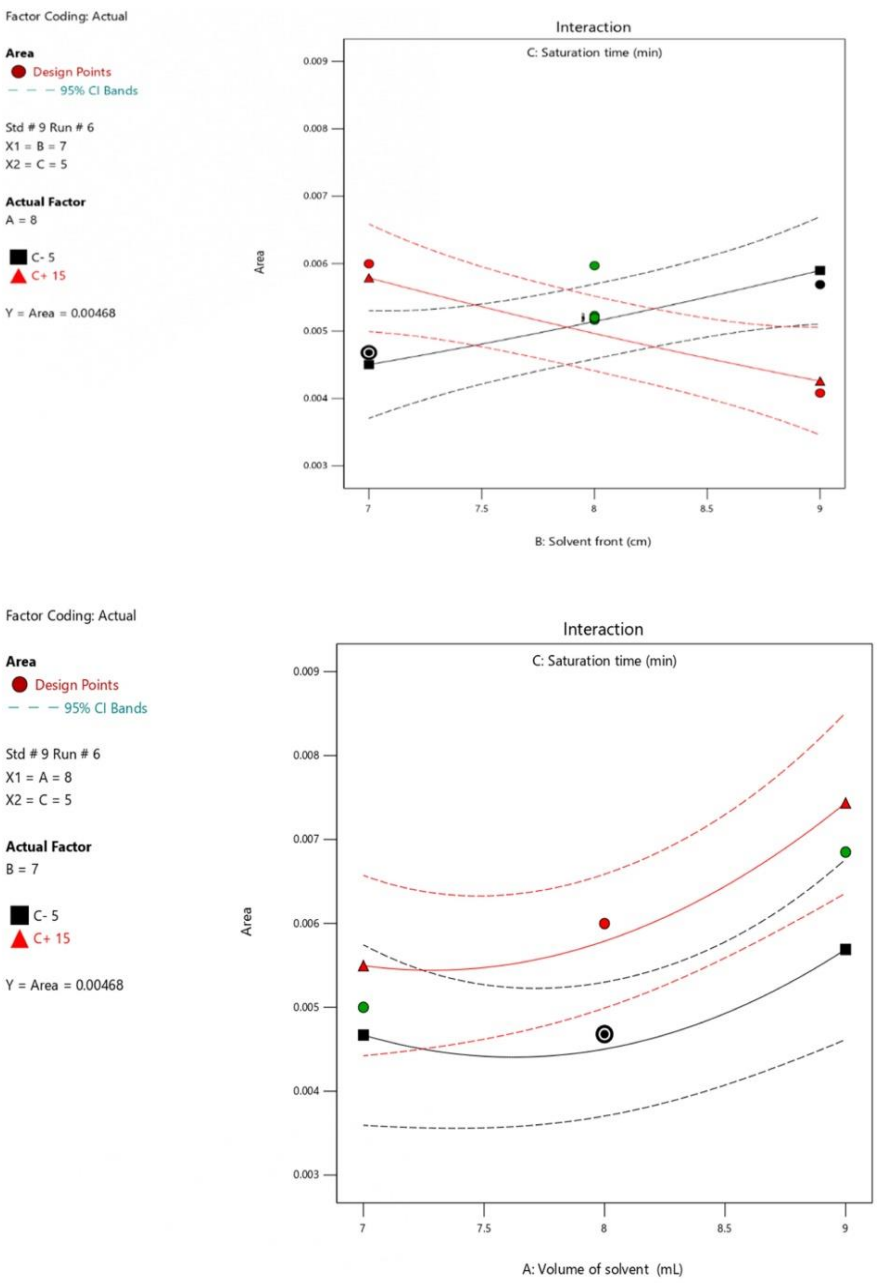
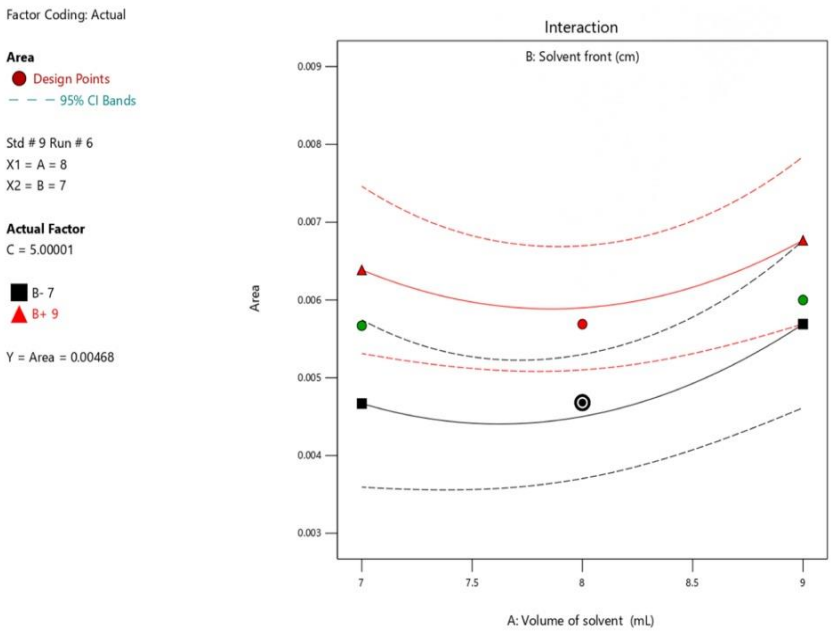


