
**“FORMULATION AND EVALUATION OF ANTI-
INFLAMMATORY HERBAL PREPARATION BY
QUALITY BY DESIGN APPROACH”**

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**For the award of the degree of
Doctor of Philosophy
In the Faculty of
Pharmacy**

By

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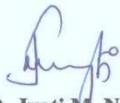
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TABLE OF CONTENTS

SL. NO.	PARTICULARS	PAGE NO.
1.	INTRODUCTION	1-6
1.1	BACKGROUND	1-3
1.2	JUSTIFICATION	4-6
1.3	OBJECTIVES	7
1.4	REVIEW OF LITERATURE	8-36
2.	MATERIAL AND METHODS	37-69
3.	RESULTS	70-122
4.	DISCUSSION	123-136
5.	SUMMARY	137-140
6.	CONCLUSION	141
7.	BIBLIOGRAPHY	142-156
8.	ANNEXURES	157-180

LIST OF ABBREVIATIONS

µg	:	Microgram
µL	:	Microliter
ANOVA	:	Analysis of Variance
ATP	:	Analytical Target Profile
BBD	:	Box-Behnken Design
CCD	:	Central Composite Design
CCS	:	Croscarmellose Sodium
CI	:	Confidence Interval
CMA s	:	Critical Material Attributes
COX	:	Cyclooxygenase
CPP	:	Critical Process Parameter
CPP s	:	Critical Process Parameters
CQA	:	Critical Quality Attributes
CRF	:	Central Research Facility
DoE	:	Design of Experiments
FTIR	:	Fourier Transform Infrared
GA	:	Gallic acid
H₂O₂	:	Hydrogen Peroxide
HCl	:	Hydrochloric Acid
HPLC	:	High-Performance Liquid Chromatography
HRBC	:	Human Red Blood Cell
ICH	:	International Conference of Harmonization
IL-1β	:	Interleukin-1 beta
IL-6	:	Interleukin-6

LC-MS/MS	:	Liquid Chromatography-Tandem Mass Spectrometry
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
LOX	:	Lipoxygenase
MCC	:	Microcrystalline Cellulose
NaOH	:	Sodium Hydroxide
NF-Kb	:	Nuclear Factor kappa B
NSAIDs	:	Non-steroidal anti-inflammatory drugs
OPA	:	Orthophosphoric Acid
PDA	:	Photodiode Array
QbD	:	Quality by Design
QC	:	Quality Control
QRM	:	Quality Risk Management
QTTP	:	Quality Target Product Profile
R & D	:	Research and Development
r²	:	Correlation Coefficient
RA	:	Risk Assessment
RA	:	Rosmarinic acid
ROS	:	Reactive Oxygen Species
RPN	:	Risk Priority Number
RSD	:	Relative Standard Deviation
RSM	:	Response Surface Method
RT	:	Retention Time
S	:	Slope
SD	:	Standard Deviation

SS	:	Stigmasterol
TCM	:	Traditional Chinese Medicine
TF	:	Tailing Factor
TNF-α	:	Tumor Necrosis Factor-alpha
TP	:	Theoretical Plates
TQP	:	Target Quality Profile
WHO	:	World Health Organization

LIST OF TABLES

SL. NO.	PARTICULARS	PAGE NO.
1	Variables and their levels used in 2^2 factorial design for simultaneous analysis gallic acid and rosmarinic acid	47
2	Chromatographic factors considered within the Box–Behnken experimental design for the proposed HPLC method	53
3	Central-composite experimental design encompassing independent and dependent variables with their levels and observed responses	61
4	Herbal raw material selected for development of anti-inflammatory formulation	70
5	Macroscopic characteristics of selected herbs	71
6	Physico-chemical parameters of selected raw materials	74
7	Preliminary phytochemical analysis of selected raw materials	75
8	Experimental design for screening of independent variables and responses	78
9	Summary of statistical parameters and polynomial equations.	79
10	Optimized chromatographic conditions	82
11	Summary of validation parameters for Gallic acid and rosmarinic acid	84
12	Quantitative estimation of Gallic acid and Rosmarinic acid	85
13	Experimental Runs, Variables, and Measured responses using response surface method (Box- Behnken) for the optimization study	87

14	The analysis of variance (ANOVA) results for the response variable Y_1 (Retention Time of Stigmasterol)	88
15	The analysis of variance (ANOVA) results for the response variable Y_2 (tailing factor of Stigmasterol)	89
16	The analysis of variance (ANOVA) results for the response variable Y_3 (theoretical plates of Stigmasterol)	90
17	Summary of validation parameters	94
18	Recovery analysis of stigmasterol	95
19	% Yield of plant extracts	97
20	Physico-chemical properties of herbal extracts	97
21	Phytochemical evaluation of herbal extracts	98
22	FT-IR spectral Analysis of medicinal plants and physical mixture	99
23	Summary of QTPP for Anti-inflammatory herbal product	102
24	CQA's for Anti-inflammatory herbal tablet formulation	103
25	Initial risk assessment and justification for the formulation variables	104-105
26	Regression equation coefficient (polynomial equations) of selected variables with 95% CI	110
27	Details of ANOVA and fit summary for dependent variables (responses)	111
28	Data represent the stability testing parameters of the optimized formulation (F-11) under both ambient and accelerated conditions	117

29	% inhibition of hemolysis from HRBC membrane stabilization method	118
30	Effect of polyherbal tablets on Anti-inflammatory activity carrageenan-induced paw edema (Reduction in paw volume)	120

LIST OF FIGURES

SL. NO.	PARTICULARS	PAGE NO.
1	<i>Terminalia chebula</i>	9
2	<i>Ocimum sanctum</i>	11
3	<i>Moringa Oleifera</i>	13
4	Steps for QbD implementation	59
5	Microscopic evaluation of Terminalia chebula fruit showing: (a) cork cells, (b) stone cells, (c) fibers, and (d) vascular bundles.	72
6	Microscopic evaluation of Ocimum Sanctum leaf showing: (a) sclereid, (b) group of lignified fibers, (c) prismatic crystals, (d) starch grain, and (e) trichomes.	73
7	Microscopic evaluation of Moringa oleifera leaf showing: (a) stomata, (b) multicellular trichome, (c) xylem vessels, (d) calcium oxalate crystals, and (e) starch grain.	73
8	Isosbestic point of gallic acid and rosmarinic acid	76
9	3D response surface plots for dependent variables, (a) tailing factor GA, (b) theoretical plates GA, (c) Tailing factor RA, and (d) theoretical plates RA	80
10	Design space overlay plot	81
11	The HPLC chromatogram of simultaneous estimation of gallic acid and rosmarinic acid	82
12	HPLC Chromatogram of Gallic acid in Terminalia chebula extract	85

13	HPLC Chromatogram of Rosmarinic acid in <i>Ocimum sanctum</i> extract	86
14	Response surface plot of response (a) Y_1 (Retention time), (b) Y_2 (Tailing factor) and (c) Y_3 (Theoretical plates)	92
15	HPLC chromatogram of stigmasterol	92
16	HPLC Chromatogram of stigmasterol in <i>Moringa oleifera</i> extract	96
17	HPLC Chromatogram of stigmasterol in <i>Moringa oleifera</i> extract	96
18	FT-IR Spectra of <i>Moringa Oleifera</i> extract	99
19	FT-IR Spectra of <i>Ocimum sanctum</i> extract	100
20	FT-IR Spectra of <i>Terminalia chebula</i> extract	100
21	FT-IR Spectra of Physical mixture (Extracts with excipients)	100
22	The 3D response surface plots illustrating the influence of Microcrystalline cellulose (X_1) and Crosscarmellose sodium (X_2) on (a) disintegration time (Y_1); (b) friability (Y_2); and (c) hardness (Y_3).	109
23	Prediction profiler illustrates the optimum values for disintegration time (Y_1), friability (Y_2), and hardness (Y_3)	112
24	The 2D Contour profiler illustrating the influence of Microcrystalline cellulose (X_1) and Crosscarmellose sodium (X_2) on (a) disintegration time (Y_1); (b) friability (Y_2); and (c) hardness (Y_3).	112
25	Prepared tablet formulations	113
26	%CDR of gallic acid, stigmasterol, and rosmarinic acid form optimized polyherbal tablet formulation	115

27	Reduction of paw volume in carrageenan-induced paw edema in rats. Values are expressed as Mean $\pm$ SEM (n = 3): analysed by two-way ANOVA followed by Tukey's Multiple comparison test whereas, ***p < 0.001 when compared to normal; **p < 0.001, **p < 0.01 when compared to diclofenac sodium.	121
28	Effect of polyherbal tablets on the histopathology of paw tissue: (A) Normal paw tissue: (a) normal, (b) control, (c) standard drug (diclofenac sodium), (d) T1 (100 mg/kg), (e) T2 (200 mg/kg), and (f) T3 (400 mg/kg); and (B) Carrageenan-induced paw tissue: (a) normal, (b) control, (c) standard drug (diclofenac sodium), (d) T1 (100 mg/kg), (e) T2 (200 mg/kg), and (f) T3 (400 mg/kg).	122

ABSTRACT

Background: Inflammatory disorders necessitate effective therapeutic interventions with minimal side effects. Polyherbal formulations offer a promising alternative due to their synergistic pharmacological activities. This study aimed to develop a scientifically validated polyherbal anti-inflammatory formulation using the Quality by Design (QbD) approach, ensuring formulation robustness and therapeutic efficacy.

Objectives: The study focused on optimizing critical formulation and process parameters for polyherbal tablet development while evaluating their anti-inflammatory potential through *in-vitro* and *in-vivo* models.

Methods: The polyherbal formulation incorporated *Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera*, selected for their well-documented anti-inflammatory properties. Phytochemical screening confirmed the presence of key bioactives, including alkaloids, flavonoids, tannins, phenols, saponins, and triterpenes. A QbD-assisted RP-HPLC method was developed for quantification of gallic acid and rosmarinic acid, employing a 2^2 factorial design to optimize chromatographic conditions. Additionally, a QbD-based RP-HPLC method for stigmasterol was optimized using Box-Behnken Design (BBD). Polyherbal tablets were formulated using a 3^2 Central Composite Design (CCD), with disintegration time, friability, and hardness as critical quality attributes. In-vitro anti-inflammatory activity was assessed via the HRBC membrane stabilization assay, while *in-vivo* efficacy was evaluated using the carrageenan-induced paw edema model in rats.

Results: Physicochemical analysis confirmed raw material quality, with moisture content and total ash values within acceptable limits. The optimized RP-HPLC

method exhibited excellent linearity ($r^2 > 0.998$), accuracy (98.4%–101.2% recovery), and precision (%RSD < 2.0). The developed polyherbal tablets demonstrated disintegration times of 19.04–28.03 min, friability of 0.38%–0.64%, and hardness of 4.6–6.3 kg/cm². Polynomial regression models and ANOVA indicated significant influences of microcrystalline cellulose and Croscarmellose Sodium on tablet properties. The HRBC membrane stabilization assay exhibited dose-dependent inhibition of hemolysis (83.63% inhibition at 500 µg/mL). In the carrageenan-induced paw edema model, polyherbal tablets significantly ($p < 0.01$) reduced edema volume, with the 400 mg/kg dose demonstrating superior efficacy.

Conclusion: The study successfully developed a scientifically validated polyherbal anti-inflammatory formulation with optimized physicochemical and pharmacological attributes. The integration of QbD principles ensured formulation robustness, while *in-vitro* and *in-vivo* evaluations confirmed significant anti-inflammatory activity. These findings substantiate the therapeutic potential of polyherbal formulations in managing inflammatory conditions and provide a rational framework for their development.

Keywords: Anti-inflammatory activity, Pharmacognostic evaluation, Polyherbal tablets, Quality by Design, High-Performance Liquid Chromatography, Human red blood cell membrane stabilization, Carrageenan-Induced Paw Edema in Rats.

1. INTRODUCTION

1.1. Background

The plant kingdom has long been recognized as a prolific reservoir of bioactive compounds and a vital source in the development and discovery of novel healing agents. In last few years, there has been a increasing awareness of the importance of medicinal plants, both for their cultural significance and their potential to address unmet medical needs. Medicinal plants are rich in diverse secondary metabolites, such as tannins, phenolics, flavonoids, and alkaloids which contribute to their pharmacological properties, including enhanced growth, innate immunity, and disease resistance. These natural products have established themselves as effective and affordable alternatives to synthetic drugs, offering therapeutic benefits for a wide spectrum of diseases ¹.

Traditionally, the applications of plants for medicinal intend back to ancient civilizations across the globe. Medicinal plants form the cornerstone of many healthcare systems, with herbal medicine encompassing herbs, herbal materials, preparations, and finished products. Phytochemical and pharmacological studies have revealed that a single plant can harbor a plethora of bioactive constituents, each capable of interacting with multiple molecular targets and pathways to exert therapeutic effects ².

The renewed interest in natural remedies, often referred to as "green medicine," is partly driven by concerns over the adverse effects of synthetic drugs on both human health and the environment. The growing demand for herbal medicines has, however, led to challenges such as adulteration and suboptimal quality. Adulteration often stems from the use of inadequate or misidentified plant parts to

meet market demands, underscoring the critical importance of proper identification and standardization of herbal raw materials ³.

Pharmacognosy, the study of medicinal plants and their constituents, has become increasingly important in recent years. Pharmacognostic investigations provide the tools necessary for the authentication and standardization of herbal materials, particularly in powdered form, where morphological identification is no longer feasible. Such outcomes are pivotal in proving the safety, efficacy, and quality of herbal medicines, as they help detect adulteration, authenticate plant material, and establish consistent standards for production ⁴.

Inflammation, a natural defense mechanism of the immune system, is a complex response to injury, infection, or irritation. While acute inflammation is vital for healing and maintaining homeostasis, chronic or uncontrolled inflammation can lead to a range of pathological conditions. Herbal remedies have gained prominence as complementary treatments for managing mild to moderate inflammation due to their richness in bioactive compounds such as alkaloids, flavonoids, steroids, and glycosides. These phytoconstituents exhibit diverse mechanisms of action, often acting synergistically to modulate inflammatory responses effectively ⁵.

India, with its rich history of traditional medicine, has extensively utilized herbs for the management of inflammation. Polyherbal formulations, a hallmark of systems such as Ayurveda and Traditional Chinese Medicine (TCM), harness the synergistic effects of multiple herbs to enhance therapeutic outcomes. However, the production of herbal medicines involves complex processes requiring meticulous control of raw materials and manufacturing parameters to ensure quality and consistency. To overcome the inherent variability associated with herbal formulations, the implementation of systematic and scientific approaches in their development is

essential. The QbD framework has emerged as a promising paradigm to address these challenges. Pursuant to the International Council for Harmonisation (ICH)'s Q8 standard, QbD is a "systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control." By employing QbD, Manufacturers may develop correlations among process parameters, material attributes, and product quality, which enables a development of robust and predictable methods of production ⁶.

QbD shifts the focus from traditional trial-and-error methods to a science-driven approach, leveraging tools such as design of experiments (DoE) and risk assessment to optimize process and product attributes ^{7, 8}. This approach is particularly valuable in the context of herbal drug development, where the complexity of chemical compositions and process variables often hinders process understanding. Through QbD, manufacturers can ensure that herbal products meet stringent quality, safety, and efficacy standards throughout their lifecycle ⁹.

In conclusion, the integration of QbD principles into the development of herbal medicines represents a transformative step forward. By fostering a deep grasping of the interplay within raw materials, manufacturing processes, and product quality, QbD not only strengthens the reliability and reproducibility of herbal formulations but also aligns with regulatory expectations for modern pharmaceutical practices ¹⁰. As the demand for herbal remedies continues to rise, adopting innovative and scientifically robust strategies will be crucial to meeting global healthcare needs while maintaining the integrity of traditional medicine.

1.2. Justification for Study

The plant kingdom has long been a prolific source of bioactive compounds, providing a foundation for therapeutic agents that have profoundly influenced modern medicine. This reservoir of natural products, primarily secondary metabolites, continues to inspire drug discovery efforts aimed at addressing the growing global burden of chronic and lifestyle-related diseases. Medicinal plants offer a promising avenue for safer, cost-effective, and sustainable therapeutic solutions, justifying the need for rigorous exploration and optimization of their potential applications. The present study seeks to contribute to this effort, with a focus on their anti-inflammatory properties and their systematic integration into contemporary healthcare systems.

Herbs have long been used for healing purposes in traditional medical systems including Ayurveda, Traditional Chinese Medicine (TCM), and Unani. To enhance efficacy, polyherbal preparations are frequently used. Despite their long-standing cultural and therapeutic significance, the transition of such formulations into modern medical practice faces challenges related to quality, safety, and efficacy. Overcoming these challenges necessitates a scientific framework, including pharmacognostic and pharmacological studies, to authenticate plant materials, elucidate their bioactive constituents, and validate their therapeutic claims. Alkaloids, flavonoids, tannins, phenolics, glycosides, and terpenoids are among the many secondary metabolites found in medicinal plants. These compounds have a wide range of pharmacological properties, such as anti-inflammatory, anti-cancer, antioxidant, and antibacterial actions. Unlike synthetic drugs, which often target specific pathways and may cause adverse effects or resistance, the multidimensional mechanisms of action exhibited by natural products allow for comprehensive therapeutic outcomes. This versatility makes medicinal plants particularly valuable for managing inflammation—a

multifaceted biological response critical to various pathological conditions, including neurodegenerative disorders, diabetes, arthritis, and cardiovascular diseases.

While synthetic anti-inflammatory drugs, such as NSAIDs (non-steroidal anti-inflammatory drugs), are effective, their long-term use is associated with significant side effects, including gastrointestinal, cardiovascular, and renal complications. This underscores the need for safer alternatives. Medicinal plants, such as *Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera*, have shown significant anti-inflammatory activity, acting on key molecular targets such as cyclooxygenase (COX), lipoxygenase (LOX), and pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6). Furthermore, these plants provide antioxidant support, mitigating oxidative stress that often exacerbates chronic inflammatory conditions.

Despite compelling preclinical evidence, the clinical translation of herbal medicines is limited by challenges such as adulteration, variability in raw material quality, and lack of standardization. Adulteration and inconsistent chemical composition, influenced by factors like geographical origin, harvesting practices, and processing methods, compromise the efficacy and safety of herbal products. These challenges highlight the necessity of pharmacognostic investigations, which use advanced techniques such as microscopy, chromatography, and spectroscopy to authenticate plant materials and establish stringent quality standards. The integration of the QbD framework into herbal medicine development offers a scientific approach to address these challenges. QbD emphasizes understanding the relationships between raw materials, processes, and product quality, enabling the development of robust, reproducible, and effective formulations. Tools such as DoE and risk assessment

facilitate the optimization of material attributes and critical process parameters ensuring consistency and compliance with regulatory standards.

By adopting QbD, the production of herbal medicines can achieve the reliability necessary for their acceptance in mainstream healthcare. India, endowed with rich biodiversity and a heritage of traditional knowledge, is uniquely positioned to lead advancements in herbal medicine. By blending traditional practices with modern scientific methodologies, the country can develop high-quality herbal products that meet global healthcare demands. The integration of QbD principles into the development of herbal formulations represents a transformative step toward sustainable and eco-friendly healthcare solutions, addressing the rising prevalence of chronic diseases and the limitations of synthetic drugs.

1.3. OBJECTIVES

❖ **Primary objective:**

- To develop polyherbal Anti-inflammatory formulation by QbD approach.

❖ **Secondary objective:**

- To perform the *in-vitro* Anti-inflammatory activity of the developed polyherbal formulation.

1.4. REVIEW OF LITERATURE

Herbs as a Source of Anti-Inflammatory Agents

The body's natural responses to injury, infection, or harmful stimuli is inflammation. The immune system, blood vessels, and other cells and molecules are all involved in this complex biological process. The goal of inflammation is to protect and heal the body, but when it becomes chronic or excessive, it can lead to various diseases and conditions. These herbs have shown promise in reducing inflammation through various mechanisms, including antioxidant effects, enzyme inhibition, and immune modulation. While many of these herbs are available as supplements or teas, before using them, it's important to discuss it with a healthcare professional, particularly if you have chronic diseases or are taking other medications ¹⁰.

Terminalia Chebula



Figure 1. *Terminalia chebula*

Family: Combretaceae

Synonyms: Haritaki, Harad, Black Myrobalan

Parts used: Dry fruits

Phytoconstituents: Gallic acid, tannic acid, polyphenolic corilagin, chemicals, triterpenoids, ascorbate, ellagic acid, chebulinic acid, chebulagic acid, and so on.

Uses: Anti-inflammatory, antiparasitic, gastroprotective, anti-arthritic, anti-microbial, anti-diabetic, anti-hyperlipidemic, antioxidant, hepatoprotective, neuroprotective, wound healing, and anti-aging properties, etc.

Terminalia chebula, commonly known as Haritaki, is a deciduous tree native to South Asia, revered in traditional medical systems like Unani and Ayurveda. Its dried fruit has been extensively utilized for its broad therapeutic potential, particularly in managing inflammatory and oxidative stress-related conditions. Emerging

scientific evidence supports its traditional use, with studies highlighting its potent antioxidant and anti-inflammatory properties ¹¹. The anti-inflammatory effects of *Terminalia chebula* are primarily attributed to its ability to inhibit key enzymes involved in inflammatory pathways, including cyclooxygenase (COX) and lipoxygenase (LOX). These enzymes catalyze the synthesis of pro-inflammatory mediators such as prostaglandins and leukotrienes, which are implicated in the initiation and amplification of inflammatory responses. By suppressing COX and LOX activities, *T. chebula* effectively reduces the production of these mediators, thereby attenuating inflammation ¹².

In addition to enzyme inhibition, *T. chebula* exhibits robust antioxidant activity, scavenging reactive oxygen species (ROS) and alleviating oxidative stress—a critical factor in chronic inflammation. The fruit is rich in bioactive phytochemicals such as tannins, flavonoids, and chebulinic acid, which provides to its pharmacological profile. Furthermore, its immunomodulatory properties enhance its ability to regulate inflammatory cascades and support immune homeostasis. While preclinical studies and traditional usage provide compelling evidence of its anti-inflammatory efficacy, the translation of these findings into clinical practice remains limited ¹³. Rigorous human clinical trials are essential to validate its curative powers, determine optimal dosages, and ensure safety profiles for long-term use. As such, *T. chebula* represents a promising candidate for natural anti-inflammatory interventions, warranting further investigation to establish its role in contemporary medicine ¹⁴.

Ocimum Sanctum



Figure 2. *Ocimum sanctum*

Family: Lamiaceae

Synonyms: Tulsi, Holy basil

Parts used: Leaves

Phytoconstituents: Eugenol, Carvacrol, Oleic acid, Luteolin, Rosmarinic acid, Ursolic acid, β -sitosterol, Carnosic acid etc.

Uses: Stress relief, Anti-inflammatory, Wound healing, Antioxidant, Antimicrobial, Antidiabetic, Digestion enhancer etc.

In ancient Indian medicine, notably Ayurveda, *Ocimum sanctum* also referred to as Holy Basil or Tulsi. It is a highly appreciated medicinal plant that is valued for both its therapeutic and spiritual properties¹⁵. Renowned for its broad pharmacological properties, *O. sanctum* has been thoroughly scrutinized for its anti-inflammatory potential, demonstrating efficacy in addressing both acute and chronic inflammatory conditions. Tulsi's ability to manage significant pro-inflammatory

cytokines, such as interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α), mediates its anti-inflammatory benefits. These cytokines play a decisive role in the initiation, amplification, and persistence of inflammatory responses, particularly such as autoimmune disorders, rheumatoid arthritis, and inflammatory bowel diseases. By suppressing the production and activity of these cytokines, *O. sanctum* mitigates the inflammatory cascade and reduces tissue damage associated with chronic inflammation ¹⁶.

Phytochemical investigations have identified several bioactive constituents in Tulsi, including eugenol, ursolic acid, rosmarinic acid, and apigenin, which contribute to its anti-inflammatory as well as immunomodulatory effects ¹⁷. These compounds inhibit the activation of nuclear factor kappa B (NF- κ B) and other transcription factors, thereby downregulating inflammatory gene expression. Additionally, Tulsi exhibits antioxidant properties, neutralizing reactive oxygen species (ROS) that exacerbate inflammation and oxidative stress. Preclinical studies have demonstrated the efficacy of *O. sanctum* in experimental models of arthritis, asthma, and cardiovascular inflammation. While these findings highlight its therapeutic potential, robust clinical trials are essential to confirm its efficacy, safety, and optimal dosing in human populations. Given its multifaceted mechanisms of action and its role in traditional medicine, Tulsi holds significant promise as a natural anti-inflammatory agent, particularly as an adjunctive therapy in managing chronic inflammatory and autoimmune conditions ¹⁸.

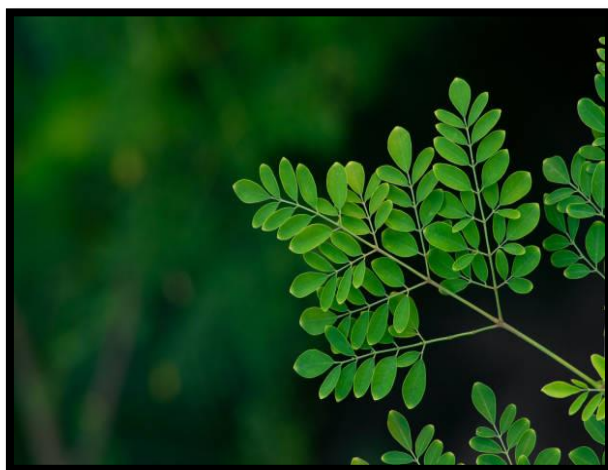
Moringa Oleifera

Figure 3. *Moringa Oleifera*

Family: Moringaceae

Synonyms: Tree of Life, Miracle Tree

Parts used: Leaves

Phytoconstituents: Alkaloids, Flavonoids, Phenolic acids, Stigmasterol, Saponins, Tannins, Glucosinolates, Isothiocyanates, Carotenoids

Uses: Antifungal, Anti-inflammatory, Anti-diabetic, Cardioprotective, Anti-cancer, Anti-asthmatic

The versatile plant *Moringa oleifera*, sometimes called as the miracle tree or drumstick tree, is native to parts of Asia and Africa. Its leaves, seeds, pods, and roots have been extensively utilized in traditional medicine for their broad spectrum of therapeutic benefits. Among these, the plant's potent anti-inflammatory properties have garnered significant scientific interest, highlighting its potential as natural remedy for inflammation-related disorders ¹⁹. *M. oleifera*'s potential to inhibit cyclooxygenase enzymes (COX-1 and COX-2), which are essential for the synthesis

of pro-inflammatory prostaglandins, is primarily responsible for its anti-inflammatory properties. This mechanism is analogous to the action of NSAIDs, positioning *M. oleifera* as a natural alternative with potentially fewer side effects. The plant helps for ailments including arthritis, inflammatory bowel disease, and other chronic inflammatory disorders since it effectively inhibits these enzymes, which reduces inflammation, discomfort, and swelling ²⁰.

The plant's anti-inflammatory properties are further enhanced by its rich phytochemical profile, which includes antioxidants, flavonoids (e.g., quercetin and kaempferol), isothiocyanates, and phenolic acids. These bioactive compounds not only suppress inflammatory pathways but also neutralize reactive oxygen species (ROS), thereby reducing oxidative stress—a key contributor to chronic inflammation. Furthermore, *M. oleifera* affords a variety of approaches to inflammation treatment by modulating inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) ²¹.

Preclinical studies have revealed the efficacy of *M. oleifera* in pilot models of arthritis, asthma, and dermal inflammation, underscoring its broad therapeutic potential. Its use in traditional medicine is often complemented by modern formulations, including powders, capsules, oils, and teas, enabling easy incorporation into daily health regimens. To confirm its safety, effectiveness, and optimal dosage, however, extensive human clinical trials are required with its positive pharmacological profile ²².

Moringa oleifera stands out as a powerful natural anti-inflammatory agent, with the added advantage of providing numerous ancillary health benefits, including improved immune function and antioxidant support. Nonetheless, individuals considering *M. oleifera* as a supplement should consult healthcare professionals,

particularly if they are on concurrent medications or managing chronic health conditions, to avoid potential interactions or adverse effects²³.

Colica, C. et al., (2018) investigated the secondary metabolites of *Rosmarinus officinalis*, a widely utilized plant in phytotherapy, cosmetics, traditional medicine, and as essence in food industry. The compound rosmarinic acid, known for its potent antioxidant, hepatoprotective, antibacterial, anti-nociceptive, and anti-inflammatory properties, has been widely investigated in the scientific literature²⁴.

Luo. et al., (2020) reviewed the potential of herbal medicines in reducing inflammation, which has garnered significant interest in recent years. One such water-soluble phenolic molecule, rosmarinic acid (RosA), is an ester of 3, 4-dihydroxyphenyl lactic acid and caffeic acid. It is found in numerous plants across the Lamiaceae and Boraginaceae families. The pharmacological actions of RosA are broad, encompassing anti-inflammatory, anti-tumorigenic, antioxidative, and anti-apoptotic activities. RosA's anti-inflammatory properties have been emphasized by *in-vitro* and *in-vivo* studies of a variety of inflammatory diseases such as atopic dermatitis, colitis, and arthritis. This article primarily discusses the preclinical studies of RosA in inflammatory conditions, with limited clinical research data. The intension of this findings is to explore RosA's anti-inflammatory mechanisms in inflammatory diseases²⁵.

Yatoo. et al., (2018) assessed the properties of anti-inflammatory in many natural herbs, including *Rosmarinus officinalis*, *Zingiber officinale*, *Curcuma longa*, *Urtica dioica*, *Uncaria tomentosa*, *Borago officinalis*, *Olea europaea*, and *Vaccinium myrtillus*. These herbs are considered safer, more effective, and superior to their synthetic counterparts. Recent patents on herbal plants and anti-inflammatory drugs are discussed, providing valuable insights into the current landscape and future

potential. These plants' ranged phytoconstituents such as glycosides, steroids, alkaloids, flavonoids, phenolics, terpenoids, polysaccharides, fatty acids, and cannabinoids —all have anti-inflammatory and anti-inflammatory-modulating properties, which are responsible for their therapeutic effectiveness²⁶.

Chouhan, Y. et al., (2014) assessed the anti-inflammatory activity of *Brassica juncea* methanolic seed extract using in-vivo techniques. *Brassica juncea*, commonly known as mustard, has been utilized since ancient times for its therapeutic benefits. This study was aimed to find out the anti-inflammatory effects of *Brassica juncea* methanolic extract at two dosages (500 and 1000 mg/kg body weight) against the carrageenan-induced paw edema model in rats. The extract demonstrated significant anti-inflammatory efficacy at a dose of 1000 mg/kg body weight, showing a 65.98% depletion in paw volume at 5 hours post-administration, which was statistically comparable ($P < 0.05$) to the 81.96% decrease achieved by the standard anti-inflammatory drug, indomethacin. The findings support the potential application of *Brassica juncea* in managing inflammatory conditions²⁷.

Cong, H. et al., (2015) explored the pharmacological control of inflammation through the use of NSAIDs, steroids, and other pharmacological agents. The carrageenan rat paw edema model is commonly employed to discover and develop novel NSAIDs, with effects typically evaluated three to five hours post-edema induction, neglecting longer-term outcomes. In this study, 18 rats were subplantar injected with 0.1 mL of a 1% aqueous solution of carrageenan- λ into their right hind paw to induce paw edema. Group 1 was used as the solvent control, Group 2 was given 15 mg/kg of naproxen, and Group 3 was given 10 mg/kg of indomethacin. Each group included six rats. Microsoft Office Excel 2007 was used to execute statistical analysis, computing standard deviations (δ), arithmetic means (M), and standard errors (m)²⁸.

Quality assessment of herbal drugs

The quality of herbal drugs is determined by defining their identity, purity, and the constituents they contain, as well as other physical, chemical, or biological attributes they exhibit. The quality benchmarks for herbal medicinal products are contingent upon a comprehensive scientific characterization of the raw materials used. The quality of herbal raw materials directly influences the efficacy and safety of the final herbal medicinal products. Therefore, authenticity, purity, and assay are considered crucial aspects of the quality assessment of herbal drugs ²⁹.

Extraction of Herbal Drugs

Herbal drug extraction is a crucial process in pharmaceutical and traditional medicine research, involving advanced procedures to separate bioactive chemicals from plant materials. Different extraction techniques are employed depending on the particular characteristics of the plant and the desired chemical features. The most commonly used method is still solvent extraction, utilizing organic solvents such as methanol and ethanol to dissolve active ingredients through differential solubility. A traditional method, maceration, involves soaking plant materials in solvents at low temperatures to retain chemicals sensitive to heat, though it is relatively slow. Sophisticated techniques like Soxhlet extraction enhance extraction efficiency through continuous solvent circulation, particularly for molecules with limited solubility ³⁰.

Steam distillation is a useful method for removing volatile substances—especially essential oils—while preserving molecular integrity. Modern carbon dioxide supercritical fluid extraction is an eco-friendly method that yields high-purity extracts without heat-induced degradation. Researchers must carefully consider the composition of the plant material, the properties of the target compounds, extract

quality criteria, and economic factors when selecting an extraction technique. The ultimate goal is to optimize the extraction of bioactive components while retaining their structural and functional properties, ensuring comprehensive investigation of the potential therapeutic benefits of herbal medicines³¹.

Zhang. et al., (2018) examined effective and specific techniques for the extraction and separation of bioactive natural compounds. The study explains the outline of techniques utilized in an extraction and separation of natural materials, highlighting both traditional and contemporary methods. The benefits, limitations, and practical applications of these methods in natural product research are thoroughly discussed. This includes a critical analysis of their utility, efficiency, and relevance in the isolation of bioactive constituents, offering valuable insights into their real-world applications³².

Formulation Development

The development of herbal drug formulations is a meticulous and systematic process aimed at converting raw plant extracts into stable and effective pharmaceutical products. This process involves precise extraction techniques designed to enhance the separation of bioactive compounds while preserving their structural integrity. Preformulation studies are crucial in evaluating solubility, stability, and potential delivery challenges by carefully assessing physicochemical parameters. Researchers select appropriate dosage forms, such as tablets, capsules, or novel delivery systems, to improve drug absorption and patient compliance. The selection of excipients is critical to ensure chemical compatibility, enhance bioavailability, and prevent adverse reactions. Comprehensive stability testing, encompassing both short-term and long-term analyses, is essential to determine optimal storage conditions and establish accurate expiry dates. Rigorous standards for

quality control are adhered to, with reliable analytical techniques developed to ensure batch-to-batch consistency and compliance with stringent regulatory requirements. The ultimate goal is to develop standardized, safe, and effective herbal drug formulations that meet the specific standards of the pharmaceutical industry while preserving the inherent medicinal potential of botanical sources^{33, 34}.

Paul S. *et al.*, (2023) developed and evaluated a polyherbal tablet incorporating extracts of ginger, turmeric, tulsi, and neem. Analytical standard evaluations, including qualitative and quantitative determination, were conducted to prepare the extract and formulate the polyherbal tablet using a systematic processing approach. Each tablet was individually weighed, and assessments were made for its thickness, hardness, and friability, as well as its disintegration time. The tablets exhibited a characteristic brownish-yellow color and were odorless. Blending different herbs in specific ratios was found to enhance the therapeutic effect while reducing toxicity. This review emphasizes on the relevance of polyherbalism and its therapeutic effect, emphasizing the integration of multiple herbal extracts to optimize medicinal benefits³⁵.

Rezghi M. *et al.*, (2021) stated that herbal medicines are considered as a productive approach for treating a range of illness. The feasibility of adapting conventional drugs to meet pharmacopeial standards for existing medications was explored. For the selected formulation (F2), key quality attributes were assessed: hardness of 6.78 ± 0.28 kg/cm², weight variation of 714.36 ± 1.71 mg, friability of $0.79 \pm 0.05\%$, disintegration time of 10.18 ± 0.39 minutes, and a drug release rate of $97.67 \pm 0.16\%$ within one hour. Each tablet was found to contain 25.15 ± 0.13 mg of total phenols. Microbial counts met the required standards, and no significant changes were observed in accelerated stability testing. Over a six-month period, the total phenol

concentration decreased by only 1.03%. Following further evaluation, including in vivo and clinical trials, the developed tablet (F2) may be considered for large-scale production³⁶.

Ladignon EA. et al., (2020) formulated the directly compressible tablets containing ethanolic leaf extracts from *Moringa oleifera*, *Mangifera indica*, and *Psidium guajava* using the QbD approach. This was achieved by identifying the CQA from the QTPP through risk assessment, a core component of QbD. The formulations met the acceptance criteria for several quality control metrics, demonstrating the effectiveness of this approach in ensuring the desired product quality³⁷.

Balekundri. et al., (2020) developed a polyherbal tablet formulation using the Design Expert tool. Among the various formulations developed, the formulation demonstrated superior outcomes according to both pre-compression and post-compression tests. The polyherbal tablets exhibited stability throughout the duration of the stability study. This strategy is endorsed by the QbD concept, which emphasizes that quality should be perceived as a basic pharmaceutical quality model that is essential to the development of pharmaceutical processes and products³⁸.

Khan. et al., (2019) investigated compressed tablets for quality attributes including friability (%), disintegration time (s), wetting time (s), thickness (mm), hardness (kg/cm²), weight variation (mg), drug content (%), and in-vitro drug release (%). To comprehend the important effects of both formulation factors on the selected responds, sophisticated statistical techniques like overlay plots, multiple regression analysis, analysis of variance (ANOVA), and 3D response graphs were effectively used. The formulation of Methylphenidate HCl fast-dissolving tablet (FDT) was developed using the Quality by Design (QbD) approach, enhancing the dissolution rate and decreasing disintegration time for rapid mouth dissolution. This approach

increases efficiency and provides robust regulatory support for the pharmaceutical development process³⁹.

HPLC Method Development and Validation

Sun Y. et al., (2020) demonstrated the analysis of *Elaeagnus angustifolia* L. leaves from different territory and seasons in Xinjiang using HPLC and hierarchical cluster investigation. R software was employed to quantify the levels of ellagic acid and gallic acid in samples collected from multiple sites in Xinjiang. A Diamonsil C18 chromatographic column (4.6 × 250 mm, 5 µm particle size) was used for separation, along with a mobile phase composed of methanol (A) and a 0.1% phosphoric acid aqueous solution (B). At a column temperature of 35°C, the injection volume was 5 µL, the flow rate was 1 mL/min, and the detection wavelength was 260 nm⁴⁰.

Mukhtar. et al., (2018) designed a method to quantify stigmasterol in *Ficusdeltoidea* plant extract. Using isocratic conditions, the separation was accomplished at a wavelength of 210 nm using acetonitrile and acetic acid with water (75:25% v/v). At a retention time of 3.17 minutes, a clear single peak for standard stigmasterol was observed, which coincided with the plant extract peak at 3.14 minutes. With a high correlation coefficient of 0.998, the calibration curve for stigmasterol was linear within the concentration range of 2–10 µg/mL. The LOD and LOQ values were found as 1.50 µg/mL and 4.55 µg/mL respectively. The recovery of stigmasterol was between 99.6% and 100.1%, with RSD values in both intra-day and inter-day precision assays being less than 0.340%, authenticating the accuracy and precision of the method. The robustness study further revealed that slight modifications in the detection wavelength and mobile phase flow rate had no discernible impact on the approach⁴¹.

Choi S. et al., (2019) discussed the development and validation of a HPLC (high-performance liquid chromatography) method for analyzing rosemary extract's primary components—carnosol, carnosic acid, and rosmarinic acid—in food matrices. It evaluated rosemary extract's antioxidant activity in food. The HPLC method showed high sensitivity, precision, and accuracy for detecting rosemary extract in edible oils, processed meat, and dressings. Among rosemary extract components, carnosic acid demonstrated superior antioxidant stability and activity compared to carnosol. Rosemary extracts effectively reduced oxidation in lard oil, though their thermal stability was lower than synthetic antioxidants like BHA (butylated hydroxyanisole). In conclusion, the validated HPLC method is suitable for detecting rosemary extract in foods, and rosemary extract can serve as a natural antioxidant, with carnosic acid being the most effective component ⁴².

Formulation development by Quality by Design approach

Pharmaceutical companies are currently driven by regulatory bodies to adopt innovative strategies that ensure enhanced standards for product safety and quality. Using the Quality by Design (QbD) approach renders it possible to efficiently achieve the desired performance and quality of products. QbD is a systematic methodology that minimizes manufacturing process errors and enables the creation of products with predefined specifications ⁴³. Originally conceptualized by Joseph M. Juran in 1992, the QbD concept was developed in response to quality control challenges in manufacturing processes. As per the ICH Q8 guidelines, QbD is “A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management.” This philosophy shifts the traditional quality assessment

paradigm from evaluating the product solely at the end stage, to ensuring that the product's quality is embedded from its inception ⁴⁴.

In contrast to the traditional Quality by Testing (QbT) methodology, which examines only the finished product for quality attributes, QbD focuses on evaluating quality attributes throughout every stage of the product's lifecycle—beginning with its design, progressing through the manufacturing process, and extending to its final formulation. By emphasizing the integration of quality considerations into the initial development and manufacturing processes, QbD helps mitigate potential issues that could compromise the final product's quality, ultimately leading to a more robust and reliable pharmaceutical product. According to the QbD concept, qualities that are considered necessary for the product's quality are identified, converted into qualities that the final product should have, and the variability between the crucial process parameters is determined in order to consistently produce a drug product with predetermined qualities ⁴⁵.

Quality Target Product Profile (QTPP)

The QTPP is the first and most pivotal component of the QbD strategy. As claimed by ICH Q8 (R2) guidelines, the QTPP is “A prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account both safety and efficacy of the drug product.” Enlisting the essential quality attributes that are desired in the finished product marks the initial step in designing the QTPP. The predetermined quality requirements of the drug product, or QTPP, serve as a roadmap for the development of novel drug products.

By clearly defining these attributes, the QTPP establishes a benchmark against which all subsequent development activities are aligned. This includes formulation development, process optimization, and risk management strategies, all of which are integral to achieving the desired product quality. The QTPP acts as a guiding document throughout the drug development lifecycle, ensuring that the development process remains focused on meeting the predefined quality goals. It helps identify critical quality attributes (CQAs) early on, allowing for informed decisions regarding formulation, process parameters, and control strategies. In essence, the QTPP not only outlines the intended quality product but also directs the overall development process towards delivering a high-quality drug product that meets regulatory standards and patient needs ⁴⁶.

Critical Quality Attributes (CQA)

As per ICH Q8 (R2), CQA is defined as “A physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality.” CQAs are extracted from the predetermined QTPP and represent the critical drug product characteristics that must be precisely monitored to meet the criteria of the QTPP.

These quality parameters are essential because they directly influence the product’s safety, efficacy, and overall quality. By identifying CQAs from the QTPP, pharmaceutical developers can establish appropriate limits, ranges, or distributions for these characteristics early in the development process. This proactive approach ensures that the product meets its intended quality attributes throughout its lifecycle, from initial formulation to final production and release. The precise monitoring of CQAs enables the detection and correction of potential issues before they impact the

product's quality, thereby enhancing process robustness and ensuring compliance with regulatory requirements ⁴⁷.

Risk Assessment

Knowledge on managing and reducing risks throughout the product development stage is provided by quality risk management (QRM). According to the ICH Q9 guideline, "Risk assessment consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards." The first step in any quality risk assessment is an in-depth risk question or problem description ⁴⁸.

The concept of quality risk assessment is underpinned by three basic principles:

What might go wrong? This involves identifying potential hazards that could affect the product's quality.

What is the likelihood/probability that will go wrong? This step evaluates the probability of each identified risk occurring.

What are the consequences (severity) if it does go wrong? This assesses the impact of the risk on the finished product's quality.

In summary, a risk assessment procedure identifies all potential high-risk factors that could jeopardize the quality of the finished product. By systematically analyzing essential characteristics and process parameters, this procedure helps to identify any potential quality problems before they manifest, enabling proactive risk mitigation strategies. Effective quality risk management ensures that resources are allocated appropriately to control these risks, thereby maintaining product quality and safety throughout its lifecycle ⁴⁹.

Design of experiments (DoE)

A main component of the QbD philosophy is the statistical approach known as the DoE. DoE provides a structured and systematic framework for comprehending the relationships between the variables affecting a process and the results that are achieved. It involves the deliberate and systematic variation of controlled input parameters, also known as independent variables, in order to assess their influence on the responses (dependent variables).

Through this structured experimentation, DoE enables it feasible to identify key variables and look into complex relationships. By varying these independent variables, it becomes possible to determine their influence on the process outcomes, leading to a deeper understanding of the underlying mechanisms. This systematic technique guarantees that the desired quality features of the product are continually attained in addition to assisting in process performance optimization. DoE is essentially a cornerstone of the QbD design, allowing for the development of reliable and consistent procedures that complement predetermined goals for product quality ⁵⁰.

Koli R. et al., (2022) generated directly compressible polyherbal tablets using QbD strategy and a novel immuno-modulatory component. In this study, utilizing a QbD method, a formulation for a polyherbal tablet was developed and optimized. The Risk Priority Number (RPN) was used for the initial risk evaluations of critical materials, and a randomized complete factorial design was used to examine the effects of important factors including the concentration of CCS and MCC. Four trial formulations were subsequently developed via a DoE approach. The independent variables studied were microcrystalline cellulose (MCC) ranging from 5% to 15% and croscarmellose sodium (CCS) from 0.5% to 5%. The responses measured were friability and disintegration time, which varied between 0.35% and 0.91% and 11.4 to

15.5 minutes, respectively. The formulation factors showed that both Y1: Disintegration time (min) and Y2: Friability (%) were significantly affected by the independent variables, X1: microcrystalline cellulose and X2: croscarmellose sodium⁵¹.

Design Space

Based on ICH Q8 (R2) guidelines, the design space means “the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality.” The design space represents the integrated area resulting from the interplay of specific variables and CQAs, where all predetermined quality standards for a drug product are met, indicating the optimal settings for consistently producing high-quality products.

The QbD technique ensures the quality of drugs by providing a structured framework to handle and understand the critical elements involved in formulation development. Extensive research involving numerous formulations has highlighted the merits of the QbD approach in this regard. One study demonstrated the successful application of the QbD technique in developing ODT. Tools like a preliminary hazard analysis tool and the Ishikawa (fishbone) diagram were employed for risk assessment. Additionally, the Box-Behnken design was utilized to investigate how key factors influenced various properties of the ODT⁵².

The use of QbD in these studies not only aids in the identification and control of critical process parameters but also enhances the ability to predict and manage risks effectively. This approach facilitates the development of robust formulations that meet desired quality attributes, leading to greater assurance of product quality and

process efficiency. By defining the design space, the QbD methodology ensures that all critical factors are optimized to develop a product that invariably meets its quality demands throughout its lifecycle.

Darkunde S. *et al.*, (2020) reviewed the QbD framework as a systematic technique to ensuring pharmaceutical quality. QbD emphasizes process understanding and control based on scientific principles and risk management, with a focus on predetermined objectives. It involves identifying CQAs and utilizing tools such as DoE and risk assessment to optimize manufacturing processes. Establishing design spaces for controlled operations ensures robust method development, enabling consistent quality despite variable conditions. This facilitates regulatory compliance and minimizes post-approval modifications. In the context of analytical methods, QbD encompasses impurity determination, dissolution studies, and advanced techniques like liquid chromatography-mass spectrometry (LC-MS) and ultra-high-performance liquid chromatography (UHPLC). Analytical Quality by Design (AQbD) integrates continuous improvement and flexibility, offering robust solutions for analytical challenges in the pharmaceutical production ⁵³.

Benjasirimongkol P. *et al.*, (2019) described the application of the QbD method in the formulating of a spray-dried emulsion of resveratrol. They employed an Ishikawa diagram and a risk assessment technique to evaluate process variables that affected the quality of the spray-dried emulsions. The Plackett–Burman design was employed to assess how the excipients used influenced the quality of the final product. Furthermore, the integration of Plackett-Burman method and risk assessment reduced risks and identified critical factors affecting the spray-drying process and the quality of the resveratrol spray-dried emulsions. QbD serves as a systematic approach in pharmaceutical development to ensure product quality, encompassing DoE, CQAs,

and risk assessment. Applications extend to analytical method development, chromatographic techniques, and process optimization, emphasizing compliance with International Council for Harmonisation (ICH) guidelines⁵⁴.

Gaonkar. et al., (2020) applied the QbD strategy for the development of a herbal antioxidant formulation. The vital steps involved defining the QTPP and CQA for the herbal supplements. RPN were assigned to formulation parameters to evaluate risks, and a CCD was utilized for DoE. The study assessed the effects of starch and crosscarmellose sodium concentrations on disintegration time and friability. The findings demonstrated the effectiveness of QbD in optimizing quality parameters for herbal supplements, ensuring product quality and consistency.

The QbD approach was employed to optimize herbal tablets containing *Psidium guajava*, *Mangifera indica*, and *Moringa oleifera*, showcasing its broad applicability in herbal product development. The study explored the enhancement of resveratrol's (RVT) properties by loading it onto porous calcium silicate (PCS) powders. Using a Box-Behnken design, the effects of low- ethyl acetate (EA), methoxyl pectin (LMP), and PCS were investigated on encapsulation efficiency, drug dissolution, and drug loading capacity. Key findings indicated that the amount of EA and PCS effectively impacted drug loading capacity, while EA amount influenced encapsulation efficiency, and the PCS amount notably influenced the dissolution rate. The optimal formulation composition was determined to be 19% w/w EA, 2.6% w/w LMP, and 13% w/w PCS. This demonstrated improved dissolution and photostability of resveratrol, highlighting the development of a method to enhance its pharmaceutical properties. The research established a design space with mathematical model validation, providing a promising approach to improve resveratrol's stability and performance in pharmaceutical applications⁵⁵.

Chippada SC. et al., (2011) measured the lower level of hypotonicity-induced HRBC membrane lysis to assess anti-inflammatory effectiveness. Different concentrations of methanolic extract and diclofenac sodium were tested to find the percentage of membrane stabilization. At a dosage of 2000 µg/ml, the highest membrane stabilization observed for *Centella asiatica* extracts was 94.97%. This research supports the isolation and application of *Centella asiatica*'s active ingredients for the treatment of inflammation⁵⁶.

Leon. et al., (2018) examined the ethereal, aqueous, and ethanolic extracts of the stems, leaves, and rhizomes of *A. vesicaria* for their anti-inflammatory qualities *in vitro*. Each dry extract of the plant sections was serially diluted at 125, 250, and 500 µg/mL using diclofenac sodium as a reference medication. Subsequently these concentrations were used to analyze the anti-inflammatory efficacy *in-vitro* using the HRBC membrane stabilization method. At various concentrations, all extracts demonstrated greater efficacy than diclofenac sodium and were capable of stabilizing the erythrocyte membrane in a hypotonic solution⁵⁷.

Parameswari. et al., (2018) evaluated the leaf extract and found the highest protection of HRBC (human red blood cells) at a concentration of 20 µg/mL, showing 74.63% stabilization, while the lowest hemolysis observed was 25.37% in a hypotonic solution, with Diclofenac sodium serving as the control⁵⁸.

Yesmin et al., (2020) studied the *in vitro* anti-inflammatory efficacy of *Piper chaba*. The *P. chaba* plant from the genus *Piper* exhibits promising anti-inflammatory properties. Utilizing the heat-induced hemolysis, HRBC membrane stabilization method, and hypotonicity-induced hemolysis, as well as an additional method involving egg albumin denaturation assay, the study evaluated its anti-inflammatory potential⁵⁹.

Kazkayasi I. et al., (2022) investigated in vivo anti-inflammatory activity by using the carrageenan-induced paw edema technique in mice. Mice were administered a single oral gavage dose of 2, 20, or 200 mg/kg metronidazole. One hour later, the hind paws were subplantar injected with 2% carrageenan. Paw thickness was recorded using a dial thickness gauge at baseline, and then at 1, 2, 3, 4, 24, and 48 hours post-injection. Additionally, a separate group of mice was treated with indomethacin as a reference drug for comparison. The levels of TNF- α , IL-1 β , and pro-inflammatory cytokines in the mouse paws were quantified using the ELISA technique. For statistical analysis ANOVA (post-hoc Bonferroni) and Student's t-tests were utilized⁶⁰.

Zang X., et al., (2022) suggested that alloferon-1's anti-inflammatory qualities might prevent acute inflammation and histological abnormalities in mice with paw edema brought on by λ -carrageenan. Vascular permeability and paw thickness were significantly reduced in mice given intraperitoneal alloferon-1 (22.0 mg/kg) as a systemic pretreatment. Alloferon-1 prevented neutrophil migration into carrageenan-stimulated air pouches and their inflow into the mice's pleura, as well as the exudation caused by λ -carrageenan, according to histological analysis of the paw tissues. According to the Luminex liquid chip, the treatment of alloferon-1 also markedly decreased the production of inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), monocyte chemo attractant protein 1 (MCP1), and interleukin-5 (IL-5), in the tissues of the inflamed paws. Overall, this study provides compelling evidence of alloferon-1's potent anti-inflammatory effects, suggesting potential therapeutic alternatives for acute inflammatory diseases⁶¹.

Silva M. et al., (2021) investigated three different *Pediastrum boryanum* green microalga extracts' antioxidant and anti-inflammatory qualities in a rat model of acute inflammation. Rats were given phenolic compounds, lyophilized biomass, and extracellular extracts of *P. boryanum* intraperitoneally after receiving an intraplantar injection of carrageenan to cause paw edema. Interleukin-1 β and tumor necrosis factor- α levels were measured, together with the degree of paw edema. Oxidative stress indicators were examined in the liver, and blood tests were performed to assess hepatic toxicity and genetic damage. The findings demonstrated that all three *P. boryanum* extracts exhibited strong anti-oedematous effects and significantly reduced cytokine levels. Among these, the phenolic compound extract, comprising gallic, chlorogenic, protocatechuic, and vanillic acids, yielded the most pronounced results. Treatment with lyophilized biomass resulted in decreased lipid peroxidation, while the extracellular extract enhanced the overall antioxidant capacity of the liver. Moreover, once *P. boryanum* extracts were administered, no symptoms of DNA damage or liver toxicity were seen. According to these results, *P. boryanum* could be a good source of anti-inflammatory compounds ⁶².

Kim K. et al., (2020) investigated if LIUS may reduce the development of paw edema in rats caused by carrageenan in relation to vascular permeability and inflammation. Rat paws were subjected to LIUS for 10 minutes right after the carrageenan injection at a frequency of 1 MHz and intensities of 50, 100, or 200 mW/cm². Paw edema brought on by a carrageenan injection peaked at 6 hours and then progressively subsided to almost the original baseline levels after 72 hours. At 2 and 6 hours, LIUS demonstrated a substantial decrease in the production of paw edema at all evaluated intensities. The intensity of 50 mW/cm² produced the greatest decrease. Histological examinations verified that LIUS significantly reduced the

infiltration of polymorphonuclear leukocytes and the enlargement of the interstitial space under the paw skin caused by carrageenan. Furthermore, LIUS consequently reduced vascular permeability, according to Evans Blue extravasation studies. Subsequently, immunohistochemical labeling showed that following LIUS stimulation, the expression of pro-inflammatory proteins, specifically cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which was raised by carrageenan injection, reverted to normal⁶³.

Patil K. et al., (2019) reviewed various techniques for anti-inflammatory drug screening, detailing their advantages and limitations. The article elaborates on the biological targets of phytoconstituents in inflammation and the biomarkers used to predict adverse effects of anti-inflammatory drugs. The systematic approach proposed in this article facilitates the rational identification of novel phytoconstituents during the initial stages of drug screening programs. This framework enhances the efficiency and effectiveness of the drug development process by providing a structured method for evaluating anti-inflammatory compounds⁶⁴.

Summary of Literature Survey

The literature review examines critical aspects of the development, formulation, and evaluation of polyherbal tablets, encompassing the collection of raw materials, analytical method development, validation, and testing for biological activity. It systematically organizes these aspects into the following key areas:

i. Collection of Herbal Materials

The sourcing of high-quality herbal raw materials is emphasized as a cornerstone for effective polyherbal formulations. The literature highlights the necessity of ensuring authenticity, contamination-free status, and compliance with

regulatory standards. Methods for sustainable collection, processing, and storage are discussed, with an emphasis on the significance of thorough documentation for maintaining batch-to-batch consistency and ensuring the therapeutic efficacy of herbal ingredients.

ii. Evaluation of Herbal Materials

A comprehensive assessment of herbal materials is critical for understanding their chemical and therapeutic potential. The review explores methods for qualitative and quantitative phytochemical analysis, focusing on the determination of bioactive constituents, antioxidant activity, and other pharmacological properties. Such evaluations establish the foundation for the efficacy and safety of polyherbal formulations.

iii. HPLC Method Development and Validation

HPLC is pivotal for analyzing and quantifying bioactive compounds in herbal formulations. The review discusses the selection of stationary and mobile phases, optimization of chromatographic conditions, and the validation of analytical methods. To guarantee reliable and reproducible outcomes, validation features including accuracy, precision, specificity, linearity, and robustness are investigated. Emphasis is placed on the role of validated methods in complying with regulatory requirements and supporting the quality assurance of herbal products.

iv. Formulation of Polyherbal Tablets

The formulation process integrates multiple herbs to achieve enhanced therapeutic outcomes. The review highlights the selection and role of binders, excipients, and disintegrants in achieving desired tablet properties, such as

disintegration time, hardness, and bioavailability. It also addresses the challenges in harmonizing the diverse properties of individual herbs and excipients to ensure the efficacy, stability, and safety of the final product.

v. Quality by Design (QbD) Approach

The application of the QbD framework in polyherbal formulations and HPLC method development provides a systematic and scientific approach to product design. The literature underscores the importance of identifying CQAs, performing risk assessments, and optimizing CPPs. The integration of QbD ensures that formulations and analytical methods are robust, consistent, and aligned with regulatory expectations, fostering reproducibility and high-quality outcomes.

vi. HRBC (HRBC) Membrane Stabilization Test

A key in vitro technique to evaluate the anti-inflammatory properties of herbal preparations is the HRBC membrane stabilization assay. The review highlights the role of this method in assessing the ability of polyherbal formulations to stabilize red blood cell membranes, a marker of their anti-inflammatory and analgesic efficacy. This test serves as a reliable indicator of the therapeutic potential of herbal tablets in managing inflammation and pain.

vii. Carrageenan-Induced Paw Edema Model

The carrageenan-induced paw edema paradigm is widely used to assess anti-inflammatory efficacy in vivo. The review discusses the mechanism of inflammation induced by carrageenan and its use in testing the efficacy of herbal and polyherbal formulations. The ability of these formulations to modulate inflammatory mediators and reduce edema and pain is critically examined, providing insights into their

therapeutic applicability. In conclusion, the review synthesizes current methodologies for the collection, analysis, and formulation of polyherbal tablets, providing insights into their therapeutic potential. It also discusses the significance of robust testing methods like HPLC and biological assays such as HRBC and carrageenan-induced paw edema in evaluating the efficacy of polyherbal formulations for inflammation-related conditions.

MATERIALS AND METHODS

Collection of Medicinal Plants Raw Material

The raw materials, comprising *Ocimum sanctum*, *Terminalia chebula*, and *Moringa oleifera*, were acquired from Shahapur, Belagavi's BMK Ayurveda Pharmacy. The botanical identity of these plant specimens was verified and authenticated by the Central Research Facility (CRF) at BMK Ayurveda Mahavidyalaya, Shahapur, Belagavi. The collected plant materials have been subjected to shade drying in order to maintain the integrity of their phytochemicals. A coarse powder was subsequently made from the dried ingredients, which were then kept in airtight containers to prevent moisture absorption and degradation of bioactive constituents.

Quality Assessment of Raw Material

1. Botanical Evaluation

i. Macroscopic Evaluation

Organoleptic evaluation refers to the qualitative assessment of drugs based on their sensory attributes, including flavor, size, form, color, odor, and distinguishing characteristics like texture and sense. This type of evaluation relies on the organs of special senses and serves to identify specific characteristics of the material. As a preliminary step in the quality control process, organoleptic evaluation aids in identifying the drug and figuring out its level of purity, forming the foundation for further physicochemical and analytical investigations⁶⁵.

ii. Microscopic Evaluation

The microscopical parameters analyzed included the type and arrangement of epidermal cells, the presence of stone cells, and the characteristics of testa and endosperm. Additionally, the presence and morphology of crystalline structures such as calcium oxalate and starch granules were examined. Other notable observations included the presence of oil globules, aleurone grains, and trichomes. All microscopic evaluations were conducted using a trinocular compound microscope (magnifications: 10× and 40×) equipped with a camera for precise imaging and documentation ⁶⁶.

2. Physico-Chemical Evaluation

i. Ash Value

To assess the plant materials' inorganic content and quality, their ash value was obtained. As part of the analysis, the amounts of water-soluble, acid-insoluble, and total ash were determined ⁶⁷.