Fig. 5. Numerical optimization.

the quantification of the Piperine in piper species. The quantitative estimation of Piperine was found to be 2.712, 3.483, 0.621 and 0.8719% w/w in *Piper longum, Piper nigrum*, Sitopaldi churna and Talisadi Churna respectively. [12–16]

HPTLC DPPH

The HP-TLC DPPH assay was carried out for anti-oxidant potential determinations. The HPTLC DPPH method showed that the yellow bands



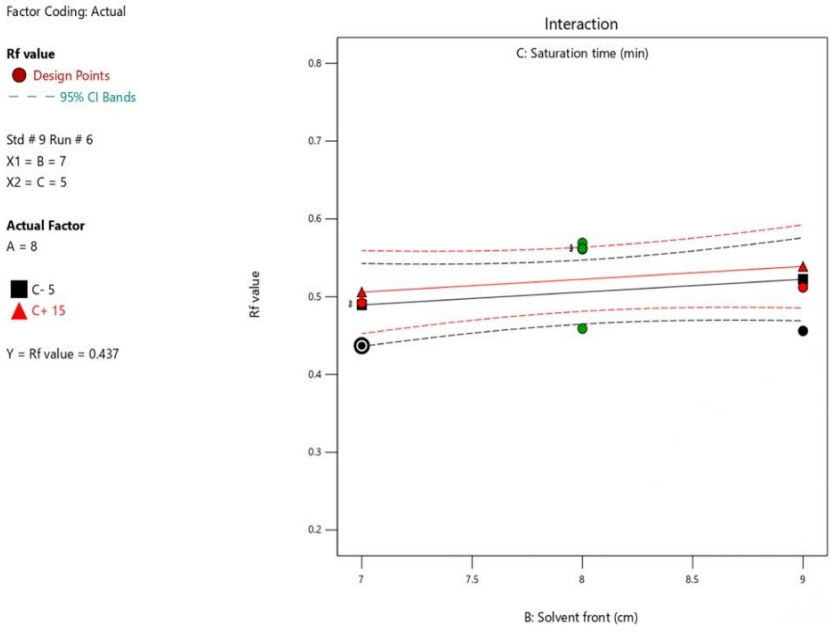
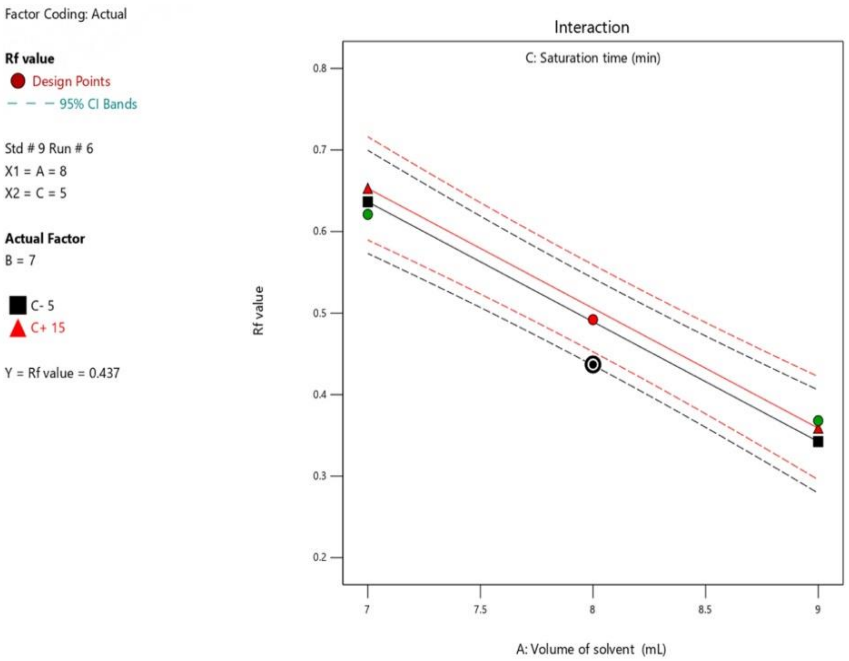