Total Ash Value

A precisely weighed 2 g portion of the powdered drug was added in a silicon crucible that had been earlier tarred. To ensure full combustion of the organic material, the sample was heated to 450°C in a muffle furnace until an equilibrium weight was obtained. The crucible was then cooled to reweigh at the ambient level in a desiccator. The proportion of total ash was calculated using the weight of the air-dried material. This procedure provides an estimate of the total mineral content and non-volatile inorganic matter found in the drug the material.

Determination of Total Ash Value Formula:

$$\% \text{ Ash Value} = \frac{\text{weight of the dish} + \text{ash (after complete incineration)} - \text{weight of empty dish}}{\text{weight of the drug taken}} \times 100$$

Acid Insoluble Ash

To dissolve the acid-soluble components, the ash from the total ash determination was mixed with 25 milliliters of diluted hydrochloric acid and gently heated for a few minutes. Ashless filter paper was used to filter the resultant combination, and the filter paper containing the insoluble residue was carefully placed within a previously tarred silica crucible. The insoluble matter was thoroughly washed with hot water to remove any residual acid and subsequently ignited in a muffle furnace till the weight remained constant. The crucible was reweighed after cooling in a desiccator. An estimate of the amount of siliceous and other insoluble impurities in the drug material was obtained by calculating the proportion of acid-insoluble ash to the sample's weight after air drying ⁶⁸.

Water Soluble Ash

25 milliliters of distilled water were used to treat the ash that originated from the total ash determination and boiled gently for five minutes to dissolve the water-soluble components. Ashless filter paper was employed to filter the resultant mixture, and the residue was carefully moved into a silica crucible that had been freshly tarred. After that, the residue was burned in a muffle furnace at 450°C until its weight remained constant. After being allowed to cool to ambient temperature in a desiccator, the crucible was weighed. The water-soluble ash was indicated by the weight difference before and after combustion. In order to determine the material's soluble

mineral content, the weight of the air-dried sample was used to calculate the proportion of water-soluble ash ⁶⁹.

ii. Determination of Moisture Content

The hot air oven process was employed for assessing the sample's moisture content. A weighed amount of the sample was dried at a predetermined temperature (usually between 100 and 105°C) in a hot air oven until the weight remained constant ⁷⁰. The percentage of dry matter was measured using an electronic balance, and the moisture content was calculated using the following equation:

% Moisture Content

$$= \frac{\text{Initial weight of sample} - \text{Final weight of sample after drying}}{\text{Initial weight of sample}} \times 100$$

iii. Extractive Value

Alcohol-Soluble Extractive

100 mL of 90% ethanol was added to a stoppered conical flask containing a carefully measured 5 g sample of powdered drug. An electric shaker was used to continuously shake the mixture for six hours, after which it was left to macerate overnight. Following the maceration process, the solution was carefully filtered. Under carefully monitored conditions, the filtrate was evaporated until it was completely dry, and the weight of the dried extract was noted ⁷¹. The percentage of ethanol-soluble extractive was calculated using the following formula:

$$\% \text{ Ethanol} - \text{soluble extractive} = \frac{\text{Weight of dried extract}}{\text{Weight of powdered drug}} \times 100$$

Water Soluble Extractive

100 mL of chloroform water was added to a carefully weighed 5 g sample of powdered drug that had been placed into a stoppered conical flask. An electric shaker was used to continuously shake the mixture for six hours, after it was left to macerate overnight. After the maceration period, the solution was carefully filtered. After the filtrate was dried by evaporation, the weight of the dried extract was noted ⁷². The following formula was used to determine the proportion of chloroform water-soluble extractive:

$$\% \text{ Water – soluble extractive} = \frac{\text{Weight of dried extract}}{\text{Weight of powdered drug}} \times 100$$

3. Phytochemical Evaluation

The powdered crude drugs underwent initial screening for phytochemicals to qualitatively assess the chemical composition of the selected plant materials using standard analytical methods. The analysis was conducted to assess whether different secondary metabolites are present, including alkaloids, glycosides, tannins, phenols, flavonoids, saponins, steroids, and triterpenes. These bioactive compounds are essential for understanding the therapeutic potential and pharmacological activities of the crude drugs ⁷³.

4. Chromatographic Evaluation

Procurement of Marker Compounds

The standard marker compounds of the selected medicinal plants were generously provided as gift samples by Himalaya Wellness, Bengaluru, and stored in appropriate containers under recommended storage conditions to maintain their integrity. The three phytoconstituents chosen for analysis included gallic acid, derived

from *Terminalia chebula*; stigmasterol, isolated from *Moringa oleifera*; and rosmarinic acid, obtained from *Ocimum sanctum*. These marker compounds serve as reference standards (Purity of 99%) for the assessment of the plant materials, qualitatively as well as quantitatively, enabling the procedures of standardization and authentication.

Chemicals and Reagents

The chemicals and reagents employed for the chromatographic analysis were of analytical-grade and suitable for HPLC. Methanol, acetonitrile, formic acid, and o-phosphoric acid of HPLC-grade, characterized by their high purity and analytical quality, were procured from Merck India Ltd. Additionally, ultrapure water, prepared using a Milli-Q purification system, was sourced from the KLE College of Pharmacy, Belagavi. These materials ensured optimal performance and accuracy in the chromatographic evaluation.

Instruments and Apparatus

UV-spectrophotometric analysis was performed using a Shimadzu UV-1900 spectrophotometer, providing excellent sensitivity and precision for analyte measurement. The optimization and validation of HPLC methods were carried out using advanced chromatographic instrumentation, including the ChemStation software is included with the Agilent 1220 Infinity II HPLC devices for data collection and analysis. In addition to the spectrophotometric and chromatographic systems, various essential laboratory instruments were employed to ensure robust analytical procedures. Precise weighing of analytes was achieved using a Sartorius electronic analytical balance, while solution preparation and homogenization were facilitated using an Ultrasonic Bath Sonicator-Ultrasonic Cleaner.

General Methodology

For the chromatographic evaluation aimed at the standardization of herbal medicines, three marker compounds were selected: gallic acid, derived from *Terminalia chebula*; stigmasterol, isolated from *Moringa oleifera*; and rosmarinic acid, obtained from *Ocimum sanctum*. Two HPLC methods were developed utilizing the QbD methodology: one for the simultaneous analysis of rosmarinic acid and gallic acid, and another for the single-component analysis of stigmasterol. These optimized methods were validated and subsequently applied to quantify the selected marker compounds in their respective medicinal plant extracts and in a prepared tablet dosage form. The methods ensured precise and accurate quantification, thereby supporting the standardization and evaluation of herbal compositions' quality.

Simultaneous Analysis of Rosmarinic Acid and Gallic Acid**i. Chromatography Instrument and Conditions**

An Agilent 1220 Infinity II HPLC equipment (LC-20AD, Japan) with a quaternary pump with degasser (G7111A), auto-injector (G7129A), and photodiode array (PDA) detector (G7115A) was utilized for the analysis. The sophisticated OpenLab CDS program was used to interpret and analyze the data. A premium Phenomenex Luna C18 analytical column with the following parameters was used to accomplish chromatographic separation and analysis: C-18(2), 100 Å, internal diameter of 250 mm, length of 4.6 mm, and particle size of 5 µm. An optimum mobile phase comprising methanol and water in a 55:45 (v/v) ratio was made in order to analyze the marker chemicals simultaneously. It was then pumped across the column at a steady flow rate of 1 mL/min. An injection volume of 10 µL was consistently used throughout the sample analysis, with detection occurring at an isosbestic wavelength of 298 nm⁷⁴.

ii. Preparation of Standard Solutions

10 milligrams of each bioactive component were precisely weighed into two distinct 10 mL volumetric flasks to create individual stock solutions of gallic acid and rosmarinic acid. After that, methanol was used to dissolve each component and dilute it to volume, giving each solution a final concentration of 1000 µg/mL. We diluted the separate stock solutions of gallic acid and rosmarinic acid with the mobile phase to create the working standard solutions at a concentration of 10 µg/mL. A combined stock solution containing both rosmarinic acid and gallic acid was then prepared by adding the required volumes of each stock solution into a single 10 mL volumetric flask, which was then filled with the mobile phase until it reached the mark. A set of working standards with concentrations ranging from 5 to 30 µg/mL were created by successively diluting the combined stock solution. To ensure clarity and avoid any possible interference during the measurement procedure, before chromatographic analysis, these substances were filtered via a 0.22 µm syringe filter.

Preliminary Method Development Study

A comprehensive literature survey revealed that methanol is a commonly employed organic solvent, frequently used in combination with buffer solutions for the chromatographic analysis of both rosmarinic acid and gallic acid. A comparison of methanol and acetonitrile as the organic phases was carried out in order to achieve optimal chromatographic conditions. In the end, methanol was chosen because of its exceptional solubility characteristics for both rosmarinic acid and gallic acid, leading to better peak resolution and improved sensitivity during analysis. Water was utilized for the aqueous phase at first, however this exhibited less-than-ideal peak characteristics, including broadening and diminished resolution. To further refine the chromatographic conditions, a QbD-enabled DoE optimization approach was utilized.

This strategy allowed for the systematic evaluation and optimization of crucial method factors, including gradient conditions, flow rate, and organic phase compositions. By employing this QbD approach, the desired chromatographic performance was achieved, ensuring optimal peak shapes, retention times, and resolution for both bioactive compounds.

iii. Determination of Isosbestic point by UV Spectroscopy

To determine the isosbestic point, a spectral scan was carried out across the wavelength range of 800–200 nm to identify the maximum absorbance (λ_{max}) for the analytes. Analysis of the spectral data revealed that the isosbestic point occurred at 298 nm, where both rosmarinic acid and gallic acid demonstrated significant absorbance overlap. This finding allowed for the identification of a wavelength at which both analytes exhibit equivalent absorbance, thereby enabling accurate and simultaneous quantification during subsequent analyses. All further measurements and quantification were performed at this isosbestic point to maintain analytical precision and ensure reliable results.

iv. Quality by Design Approach

The QbD-based method optimization was systematically executed through a series of well-defined steps to ensure a robust and reliable method development⁷⁵⁻⁷⁷.

Risk Assessment

This study's principal goal was to systematically examine the various factors influencing the Critical Quality Attributes (CQAs). CAAs were used to illustrate the connections between the technique parameters and the CQAs, providing a comprehensive understanding prior to conducting a formal risk assessment. The

specific goals of the study included categorizing potential causes of challenges in meeting the desired quality attributes and identifying underlying reasons for deficiencies, discrepancies, and defects in the analytical method. Additionally, the study aimed to pinpoint the critical factors essential for achieving the CQAs, thereby ensuring robust method performance and consistent quality outcomes.

The factors influencing the target responses were systematically classified into two levels: low (-1) and high (+1). Initially, five factors were identified and assessed for their risk levels. A thorough evaluation allowed the identification of critical factors, narrowing the selection down to two key variables. These critical factors were further optimized using experimental design approaches. The study established the Target Quality Profile (TQP), emphasizing tailing factor (TF) and theoretical plates (TP) as the primary dependent variables. These parameters were prioritized to ensure method robustness, accuracy, and reproducibility, aligning with the analytical objectives.

Optimization by DoE

The Analytical Quality by Design (AQbD) approach was applied in order to methodically assess how independent variables affected several important responses. In order to execute this, Design Expert software (version 13.0) was used to choose a 2^2 full-factorial design. This approach is advantageous due to its ability to efficiently generate optimal results with relatively few experimental trials. The independent variables considered in this study included: The ratio of the mobile phase (A) and the flow rate (mL/min) (B). Their individual and interactive effects were evaluated for the following responses: Y_1 (Tailing Factor for Gallic Acid), Y_2 (Theoretical Plates for Gallic Acid), Y_3 (Tailing Factor for Rosmarinic Acid), and Y_4 (Theoretical Plates for Rosmarinic Acid) (**Table 1**).

To determine the ideal set of variables, four experimental runs were carried out. ANOVA, Polynomial equations and three-dimensional response surface plots are employed to demonstrate how these factors affected responses. These graphical and mathematical tools provide valuable insights into the way the independent variables interact as they impact the desired responses, thereby guiding the method optimization process. By understanding these relationships, the study intended to improve the analytical method's reproducibility, accuracy, and robustness.

TABLE 1. Variables and their levels used in 2² factorial design for simultaneous analysis gallic acid and rosmarinic acid.

Factors	Levels used, actual (coded)	
	Low (-1)	High (+1)
Independent variables		
A: Mobile phase ratio	55:45 (-1)	50:50(+1)
B: Flow rate (ml/min)	1.0 (-1)	1.1 (+1)
Dependent variables		
Y ₁ & Y ₃ : Tailing factor	Minimize	
Y ₂ & Y ₄ : Theoretical plates	Maximize	

v. Analytical Method Validation

To ensure its repeatability and appropriateness for analytical applications, the newly established RP-HPLC approach for the simultaneous evaluation of gallic acid and rosmarinic acid was verified in accordance with current regulatory criteria. The validation process encompassed the assessment of key performance parameters, including: linearity, range, LOD, LOQ, system suitability, precision, accuracy, and robustness^{78, 79}.

Linearity and Range

Gallic acid and rosmarinic acid standard solutions were prepared at amounts varying from 5 µg/mL to 30 µg/mL in order to evaluate the linearity as well as range of the established RP-HPLC method. These solutions were made by gradually diluting the suitable stock solution with the mobile phase, which is a 55:45 (v/v) mixture of methanol and water. Each solution was subjected to six replicates (n = 6) and analyzed under identical chromatographic conditions to ensure the reproducibility and reliability of the measurements.

LOD & LOQ

Statistical techniques based on the response standard deviation (σ) and the calibration curve slope (S) were used to calculate the LOD and LOQ for the proposed HPLC method. The calculations were performed using the following formulas:

$$\text{LOD} = \frac{3.3 \times \text{Standard Deviation of Y intercept}}{\text{Slop of the calibration}}$$

$$\text{LOQ} = \frac{10 \times \text{Standard Deviation of Y intercept}}{\text{Slop of the calibration}}$$

System suitability

System suitability criteria are essential for validating HPLC methods since they validate the chromatographic system's reliability and efficacy. For the developed method, system suitability was evaluated by analyzing a concentration of 10 µg/mL of both gallic acid and rosmarinic acid in six replicates under the optimized chromatographic conditions. Parameters like tailing factor, peak area, theoretical plate

number, and retention time were evaluated, which were assessed to ensure consistency, accuracy, and efficiency of the analytical technique.

Precision

One crucial factor that indicates the degree of scatter or the degree of agreement between a set of measurements made from several samplings of a single homogenous sample under particular circumstances is the analytical procedure's precision. Precision was evaluated through several approaches: system precision, intraday precision (assessed three times within a single day), and interday precision (assessed over three consecutive days) 5 µg/mL, 15 µg/mL, and 25 µg/mL at different amounts for both substances. This study aimed to establish the reproducibility of results both on a single day and on distinct days. The percentage %RSD for the obtained data was consistently less than 2%, demonstrating excellent precision and reliability of the method.

Accuracy

To assess accuracy, the conventional addition approach was employed. A sample solution containing gallic acid and rosmarinic acid at a concentration of 5 µg/mL was spiked with standard concentrations corresponding to 50%, 100%, and 150% of the nominal value. The spiked samples were then analyzed, and the recovered amounts were quantified. The percentage recovery and the %RSD were calculated to assess the accuracy and dependability of the analytical method. These results provided a measure of the method's capability to accurately reflect the true concentration of the analytes in the sample.

Robustness

Through intentional, small adjustments to the chromatographic settings, the new method's robustness was carefully evaluated. These variations included adjustments to the mobile phase composition (comprising 53:47% and 57:43% ratios), detecting wavelength (296 nm and 300 nm), and flow rate (0.8 mL/min and 1.2 mL/min). These tests were designed to evaluate the method's ability to remain unaffected by small, intended changes in operational conditions, thereby demonstrating its reliability and consistency under different circumstances. These tests' outcomes demonstrated the method's robustness and its capacity to yield trustworthy analytical results even with slight modifications to the experimental setup.

Quantitative Analysis of Gallic Acid and Rosmarinic Acid

Gallic acid and rosmarinic acid were determined using an optimized RP-HPLC technique, in their respective medicinal plant extracts and prepared tablet dosage forms. For the standardization of herbal medicine, 50 mg of each respective plant extract was precisely weighed, dissolved in 100 milliliters of methanol, and then sonicated for 1 hour to ensure complete dissolution. The mobile phase was appropriately diluted to provide a 10 µg/mL extract solution. For the tablet dosage form, a single 500 mg tablet was completely dispersed in 250 milliliters of methanol, then subjected to a 30-minute sonication to achieve complete dissolution of the tablet contents. A 20 mL volume of the mixture was taken out after sonication and mixed with the mobile phase to attain a final concentration of 10 µg/mL. The marker content in the corresponding plant materials and tablet dosage forms was then ascertained by analyzing these diluted solutions using the suggested HPLC approach.

HPLC Analysis of Stigmasterol⁸⁰**i. Chromatography Instrument and Conditions**

An Agilent 1220 Infinity II HPLC system (LC-20AD, Japan) equipped with a photodiode array (PDA) detector (model G7115A), an auto-sampler (model G7129A), and a quaternary pump integrated with a degasser (model G7111A) was used for the chromatographic analysis. The reliable and sophisticated OpenLab CDS software was used for data collection, processing, and interpretation, guaranteeing accurate and repeatable outcomes. A efficient Phenomenex Luna C18 analytical column was employed for chromatographic separation and quantification, with the following specifications: C18(2) stationary phase, pore size of 100 Å, internal diameter of 4.6 mm, length of 250 mm, and particle size of 5 µm. This column was selected for its superior separation efficiency and compatibility with the analyte under investigation. A mobile phase made up of methanol and 0.1% formic acid in a volumetric mixture of 80:20 (v/v) was used to optimize the analysis of stigmasterol. To ensure uniform analyte elution, the mobile phase was degassed and provided across the column at a steady flow rate of 1.0 mL/min. A precise 10 µL volume for injection was maintained for each sample to ensure accuracy and reproducibility. To improve separation efficiency and preserve analyte stability, the column temperature was carefully regulated at 35°C. At 210 nm, the detecting wavelength was established based on the maximum absorbance of stigmasterol, to provide high sensitivity and selectivity.

ii. Preparation of Standard solutions

To achieve an extent of concentration 1 mg/mL, 1 mg of stigmasterol was dissolved in 1 mL of methanol to generate the primary stock solution. Working standard solutions with concentrations ranging from 5 to 30 µg/mL were then

obtained by serially diluting this stock solution using the mobile phase. Prior to HPLC analysis, all prepared standard solutions were transferred to tightly sealed volumetric flasks and stored at 4 °C to maintain stability and prevent degradation.

iii. Preliminary Method Development Studies

Stigmasterol, due to its limited solubility in aqueous phases, needed the application of organic solvents, like methanol and acetonitrile for dissolution. Methanol was identified as the optimal solvent, as it allowed complete dissolution of stigmasterol and was consequently selected for the initial chromatographic analysis. However, early attempts employing methanol-water as the mobile phase did not produce discernible peaks in the chromatogram, even after a 30-minute runtime. To address this, water was substituted with ortho-phosphoric acid (OPA), which resulted in the appearance of peaks. Despite this improvement, the chromatographic peaks exhibited excessive tailing and a reduced number of theoretical plates, reflecting inadequate separation.

Subsequently, 0.1% formic acid in water was employed in place with OPA, at a 70:30 ratio (organic: aqueous). This modification produced stigmasterol peaks but with persistent tailing and prolonged retention times exceeding 35 minutes. To overcome these challenges and achieve optimal chromatographic performance, a QbD approach was adopted, leveraging DoE methodologies. This systematic strategy facilitated the fine-tuning of chromatographic parameters, ensuring robust separation and superior peak characteristics, thereby enhancing the reliability and reproducibility of the analytical method.

iv. Development of RP-HPLC Method Assisted By Box-Behnken Design

The current study employs Response Surface Methodology (RSM) integrated with a robust HPLC method to optimize chromatographic conditions. The optimization process utilized the BBD, a specialized experimental design featured in Version 13.0 of Design of Experiments (DoE) software. BBD was selected over the Central Composite Design (CCD) due to its superior capability in optimizing responses with fewer experimental runs, enhancing efficiency without compromising accuracy. Three independent variables were systematically varied to evaluate their effects on key chromatographic performance metrics. These variables included column temperature (X_3), flow rate (X_2), and the proportion of the organic phase (X_1). The corresponding dependent variables are described in detail in Table 2 and are retention time (Y_1), tailing factor (Y_2), and the number of theoretical plates (Y_3)⁸¹.

TABLE 2. Chromatographic factors considered within the Box–Behnken experimental design for the proposed HPLC method.

Independent variables	Levels		
	Low (-1)	Medium (0)	High (+1)
X1= organic Phase % (v/v)	70.0	80.0	90.0
X2= flow rate (mL/min)	0.8	1.0	1.2
X3= column oven temp (°C)	30	35	40
Dependent variables			
Y_1 = RT	Minimum	+++	
Y_2 = Tailing factor	Minimum	+++	
Y_3 = theoretical plates	Maximum	+++	

The experimental conditions for optimization were defined as follows: the percentage of organic phase (X_1) was investigated at three levels (70%, 80%, and 90%), the column temperature (X_3) was altered at 30 °C, 35 °C, and 40 °C, while the flow rate (X_2) was set to 0.8, 1.0, and 1.2 mL/min. To demonstrate the relation with the responses and the independent variables, polynomial equations were generated. To get the ideal values of X_1 , X_2 , and X_3 , these equations have been applied through ANOVA that simultaneously satisfied the desired chromatographic outcomes. The model's adequacy was rigorously validated by assessing its predictions for the dependent variables: retention time (Y_1), tailing factor (Y_2), and theoretical plate count (Y_3). The results show that the application of BBD within the RSM framework offers a systematic and efficient approach to optimizing HPLC conditions, ensuring high precision and reliability in chromatographic analyses.

v. Analytical Method Validation

Method validation is a critical component of analytical development, ensuring the reliability and accuracy of results obtained through HPLC. Conducted in accordance with ICH guidelines, validation involves a comprehensive assessment of key parameters, including linearity, LOD, LOQ, system suitability, precision, accuracy, and robustness^{82, 83}.

Linearity

Utilizing a mobile phase containing methanol and 0.1% formic acid, a series of stigmasterol stock solutions were generated at concentrations that varied from 5 µg/mL to 30 µg/mL in order to assess the linearity of the established technique. Each concentration was analyzed in six replicates ($n = 6$). The least squares regression method was used to evaluate the linear association between the variables after the

peak areas from the chromatographic analysis were plotted against the respective concentrations. By computing the regression coefficient (R^2) and analyzing the y-intercept of the regression line, which was obtained from the plot of peak area vs analyte concentration, the linearity of the procedure was verified.

LOD & LOQ

Statistical techniques based on the response standard deviation (σ) and the calibration curve slope (S) were used to calculate the LOD and LOQ for the proposed HPLC method. The calculations were performed using the following formulas:

$$\text{LOD} = \frac{3.3 \times \text{Standard Deviation of Y intercept}}{\text{Slop of the calibration}}$$

$$\text{LOQ} = \frac{10 \times \text{Standard Deviation of Y intercept}}{\text{Slop of the calibration}}$$

System Suitability

System suitability parameters play a pivotal role in validating HPLC methods, establishing assured that the chromatographic system performs as efficiently as possible and generates accurate analytical results. To assess the system's performance, important metrics were assessed for stigmasterol, including peak area, theoretical plates, and tailing factor. Six injections of the material were used to determine these parameters, demonstrating the chromatographic system's reliability and consistency.

Precision

The precision parameter in HPLC method validation evaluates the reproducibility and repeatability of analytical results under specified conditions.

Intra-day Precision (Repeatability): Standard solutions at 10, 15, and 20 µg/mL concentrations were prepared in triplicate and analyzed at three distinct time intervals within the same day. This ensured consistency in results over a short duration.

Inter-day Precision (Intermediate Precision): Over a period of three days, standard solutions with the same concentrations (10, 15, and 20 µg/mL) were produced in triplicate and analyzed. Samples were stored in tightly sealed containers, protected from light, to maintain stability throughout the study.

Accuracy

By using the suggested HPLC method to analyze placebos with preset amounts of stigmasterol throughout the method's linear range, accuracy was evaluated. These quantities corresponded to 50%, 100%, and 150% of the nominal levels. The percentage recovery (%), a key indicator of accuracy, was calculated for both standard stigmasterol and for samples of *moringa oleifera* extract and prepared tablet formulations.

Robustness

Minimal modifications to the chromatographic conditions were used to assess the robustness of the established HPLC technique. These comprised adjustments to the flow rate (0.8 and 1.2 mL/min), detection wavelength (208 and 212 nm), and mobile phase composition (ratios of 78:22 and 82:18%). These modifications were intended to assess the method's reliability under slight experimental changes, ensuring consistent performance across different settings.

Quantitative Analysis of Stigmasterol

The amount of stigmasterol in developed tablet dosage forms and *moringa oleifera* extract was measured using an optimized RP-HPLC method. 50 mg of the plant extract was precisely weighed, diluted in 100 mL of methanol, and sonicated for an hour to ensure thorough dissolution in order to standardize the herbal medicine. The extract was then appropriately diluted with the mobile phase to provide a 10 mg/mL extract solution. A 500 mg tablet was fully dissolved in 250 mL of methanol, with sonication applied for 30 minutes to ensure complete dissolution of the tablet components. A 20 mL portion of this solution was withdrawn and diluted with the mobile phase to achieve a final concentration of 10 µg/mL. The stigmasterol content of the plant material and the tablet dosage forms was then ascertained by analyzing these diluted solutions using the suggested HPLC approach.

Extraction

Various extraction techniques were employed to prepare three distinct plant extracts according to standardized procedures. *Terminalia chebula* and *Moringa oleifera* extracts were prepared using two conventional techniques: Soxhlet extraction and cold maceration. A quantity of 50 grams of powdered material was subjected to maceration with 70% v/v ethanol for a period of 24 hours. The marc was subsequently extracted using the Soxhlet apparatus with 95% v/v ethanol. After combining the extracts from the two methods, to eliminate the solvent, a rotating evaporator was employed at a lower pressure. After drying, the resulting extracts were kept out of direct sunlight in tightly sealed containers. In contrast, *Ocimum sanctum* extract was prepared using a simple maceration method, where 50 grams of powdered drug were soaked in a mixture of ethanol and water (75:25% v/v) for seven days. A rotary

evaporator was then used to filter and evaporate the extract until it was completely dry. All of the crude extracts were weighed and kept for later use in a deep freezer ⁸⁴.

Quality Assessment of Herbal Extract

The quality assessment of the herbal drug extracts was conducted to determine the quality attributes of each extract. Each plant extract was evaluated according to the quality control parameters specified in the World Health Organization (WHO) guidelines. These parameters included phytochemical analysis, ash value, and moisture content, which were assessed using standardized procedures ⁸⁵.

Formulation Development

Compatibility Study

Fourier Transform Infrared (FTIR) spectroscopy analysis was employed to examine the herbal extracts' compatibility with excipients. This method was used to determine the chemical compounds within the extracts and evaluate their compatibility with other excipients used in the formulation of polyherbal tablets. FTIR spectra facilitate the identification of specific compounds based on the presence of various functional groups, providing valuable insights into potential interactions between the herbal extracts and excipients ⁸⁶.

QbD Approach for Formulation Development

A methodical approach is used in the QbD philosophy to achieve a comprehensive understanding of the process and product. Determining the CQAs, CMAs, CPPs, and defining the QTPP are important components. This strategy includes choosing suitable optimization and screening designs, determining the

optimal formulation by identifying design and control spaces, and developing a control strategy for ongoing process improvement (**Figure 4**)⁸⁷.

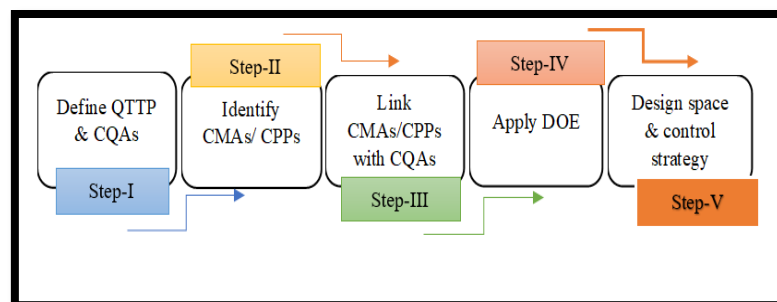


Figure 4. Steps for QbD implementation

i. Quality Target Product Profile (QTPP)

Determining the goals to attain the target product's targeted qualitative attributes is the first stage of the drug product development plan. A depicted and dynamic summary of the drugs product's quality features is known as the QTPP. It encompasses a set of elements that outline the essential characteristics of the drug product, serving as a guiding framework for development. The QTPP establishes the objective or target in advance, providing a clear roadmap for the development process.

ii. Critical Quality Attributes (CQA)

Establishing the drugs product's major quality determinants or metrics, or CQAs, is a crucial next step after defining the QTPP. These characteristics, which come from the QTPP's components, have a direct bearing on the drug's efficacy and safety. The physical, chemical, biological, and microbiological properties of the product are examples of CQAs. Maintaining these attributes within defined limits, ranges, or distributions is crucial to achieving and ensuring consistent product quality.

iii. Critical Material Attributes (CMA) and Critical Process Parameters (CPPs)

Material attributes (MAs) and process parameters (PPs), which have a substantial independent influence on both product and process performance, are commonly included as input variables linked to a product's CQAs. While process parameters are variables related to the unit operations during manufacture, material attributes are features of the drug substance and excipients. By leveraging literature reviews, expert insights, and experience with similar or related products, critical MAs and PPs can be identified and prioritized effectively for any pharmaceutical product.

iv. Quality Risk Management (QRM) Approach

By giving formal risk scores to elements associated to products and processes, QRM is a systematic method used to prioritize CMAs and CPPs. Through QRM, factors critical to the quality of intermediate or finished products are identified and classified into distinct risk levels: low risk (minimal and acceptable, requiring no mitigation), medium risk (moderate and acceptable, with potential mitigation needs), and high risk (severe and unacceptable, demanding mitigation)⁸⁸.

v. Design of Experiments

In the current study, the Design of Experiments (DoE) approach was utilized to formulate polyherbal tablets using a 3^2 central-composite design (CCD). Preliminary investigations were conducted to determine the optimal composition for the tablets. The selected independent variables, or factors, for this study were the concentrations of microcrystalline cellulose (MCC, X1) and croscarmellose sodium (CCS, X2). The dependent variables, or responses, considered were disintegration time (Y1), friability (Y2), and tablet hardness (Y3). **Table 3** presents a comprehensive overview of the experimental design, detailing the factors, their respective levels, and

the corresponding responses. The DoE was conducted using the statistical software JMP® (Version 18.0.1, SAS Institute, Inc., Cary, NC). Model screening was carried out through ANOVA, and MLRA equations were subsequently analyzed to evaluate their direct impact on the responses and the interactive effects between the factors^{89, 90}.