Fig. 5. (*continued*).



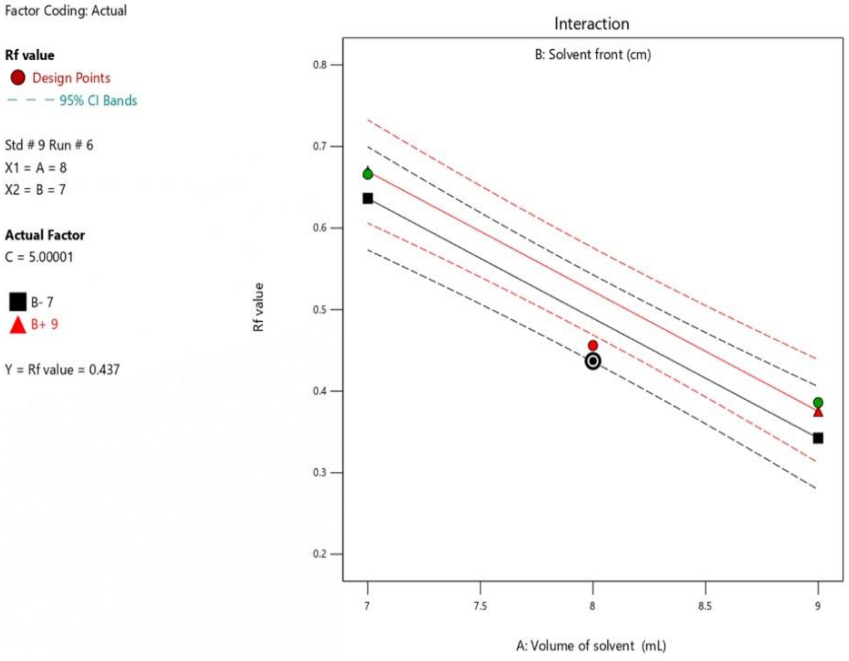


Fig. 5. (*continued*).

Table 4a
ANOVA for Quadratic model Response 1: Rf value.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1879	9	0.0209	10.89	0.0024	significant
A Volume of solvent	0.1729	1	0.1729	90.15	< 0.0001	
B Solvent front	0.0022	1	0.0022	1.14	0.3219	
C-Saturation time	0.0005	1	0.0005	0.2839	0.6106	
AB	0.0003	1	0.0003	0.1420	0.7175	
AC	0.0015	1	0.0015	0.7729	0.4085	
BC	2.500E-07	1	2.500E-07	0.0001	0.9912	
A^2	0.0003	1	0.0003	0.1310	0.7281	
B^2	0.0045	1	0.0045	2.36	0.1685	
C^2	0.0054	1	0.0054	2.81	0.1376	
Residual	0.0134	7	0.0019			
Lack of Fit	0.0046	3	0.0015	0.6967	0.6009	not significant
Pure Error	0.0088	4	0.0022			
Cor Total	0.2013	16				