TABLE 3. Central-composite experimental design encompassing independent and dependent variables with their levels and observed responses.

Independent variables (Factors)			Dependent variables (Responses)		
Batch code	Microcrystalline cellulose (X_1)	Croscarmellose sodium (X_2)	Disintegration time (min, Y_1)	Friability (% , Y_2)	Hardness (kg/cm^3 , Y_3)
F1	50	30	25.1	0.52	5.6
F2	75	20	19.04	0.42	4.6
F3	100	30	24.3	0.48	5.7
F4	75	20	19.21	0.42	4.7
F5	75	30	22.04	0.4	5
F6	100	10	24.2	0.64	6.3
F7	75	20	19.24	0.41	4.7
F8	50	20	26.1	0.42	5.3
F9	75	10	21.06	0.38	4.9
F10	50	10	28.03	0.38	5.2
F11	75	20	19.4	0.41	4.6
F12	75	20	19.17	0.42	4.7
F13	100	20	23.12	0.56	6.1

vi. Prediction Profiler

The Prediction Profiler in JMP software is an interactive tool designed to visualize and explore the impact of multiple factors on a response variable. It allows users to manipulate predictor values and immediately observe the corresponding predicted response, thus facilitating the identification of optimal conditions and enhancing the understanding of model behavior. The formulation's intended results and target responses served as the basis for defining the desirability function. The Prediction Profiler offers a range of values for each factor and their corresponding responses, though not all potential combinations are assessed. The profiler plot illustrates the predicted values for each target response. In this study, the Prediction Profiler was utilized to identify the optimal formulation for a polyherbal tablet. Prediction traces were analyzed for key responses, including disintegration time (in minutes), friability (in %), and tablet hardness (in kg/cm³)⁹¹.

Preparation of Polyherbal Tablets

The extracts of *Terminalia chebula* (200 mg), *Moringa oleifera* (100 mg), and *Ocimum sanctum* (200 mg) were precisely weighed and thoroughly combined with excipients, including microcrystalline cellulose (MCC), croscarmellose sodium (CCS), and lactose monohydrate. Twenty tablets per formulation were prepared, ensuring that all components passed through a mesh sieve number 60. The excipients were uniformly blended with the extracts for a duration of 30 minutes using a pestle and mortar, with the exception of the lubricant. Magnesium stearate, serving as the lubricant, was sieved and accurately weighed prior to incorporation. Talcum powder was subsequently incorporated into the mixture, and the mixture was stirred for an additional 10 minutes⁹².

The final blend was compressed into tablets using a direct compression method with a single-station Rimek 10-station tablet press machine. Each tablet had a total weight of 650 mg, including the excipients. Thorough tests were performed following standard reference procedures to assess the powder blends both after mixing and before compression. These tests included measurements of loose bulk density, tapped bulk density, Carr's index, Hausner's ratio, and the angle of repose⁹³.

Characterization Parameters

After compression, the tablets underwent a thorough evaluation process, which included parameters such as appearance, hardness, thickness, friability, weight variation, and disintegration time. The appearance was visually inspected to ensure uniformity of color. Hardness was measured using a Monsanto hardness tester, while the thickness was determined with a Mitutoyo digital Vernier caliper. For friability testing, ten randomly selected tablets were placed in a tablet friability tester drum, the device was operated at 25 rpm for 4 minutes. After the specified rotation, the tablets were removed, and their weight was recorded to calculate the percentage weight loss. Furthermore, the tablets were assessed for weight variation and disintegration time using a standardized tablet disintegration apparatus^{94, 95}.

Drug Content Analysis

For drug content analysis of tablets derived from three different plant extracts, three specific phytoconstituents were selected: gallic acid from *Terminalia chebula*, stigmasterol from *Moringa oleifera*, and rosmarinic acid from *Ocimum sanctum*. A total of ten tablets were randomly chosen and finely ground. An equivalent of 50 mg from each powdered extract was carefully measured and placed into 150 mL of 0.1 N HCl. For an hour, the mixture was agitated using a magnetic stirrer to ensure complete

dissolution. Afterward, the solution was filtered, and a 5 mL portion of the filtrate was transferred to a 10 mL volumetric flask and diluted with methanol. The concentrations of the phytoconstituents were analyzed using a UV-Visible spectrophotometer (Shimadzu UV-1800) at wavelengths of 271 nm for gallic acid, 328 nm for rosmarinic acid, and 210 nm for stigmasterol ⁹⁶.

***In-Vitro* Drug Release Analysis**

Using a USP Type II dissolution device set at 50 rpm and 900 mL of 0.1 N HCl (pH 1.2) as the dissolution medium, *in-vitro* drug release tests of the tablets were carried out. Throughout the process, the temperature was maintained at 37°C. A formulation containing 50 mg of each extract was introduced into the dissolution medium for the release testing. At regular intervals of 0, 15, 30, 60, 90, 120, 150, and 180 minutes, 5 mL aliquots were withdrawn. To get rid of any particles, the samples were then filtered via Whatman filter paper. Every extracted sample was immediately replaced with an equivalent volume of fresh dissolving medium to ensure ideal sink conditions. The aliquots were then diluted to 10 mL with methanol and analyzed using a UV-Visible spectrophotometer (Shimadzu UV-1800) at wavelengths of 271 nm for gallic acid, 328 nm for rosmarinic acid, and 210 nm for stigmasterol ⁹⁷.

Stability Study

Stability testing was carried out on a statistically optimized tablet formulation following the Q1A (R2) guidelines set by the ICH. The samples were stored in capped high-density polyethylene (HDPE) bottles for a duration of 60 days, under both ambient conditions (25 ± 2°C, RH 65%) and accelerated conditions (40 ± 2°C, RH 75%). The tablets were evaluated periodically at 15, 30, 45, and 60 days for critical parameters including hardness, disintegration, and friability ⁹⁸.

Anti-Inflammatory Activity

The standardized methods outlined below were utilized for both *in-vitro* and *in-vivo* anti-inflammatory assessments of the prepared polyherbal tablets.

***In-Vitro* Human Red Blood Cell (HRBC) Membrane Stabilization Assay**

The *in-vitro* anti-inflammatory potential of the formulated tablets was assessed using the HRBC membrane stabilization assay. This assay, widely used for screening anti-inflammatory agents, assesses the protective effect of test formulations against red blood cell (RBC) lysis. NSAIDs were used as the reference standard for comparison. The degree of anti-inflammatory activity was quantitatively represented as the percentage inhibition of RBC hemolysis. Hemoglobin release into the suspension, indicative of membrane rupture, was measured spectrophotometrically at 560 nm⁹⁹.

Blood samples for the study were obtained from healthy volunteers, with the primary exclusion criterion being the intake of NSAIDs within two weeks prior to sample collection. Sodium oxalate was utilized as an anticoagulant to prevent coagulation, ensuring the integrity of erythrocytes. Before being processed further, the obtained blood samples were kept for 24 hours at 4°C. The samples were centrifuged for 15 minutes at 3000 rpm in order to separate the erythrocytes, and the supernatant was disposed of. Following three rounds of washing with sterile saline solution (0.9% w/v NaCl), the erythrocytes were centrifuged for fifteen minutes at 3000 rpm. The packed cell volume (PCV) was measured following the last wash.

For reconstitution, a 40% (v/v) erythrocyte suspension was made in phosphate-buffered saline (PBS; 10 mM, pH 7.4), and the volume was adjusted with distilled water to 1 L. This erythrocyte suspension served as the working solution for

the assay. Test solutions of the formulation and at concentrations of 100, 200, 300, 400, and 500 µg/mL, reference standards were made. The assay involved incubating these solutions with the erythrocyte suspension under controlled conditions¹⁰⁰. The degree of RBC lysis was evaluated by measuring the hemoglobin content in the supernatant¹⁰¹. Hemolysis observed in the control group (incubated without the test formulation or reference standard) was set as 100%. Indicative of anti-inflammatory activity, the formula below was used to determine the % inhibition of RBC hemolysis:

$$\begin{aligned} & \% \text{ membrane stabilization} \\ &= \left(100 - \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \right) \times 100 \end{aligned}$$

In-Vivo Anti-Inflammatory Activity

Experimental Animals

Male Wistar rats, with weights ranging from 180 to 220 g, were used as experimental subjects in this study. The animals were obtained from a certified animal facility and housed under controlled environmental conditions. The ambient temperature was kept at 25 ± 1°C, with a 12-hour light/dark cycle to maintain a stable circadian rhythm. The animals were given ad libitum access to water and a standard laboratory diet, formulated to meet their nutritional needs. Prior to the commencement of experimental procedures, all rats were acclimatized to the housing conditions for a minimum of one week to minimize stress-related variables.

For studies involving the oral administration of tablets or drugs, the animals were subjected to an 18-hour fasting period, during which water was freely available to prevent dehydration. This fasting protocol ensured consistent gastric emptying across subjects, optimizing the reliability of oral drug absorption and pharmacokinetic

analyses. All experimental protocols were conducted in full compliance with the official standards outlined by the OECD concerning the care and ethical use of laboratory animals. The IAEC provided ethical approval for the study, ensuring adherence to the guidelines established by the CPCSEA, ensuring compliance with internationally recognized standards for animal welfare. The study was conducted in 2024 under IAEC approval number 221/Po/Re/S/2000/CPCSEA.

Carrageenan-Induced Paw Edema in Rats

The anti-inflammatory properties of the polyherbal tablets were assessed using a carrageenan-induced paw edema model in male *Wistar* rats. This method, a widely accepted preclinical model for evaluating acute inflammation, involves the subplantar administration of a pro-inflammatory agent to induce localized edema. In this study, inflammation was induced in all experimental groups by subplantar administration of 0.1 mL of a freshly prepared 1% (w/v) carrageenan suspension into the right hind paw of each rat. The untreated left hind paw was used as an internal control to measure changes in paw thickness. Carrageenan injection induced observable redness and significant edema, which became prominent within 2 hours and persisted throughout the 5-hour experimental period ¹⁰². Paw edema progression was quantitatively measured at 0, 1, 2, 3, 4, and 5 hours after carrageenan injection using a Vernier digital caliper, ensuring precise assessment of paw thickness. The animals, acclimatized to controlled environmental conditions, were randomly assigned to five groups, each comprising six rats (n = 6). One hour prior to carrageenan injection, the first three groups were pre-treated with the polyherbal tablets at doses equivalent to 1/5th, 1/10th, and 1/20th of the median lethal dose (LD50), respectively. The remaining two groups were assigned as the control group, receiving distilled water at

a dose of 10 mL/kg, and the reference group, treated with Diclofenac sodium (10 mg/kg), a standard non-steroidal anti-inflammatory drug¹⁰³.

The extent of inflammation was evaluated by determining the percentage inhibition of paw edema compared to the untreated control group, using the formula:

$$\% \text{ inhibition} = \frac{\text{Change in control} - \text{Change in treatment} \times 100}{\text{Change in control}}$$

In this calculation, the change in paw thickness was measured as the difference between the thickness of the treated (right) hind paw and the untreated (left) hind paw. The polyherbal tablet formulations exhibited a dose-dependent decrease in paw edema, with their effects compared to the control and reference groups to evaluate their anti-inflammatory efficacy.

Histopathology

For histopathological analysis, the excised paw tissues were promptly fixed in 10% neutral buffered formalin to maintain cellular and structural integrity. After fixation, the tissues underwent dehydration in a series of graded alcohols, followed by clearing in xylene, and were then embedded in paraffin wax to aid in sectioning. Using a microtome, thin tissue slices (about 4–5 µm) were created and placed onto glass slides for additional analysis.

The mounted tissue sections were stained with hematoxylin and eosin (H&E), a routine staining protocol that enables clear visualization of cellular and tissue architecture. Hematoxylin selectively stains nuclear components blue, while eosin imparts a pink hue to cytoplasmic and extracellular matrix components, allowing for the differentiation of various tissue elements. The stained slides were observed under a light microscope to assess histopathological changes associated with inflammation,

including cellular infiltration, edema, and tissue damage. A comparative analysis was performed between the treatment groups, the control group, and the reference group (Diclofenac sodium), offering insights into the anti-inflammatory effects of the polyherbal tablet formulations at the histological level ¹⁰⁴.

Statistical Analysis

Statistical analysis was carried out to assess the significance of differences between experimental groups using one-way ANOVA, an effective method for comparing means across multiple groups. The data within each group are presented as the mean $\pm$ SEM to ensure clear and reliable data reporting. GraphPad Prism software (version 8.0.2), a popular tool for biological data analysis, was utilized to conduct statistical analyses. When necessary, post-hoc analyses were performed to pinpoint precise group differences. Statistical significance was defined as a p-value of less than 0.05 ($p < 0.05$), which denotes a probability of less than 5% that the observed differences were the result of chance.

RESULTS

Selection of Anti-inflammatory Herbs

The raw materials were selected based on predefined criteria, emphasizing anti-inflammatory potential. Several herbs reported in the literature for their anti-inflammatory properties were initially identified. A comprehensive literature review of the selected herbs was subsequently performed, focusing on the following aspects: (a) quality and authenticity, (b) therapeutic efficacy, (c) mechanisms of action and potential synergistic effects, and (d) availability, including the presence of marker compounds and cost considerations. Based on this rigorous evaluation, three herbs exhibiting significant anti-inflammatory activity were chosen for formulation development. The selected herbs are summarized in **Table 4**.

TABLE 4. Herbal raw material selected for development of anti-inflammatory formulation

Herbs Name	Family	Active ingredient
<i>Terminalia chebula</i>	Combretaceae	Gallic acid
<i>Ocimum sanctum</i>	Laminaceae	Rosmarinic acid
<i>Moringa oleifera</i>	Moringaceae	Stigmasterol

Quality Assessment of Herbal Raw Material

1. Botanical Evaluation

i. Macroscopic Evaluation

The macroscopic characteristics of the selected herbal raw materials—*Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera*—were evaluated and are presented in **Table 5**. The assessment includes key parameters such as color, shape, odor, size, and taste of the raw materials.

TABLE 5. Macroscopic characteristics of selected herbs

Parameters	<i>Terminalia Chebula</i> (fruit)	<i>Ocimum Sanctum</i> (leaf)	<i>Moringa Oleifera</i> (leaf)
Colour	Yellowish Brown	Blackish Green	Yellowish Green
Odour	Odorless	Aromatic	Pungent
Taste	Astringent	Characteristic	Acrid
Size	3.8cm length, 1.4 -2.0 cm width	3.8cm in length 2.4cm in width	2.7 cm in length 1.4 cm in width
Shape	Ovoid	Oblong	Ovate

ii. Microscopic Evaluation

The microscopic examination of the selected herbs, depicted in **Figures 5–7**, reveals important structural characteristics, including the presence of cork cells, stone cells, fibers, vascular bundles, and other features. These findings are essential for confirming the identity and purity of the crude drugs.

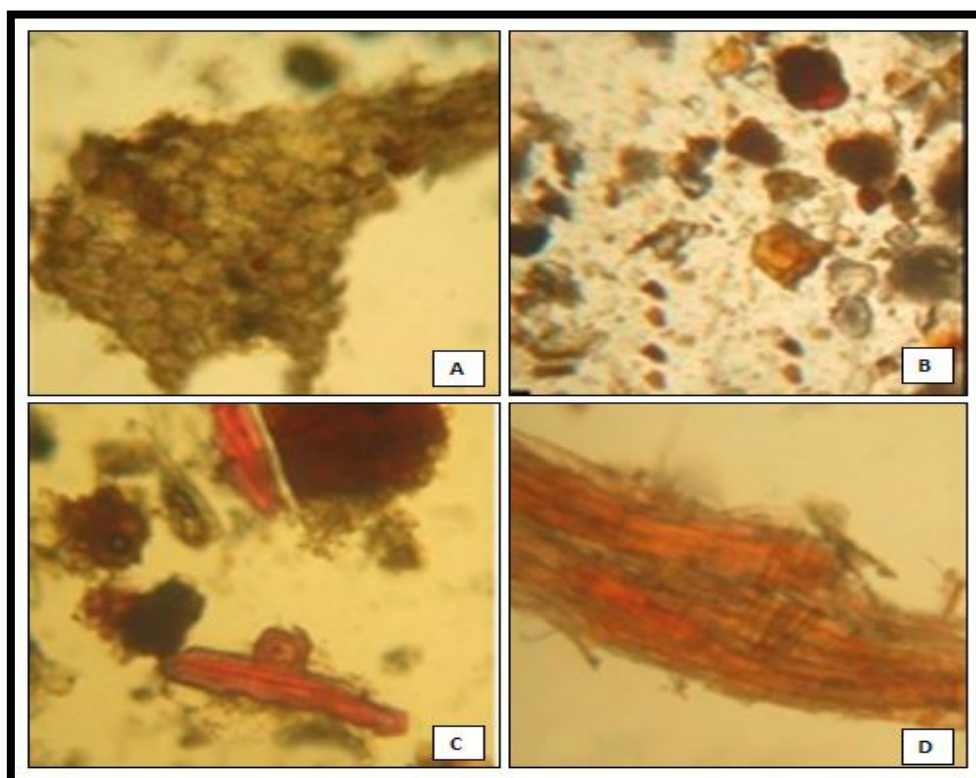


Figure 5. Microscopic evaluation of *Terminalia chebula* fruit showing:
(a) cork cells, (b) stone cells, (c) fibers, and (d) vascular bundles.

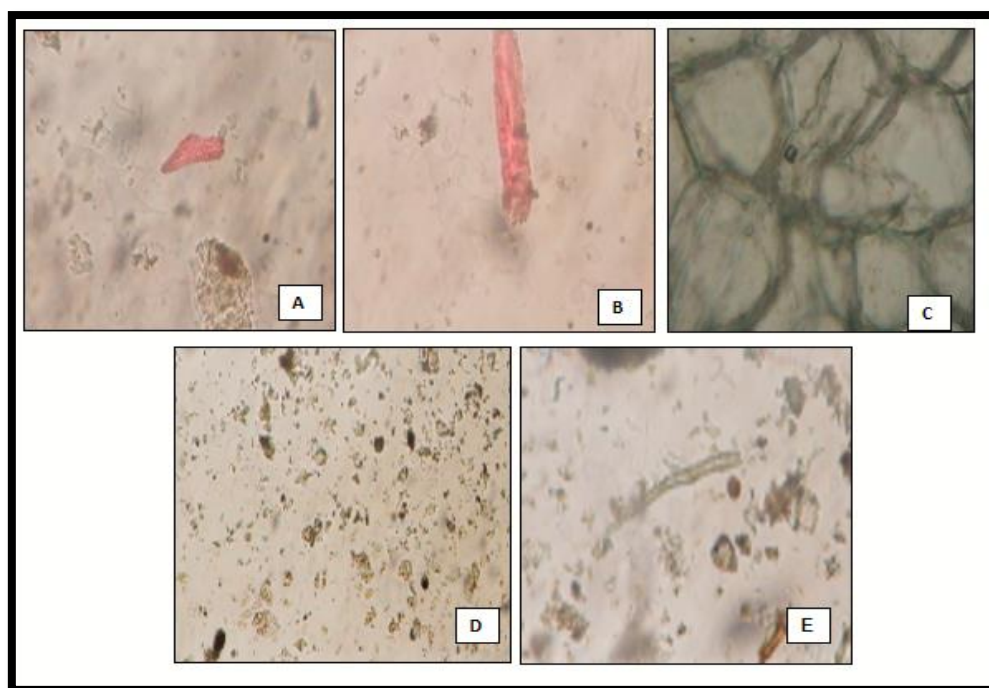


Figure 6. Microscopic evaluation of *Ocimum Sanctum* leaf showing: (a) sclereid, (b) group of lignified fibers, (c) prismatic crystals, (d) starch grain, and (e) trichomes.

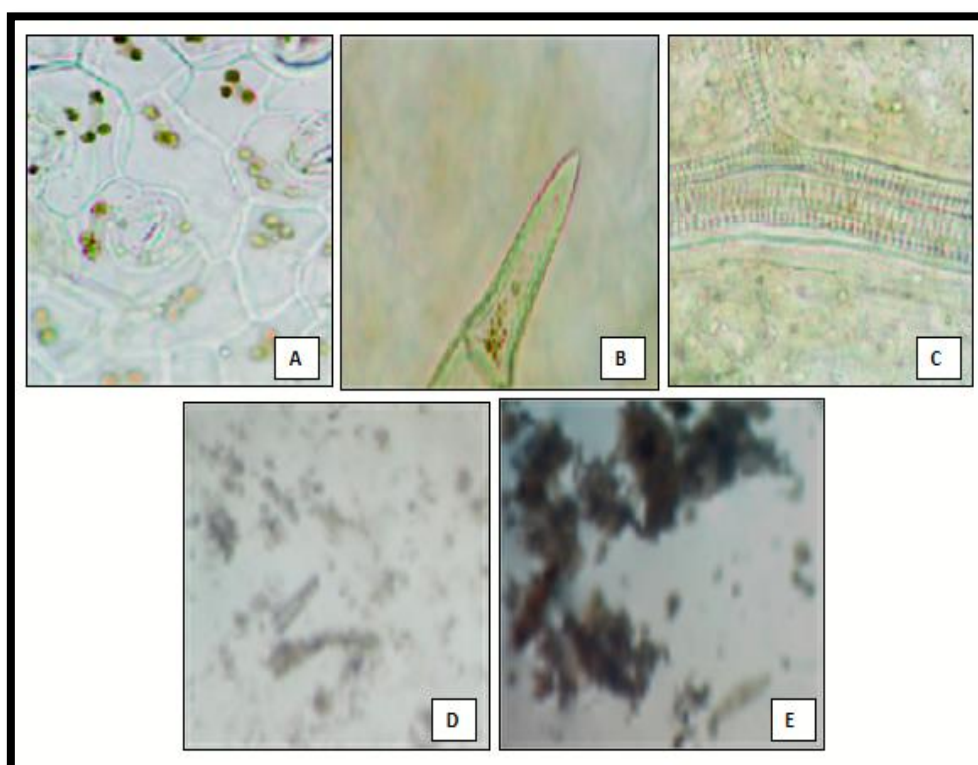


Figure 7. Microscopic evaluation of *Moringa oleifera* leaf showing: (a) stomata, (b) multicellular trichome, (c) xylem vessels, (d) calcium oxalate crystals, and (e) starch grain.

2. Physico-Chemical Evaluation

Physicochemical parameters, such as total ash value, acid-insoluble ash, water-soluble ash, moisture content, and extractive values (alcohol-soluble and water-soluble), were assessed for each herbal raw material. All parameters fell within the specified limits. A summary of these physicochemical characteristics is provided in **Table 6**.

TABLE 6. Physico-chemical parameters of selected raw materials.

Parameters	<i>Terminalia Chebula</i> (fruit)	<i>Ocimum Sanctum</i> (leaf)	<i>Moringa Oleifera</i> (leaf)
Total Ash value	3.65±0.393	8.66±1.312	10.41±1.55
Acid insoluble ash	0.28±0.102	0.76±0.205	2.75±0.735
Water soluble ash	0.83±0.0623	3.36±0.277	1.6±0.294
Moisture content	8±0.571	0.75±0.215	2.58±0.289
Alcohol-soluble extractive	43.8±0.571	8.7±0.163	13.2±0.616
Water Soluble extractive	32.8±0.8164	4.1±0.282	8.63±1.06
Ether Soluble Extractive	12.1±0.616	3.9±0.276	3.48±0.169

Results are expressed in % Mean± SD; n=3

3. Phytochemical Evaluation

A preliminary phytochemical analysis was performed to identify the secondary metabolites present in the selected herbal raw materials. The detected secondary metabolites for each raw material are summarized in **Table 7**.

TABLE 7. Preliminary phytochemical analysis of selected raw materials.

Parameters	<i>Terminalia Chebula</i>	<i>Ocimum Sanctum</i>	<i>Moringa oleifera</i>
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	+	+	-
Phenols	+	+	+
Astringents	+	+	+

4. Chromatographic Evaluation

Simultaneous Analysis of Gallic Acid and Rosmarinic Acid

Determination of isosbestic point by UV spectroscopy

The isosbestic point was determined through UV spectroscopy, revealing a maximum absorbance (λ_{max}) at 298 nm within the detection range of 200–800 nm. The UV spectrum corresponding to this observation is illustrated in **Figure 8**.

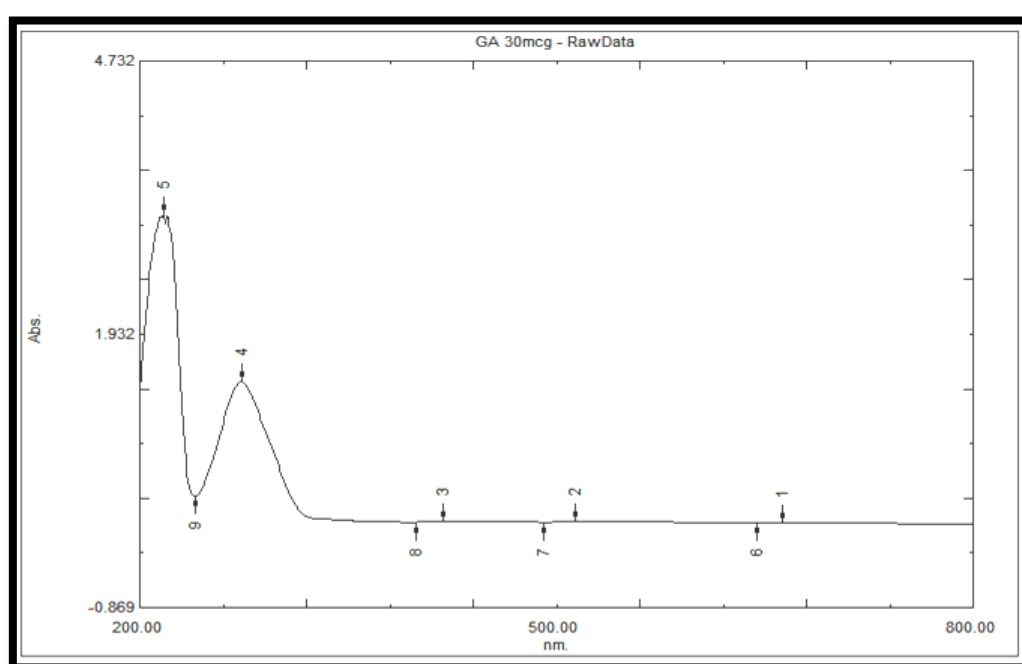


Figure 8. Isosbestic point of gallic acid and rosmarinic acid

QbD-Assisted RP-HPLC Method Development

The ATP outlines key characteristics that reflect the method's performance and its implications, serving as a critical component in method development. The choice of an ATP is inherently aligned with the intended purpose of the method. Within the framework of Analytical QbD, defining the ATP based on quality attributes is the initial and critical step. In this study, the ATP for the proposed analytical method was designed to ensure effective separation and quantification of

gallic acid and rosmarinic acid, with minimal tailing factor and peak width, while maintaining a reasonable analysis time. Based on this ATP, the CQAs were established, including a tailing factor not exceeding 2 and a theoretical plate count of at least 3000. The rationale for selecting these CQAs stems from their direct influence on the accuracy of quantitation for each marker compound. Elevated tailing factors can compromise quantitation accuracy by affecting peak integration. Thus, maintaining a low tailing factor and ensuring adequate theoretical plates are pivotal for achieving precise and reproducible quantification of the selected marker compounds. These criteria collectively support the method's robustness and reliability.

Method Optimization by DoE

A 2^2 full factorial design was utilized to examine how two independent factors—mobile phase ratio (X_1) and flow rate (X_2)—influence the performance of the analytical method. The independent variables were assessed at two levels, designated as low (−1) and high (+1). The response variables, namely the tailing factor and theoretical plates, were chosen as the key outputs for the experimental trials.

The results from the four chromatographic trials conducted within the factorial design are presented in **Table 8**, which outlines the observed values for both the tailing factor and theoretical plates. Following this, statistical optimization of the analytical method was carried out using Design-Expert Software (Version 13.0), where various statistical parameters were assessed to refine the method. The statistical outcomes of the design are provided in **Table 9**.

TABLE 8. Experimental design for screening of independent variables and responses

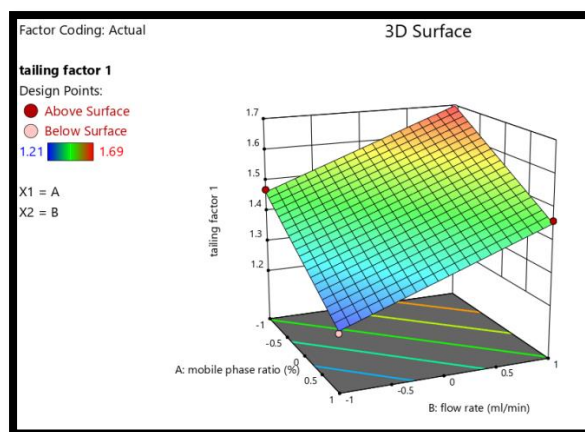
Responses								
	Coded levels		Actual levels		Tailing factor GA	Theoretical plates GA	Tailing factor RA	Theoretical plates RA
Code	X ₁	X ₂	X ₁	X ₂	Y ₁	Y ₂	Y ₃	Y ₄
Trail 1	1	1	55:45	1.1	1.46	6263	1.48	6161
Trail 2	1	-1	55:45	1.0	1.21	6624	1.29	6641
Trail 3	-1	1	50:50	1.0	1.69	4798	1.62	4329
Trail 4	-1	1	50:50	1.1	1.47	5012	1.45	4976

Polynomial equations were formulated to explain the relationship between the independent variables (X₁: mobile phase ratio, X₂: flow rate) and the response variables (Y₁: tailing factor of gallic acid, Y₂: theoretical plates of gallic acid, Y₃: tailing factor of rosmarinic acid, Y₄: theoretical plates of rosmarinic acid). These equations offered a mathematical framework for understanding the impact of the independent variables on chromatographic performance, thereby aiding in the optimization of the method.

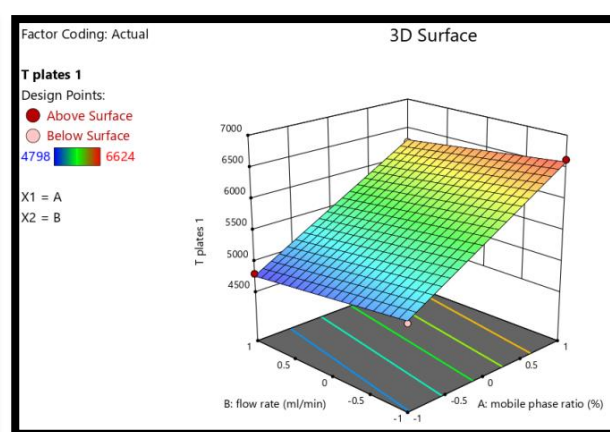
TABLE 9. Summary of statistical parameters and polynomial equations.

Response		P-value	Model significance	Polynomial equation
G A	Y ₁	0.0441	Significance	+1.45-0.122*X ₁ +0.1175*X ₂
	Y ₂	0.0469	Significance	+5674.2+769.2*X ₁ -143.7*X ₂
R A	Y ₃	0.0426	Significance	+1.46-0.075*X ₁ +0.0900*X ₂
	Y ₄	0.0454	Significance	+5526.7+874.25*X ₁ -281.75*X ₂

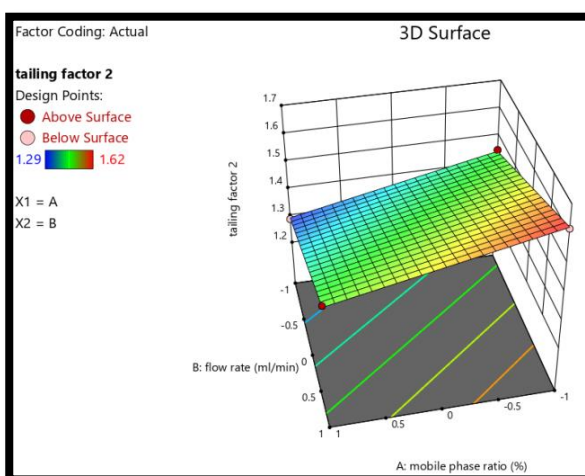
The sign of the coefficient in the polynomial equation reveals the type of influence the independent variable has on the response: a positive coefficient indicates a synergistic effect, whereas a negative coefficient signifies an antagonistic effect. The magnitude of the coefficient reflects the strength of the variable's effect, with larger coefficients signifying a stronger influence on the response. To provide a visual representation of how the independent variables affect the responses, response surface plots were created, as depicted in **Figure 9**. These plots visually illustrate the relationship between the dependent variables (CQAs) and the independent variables. The response surface plots show that an increase in both the mobile phase ratio and flow rate leads to a rise in the tailing factor. In contrast, an increase in the mobile phase ratio and flow rate causes a decrease in the theoretical plates. These trends emphasize the inverse relationship between the independent variables and their corresponding CQAs, providing crucial insights for optimizing the method.



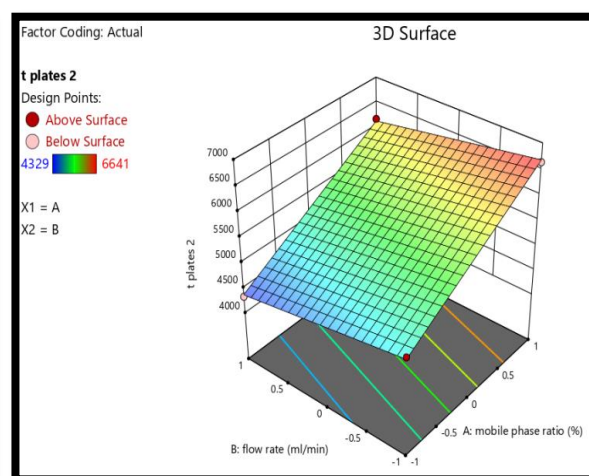
(A)



(B)



(C)



(D)

Figure 9. 3D response surface plots for dependent variables, (a) tailing factor GA, (b) theoretical plates GA, (c) Tailing factor RA, and (d) theoretical plates RA.

Establishment of MODR

Based on the MODR, Analytical Trial 2, utilizing a methanol-to-water ratio of 55:45, was identified as falling within the successful operating range and meeting the criteria outlined in the ATP and CQAs for the HPLC method (**Figure 10**). The selection of the optimal run was guided by chromatographic conditions that minimized the tailing factor and peak width. Based on the evaluation, Trial 2, which employed a methanol-to-water ratio of 55:45, was chosen as the optimized HPLC

method (Table 10). The corresponding HPLC chromatograms for the standard samples of gallic acid and rosmarinic acid, shown in **Figure 11**, illustrate the effectiveness of the selected conditions in achieving the desired analytical performance.

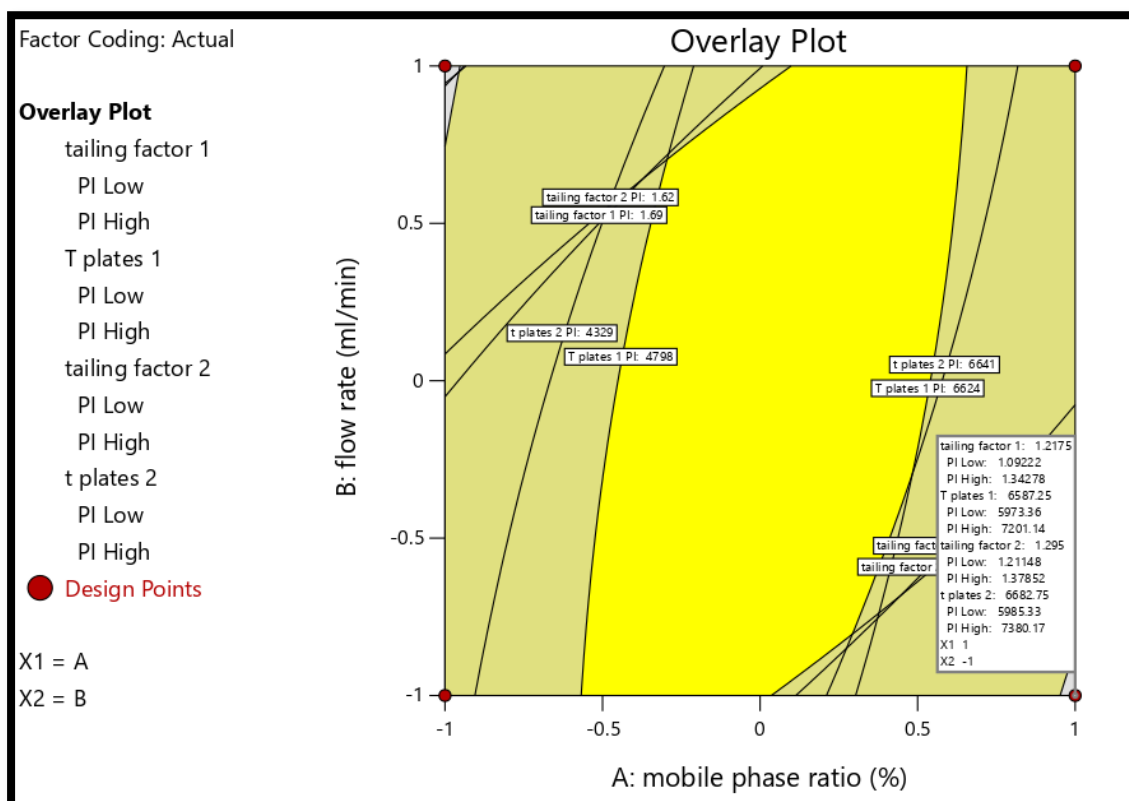


Figure 10. Design space overlay plot

TABLE 10. Optimized chromatographic conditions

Parameters	Chromatographic conditions
Stationary phase	Phenomenex C18 (250mm 4.6mm, 5m) column
Mobile phase	Methanol: Water
Mobile phase ratio	55:45
Flow rate	1 mL/min
Detection wavelength	298nm
Injection volume	10 μ L
Retention time of Gallic acid	2.6 min
Retention time of Rosmarinic acid	4.3 min

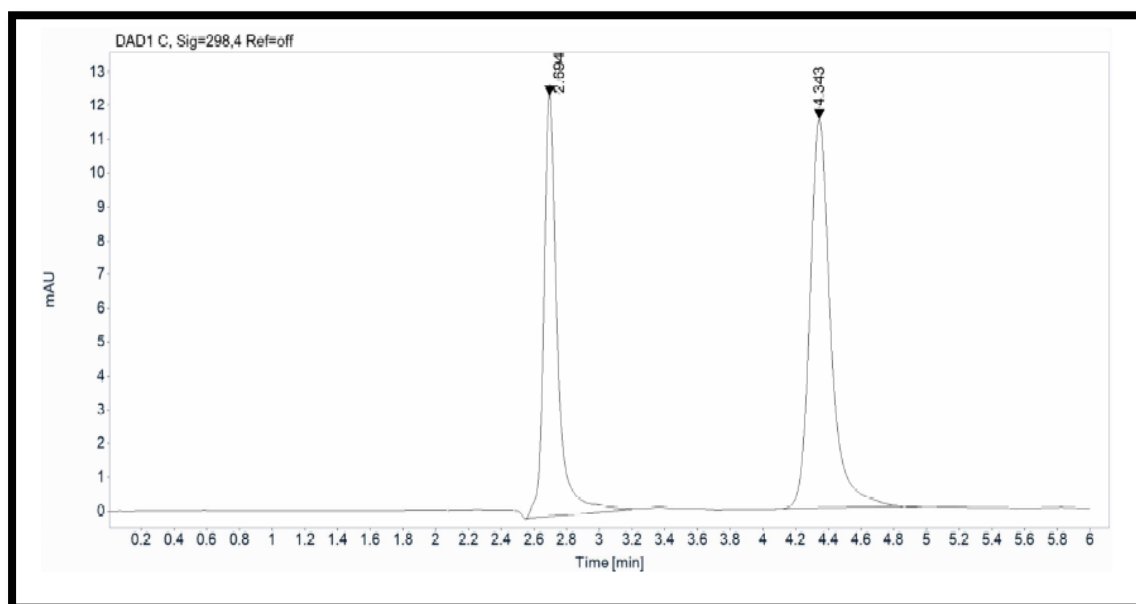


Figure 11. The HPLC chromatogram of simultaneous estimation of gallic acid and rosmarinic acid.

Analytical Method Validation

The validation data for the developed RP-HPLC method are presented in **Table 11**. System suitability was assessed by determining the % RSD of key parameters such as peak area, retention time, theoretical plates, and tailing factor. All evaluated parameters exhibited a % RSD of less than 2%, indicating that the method meets the required acceptance criteria. A linear calibration curve was established for gallic acid and rosmarinic acid over the tested concentration ranges, demonstrating excellent linearity. The LOD and LOQ were calculated using the linear regression equations obtained from the calibration curves. Precision was evaluated by conducting intra- and inter-day variability tests, with % RSD values for both gallic acid and rosmarinic acid staying below the acceptable limit, demonstrating the method's reliability and consistency.

Accuracy was evaluated via recovery studies by spiking samples with known concentrations of analytes at three levels (50%, 100%, and 150%). The high recovery rates obtained demonstrate the method's accuracy. Robustness testing involved deliberate minor variations in key chromatographic parameters, including wavelength, flow rate, and mobile phase composition. The consistency of the results under these conditions confirmed the robustness of the developed RP-HPLC method.

TABLE 11. Summary of validation parameters for Gallic acid and rosmarinic acid

Validation parameters	Gallic acid	Rosmarinic acid
System suitability		
Retention time Mean $\pm$ SD (%RSD)	2.68 $\pm$ 0.0168 (0.89)	4.27 $\pm$ 0.127 (1.02)
Peak area	238 $\pm$ 0.042 (0.72)	198 $\pm$ 0.45 (0.98)
Theoretical plates Mean $\pm$ SD (%RSD)	6624 $\pm$ 0.0324 (0.68)	6641 $\pm$ 0.0781 (1.12)
Tailing factor Mean $\pm$ SD (%RSD)	1.510 $\pm$ 0.0254 (0.85)	1.358 $\pm$ 0.0874 (0.65)
Linearity		
Linearity range μ g/mL	5-25	5-25
Slope	145.6	1358.4
Intercept	2142	3254
Correlation coefficient	0.9983	0.9984
LOD (μ g/mL)	0.147	0.146
LOQ (μ g/mL)	0.45	0.44
Precision		
Intra-day (% RSD)	1.24	1.54
Inter-day (% RSD)	1.08	0.35
Accuracy		
50% recovery	95.70182	96.44044
100% recovery	99.02062	98.86455
150% recovery	98.8373	99.3223
Robustness		
Change in mobile phase ration (% RSD)	0.475	1.358
Change in flow rate (% RSD)	0.687	1.240
Change in wavelength (%RSD)	1.54	1.578

Quantitative Analysis of Gallic Acid and Rosmarinic Acid

The optimized analytical method was effectively used for the quantitative analysis of gallic acid in crude extracts of *Terminalia chebula* and rosmarinic acid in *Ocimum sanctum* extracts. The quantities of these phytochemicals in the respective plant materials are presented in **Table 12**. Representative chromatograms illustrating the detection of the standard markers within the crude extracts are shown in **Figures 12 and 13**.

TABLE 12. Quantitative estimation of Gallic acid and Rosmarinic acid

Marker compound	Raw material taken ($\mu\text{g/mL}$)	Amount of marker obtained ($\mu\text{g/mL}$)	% Accuracy
Gallic acid	10	2.51	99.02 $\pm$ 0.06
Rosmarinic acid	10	3.61	98.65 $\pm$ 0.02

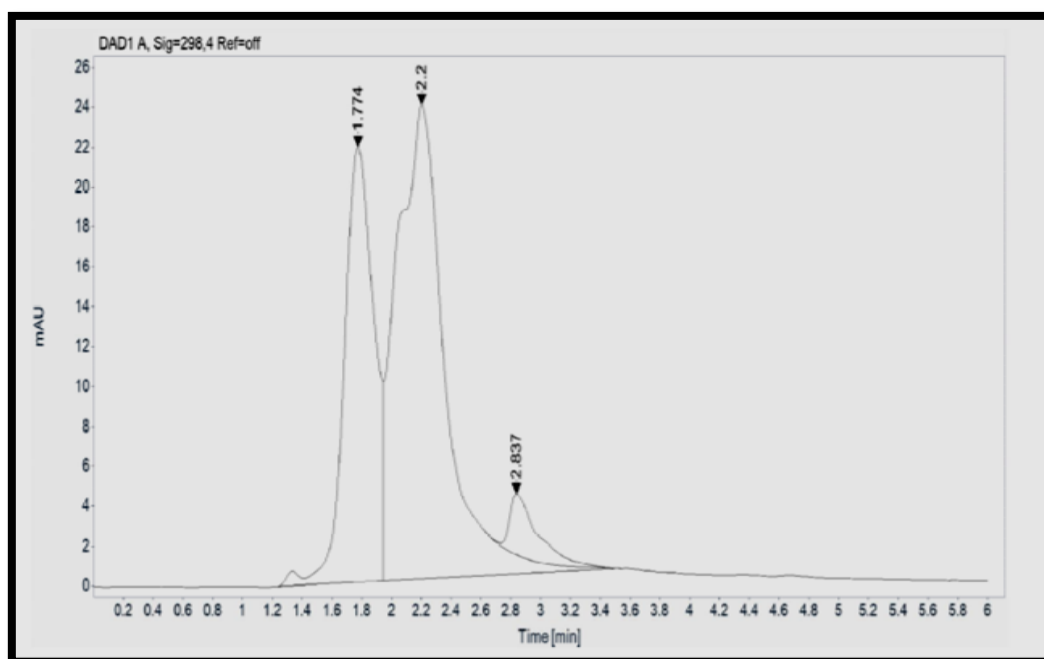


Figure 12. HPLC Chromatogram of Gallic acid in *Terminalia chebula* extract

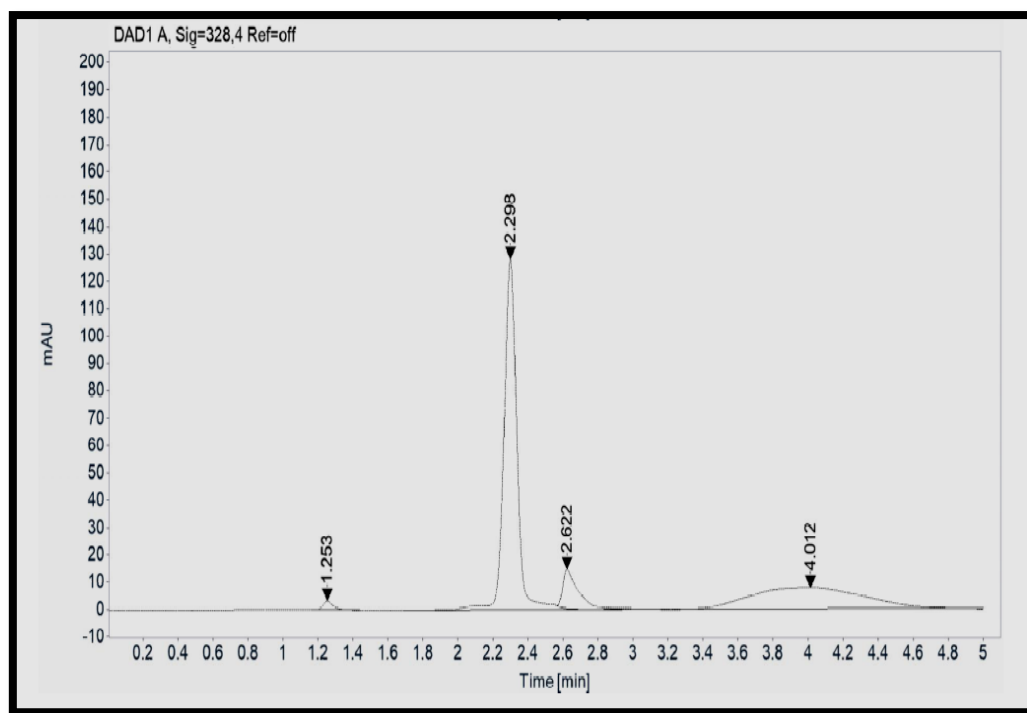


Figure 13. HPLC Chromatogram of Rosmarinic acid in *Ocimum sanctum* extract

HPLC Analysis of Stigmasterol

Development of RP-HPLC Method Assisted By Box-Behnken Design

The BBD was utilized to optimize the chromatographic parameters, selecting the organic phase volume, flow rate, and column temperature as independent variables. The response variables were defined as retention time, tailing factor, and theoretical plates. The experimental runs were designed and analyzed using Design-Expert software (version 13.0), which generated a total of 15 experimental runs, including three center points (**Table 13**).

TABLE 13. Experimental Runs, Variables, and Measured responses using response surface method (Box- Behnken) for the optimization study

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
	A:Organic Phase	B:Flow rate	C:Column Temp	Rt	TF	TP
	%	ml/min	C	min		
1	80	0.8	30	17.6	1.1	7253
2	70	0.8	35	22.5	1	6587
3	90	0.8	35	13.4	1.39	7265
4	90	1	40	12.8	1.43	7298
5	80	1	35	15.04	1.22	8764
6	70	1	40	20.9	1.02	6642
7	80	0.8	40	16.8	1.05	7569
8	80	1.2	30	14.21	1.3	7542
9	80	1	35	15.02	1.21	8762
10	70	1.2	35	19.1	1.09	6720
11	70	1	30	21.2	1.08	6652
12	90	1	30	13	1.41	7165
13	90	1.2	35	12.1	1.44	7192
14	80	1.2	40	14	1.31	7432
15	80	1	35	14.98	1.23	8738

The experimental data were subjected to ANOVA to assess the impact of individual factors and their interactions on the responses. The statistical parameters of the model are presented in Tables 14, 15, and 16. Polynomial equations were

developed to describe the relationship between the independent and response variables. Positive coefficients in the equations indicate synergistic effects, whereas negative coefficients signify antagonistic effects. Strong correlations in the generated models confirmed the reliability of the design, enabling the prediction of optimal conditions for the response variables (Equations 1-3).

TABLE 14. The analysis of variance (ANOVA) results for the response variable Y1 (Retention Time of Stigmasterol)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	156.66	9	17.41	274.65	< 0.0001	significant
A-Organic Phase	131.22	1	131.22	2070.42	< 0.0001	
B-Flow rate	14.82	1	14.82	233.90	< 0.0001	
C-Column Temp	0.2850	1	0.2850	4.50	0.0874	
AB	1.10	1	1.10	17.40	0.0087	
AC	0.0025	1	0.0025	0.0394	0.8504	
BC	0.0870	1	0.0870	1.37	0.2941	
A ²	8.78	1	8.78	138.54	< 0.0001	
B ²	0.1780	1	0.1780	2.81	0.1546	
C ²	0.6500	1	0.6500	10.26	0.0239	
Residual	0.3169	5	0.0634			
Lack of Fit	0.3150	3	0.1050	112.51	0.0088	not significant
Pure Error	0.0019	2	0.0009			
Cor Total	156.98	14				

**TABLE 15. The analysis of variance (ANOVA) results for the response variable
Y2 (tailing factor of Stigmasterol)**

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.3274	9	0.0364	13.99	0.0048	significant
A-Organic Phase	0.2738	1	0.2738	105.31	0.0002	
B-Flow rate	0.0450	1	0.0450	17.31	0.0088	
C-Column Temp	0.0008	1	0.0008	0.3077	0.6030	
AB	0.0004	1	0.0004	0.1538	0.7110	
AC	0.0016	1	0.0016	0.6154	0.4683	
BC	0.0009	1	0.0009	0.3462	0.5819	
A ²	0.0028	1	0.0028	1.07	0.3475	
B ²	0.0011	1	0.0011	0.4349	0.5387	
C ²	0.0006	1	0.0006	0.2219	0.6574	
Residual	0.0130	5	0.0026			
Lack of Fit	0.0128	3	0.0043	42.67	0.0230	not significant
Pure Error	0.0002	2	0.0001			
Cor Total	0.3404	14				

**TABLE 16. The analysis of variance (ANOVA) results for the response variable
Y3 (theoretical plates of Stigmasterol)**

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7.937E+06	9	8.819E+05	1850.40	< 0.0001	significant
A-Organic Phase	6.722E+05	1	6.722E+05	1410.50	< 0.0001	
B-Flow rate	5618.00	1	5618.00	11.79	0.0186	
C-Column Temp	13530.13	1	13530.13	28.39	0.0031	
AB	10609.00	1	10609.00	22.26	0.0053	
AC	5112.25	1	5112.25	10.73	0.0221	
BC	45369.00	1	45369.00	95.20	0.0002	
A ²	4.983E+06	1	4.983E+06	10455.70	< 0.0001	
B ²	1.569E+06	1	1.569E+06	3293.05	< 0.0001	
C ²	1.578E+06	1	1.578E+06	3310.76	< 0.0001	
Residual	2382.92	5	476.58			
Lack of Fit	1964.25	3	654.75	3.13	0.2516	not significant
Pure Error	418.67	2	209.33			
Cor Total	7.939E+06	14				

1. $RT = +15.01 - 4.05 * A - 1.36 * B - 0.1888 * C + 0.5250 * AB + 0.0250 * AC + 0.1475 * BC + 1.54 * A^2 + 0.2196 * B^2 + 0.4196 * C^2$
2. $TF = +1.22 + 0.1850 * A + 0.0750 * B - 0.0100 * C - 0.0100 * AB + 0.0200 * AC + 0.0150 * BC + 0.0275 * A^2 - 0.0175 * B^2 - 0.0125C^2$
3. $TP = +8754.67 + 289.87 * A + 26.50 * B + 41.13 * C - 51.50 * AB + 35.75 * AC - 106.50 * BC - 1161.71 * A^2 - 651.96 * B^2 - 653.71 * C^2$

The response surface analysis for Y_1 (retention time) reveals an inverse relationship with the organic phase concentration, as retention time decreases with increasing organic content. Conversely, retention time exhibits a positive correlation with the flow rate, increasing as flow rate rises. The influence of column temperature is negligible, with a marginal reduction in retention time observed at higher temperatures (**Figure 14a**). For Y_2 (tailing factor), a direct relationship was noted; higher concentrations of the organic phase and elevated flow rates resulted in increased tailing factors, indicating a pronounced correlation (**Figure 14b**). Column temperature showed minimal impact on the tailing factor. For Y_3 (theoretical plates), both the organic phase concentration and flow rate significantly increased the number of theoretical plates, with optimal values at 80% v/v and 1 mL/min, respectively, suggesting enhanced separation efficiency. Beyond these thresholds, a decline in theoretical plate count was observed (**Figure 14c**). The column temperature exhibited minimal variation across Y_3 , maintaining relative consistency.

The optimal chromatographic conditions were determined using Design-Expert software version 13.0 (Stat-Ease, Inc.), with the ideal settings being 80% v/v organic phase, a flow rate of 1 mL/min, and a column temperature of 35 °C. These optimized parameters yielded predictions that closely aligned with experimental outcomes, particularly in run 9, which exhibited a low prediction error of 5%. Under the optimized conditions, excellent chromatographic separation of the drug was achieved (**Figure 15**).

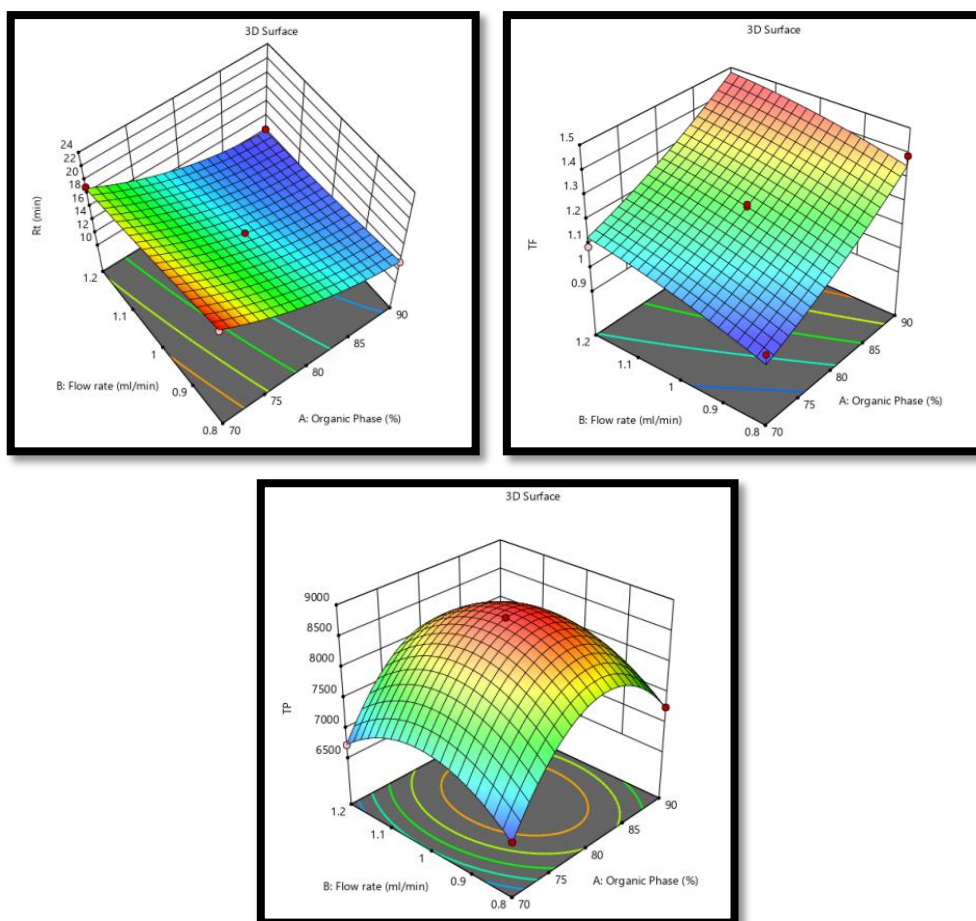


FIGURE 14 (a). Response surface plot of response Y1 (Retention time), (b) Y2 (Tailing factor) and (c) Y3 (Theoretical plates)

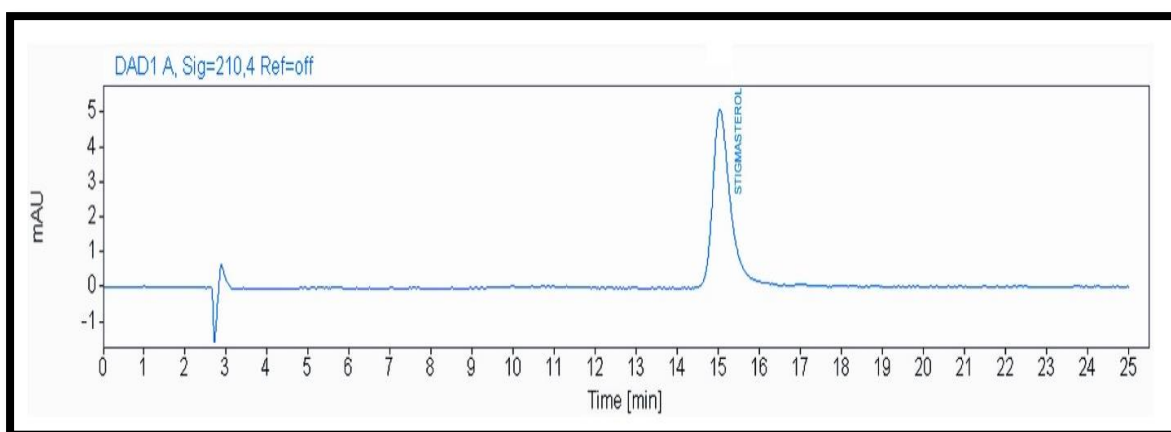


Figure 15. HPLC chromatogram of stigmasterol

Analytical Method Validation

A linear correlation was established via a calibration curve, where the mean peak area of stigmasterol was plotted against its corresponding concentrations in the range of 5–30 µg/mL. The linear regression equation derived from this curve was instrumental in determining the LOD and LOD. To validate the method's suitability, the %RSD was assessed for key parameters such as peak area, retention time, theoretical plates, and tailing factor. All %RSD values were below 2%, confirming the method's precision and compliance with acceptance criteria.