Table 4b
ANOVA for Quadratic model Response 2: Area.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7.463E-06	9	8.293E-07	5.46	0.0179	significant
A-Volume of solvent	2.689E-06	1	2.689E-06	17.72	0.0040	
B Solvent front	8.978E-09	1	8.978E-09	0.0592	0.8148	
C Saturation time	6.301E-08	1	6.301E-08	0.4152	0.5399	
AB	1.024E-07	1	1.024E-07	0.6747	0.4385	
AC	2.107E-07	1	2.107E-07	1.39	0.2772	
BC	2.143E-06	1	2.143E-06	14.12	0.0071	
A^2	1.934E-06	1	1.934E-06	12.74	0.0091	
B^2	1.478E-08	1	1.478E-08	0.0974	0.7641	
C^2	3.670E-07	1	3.670E-07	2.42	0.1639	
Residual	1.062E-06	7	1.518E-07			
Lack of Fit	5.761E-07	3	1.920E-07	1.58	0.3265	not significant
Pure Error	4.863E-07	4	1.216E-07			
Cor Total	8.526E-06	16				

Table 5
Chromatographic criteria's of the optimized method.

Parameters	Conditions
Stationary Phase	TLC silica gel F254 plate with aluminum backing
Mobile Phase	Ethanol: Water: Formic acid (8:2:0.1) v/v/v
Saturation time	5mins
Distance travelled by solvent front	7 cm

have appeared on the purple plate background after derivatization with the DPPH reagent which shows the presence of the antioxidant potential of the samples. The more antioxidant potential is shown by the *Piper nigrum* then the *Piper longum* species (Fig. 8) (Supplementary Fig. 3).

Table 6
Validation parameter results.

Parameters	Piperine
Linearity	$y = 0.0021x + 0.0199$
Range (µL/band)	2–10 mcg
R^2	0.992
LOD(µg/mL)	1.033293
LOQ(µg/mL)	3.131192
Inter-day Precision (%RSD)	
Rf value	1.018
Area under curve	0.857
Intra-day Precision (%RSD)	
Rf value	0.9651
Area under curve	0.5260
Robustness (Rf value)%RSD	
Mobile phase composition	0.5697
Mobile phase volume	0.3591
Duration of chamber saturation	0.4789

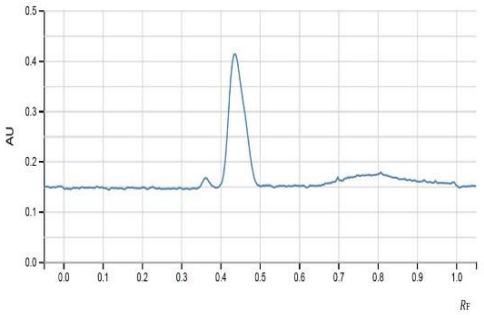


Fig. 6a. : Chromatogram of Standard Piperine

[17]

Greenness scale

The AGREE scale was employed to determine the greenness score of the developed method. The score of the method was found to be 0.80 after evaluating all the twelve Green analytical chemistry components (GAC) and the Fig. 9 shows the pictorial representation of the scale. scale. A higher score on the AGREE scale would indicate a greener or more environmentally friendly method, while a lower score might suggest a higher environmental impact. [18–23]

Conclusion

In the present study the quality assessment of *Piper longum* and *Piper nigrum* was carried out which showed good results in accordance with the Ayurvedic Pharmacopeia of India guidelines. The implementation of the analytical Quality by design approach for the development of simple, fast and robust HPTLC method was developed. The developed method was also employed for quantification of the Piperine and few addition of the steps were done to carry out the HPTLC-DPPH method for anti-oxidant activity. The developed method was found to be cost effective and also an approach towards eco-friendly technique. The DoE tool has helped in adding up to the quality of the developed method along with validation of the method which was in accordance with ICH guidelines. It can be concluded that the developed method is time and cost effective along with simple, robust and eco-friendly. The developed method can be employed for routine analysis.