The robustness of the analytical procedure was evaluated by making slight modifications to HPLC parameters, including detection wavelength, column oven temperature, and mobile phase composition. The %RSD values for both peak area and retention time of the analyte remained below the acceptable limit of $\leq 2\%$, indicating the method's robustness. Precision studies, encompassing both intra-day and inter-day variations, demonstrated the method's high reproducibility. Accuracy, assessed through percent recovery at concentrations of 50%, 100%, and 150%, showed excellent precision. Together, these findings confirm the reliability and robustness of the developed HPLC method, as detailed in **Table 17**.

TABLE 17. Summary of validation parameters

Sr. no	Validation parameters	Stigmasterol
1	Linearity	
	Linearity range ($\mu\text{g/mL}$)	5-30
	Slope	6.2857x
	Intercept	2.4333
	Correlation-coefficient	0.9994
2	LOD ($\mu\text{g/mL}$)	1.52
	LOQ($\mu\text{g/mL}$)	4.592
4	System suitability	
	Peak area (%RSD)	1.2
	Retention time (%RSD)	0.32
	Theoretical plates (%RSD)	0.69
	Tailing factor (%RSD)	1.31
3	Precision	
	Intra-day (%RSD)	0.85
	Inter-day (%RSD)	0.98
4	Robustness	
	Mobile phase ratio (%RSD)	0.85
	Change in the flow rate(%RSD)	1.74
	Column oven temp (%RSD)	1.34
5	Accuracy	
	50% recovery	96.72
	100% recovery	97.68
	150% recovery	99.01

Quantitative Analysis of Stigmasterol

The optimized analytical technique was successfully utilized to quantitatively measure stigmasterol in both the crude extract of *Moringa oleifera* and its tablet formulation. The method demonstrated favorable recovery rates for stigmasterol in both the plant material and the formulated dosage form, as summarized in **Table 18**. Representative chromatograms depicting the detection of stigmasterol in the crude extract and tablet dosage form are shown in **Figures 16 and 17**, respectively.

TABLE 18. Recovery analysis of stigmasterol

Sample	Excess samples added to analytes (%)	Spiked (µg/ml)	Found (µg/ml)	Recovery (%)
Extract	50	15	14.79±0.21	98.6
	100	20	19.22±0.43	96.1
	150	25	24.34±0.03	97.3
Tablets	50	15	13.82±0.12	87.2
	100	20	16.2±0.05	81.1
	150	25	20.5±0.23	82.2

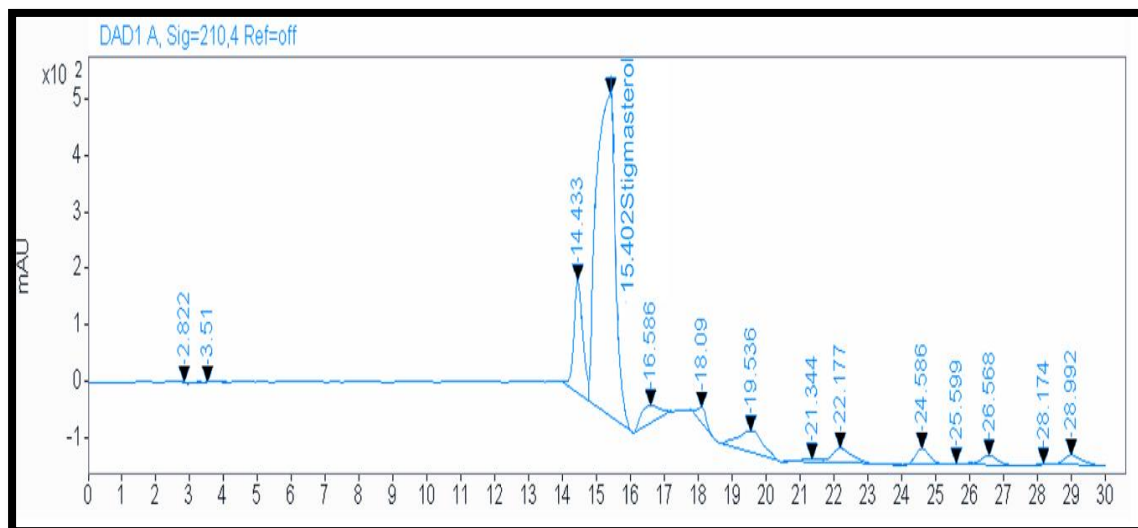


Figure 16. HPLC Chromatogram of stigmasterol in *Moringa oleifera* extract

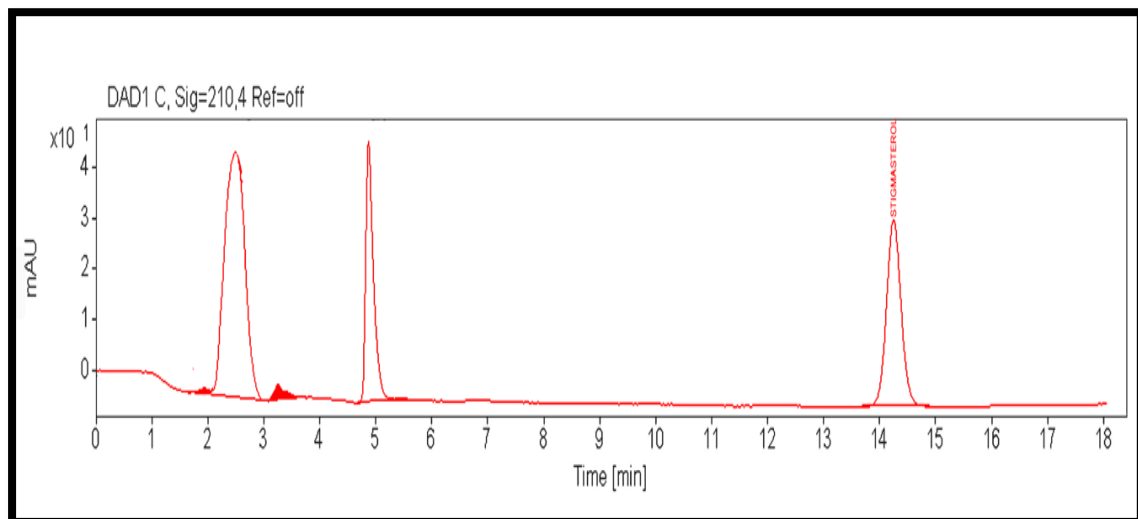


Figure 17. HPLC Chromatogram of stigmasterol in Tablet dosage form

Extraction

The extraction of *Terminalia chebula* dry fruits, *Ocimum sanctum* leaves, and *Moringa oleifera* leaves was performed following the prescribed procedure. The percentage yield of each extract was determined by weighing the air-dried plant material. A summary of the extraction yields for the chosen crude drugs is presented in **Table 19**.

TABLE 19. % Yield of plant extracts

Crude drug	% Yield
<i>Terminalia chebula</i>	32.01%
<i>Ocimum sanctum</i>	26.87%
<i>Moringa oleifera</i>	19.05%

Quality Assessment of Herbal Extract

The quality assessment of the herbal drug substances, specifically the plant extracts, was conducted by evaluating their moisture content and ash values. The results, summarized in **Table 20**, were found to be within the standard limits. Furthermore, phytochemical screening was conducted on each plant extract to detect the presence of secondary metabolites, and the results are shown in **Table 21**.

TABLE 20. Physico-chemical properties of herbal extracts

Parameters	<i>Terminalia chebula</i>	<i>Ocimum sanctum</i>	<i>Moringa oleifera</i>
Moisture content (%)	2.11± 0.20	1.52 ± 0.08	3.63 ± 0.25
Ash value (%)	2.81± 0.076	0.91 ± 0.062	4.05 ± 0.12

TABLE 21. Phytochemical evaluation of herbal extracts

Phytochemicals	<i>Terminalia chebula</i>	<i>Ocimum sanctum</i>	<i>Moringa oleifera</i>
Alkaloids	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Phenols	+	+	+
Saponins	+	+	+
Triterpenes	+	+	+
Glycosides	+	+	-

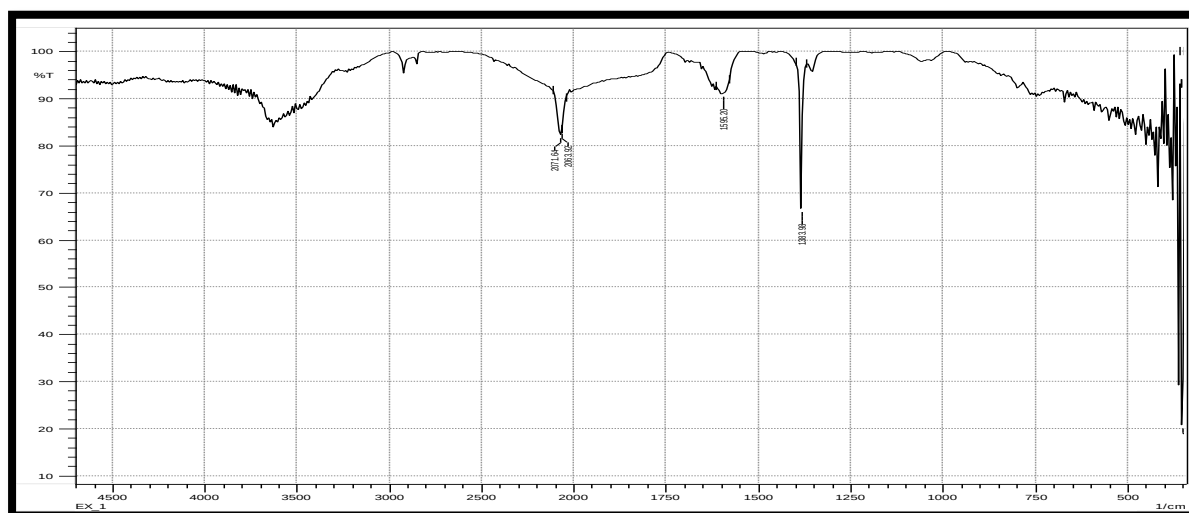
Formulation Development

Compatibility Study

FTIR spectroscopy was used to assess the compatibility of the plant extracts with the excipients incorporated in the polyherbal tablet formulation. The FTIR spectra of the individual plant extracts and the mixture of extracts with excipients were recorded and analyzed (**Figures 18–21**). A comparative analysis of the spectra showed that the characteristic peaks of the functional groups in the plant extracts were maintained, with no noticeable shifts or changes in intensity. These results suggest that the functional groups of the plant extracts stayed within their expected spectral ranges, implying that no chemical interactions occurred between the extracts and the excipients. Therefore, it can be concluded that the excipients selected for the polyherbal tablet formulation are compatible with the plant extracts (**Table 22**), ensuring the stability and integrity of the final product.

TABLE 22. FT-IR spectral Analysis of medicinal plants and physical mixture

Functional group	Extracts			Physical mixture
	<i>Moringa oleifera</i>	<i>Ocimum sanctum</i>	<i>Terminalia chebula</i>	
O-H stretch	-----	3533.74 to 3330.22 cm ⁻¹	3440.20 to 3350.22 cm ⁻¹	3541.46 to 3390.04 cm ⁻¹
CH	1383.98cm ⁻¹	-----	-----	-----
C=O stretch		1616.42 cm ⁻¹	1653.07 cm ⁻¹	1617.38 cm ⁻¹
C=C stretch	1595.20 cm ⁻¹	-----	-----	-----
C-N	-----	2070.67 cm ⁻¹	2069.71 cm ⁻¹	2069.71 cm ⁻¹
NH	-----	3452.73 cm ⁻¹	3452.73 cm ⁻¹	3402.58 cm ⁻¹

Figure 18. FT-IR Spectra of *Moringa Oleifera* extract

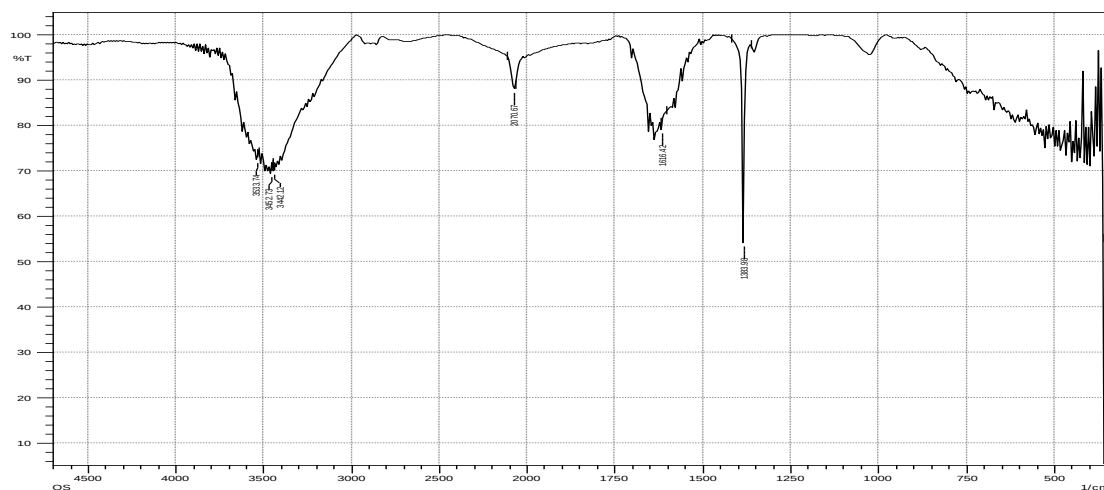


Figure 19. FT-IR Spectra of *Ocimum sanctum* extract

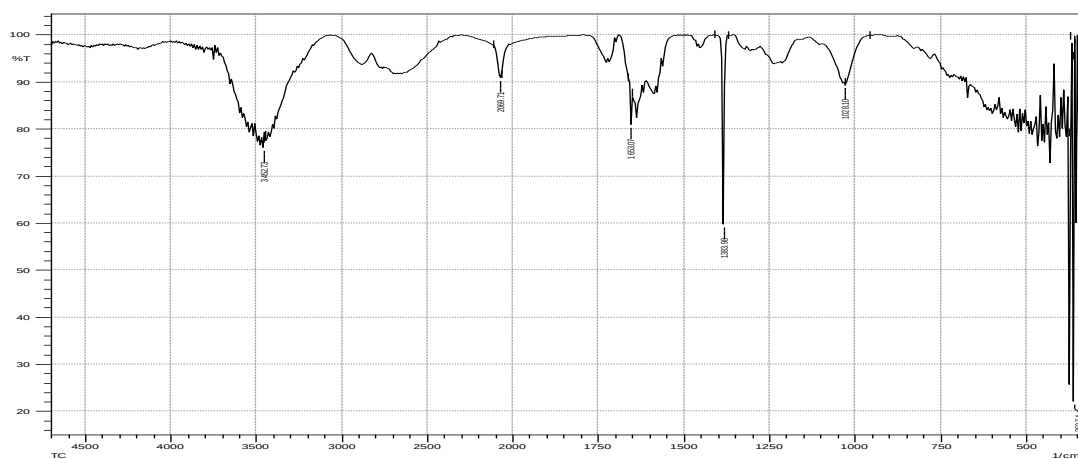


Figure 20. FT-IR Spectra of *Terminalia chebula* extract

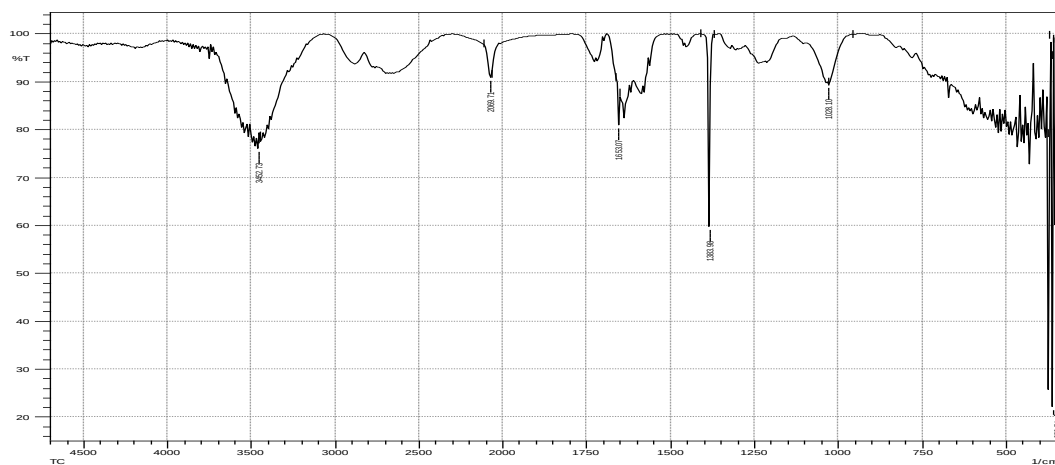


Figure 21. FT-IR Spectra of Physical mixture (Extracts with excipients)

QbD Approach for Formulation Development

Quality Target Product Profile

The QTPP for the anti-inflammatory herbal tablets was carefully defined, considering the essential quality attributes necessary to guarantee the desired properties of the final product. This systematic approach ensures that the formulation meets predefined quality specifications, aligning with the intended therapeutic purpose and patient requirements. **Table 23** provides a comprehensive summary of the QTPP for the development of the anti-inflammatory herbal drug product.

TABLE 23. Summary of QTPP for Anti-inflammatory herbal product

QTPP Elements		Target	Justification
Dosage form		Tablet	Ensures patient acceptability
Dosage design		Conventional release tablet	Promotes patient acceptability and compliance.
Route of Administration		Oral	Designed for oral administration for ease of use and convenience.
Dosage strength		650mg	Provides the therapeutic dose necessary for efficacy.
Drug product Quality Attributes	Appearance	Tablet confirming to description shape and size	Ensures patient acceptability and compliance.
	Hardness	3-6 kg	Maintains tablet quality as per product specifications.
	Friability	NMT 1.0%	Ensures physical integrity during handling and transport.
	Disintegration	NMT 30 min	Ensures appropriate release of the active components for therapeutic action.
Container closure system		Container closure system qualified as suitable for this drug product	Ensures the target shelf life and protects tablet integrity during storage.
Storage condition		Store in air-tight container in a cool place.	Maintains product integrity and quality during storage.

Identification of Critical Quality Attributes (CQAs)

From the established QTPP, the CQAs for the anti-inflammatory herbal formulations were identified and defined (**Table 24**). These CQAs represent the quality attributes that, when altered, are most likely to impact the overall quality, safety, or efficacy of the final product. Attributes deemed critical from a formulation development perspective were prioritized, ensuring the robust design and consistent performance of the herbal formulation.

TABLE 24. CQA's for Anti-inflammatory herbal tablet formulation

Quality Attributes of Drug product	Target	Is this a CQA?	Justification
Disintegration time	NMT 30 min	Yes	Ensures compliance with Pharmacopoeial specifications to facilitate proper drug release and action.
Friability	NMT 1.0%	Yes	Ensures compliance with Pharmacopoeial specifications to maintain physical integrity during handling.
Hardness	3-6 kg/cm ²	Yes	Prevents excessively hard tablets that may hinder disintegration and dissolution, while avoiding weak tablets prone to breakage or increased friability.

Quality Risk Management (QRM) Approach

An initial risk assessment was conducted to ensure that the QTPP for the anti-inflammatory herbal tablet formulation was met. This assessment evaluated the potential impact of the drug substances (herbal extracts) and formulation variables (excipients) on the CQAs. The findings from the initial risk assessment, along with their justifications, are summarized in **Table 25**. This systematic evaluation helps identify and prioritize critical formulation factors, guiding the development process toward achieving the desired product quality.

TABLE 25. Initial risk assessment and justification for the formulation variables.

Formulation variables	Drug product CQA	Justification	Risk level
MCC Level	Disintegration	MCC acts as a diluent. Variations in its level can significantly impact disintegration time by affecting tablet hardness.	High
	Friability	MCC's role as a diluent means changes in its level can greatly influence tablet friability.	High
	Hardness	Changes in MCC level directly influence tablet hardness, affecting mechanical strength and disintegration.	High
CCS Level	Disintegration	CCS is a disintegrant. Variations in its level can have a pronounced effect on disintegration time.	High
	Friability	Higher levels of CCS may increase friability but can be managed by adjusting tablet hardness during compression.	Medium
	Hardness	CCS level can affect hardness indirectly by altering compression dynamics.	Medium

Lactose Level	Disintegration	Lactose is filler and generally does not affect disintegration time.	Low
	friability	Lactose typically has no significant impact on friability.	Low
	Hardness	Lactose has minimal impact on tablet hardness due to its role as a neutral filler.	Low
Talc Level	Disintegration	Talc has minimal effect on disintegration. The low levels used are unlikely to affect this parameter.	Low
	Friability	Talc has no appreciable effect on friability.	Low
	Hardness	Talc does not contribute to or affect tablet hardness significantly.	Low
Magnesium stearate Level	Disintegration	The small quantities of magnesium stearate used are not expected to impact disintegration time.	Low
	Friability	Magnesium stearate has negligible influence on tablet friability.	Low
	Hardness	Excessive magnesium stearate may reduce hardness by creating a lubricating barrier, but at low levels, its impact is minimal.	Low

Design of Experiment

The DoE represents a powerful and systematic approach for exploring and quantifying the relationships between dependent and independent variables, particularly for identifying significant interaction effects. In the current study, a 3^2 CCD was employed as the experimental framework for the formulation and optimization of polyherbal tablets. A total of thirteen formulation batches were prepared, encompassing five replicates at the central point to ensure model reliability and reproducibility. The formulated batches were systematically evaluated for critical quality attributes, including disintegration time (Y_1), friability (Y_2), and hardness (Y_3). The influence of formulation variables on these responses was analyzed using Multiple Linear Regression Analysis (MLRA). Polynomial regression models, incorporating linear, interaction, and quadratic terms, were generated to describe each response. Confidence intervals (95%) were included to validate the precision of the predicted models.

RSM was used to explore the interaction effects and optimize the formulations. 3D response surface plots and 2D contour plots were generated, offering detailed insights into the interactions between the independent variables and their collective impact on the responses. Model adequacy and predictability were confirmed through ANOVA, which evaluated the statistical significance of regression coefficients, goodness-of-fit (R^2), and associated p-values. This approach ensured robust model validation, enabling the optimization of the polyherbal tablet formulations with confidence.

Effect on Disintegration Time

The disintegration time for the thirteen formulated batches (F1–F13) was observed to vary between 19.04 and 28.03 minutes, as presented in **Table 3**. The formulation variables, specifically MCC and CCS, demonstrated a pronounced influence on disintegration time, as depicted in **Figure 22a**. An initial increase in the levels of MCC and CCS resulted in a reduction in disintegration time, indicating enhanced tablet breakdown. However, further elevation beyond optimal concentrations led to an unexpected increase in disintegration time, likely due to structural compaction effects that hindered tablet disintegration.

To elucidate the interaction effects between MCC, CCS, and disintegration time, polynomial regression equations were developed. These equations capture the linear, quadratic, and interaction terms, providing a comprehensive model of the observed effects. **Table 26** details the polynomial equations alongside the corresponding 95% confidence intervals for each interaction term, offering insights into the statistical significance and reliability of the interactions. These findings underscore the critical role of MCC and CCS concentrations in optimizing tablet disintegration properties.

Effect on Friability

The percentage friability of the thirteen tablet formulations (F1–F13) ranged from 0.38% to 0.64%, as detailed in **Table 26**. Friability, a critical quality attribute reflecting the mechanical strength of the tablets, was significantly influenced by the concentrations of MCC and CCS, as illustrated in **Figure 22b**. Raising the levels of both MCC and CCS led to significant changes in friability. The three-dimensional response surface analysis indicated that the influence of MCC on friability was more

substantial compared to that of CCS. This could be attributed to MCC's role as a binder, which directly impacts the tablet's mechanical integrity. In contrast, the influence of CCS, a superdisintegrant, was comparatively less significant, likely due to its primary role in facilitating tablet disintegration rather than contributing to structural robustness.

The interactions between MCC and CCS concentrations were further explored using polynomial regression models, incorporating linear, interaction, and quadratic terms. These equations are summarized in **Table 26**, alongside their corresponding 95% confidence intervals, providing a detailed statistical representation of the observed effects. The results emphasize the dominant role of MCC in modulating friability and its interplay with CCS in determining the overall mechanical properties of the formulations.

Effect on Hardness

The hardness of the tablet formulations across all thirteen batches (F1–F13) ranged from 4.6 to 6.3 kg/cm², as reported in **Table 3**. Hardness, a key parameter reflecting the mechanical strength and compaction properties of the tablets, exhibited significant dependence on the concentrations of MCC and CCS. As depicted in **Figure 22c**, an increase in the levels of MCC and CCS initially led to a reduction in tablet hardness. This trend can be attributed to the disruption of the tablet matrix structure due to increased disintegrant levels (CCS) or reduced binder-to-lubricant balance (MCC). However, beyond specific concentration thresholds, further increases in both MCC and CCS concentrations contributed to enhanced hardness. This rebound effect suggests that higher MCC levels promote greater binding forces, while CCS, at elevated concentrations, may indirectly aid compaction through matrix reorganization.

Among the two factors, MCC demonstrated a more pronounced influence on hardness, underscoring its critical role in tablet compaction and mechanical strength. The interactions and combined effects of these variables were further analyzed using polynomial regression models. The equations, incorporating linear, quadratic, and interaction terms, are summarized in **Table 26** alongside their corresponding 95% confidence intervals, providing a robust statistical framework for understanding these relationships.

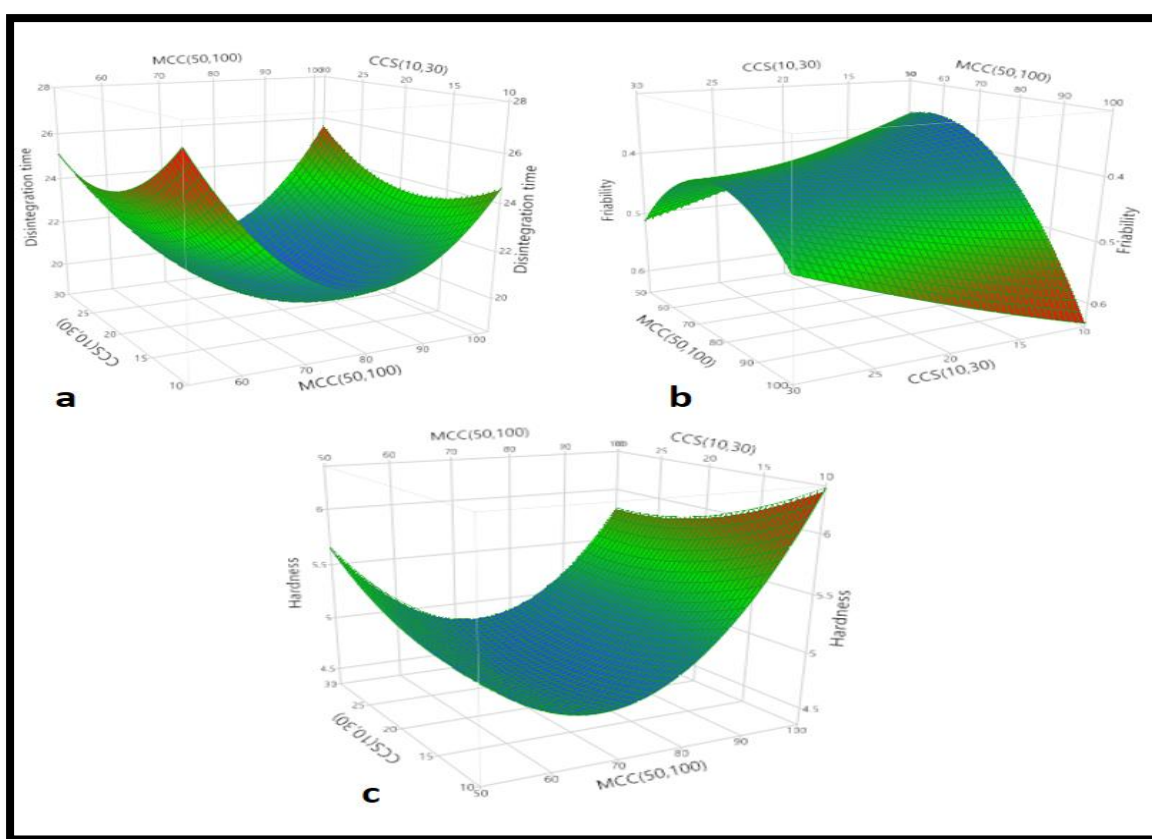


Figure 22. The 3D response surface plots illustrating the influence of Microcrystalline cellulose (X_1) and Crosscarmellose sodium (X_2) on (a) disintegration time (Y_1); (b) friability (Y_2); and (c) hardness (Y_3).

TABLE 26. Regression equation coefficient (polynomial equations) of selected variables with 95% CI.

Sr. No	Variables	Constant	A	B	AB	A ²	B ²
1	Disintegration time (min)	19.42	1.26	-0.30	0.75	4.65	1.59
	(95% CI)	(18.7, 20.1)	(1.97, 0.56)	(-1.01, 0.39)	(-0.10, 1.62)	(3.61, 5.69)	(0.55, 2.63)
2	Friability (%)	0.41	0.06	0.00	-0.07	0.09	-0.006
	(95% CI)	(0.39, 0.42)	(0.04, 0.07)	(-0.01, 0.01)	(-0.09, 0.05)	(0.07, 0.11)	(-0.02, 0.01)
3	Hardness (kg/cm ³)	4.7	0.33	-0.01	-0.25	0.9	0.15
	(95% CI)	(4.58, 4.81)	(0.21, 0.44)	(-0.13, 0.09)	(-0.38, -0.11)	(0.73, 1.06)	(-0.01, 0.31)

Prediction Profiler and Validation

An ANOVA was conducted to evaluate the adequacy and fitness of the proposed quadratic model, with a detailed summary of the ANOVA results and model parameters provided in **Table 27**. The coefficients of the polynomial equations derived through MLRA demonstrated an excellent fit to the experimental data. This was evidenced by a statistically significant F-value ($p < 0.05$), underscoring the reliability of the model in predicting the responses. The Prediction Profiler (PP), a powerful statistical tool, was employed to forecast the optimal formulation composition by aligning factor levels with desired response values. **Figure 23** presents the profiler output for disintegration time (Y_1), friability (Y_2), and hardness

(Y_3). The PP offers an interactive visualization of cross-sectional relationships among the factors and responses. The vertical dotted red line on the profiler graph indicates the current factor levels ($X_1 = 75$ mg, $X_2 = 20$ mg), with these values displayed below the horizontal axis. Corresponding predicted response values—disintegration time (Y_1) = 19.42 min, friability (Y_2) = 0.41%, and hardness (Y_3) = 4.7 kg/cm²—are shown in red on the vertical axes.

TABLE 27. Details of ANOVA and fit summary for dependent variables (responses).

Responses	DF	Sum of square	Mean square	F ratio	Adjusted R^2	Predicted R^2	P values Model Lack of fit	
Disintegration time	5	108.9	21.7	40.54	0.9427	0.9666	<0.0001	0.0006
Friability	5	0.071	0.014	53.05	0.955	0.974	<0.0001	0.0075
Hardness	5	3.940	0.788	57.06	0.958	0.976	<0.0001	0.0277

To further optimize the responses, a desirability function was constructed to identify the most suitable combination of factors across the design space. The predicted values for disintegration time, friability, and hardness closely matched the experimental values, demonstrating minimal percentage error and confirming the high predictive accuracy of the model. Based on these findings, formulation batch 11 was identified as the optimized formulation. Additionally, **Figure 24** provides a two-dimensional (2D) graphical representation of the relationships between the selected factors and the observed responses, offering further insight into the influence of MCC and CCS concentrations on tablet characteristics. These comprehensive analyses validate the robustness of the proposed model and its utility in optimizing polyherbal tablet formulations and all prepared tablet formulations were shown in **Figure 25**.

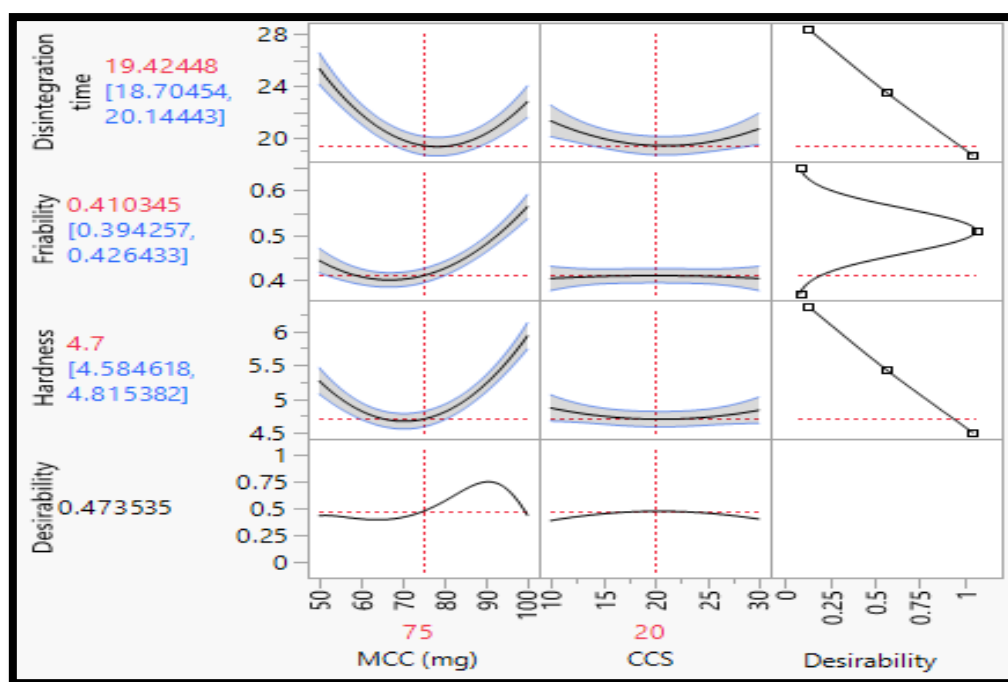


Figure 23. Prediction profiler illustrates the optimum values for disintegration time (Y_1), friability (Y_2), and hardness (Y_3).

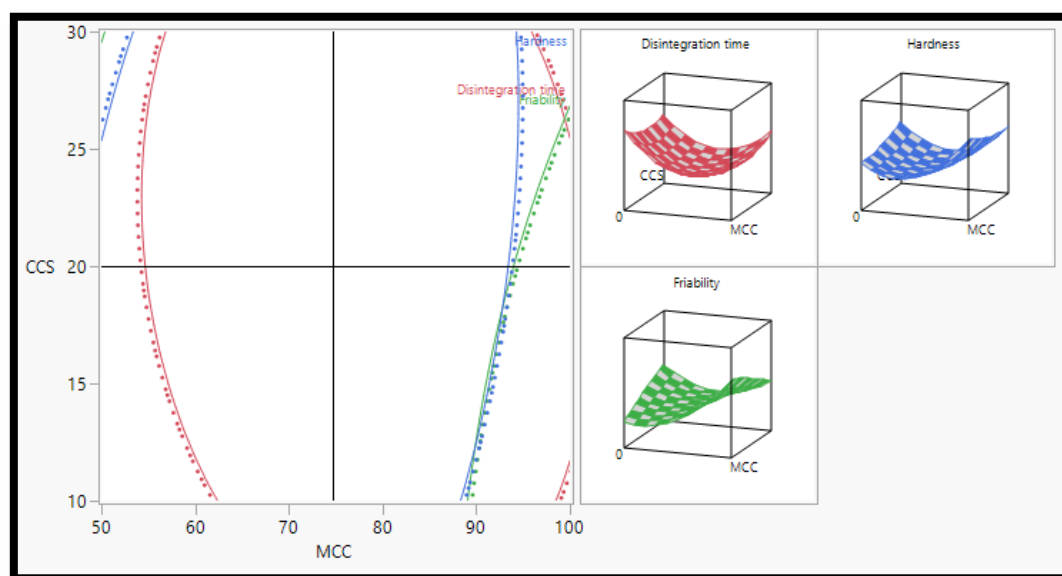


Figure 24. The 2D Contour profiler illustrating the influence of Microcrystalline cellulose (X_1) and Crosscarmellose sodium (X_2) on (a) disintegration time (Y_1); (b) friability (Y_2); and (c) hardness (Y_3).



Figure 25. Prepared tablet formulations

Pre and Post Compression Parameters

Prior to tablet compression, the powder blend was evaluated for pre-compression parameters to assess its suitability for direct compression. All measured parameters were within pharmaceutically acceptable limits, indicating excellent flow properties. The bulk density of the powder blend across the thirteen formulations (F1–F13) ranged from 0.412 ± 0.003 to 0.529 ± 0.124 g/cm³, while the tapped density varied between 0.462 ± 0.012 and 0.613 ± 0.004 g/cm³. The angle of repose, a critical indicator of flowability, was recorded between $32.4 \pm 0.002^\circ$ and $36.5 \pm 0.321^\circ$. Compressibility indices such as Carr's index and Hausner's ratio were also evaluated, with Carr's index ranging from $10.8 \pm 0.04\%$ to $16.2 \pm 0.66\%$, and Hausner's ratio between 1.12 ± 0.12 and 1.19 ± 0.23 . These results confirm the blend's suitability for tablet manufacturing via direct compression.

After compression, the polyherbal tablets were subjected to a comprehensive evaluation of post-compression parameters. The tablets exhibited a uniform brown color, characteristic odor, and bitter taste. The thickness of the compressed tablets ranged from 6.3 ± 0.003 mm to 6.8 ± 0.21 mm, ensuring consistency across the batches. The weight of the tablets was within pharmacopeial specifications, varying from 648 ± 0.014 mg to 651 ± 0.32 mg, indicating uniformity in tablet weight. All evaluated post-compression parameters, including appearance, thickness, and weight variation, met the acceptable pharmacopeial limits, confirming the quality of the compressed polyherbal tablets. These findings validate the reliability of the formulation process and ensure compliance with regulatory standards.

Drug Content Analysis

The optimized formulation batch F-11 was selected for quantitative drug content analysis, focusing on three bioactive compounds derived from distinct plant sources: gallic acid, stigmasterol, and rosmarinic acid. The polyherbal tablets were analyzed for the content of these compounds using validated spectrophotometric methods at their respective maximum absorbance wavelengths—271 nm for gallic acid, 328 nm for rosmarinic acid, and 210 nm for stigmasterol. The results of the analysis demonstrated that the tablets contained 63.2% of the labeled amount of gallic acid, 74.1% of stigmasterol, and 82.2% of rosmarinic acid. These findings indicate variations in the recovery rates of the individual components, which may be attributed to differences in extraction efficiency, compound stability, or matrix interactions within the polyherbal formulation.

***In-vitro* drug release analysis**

The *in-vitro* drug release profile of the optimized tablet formulation batch F-11 was evaluated, yielding promising results. The formulation exhibited a favorable release pattern, characterized by the sustained and controlled release of the key bioactive compounds—gallic acid, stigmasterol, and rosmarinic acid—over a range of time intervals (0 to 180 minutes). At the end of the 180-minute period, the cumulative release percentages of the bioactive compounds were determined to be 72.47% for gallic acid, 81.2% for stigmasterol, and 76.21% for rosmarinic acid, as depicted in **Figure 26**.

These results highlight the formulation's ability to achieve efficient drug release while maintaining the stability and bioavailability of the compounds. The observed release behavior suggests that the excipient composition and formulation process parameters effectively facilitated the dissolution and diffusion of the active components. This favorable release profile underscores the potential of the optimized polyherbal tablet formulation for therapeutic applications, warranting further *in-vivo* investigations to corroborate its efficacy and performance.

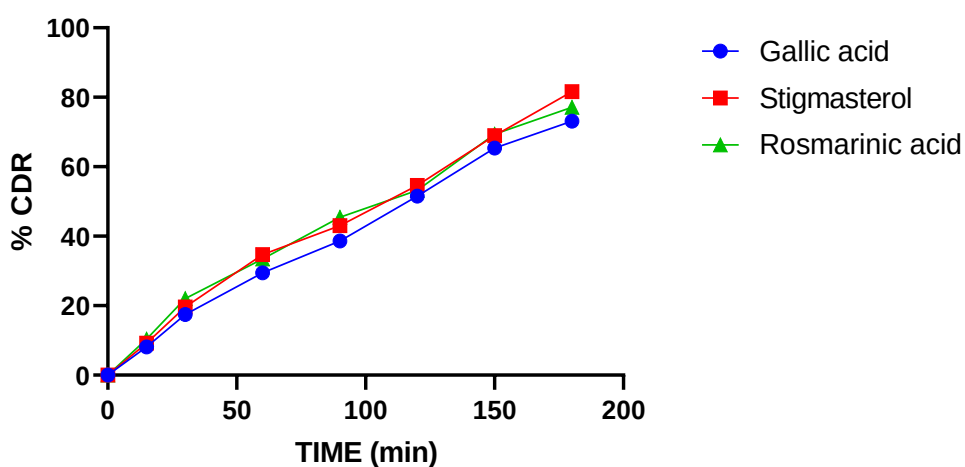


Figure 26. %CDR of gallic acid, stigmasterol, and rosmarinic acid form optimized polyherbal tablet formulation

Stability Study

The stability of the optimized polyherbal tablet formulation batch F-11 was assessed in accordance with ICH guidelines. Stability studies were conducted under ambient (25 ± 2 °C, $60 \pm 5\%$ RH) and accelerated (40 ± 2 °C, $75 \pm 5\%$ RH) storage conditions for a duration of 60 days, with results summarized in **Table 28**. The physical properties of batch F-11, including hardness, friability, and disintegration time, remained consistent throughout the study period, exhibiting only slight variations under both storage conditions. These minor deviations were within pharmacopeial and acceptable stability limits, indicating that the formulation retained its integrity and performance characteristics. The findings demonstrate that the optimized polyherbal formulation exhibits satisfactory physical stability over the evaluated period, supporting its potential for long-term storage and clinical application. Further studies over extended periods may provide additional insights into its shelf-life and robustness under diverse storage conditions.

TABLE 28. Data represent the stability testing parameters of the optimized formulation (F-11) under both ambient and accelerated conditions.

Evaluation parameters	Storage condition	0 days	15 days	30 days	45 days	60 days
Disintegration time	25 ± 2°C/ RH 65%)	19.4	19.5	19.7	19.8	20.0
	40 ± 2°C/ RH 75%	19.3	19.4	19.6	19.7	19.9
Friability	25 ± 2°C/ RH 65%)	0.41	0.41	0.43	0.47	0.47
	40 ± 2°C/ RH 75%	0.42	0.42	0.44	0.44	0.47
Hardness	25 ± 2°C/ RH 65%)	4.6	4.7	4.7	4.8	4.9
	40 ± 2°C/ RH 75%	4.6	4.7	4.8	4.8	4.9

Anti-Inflammatory Activity

In-vitro Human red blood cell (HRBC) membrane stabilization assay

The in vitro anti-inflammatory activity of the polyherbal tablets was evaluated using the HRBC membrane stabilization assay. This method assesses the ability of test samples to prevent lysis of erythrocyte membranes under hypotonic conditions, serving as an indicator of anti-inflammatory potential. The absorbance values for both the polyherbal tablet formulation and the reference standard, Diclofenac sodium, decreased with increasing concentrations, reflecting enhanced membrane stabilization. The assay was performed across a range of concentrations, with both the test and standard demonstrating a concentration-dependent increase in percent inhibition of hemolysis.

At the highest concentration tested (500 µg/mL), the polyherbal tablets exhibited remarkable membrane stabilization activity, achieving 83.63% inhibition of hemolysis, compared to 72.01% inhibition observed with Diclofenac sodium. These findings, detailed in **Table 29**, indicate the superior anti-inflammatory potential of the polyherbal formulation at this concentration. The concentration-dependent response underscores the efficacy of the polyherbal tablets in stabilizing cell membranes, likely due to the synergistic effects of bioactive compounds present in the formulation. These results suggest that the polyherbal tablets may offer a promising alternative to conventional anti-inflammatory agents, warranting further investigation to elucidate their mechanism of action and therapeutic potential.

TABLE 29. % inhibition of hemolysis from HRBC membrane stabilization method.

Concentrations (µg/ml)	Diclofenac Sodium		Polyherbal tablet	
	Absorbance (560nm)	% Rate of inhibition	Absorbance (560nm)	% Rate of inhibition
500	0.549±0.001	72.01	0.321±0.003	83.63
400	0.602±0.008	69.31	0.640±0.004	67.38
300	0.712±0.006	63.71	0.898±0.004	54.23
200	1.234±0.003	37.10	1.05±0.040	46.48
100	1.436±0.00	26.80	1.35±0.008	31.19

Carrageenan-Induced Paw Edema in Rats (*In-vivo*)

The *in-vivo* anti-inflammatory effect of the polyherbal tablets was evaluated using the carrageenan-induced paw edema model in Wistar rats. This model evaluates the ability of test substances to reduce acute inflammation induced by carrageenan injection, a widely accepted method for studying anti-inflammatory efficacy. Rats were pre-treated with polyherbal tablets at doses corresponding to 1/20th (100 mg/kg, T-I), 1/10th (200 mg/kg, T-II), and 1/5th (400 mg/kg, T-III) of the LD50 value. The results revealed a dose-dependent reduction in paw edema volume measured 24 hours post-carrageenan injection. Among the tested doses, the T-III group (400 mg/kg) demonstrated the most pronounced anti-inflammatory effect, achieving a statistically significant reduction in paw volume ($p < 0.001$) compared to the reference standard, diclofenac sodium (10 mg/kg).

At the end of the 24-hour period, the paw volume in the T-III group was recorded at 0.368 ± 0.017 mL, markedly lower than the paw volume of 0.50 ± 0.013 mL observed in the standard drug group, as detailed in **Table 30** and illustrated in **Figure 27**. These results highlight the superior efficacy of the polyherbal formulation in mitigating acute inflammation at higher doses. The significant reduction in paw edema suggests that the polyherbal tablets possess potent anti-inflammatory activity, potentially mediated through the synergistic effects of their bioactive constituents. These findings underscore the therapeutic potential of the polyherbal formulation as an effective alternative to conventional anti-inflammatory agents and warrant further investigations to explore its mechanism of action and long-term efficacy.

TABLE 30. Effect of polyherbal tablets on Anti-inflammatory activity
carrageenan-induced paw edema (Reduction in paw volume)

Groups	0 (h)	1 (h)	2 (h)	3 (h)	4 (h)	5 (h)	6 (h)	8 (h)	24 (h)
Normal	0.10± 0.003	0.10± 0.003	0.11± 0.003	0.115± 0.002	0.105± 0.003	0.11± 0.03	0.11± 0.004	0.11± 0.003	0.11± 0.003
Control	0.14± 0.018	0.72± 0.021	0.81± 0.052	1.05± 0.038	1.26± 0.07	1.12± 0.077	0.96± 0.04	0.85± 0.02	0.78± 0.02
Standard	0.13± 0.01	0.70± 0.021	0.75± 0.022	0.79± 0.10	0.80± 0.02	0.74± 0.016	0.66± 0.016	0.57± 0.023	0.50± 0.013
T1	0.14± 0.016	0.71± 0.018	0.77± 0.019	0.99± 0.030	1.11± 0.04	1.008± 0.05	0.833± 0.030	0.753± 0.02	0.675± 0.021
T2	0.13± 0.009	0.703± 0.012	0.735± 0.014	0.76± 0.017	0.78± 0.011	0.735± 0.013	0.698± 0.014	0.625± 0.008	0.553± 0.010
T3	0.121± 0.009	0.688± 0.10	0.708± 0.032	0.7533 ±0.02	0.775± 0.025	0.7133 ±0.008	0.645± 0.013	0.546± 0.015	0.368± 0.017

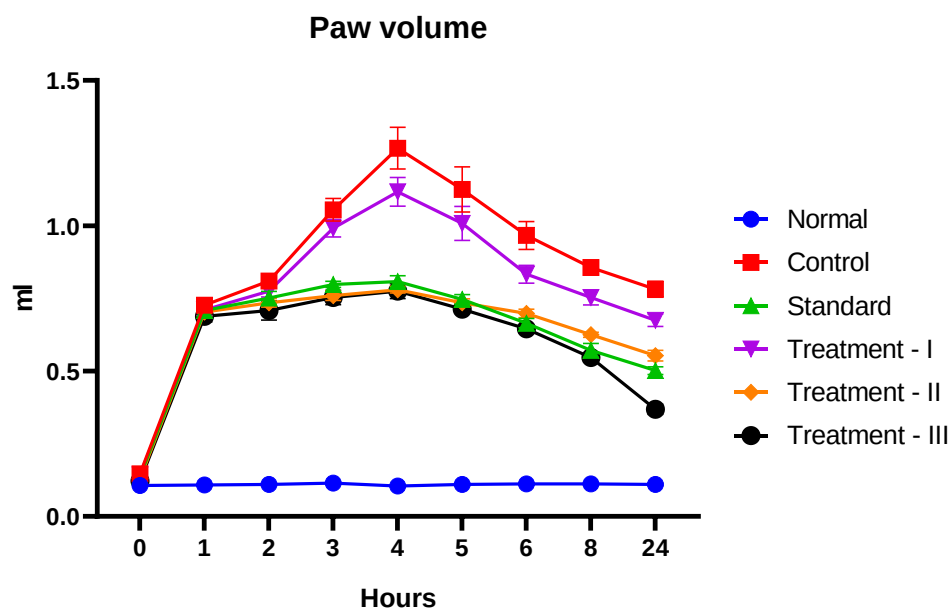


Figure 27. Reduction of paw volume in carrageenan-induced paw edema in rats.

Values are expressed as Mean $\pm$ SEM (n = 3): analysed by two-way ANOVA followed by Tukey's Multiple comparison test whereas, ***p < 0.001 when compared to normal; **p < 0.001, *p < 0.01 when compared to diclofenac sodium.

Histopathological Assessment

Histopathological assessments, presented in **Figure 28**, provided a visual representation of the inflammatory response in paw tissues across different treatment groups. In contrast, the carrageenan-induced paw tissue in the control group (b) exhibited marked edema, neutrophil infiltration, and significant tissue disorganization, indicative of acute inflammatory response. The diclofenac sodium-treated group (c) showed notable improvements, with a reduction in edema and inflammation, approaching a near-normal tissue architecture.

The polyherbal treatment groups demonstrated dose-dependent benefits. At the lowest dose, T1 (100 mg/kg) (d), there was moderate edema and reduced inflammatory cell infiltration. Increasing the dose to T2 (200 mg/kg) (e) led to further reductions in edema and enhanced tissue architecture. The highest dose, T3 (400 mg/kg) (f), resulted in nearly normal histological features, exhibiting minimal inflammation and well-preserved tissue integrity. These histopathological findings suggest that the polyherbal tablets exert a protective effect against carrageenan-induced inflammation in a dose-dependent manner, providing evidence of their potential therapeutic benefits. The observed improvements in tissue architecture underscore the efficacy of the polyherbal formulation in mitigating acute inflammation.

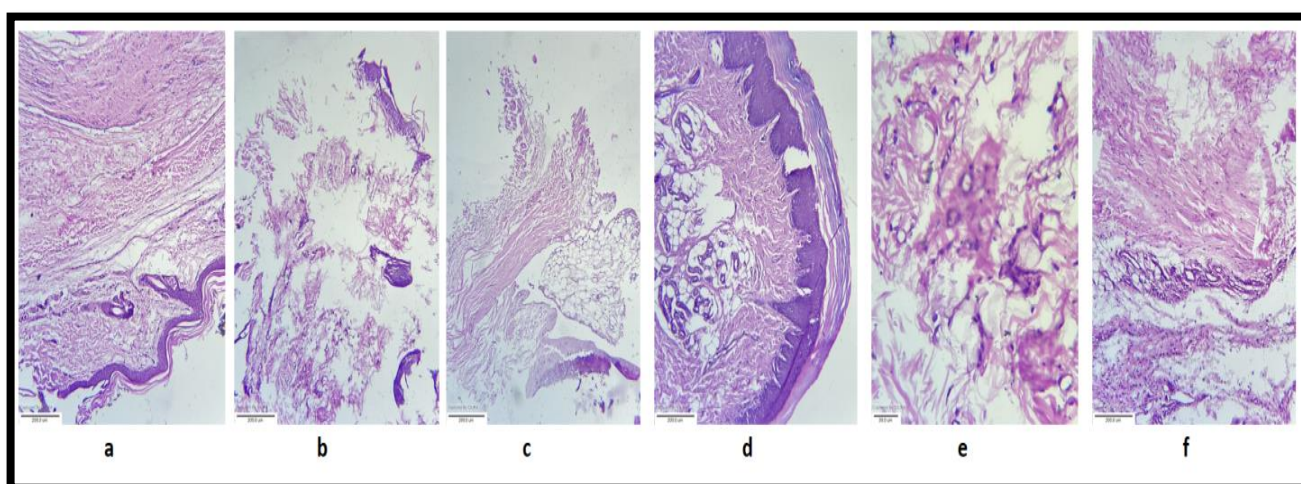


Figure 28. Effect of polyherbal tablets on the histopathology of paw tissue
Carrageenan-induced paw tissue: (a) normal, (b) control, (c) standard drug
(diclofenac sodium), (d) T1 (100 mg/kg), (e) T2 (200 mg/kg), and (f) T3 (400
mg/kg).

DISCUSSION

The objective of this study is to formulate a polyherbal anti-inflammatory product using the QbD approach and assess its anti-inflammatory activity through both *in-vitro* and *in-vivo* models. Through the application of QbD principles, the study systematically tackles the inherent variability and complexity of herbal formulations. The QbD framework aids in identifying and controlling CMAs and CPPs, thereby ensuring consistent product quality, reproducibility, and therapeutic effectiveness. The formulation incorporates selected herbal ingredients—*Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera*—which are widely recognized for their diverse pharmacological properties and have been extensively utilized in traditional medicine systems for the management of inflammation and oxidative stress.

Terminalia chebula is renowned for its strong enzyme-inhibitory and antioxidant properties, which help reduce pro-inflammatory mediators like prostaglandins and leukotrienes. *Ocimum sanctum*, commonly known as "Holy Basil," possesses immunomodulatory and anti-inflammatory effects, largely by regulating pro-inflammatory cytokines and oxidative stress pathways, making it especially beneficial for chronic inflammatory conditions. Likewise, *Moringa oleifera* exhibits significant anti-inflammatory properties by inhibiting COX enzymes and modulating inflammatory mediators, offering a natural alternative to synthetic NSAIDs.

The rationale for a polyherbal approach lies in the synergistic potential of these herbs, which capitalize on the complementary and overlapping mechanisms of their bioactive phytoconstituents. This synergy enhances the overall therapeutic efficacy and reduces the likelihood of side effects associated with single-compound treatments.

Quality Assessment of Herbal Raw Material

The comprehensive quality assessment of the herbal raw materials *Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera* was conducted through botanical, physicochemical, and phytochemical evaluations, ensuring the identity, purity, and quality essential for pharmaceutical and therapeutic applications. The botanical evaluation included both macroscopic and microscopic analyses. Macroscopic characteristics, such as color, odor, taste, size, and shape, provided distinct markers for each herb. *Terminalia chebula* fruit was yellowish-brown and ovoid with an astringent taste, *Ocimum sanctum* leaves were blackish-green, aromatic, and oblong, and *Moringa oleifera* leaves were yellowish-green, pungent, and ovate. These features establish primary identification criteria, preventing adulteration and substitution.

Microscopic analysis offered deeper insights into structural attributes critical for authentication and detecting adulterants. *Terminalia chebula* fruit exhibited cork cells, stone cells, fibers, and vascular bundles, reflecting robust structural integrity. *Ocimum sanctum* leaves demonstrated sclereids, lignified fibers, prismatic crystals, starch grains, and trichomes, consistent with its anatomical features, while *Moringa oleifera* leaves showed stomata, multicellular trichomes, xylem vessels, calcium oxalate crystals, and starch grains. These observations are invaluable for ensuring purity and quality, as deviations from these features may indicate contamination or degradation.

Physicochemical parameters such as total ash, acid-insoluble ash, moisture content, and extractive values, provided critical insights into the quality and stability of the raw materials. Ash values, which reflect mineral content and inorganic impurities, varied among the herbs, with *Moringa oleifera* showing the highest total ash (10.41%), followed by *Ocimum sanctum* (8.66%) and *Terminalia chebula*

(3.65%). Acid-insoluble ash was lowest in *Terminalia chebula* (0.28%) and highest in *Moringa oleifera* (2.75%), highlighting botanical source variations. Moisture content, crucial for stability and shelf life, was highest in *Terminalia chebula* (8%) and lowest in *Ocimum sanctum* (0.75%), indicating the latter's reduced susceptibility to microbial contamination. Extractive values demonstrated the solubility of bioactive constituents, with *Terminalia chebula* exhibiting higher alcohol-soluble (43.8%) and water-soluble extractives (32.8%), reflecting its rich phytochemical composition.

Phytochemical analysis confirmed the presence of notable secondary metabolites in the selected herbs. *Terminalia chebula* and *Ocimum sanctum* consistently exhibited alkaloids, flavonoids, saponins, tannins, phenols, and astringents, all of which are recognized for their antioxidant, antimicrobial, and anti-inflammatory activities. In contrast, the absence of tannins in *Moringa oleifera* highlighted phytochemical variations across species. The abundance of alkaloids, flavonoids, and phenols in all three herbs underscores their potential in managing oxidative stress and inflammation. The high tannin and astringent content in *Terminalia chebula* supports its use in gastrointestinal disorders, while the aromatic properties of *Ocimum sanctum* make it effective for respiratory ailments. The rich mineral and phenolic content of *Moringa oleifera* further validates its role in nutritional and therapeutic formulations.

These evaluations collectively establish the quality and authenticity of *Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera*, supporting their application in traditional medicine and modern pharmacology.

Chromatographic Evaluation

The chromatographic evaluation and simultaneous analysis of gallic acid and rosmarinic acid using a QbD-assisted RP-HPLC method present a thorough and methodical approach to analytical method development. Initial determination of the isosbestic point, identified at 298 nm through UV spectroscopy, was a pivotal step in selecting the optimal wavelength for detecting these marker compounds. This fundamental parameter ensures accurate and sensitive detection within the UV range of 200–800 nm. Within the QbD framework, the establishment of the ATP served as a strategic guideline for method development. By focusing on achieving effective separation and quantification with minimal tailing factors, narrow peak widths, and reasonable analysis times, the ATP underscored the method's robustness and precision.

The CQAs identified—such as a tailing factor not exceeding 2 and a theoretical plate count of at least 3000—were crucial for maintaining quantitation accuracy and reproducibility. The selection of these CQAs reflects their essential role in ensuring robust peak integration and chromatographic performance. Elevated tailing factors or suboptimal theoretical plate counts could significantly compromise data reliability, highlighting the importance of these parameters in method validation.

A systematic approach was employed using a 2^2 factorial design to explore the influence of independent variables—specifically, the mobile phase ratio and flow rate—on chromatographic performance. By setting these variables at high and low levels, the experimental design facilitated a nuanced understanding of their effects on the responses—tailing factors and theoretical plates for both gallic acid and rosmarinic acid. Statistical optimization using Design-Expert Software revealed the significance of these variables, as evidenced by the derived polynomial equations.

These equations not only quantified the impact of each variable but also elucidated the synergistic or antagonistic effects on the responses. Positive coefficients indicated enhancements in performance with variable increases, while negative coefficients highlighted potential trade-offs.

Response surface plots provided a visual representation of these relationships, offering an intuitive understanding of how variations in mobile phase composition and flow rate affected tailing factors and theoretical plates. The observed trends, where increases in mobile phase ratio and flow rate led to higher tailing factors but reduced theoretical plate counts, were critical for refining the method. By balancing these trade-offs, the study identified an optimal operating range within the method's design space.

The establishment of the MODR was a cornerstone of the QbD approach. Analytical Trial 2, employing a methanol-to-water ratio of 55:45, emerged as the optimal condition, aligning with the ATP and CQAs. This choice minimized tailing factors and peak widths while ensuring adequate theoretical plate counts, as corroborated by the HPLC chromatograms of gallic acid and rosmarinic acid standards.

The optimized conditions were subsequently validated through thorough method validation protocols. System suitability parameters, such as % RSD for peak area, retention time, theoretical plates, and tailing factor, remained consistently below 2%, confirming the method's precision and reliability. The method's linearity, demonstrated over a concentration range of 5–25 µg/mL for both analytes, and its low LOD and LOQ underscored its sensitivity. Precision studies, with intra- and inter-day % RSD values well within acceptable limits, validated the method's reproducibility. High recovery rates in accuracy studies confirmed its capability to accurately quantify

analytes across varied concentrations. Robustness testing, involving deliberate variations in chromatographic parameters, further reinforced the method's resilience under diverse conditions.