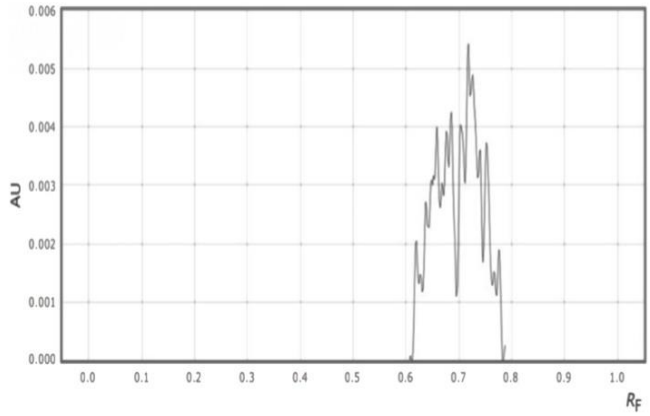


Fig. 6b. : Chromatogram of Mobile phase/blank

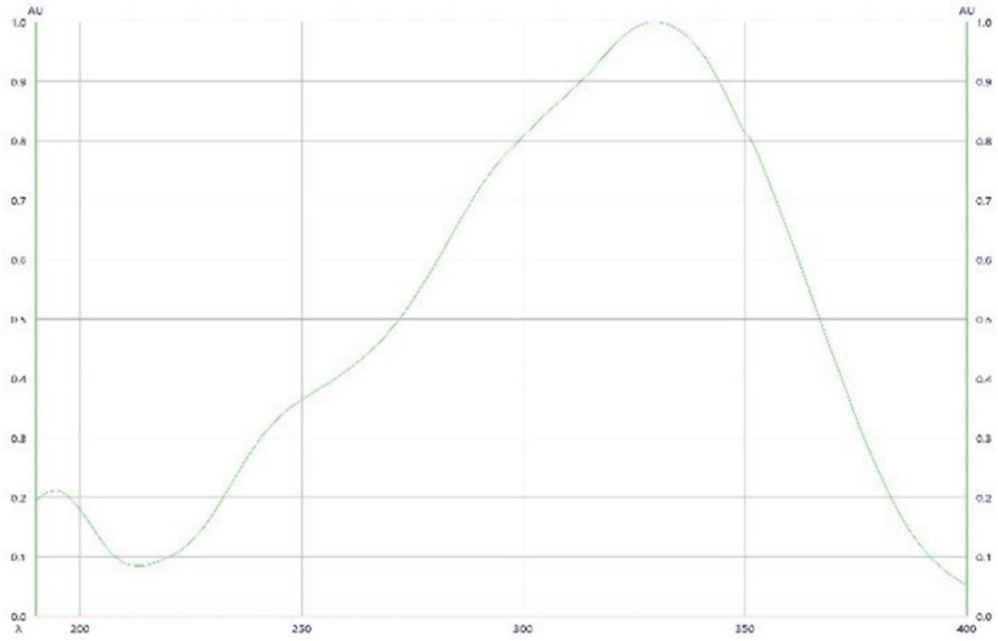


Fig. 7. Spectra of Piperine

Ethics approval and consent to participate

Not applicable

Funding

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CRediT authorship contribution statement

Amruta Balekundri: Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Pramod J Hurkadale:** Writing – review & editing, Writing – original draft, Project administration, Methodology. **Harsha Hegde:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

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.

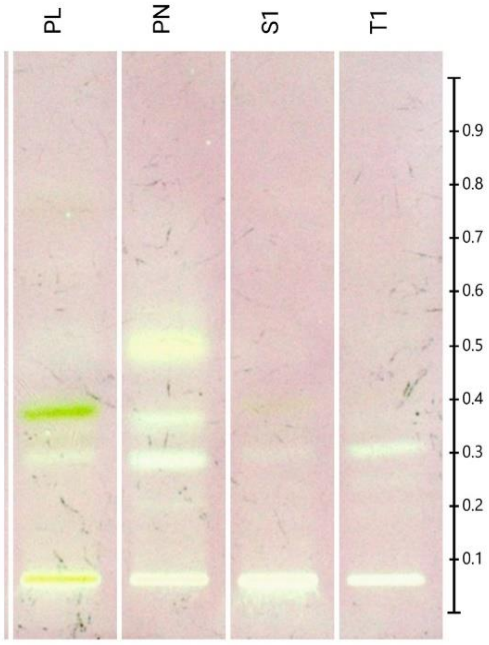

Fig. 8. HPTLC-DPPH (2,2-diphenyl-1-picrylhydrazyl) method plate image

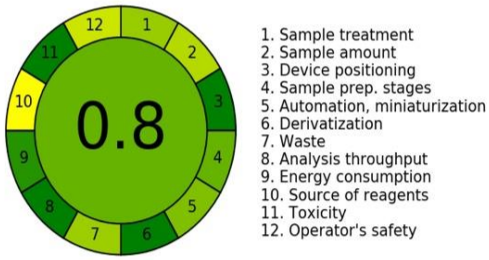

Fig. 9. Pictorial representation of the AGREE scale

Data Availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2024.100093.

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