The practical application of the optimized method to quantify gallic acid in *Terminalia chebula* and rosmarinic acid in *Ocimum sanctum* extracts exemplified its utility. The measured quantities, combined with high accuracy percentages, validated the method's effectiveness in real-world scenarios. Representative chromatograms from these analyses provided visual confirmation of the method's capability to accurately identify and quantify these marker compounds within complex matrices.

Additionally, a RP-HPLC method for stigmasterol analysis was developed using QbD principles. Optimization was achieved through the BBD, with critical factors including organic phase composition (40–60% acetonitrile), flow rate (0.8–1.2 mL/min), and column temperature (25–35°C). Retention time, tailing factor, and theoretical plates were selected as response variables to evaluate method performance. ANOVA analysis confirmed the significance of these factors, with polynomial equations indicating their individual and interactive effects. The optimized conditions—80% acetonitrile, a 1.0 mL/min flow rate, and a 35°C column temperature—yielded a well-resolved stigmasterol peak, characterized by minimal tailing and a high number of theoretical plates.

Validation followed ICH guidelines, confirming the method's reliability. System suitability tests showed %RSD $\leq$ 2% for all parameters, ensuring consistent chromatographic performance. The method demonstrated linearity ($R^2 > 0.998$) across a broad concentration range, with LOD and LOQ values of 0.15 $\mu\text{g/mL}$ and 0.45 $\mu\text{g/mL}$, respectively, indicating high sensitivity. Precision studies yielded %RSD values below 2% for both intra-day and inter-day measurements. Recovery rates from

accuracy tests ranged between 98–102%, highlighting the method's accuracy. Robustness was confirmed under varied chromatographic conditions, with negligible impact on performance.

Application of the validated method to crude extracts enabled the accurate quantification of stigmasterol. Chromatograms displayed well-defined peaks, with consistent retention times and peak areas. The method was highly reproducible and suitable for routine analysis of stigmasterol in phytochemical research and quality control. Its QbD-based optimization and compliance with regulatory guidelines ensure reliability, making it a valuable tool for standardization in herbal formulations.

Extraction and Quality Assessment of Herbal Extract

The extraction process for *Terminalia chebula* dry fruits, *Ocimum sanctum* leaves, and *Moringa oleifera* leaves was successfully carried out using the prescribed procedure, resulting in varying percentage yields for each crude drug. *Terminalia chebula* demonstrated the highest yield at 32.01%, followed by *Ocimum sanctum* with a yield of 26.87%, and *Moringa oleifera* with a lower yield of 19.05%. These yields indicate the effectiveness of the extraction process and the inherent natural composition of each plant material.

Quality assessment of these herbal extracts further validates their purity and authenticity, with moisture content and ash values remaining within acceptable limits. Phytochemical analysis identified a variety of secondary metabolites, such as alkaloids, flavonoids, tannins, phenols, saponins, triterpenes, and glycosides. The identification of these compounds is indicative of their potential therapeutic properties. These findings not only validate the extraction process but also highlight

the richness of bioactive compounds present in these medicinal plants, supporting their use in traditional medicine.

Formulation Development

The compatibility study of the plant extracts with the excipients used in the polyherbal tablet formulation was evaluated using FTIR spectroscopy. FTIR spectra were recorded and analyzed for both the individual plant extracts and their mixtures with excipients. Comparative assessment of these spectra revealed that the characteristic peaks of the functional groups within the plant extracts were preserved, with no significant shifts or changes in intensity. This preservation indicates that the functional groups within the plant extracts remain within their expected spectral limits, demonstrating the absence of chemical interactions between the extracts and the excipients. Consequently, it can be concluded that the excipients selected for the polyherbal tablet formulation are compatible with the plant extracts. Ensuring compatibility is crucial for maintaining the stability and integrity of the final product.

In formulating the anti-inflammatory herbal tablets, a QbD approach was adopted to establish the QTPP. The QTPP was meticulously defined, taking into account the CQAs necessary to achieve the desired characteristics of the final product. This systematic development of the QTPP aligns the formulation with the intended therapeutic purpose and patient requirements. A comprehensive summary of the QTPP for the anti-inflammatory herbal product is provided, detailing the dosage form, dosage design, route of administration, dosage strength, and critical quality attributes. These include tablet appearance, hardness, friability, disintegration time, and storage conditions. This structured approach ensures that the formulation meets predefined quality specifications, promoting patient acceptability and compliance while ensuring product safety and efficacy.

The identification of CQAs played a pivotal role in the QbD strategy. Based on the established QTPP, CQAs were pinpointed and ranked according to their substantial influence on the overall quality, safety, and efficacy of the final product. This risk-based approach aids in identifying and managing the factors most likely to influence product quality. Initial risk assessments for formulation variables such as MCC, CCS, lactose, talc, and magnesium stearate revealed their varying impacts on the CQAs. For instance, variations in MCC and CCS levels were identified as having high risk due to their direct influence on disintegration time, friability, and hardness. The QRM approach guides the formulation process by identifying critical factors and implementing strategies to mitigate risks, thereby ensuring the robustness of the formulation.

The application of a DoE using a 3^2 CCD framework facilitated a systematic exploration and optimization of the polyherbal tablet formulations. A total of thirteen formulation batches were developed and assessed for critical quality attributes, including disintegration time, friability, and hardness. The influence of formulation variables on these responses was analyzed using MLRA. Polynomial regression models were developed to describe the interactions between variables like MCC and CCS, capturing linear, quadratic, and interaction effects. These models provided a comprehensive understanding of how different concentrations of MCC and CCS influenced the tablet properties. The statistical significance of these regression models was validated through ANOVA, demonstrating a strong fit and reliability for the models in predicting formulation responses.

Results from the DoE study revealed significant effects of MCC and CCS on the critical quality attributes. Disintegration time varied between 19.04 to 28.03 minutes, underscoring the importance of MCC and CCS levels in tablet breakdown.

Increased concentrations of these excipients initially reduced disintegration time but beyond certain levels, led to a decrease due to structural compaction effects. Similarly, the friability of the tablets ranged from 0.38% to 0.64%, with MCC having a more pronounced effect than CCS, directly influencing tablet strength. The hardness of the formulations, which ranged from 4.6 to 6.3 kg/cm², also depended significantly on MCC and CCS. Higher levels of these excipients contributed to improved tablet hardness by enhancing binding forces and reorganization of the tablet matrix. These findings underscore the critical role of MCC as a binder and CCS as a disintegrant in determining the mechanical strength and disintegration properties of the polyherbal tablets.

The Prediction Profiler (PP) was employed to forecast the optimal formulation composition by aligning factor levels with desired response values. The profiler output provided a visual representation of the cross-sectional relationships among formulation factors and responses, offering valuable insights for optimization. Polynomial regression models and ANOVA results confirmed the validity and predictive power of the model, providing a solid foundation for further optimization. The desirability function used in conjunction with the PP helped select the formulation parameters that would yield optimal disintegration time, friability, and hardness, thereby ensuring the robustness of the formulation.

Overall, the integration of QbD principles—including risk management, DoE, and statistical modeling—provided a systematic approach to formulation development. These findings not only highlight the compatibility of plant extracts with excipients but also emphasize the importance of optimizing formulation variables to achieve desired tablet properties. The application of QbD ensures that the

anti-inflammatory herbal tablet formulation is robust, consistent, and capable of meeting the intended therapeutic objectives.

The results demonstrate the quality and suitability of the polyherbal tablet formulation F-11. Pre-compression parameters exhibited excellent flow properties, with bulk densities ranging from 0.412 to 0.529 g/cm³, tapped densities from 0.462 to 0.613 g/cm³, an angle of repose between 32.4° and 36.5°, and compressibility indices (Carr's index) ranging from 10.8% to 16.2%, along with Hausner's ratios from 1.12 to 1.19. These values confirm the suitability of the powder blend for direct compression. Post-compression, the tablets exhibited a uniform appearance with a characteristic brown color, bitter taste, and consistent thickness (6.3 to 6.8 mm) and weight (648 to 651 mg) across all batches, meeting pharmacopeial specifications. Drug content analysis revealed variations in recovery rates of gallic acid (63.2%), stigmasterol (74.1%), and rosmarinic acid (82.2%). These differences may be attributed to extraction efficiency, compound stability, or matrix interactions within the formulation.

The *in-vitro* drug release profile of batch F-11 showed a favorable sustained release pattern for the bioactive compounds—72.47% for gallic acid, 81.2% for stigmasterol, and 76.21% for rosmarinic acid—over 180 minutes. This suggests effective dissolution and diffusion of the active components, enhancing their bioavailability. Stability studies under ICH conditions revealed that physical properties such as hardness, friability, and disintegration time remained consistent over 60 days, even under accelerated conditions. These findings support the formulation's long-term stability and confirm its suitability for clinical applications. Together, these results validate the quality, effectiveness, and potential therapeutic use of the polyherbal tablet formulation.

Anti-Inflammatory Activity

The comprehensive assessment of the anti-inflammatory activity of polyherbal tablets, utilizing both *in-vitro* and *in-vivo* models, has yielded compelling evidence of their therapeutic efficacy. The HRBC membrane stabilization assay, a widely recognized preliminary screening method, revealed a dose-dependent inhibition of hemolysis by the polyherbal tablets, highlighting their ability to enhance erythrocyte membrane stabilization under hypotonic conditions—a hallmark of anti-inflammatory activity. At a concentration of 500 µg/mL, the polyherbal tablets demonstrated an 83.63% inhibition of hemolysis, which markedly exceeded the 72.01% inhibition achieved by the reference standard, Diclofenac sodium. This outcome illustrates the superior anti-inflammatory potential of the polyherbal formulation, underscoring the synergy among its bioactive components. The dose-dependent nature of the inhibition suggests that the bioactive compounds within these tablets contribute collectively to their enhanced anti-inflammatory efficacy.

The HRBC membrane stabilization assay is particularly valuable in preliminary anti-inflammatory screening due to its simplicity and reproducibility. By preventing erythrocyte membrane lysis under hypotonic conditions, this assay indirectly measures the stabilizing effect of the test substances on cell membranes, indicative of their potential to stabilize lysosomal membranes and prevent the release of hydrolytic enzymes that contribute to inflammation. The results of this assay not only establish the potent anti-inflammatory nature of the polyherbal tablets but also highlight their potential as a safer alternative to conventional synthetic anti-inflammatory drugs like Diclofenac sodium.

Further validation of these *in vitro* findings was provided by the *in vivo* carrageenan-induced paw edema model, a standard for evaluating anti-inflammatory activity due to its ability to induce acute inflammatory responses similar to those observed in human conditions. The dose-dependent reduction in paw edema following administration of the polyherbal tablets indicates that the bioactive constituents present in these tablets effectively mitigate inflammation by blocking key inflammatory pathways. Of the doses evaluated, T3 (400 mg/kg) showed the most notable reduction in paw edema, with a substantial decrease in paw volume when compared to both the control group and the standard drug, Diclofenac sodium. These findings are statistically significant, with p-values indicating a high level of confidence in the results, further substantiating the anti-inflammatory potential of the polyherbal tablets.

Histopathological evaluations further corroborated the anti-inflammatory effects observed in both the HRBC membrane stabilization assay and the carrageenan-induced paw edema model. The histological analysis of paw tissues from treated rats revealed dose-dependent improvements, with the T3 (400 mg/kg) group displaying nearly normal tissue architecture, minimal edema, and reduced infiltration of neutrophils. These histopathological changes are consistent with the biochemical data, reinforcing the conclusion that the polyherbal tablets exert a protective effect against inflammation by preserving tissue integrity and reducing cellular damage.

The anti-inflammatory effects of the polyherbal tablets are probably attributed to the synergistic action of various bioactive compounds in the formulation. These include flavonoids, tannins, and terpenoids, which are recognized for their antioxidant, anti-inflammatory, and anti-nociceptive properties. The synergistic interaction among these compounds likely enhances the anti-inflammatory effect,

resulting in a more potent therapeutic outcome than any single compound alone. The polyherbal formulation appears to modulate inflammatory mediators such as cytokines, eicosanoids, and nitric oxide, contributing to its observed anti-inflammatory activity. This is particularly evident from the dose-dependent inhibition of paw edema, which aligns with the stabilization of cell membranes and reduced release of pro-inflammatory mediators.

These findings suggest that the polyherbal tablets could serve as a viable alternative to conventional anti-inflammatory agents, offering a safer profile with fewer side effects. Given the growing concern over the adverse effects associated with the long-term use of synthetic drugs, particularly NSAIDs, the polyherbal tablets present an attractive option with their potential to mitigate inflammation without the gastrointestinal, cardiovascular, or renal side effects commonly associated with these drugs. This makes the polyherbal formulation not only an effective anti-inflammatory agent but also one with a promising safety profile for long-term use in managing chronic inflammatory conditions.

SUMMARY

This study aims to develop a polyherbal anti-inflammatory formulation using the QbD approach and evaluate its performance through *in-vitro* and *in-vivo* models. The goal is to tackle the variability and complexity associated with herbal formulations by incorporating QbD principles. QbD focuses on the systematic identification, comprehension, and control of CMAs and CPPs to guarantee consistent product quality, reproducibility, and therapeutic efficacy.

The polyherbal formulation incorporates *Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera*, each known for their pharmacological properties in traditional medicine. *Terminalia chebula*, also known as Haritaki, exhibits potent enzyme-inhibitory and antioxidant activities, reducing pro-inflammatory mediators like prostaglandins and leukotrienes. *Ocimum sanctum* (Holy Basil) shows immunomodulatory and anti-inflammatory properties by modulating pro-inflammatory cytokines and oxidative stress pathways. *Moringa oleifera*, with its significant anti-inflammatory effects, inhibits cyclooxygenase (COX) enzymes and modulates inflammatory mediators. This polyherbal approach leverages the synergistic potential of these herbs to enhance therapeutic efficacy and reduce side effects through their complementary mechanisms.

To ensure the quality of the formulation, a thorough assessment of the herbal raw materials—*Terminalia chebula* dry fruits, *Ocimum sanctum* leaves, and *Moringa oleifera* leaves—was conducted through botanical, physicochemical, and phytochemical evaluations. The moisture content and ash values of the extracts were found to be within acceptable limits, indicating the purity and authenticity of the herbal ingredients. Phytochemical screening revealed the presence of significant

secondary metabolites such as alkaloids, flavonoids, tannins, phenols, saponins, and triterpenes, confirming their potential in managing inflammation and oxidative stress. These bioactive compounds are indicative of the therapeutic properties inherent in each plant material.

A QbD-assisted RP-HPLC method was developed and validated for quantifying key marker compounds like gallic acid and rosmarinic acid. An ATP was established to guide method development, focusing on effective separation, minimal tailing factors, and narrow peak widths. A 2^2 factorial design was employed to investigate the impact of independent variables—mobile phase ratio and flow rate—on chromatographic performance. Statistical optimization through Design-Expert Software identified the optimal conditions for the method, ensuring high precision, accuracy, and robustness in accordance with ICH guidelines. The validated method was effectively utilized for routine quality control in phytochemical research and the standardization of the herbal formulation. Furthermore, a RP-HPLC method for stigmasterol analysis was developed using QbD principles. Optimization was achieved through the BBD, with critical factors including organic phase composition (40–60% acetonitrile), flow rate (0.8–1.2 mL/min), and column temperature (25–35°C).

A QbD approach was utilized in the development of polyherbal tablet formulations. A 3^2 CCD was applied to optimize the formulation. Thirteen formulation batches were prepared and assessed for critical quality attributes, including disintegration time, friability, and hardness. Polynomial regression models were developed to describe the interactions between variables like MCC and cross-linked CCS. These models captured linear, quadratic, and interaction effects, providing a comprehensive understanding of how different concentrations of MCC

and CCS influenced tablet properties. ANOVA validated the models, demonstrating a strong fit and reliability for predicting formulation responses.

The results indicated significant effects of MCC and CCS on critical quality attributes. Disintegration time varied between 19.04 to 28.03 minutes, highlighting the importance of MCC and CCS levels in tablet breakdown. The variations in friability (0.38% to 0.64%) and hardness (4.6 to 6.3 kg/cm²) of the tablets were directly influenced by MCC and CCS. Higher levels of these excipients improved tablet hardness by enhancing binding forces and reorganization of the tablet matrix, underscoring their critical role in formulation development.

The Prediction Profiler (PP) was employed to forecast the optimal formulation composition by aligning factor levels with desired response values. The profiler output provided a visual representation of the relationships among formulation factors and responses, offering valuable insights for optimization. Polynomial regression models and ANOVA results confirmed the validity and predictive power of the model, providing a solid foundation for further optimization. The integration of the desirability function with the PP helped select formulation parameters that would yield optimal disintegration time, friability, and hardness, ensuring the robustness of the formulation.

The anti-inflammatory activity of the polyherbal tablets was assessed using both *in-vitro* and *in-vivo* models. The HRBC membrane stabilization assay, an initial screening test for anti-inflammatory effects, demonstrated a dose-dependent inhibition of hemolysis by the polyherbal tablets, achieving 83.63% inhibition at a concentration of 500 µg/mL.

In the carrageenan-induced paw edema model, the reduction in paw edema was comparable to the standard drug diclofenac sodium, demonstrating effective attenuation of acute inflammation by the polyherbal tablets. The statistical analysis showed significant reduction ($p < 0.01$) in edema volume at both 200 mg/kg and 400 mg/kg doses, with the latter dose showing a superior effect. These findings align with earlier *in-vitro* results, reinforcing the anti-inflammatory potential of the formulation across multiple models. These results suggest that the formulation not only modulates inflammatory mediators but also provides a protective antioxidant effect, which is vital for mitigating oxidative damage in inflammatory conditions.

CONCLUSION

The study successfully developed and evaluated a polyherbal anti-inflammatory formulation utilizing a QbD approach. By incorporating *Terminalia chebula*, *Ocimum sanctum*, and *Moringa oleifera*, each renowned for their pharmacological benefits, the formulation harnesses their complementary mechanisms to enhance anti-inflammatory and antioxidant effects. Comprehensive assessments of the herbal raw materials, including botanical, physicochemical, and phytochemical evaluations, validated the authenticity and quality of the ingredients. The QbD-assisted RP-HPLC method developed for the quantification of key marker compounds, coupled with factorial designs and polynomial regression models, provided robust validation and optimization for routine quality control. The study demonstrated the significant impact of formulation factors, such as MCC and CCS, on critical quality attributes like disintegration time, friability, and hardness of the polyherbal tablets. ANOVA results and Prediction Profiler analysis underscored the validity and predictive power of the models, offering valuable insights for formulation optimization. The notable anti-inflammatory activity of the polyherbal tablets, observed across both *in-vitro* and *in-vivo models*, highlights the formulation's potential in attenuating inflammation and oxidative stress. These findings not only support the therapeutic potential of the polyherbal formulation but also exemplify the efficacy of the QbD approach in ensuring product quality and performance, paving the way for further developments in herbal-based therapeutics.

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ANNEXURE – I

ANIMAL ETHICAL CLEARANCE CERTIFICATE



KLE College of Pharmacy
A Constituent Unit of
KLE Academy of Higher Education and Research
(Deemed to be University)
DEPARTMENT OF PHARMACOLOGY
JNMC Campus, Nehru Nagar, Belagavi - 590 010, Karnataka, India
INSTITUTIONAL ANIMAL ETHICS COMMITTEE
Reg.No.221/PO/Re/S/2000/CPCSEA



DATE: 19/12/2023

CERTIFICATE

This is to certify that the project proposal No..... entitled, "Formulation and evaluation of Anti-inflammatory herbal preparation by quality by design approach ", submitted by Dr. / Mr. / Ms. Mahantesh Kunchanur under the guidance of Dr. Vinodh Kumar Mannur has been approved/recommended by the IAEC of KLE College of Pharmacy, Belagavi, Reg.No.221/PO/Re/S/2000/CPCSEA in its meeting dated 19/12/2023, resolution No. 36 has been sanctioned 39..... Rats/ ~~Mice~~/ Rabbits ~~Guinea pig~~ (animals) sex ~~male~~ under this proposal for a duration of next months.

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.

Authorized by	Name	Signature	Date
Member Secretary	Dr. N. A. Khatib		19/12/2023
MEMBER SECRETARY Institutional Animal Ethics Committee Reg. No. 221/PO/Re/S/2000/CPCSEA KLE College of Pharmacy, BELAGAVI.			

Main Nominee of

CPCSEA

CPCSEA Nominee
Institutional Animal Ethics Committee
Reg. No. 221/PO/Re/S/2000/CPCSEA
KLE College of Pharmacy,
BELAGAVI.

ANNEXURE – II

PUBLICATIONS

Quality assessment and QbD approached HPLC method development and validation for simultaneous estimation of Gallic acid and Rosmarinic acid

Section A-Research paper



QUALITY ASSESSMENT AND QBD APPROACHED HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF GALLIC ACID AND ROSMARINIC ACID

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ABSTRACT

The current work intends to investigate the quality of selected herbs and create a QbD assisted RP-HPLC method for the simultaneous estimation of gallic acid and rosmarinic acid. According to physicochemical, phytochemical, and toxic contaminant analysis, the quality of crude *Terminalia chebula* and *Ocimum sanctum* samples was assessed. The ATP and CQA's were determined, and then the method was optimized using a 2^2 factorial design. The tailing factor GA (R1) and theoretical plates GA (R2) were chosen as dependent variables, meanwhile the flow rate (X1) and mobile phase ratio (X2) were chosen as independent variables. The mobile phase, Methanol and Water in a 55:45 ratio, with a flow rate was selected as 1 mL/min and a detection wavelength of 298 nm, were found to be the ideal chromatographic conditions. Extrapolated from the results is that the QbD approach was employed to assure the development of a more accurate method that could generate constant, reliable, and improved data throughout the process whilst saving time.

Key Words: QbD, RP-HPLC, ATP, QTPP, CQA, Gallic acid, Rosmarinic acid

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INTRODUCTION

Medicinal plants have a substantial influence on health owing to the phytoconstituents they contain. Alkaloids, glycosides, tannins, flavonoids, and phenolic compounds are among the most significant of these elements. Phenolic acids are a broad class of compounds that include hydroxybenzoic and hydroxycinnamic acids. Plant phenolic acids include ferulic acid, ellagic acid, synergetic acid, caffeic acid, gallic acid, and rosmarinic acid, among others. They are also employed as natural antioxidant substitutes in the culinary, cosmetic, and pharmaceutical sectors. Gallic acid and Rosmarinic acid are two significant phenolic acids found in numerous plants, fruits, tea, coffee, and wine. It is generated by several species as free acids, esters, catechin derivatives, and hydrolysable tannins. Methylated gallic acids, such as syringic

acid, or galloyl conjugates of catechin derivatives, such as flavan-3-ols, or polygalloyl esters of glucose, quinic acid, or glycerol, can also be synthesised.^[1,2]

Gallic acid is categorized as a phenol. Its chemical formula is 3, 4, 5-trihydroxybenzoic acid. Its structure includes phenolic groups, which provide as a source of readily available hydrogen atoms, allowing radicals to be delocalized across the phenolic structure. Researchers are fascinated in the pharmacological activity of these compounds as radical scavengers. Gallic acid may be a viable starting molecule for novel medication development due to these properties.^[3]

Scarpati and Oriente isolated rosmarinic acid from *R. officinalis* in 1958. For the first time, Ellis and Towers described a RA biosynthetic route 12 years later. RA, a phenylpropanoid (i.e. $C_{18}H_{16}O_8$, molecular mass: 360.3 g/mol), is the caffeic acid ester of 3, 4-dihydroxyphenyllactic acid. A composition of two aromatic amino acids, phenylalanine and tyrosine the RA formed and they are joined by an ester linkage. Caffeic acid and 3, 4-dihydroxyphenyllactic acid is produced by two different pathways of the shikimic acid biosynthetic pathway. The caffeic acid component is generated primarily from phenylalanine, while the 3, 4-dihydroxyphenyllactic acid component is created exclusively from tyrosine.^[4]

The task of maintaining the quality of medicinal herbs is crucial; factors including geographical and environmental variations in growing conditions, physical constants, adulterations, and foreign components may have an impact on the herbal drug quality and consistency from batch to batch. Nevertheless, it is crucial to evaluate the quality of herbal crude drugs, particularly in terms of parameters like pharmacognostic and phytochemical, so as to demonstrate the efficacy and safety of herbal medicines. Various analytical methods have hitherto been published for the determination of

4771

Eur. Chem. Bull. 2022,11 (Regular Issue 12), 4771-4779

total and individual gallic acid and rosmarinic acid in diverse matrices, notably those utilising spectrophotometric analysis for gallic acid and rosmarinic acid determination.^[5, 6]

Effective component separation is better to prevent mutual interferences and achieve accurate quantification of GA and RA in various samples. As a result of their ability to separate substances, chromatographic techniques are thought to be the best methods for quantifying gallic acid and rosmarinic acid. For the separation of complex substances, HPLC methods are frequently used due to the method's simplicity and sensitivity. The excellent efficiency and quick separation of the HPLC process are well recognised.^[7, 8]

There are a number of HPLC techniques documented for the separation of gallic acid and rosmarinic acid. The previously described methods, in contrast side, were centered on the time-consuming principle of altering OFAT (One Factor at a Time). But at another end, as a novel idea for developing and analysing quality pharmaceutical products, the Quality by Design method is picking up steam. When used to develop an analytical technique, this methodology is known as AQbD. The ICH Q8 and Q9 recommendations, which depend heavily on the principle of variation of specific parts and defining the risk that may result in inadequate method robustness, support the QbD approach as a systemic and risk-based strategy. Generation of Design space is the core concept of AQbD, It suggests that changing the method's parameters won't have a substantial impact on the outcomes because the experimental zone has been established. While a result, this strategy adds stability to the technique as it is being developed. The FDA has been notably proactive in advocating the QbD concept, which demands integrating efficiency Rather of attempting to assess dependability after the development stage is complete, incorporate it into the process and the product in a systematic, scientific, and risk-based approach during the development stage. The previously reported HPLC-UV methods for gallic acid and rosmarinic acid have significant drawbacks, including long separation periods, low resolution, and/or complex solvent combinations with gradient elution. The current study intends to create a QbD aided simple and quick isocratic RP-HPLC technique for the simultaneous estimation of GA and RA for quality control in the form powdered raw material.

MATERIALS AND METHODS

Chemicals and reagents

The raw materials of plants *Terminalia chebula* and *Ocimum sanctum* were purchased from BMK Ayurveda Pharmacy, Shahapur, Belagavi and the plants specimen were confirmed and verified by CRF, BMK Ayurveda Mahavidyalaya, Shahapur, Belagavi. After being gathered, the raw materials were shade dried, crushed into a coarse powder, and then kept in airtight containers.

Quality assessment of *Terminalia chebula* and *Ocimum Sanctum*^[6, 11]

By implementing the quality control parameters stated in the raw samples, the quality of *Terminalia chebula* and *Ocimum sanctum* was evaluated.

The analysis of phytochemicals and physicochemical parameters following WHO criteria, total ash value, water-

soluble ash value, acid insoluble ash value, extractive value, and moisture content examples of such parameters, were carried out.

Procurement of Marker compounds

Himalaya Wellness, Bengaluru, provided the standard marker compounds of a few chosen phytoconstituents as free samples, which were then stored in the proper containers.

Preparation of standard solution^[12, 13]

The standard solutions were tried using different solvents but methanol and water was selected as optimized mobile phase for GA and RA phytoconstituents hence the different concentration solution were prepared using selected solvents & filtered using a 0.22mm membrane filter prior to being injected into the chromatographic column.

To prepare the sample, separately weighed accurately 1gm of powdered *Terminalia* and *Ocimum* samples were thoroughly refluxed for 1hr with 50mL of methanol: water. The solution was filtered & the residue was refluxed for 1 hour with 50mL of methanol and again filtered. The filtrate was homogenized and diluted to 100mL with methanol: water for further analysis.

Chromatographic instrumentation and conditions^[14, 15]

The chromatographic analysis was performed out using an Agilent 1220 Infinity II LC system (Agilent technologies, Germany), which was outfitted with a system controller, online degasser, solvent supply module, low-pressure gradient pump, and manual sample injector (injection volume ranging from 5 to 20 µL), & UV-Vis detector. The Agilent OpenLab software was used to operate the instruments and process the data. For chromatographic separation, C-18 column (5 mm, 4.6mm 250 mm, ZORBAX) was utilised. The mobile phase was thought up of different ratios of Methanol & Water. The flow rate of the samples was set to 1 mL/min, and the wavelength was fixed to 298 nm. A 20 µL sample was put into the column for each analysis.

QbD based method development^[16, 17]

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

The utilization of the QbD approach for analytical method development implies the generation of ATP. In this section, the basic qualities that are considered to be indicators of approach performance must be identified. To achieve reliable outcomes, CQA must be recognized from the specified ATP that will be beneficial in assessing the compensating performance of the developed technique.

Optimization of method using design of experiments (DoE)

The DoE was used to evaluate the influence of chosen process factors and their responses on the HPLC procedure. To discover the optimal chromatographic procedure, a basic 2² complete factorial design with two variables and two levels was used, resulting in four experimental runs. Design-Expert software version 13.0 was employed to generate the tests (Stat-Ease Inc., Minneapolis, MN, USA). To identify the critical values required to achieve the desired response of the various independent variables the DoE software was used.

Quality assessment and QbD approached HPLC method development and validation for simultaneous estimation of Gallic acid and Rosmarinic acid

Section A-Research paper

Establishment of method operable design region (MODR)

To determine its suitability, the MODR was created based on the regression models and an evaluation of the chance of failure. Furthermore, the optimal mobile phase was predicted

using the overlay plot displaying MODR. All of the specifications indicated in the ATP are met within the design region at a given risk level. The finest run was chosen based on the criteria of the designated CQAs

Validation ^[10, 18, 19]

Technique validation was carried out in compliance with ICH Q2 (R1) guidelines to validate the consistency of the optimised method. The modified technique's system applicability, linearity, LOD, LOQ, precision, and accuracy were all verified.

Where S is the mean slope of calibration curves and σ is the standard deviation of the y-intercept.

Precision ^[10]

The optimised method's precision was evaluated in terms of intra-day and inter-day precision. The intra-day precision was achieved by assessing repeatability on the same day at different time intervals and the Inter-day precision was assessed over three consecutive days for different analyte concentrations, such as low, moderate, and high concentrations.

System Suitability ^[10, 20]

The suitability of the system was determined by injecting six replicate shots of standards (20 $\mu\text{g/ml}$), accompanied by measurement of the % RSD for the CQAs.

Accuracy ^[10, 21]

The recovery studies were used to prove the method's accuracy. Known quantities of prepared samples were spiked in triplicate injections at 50, 100, and 150% concentration levels. The mean % recovery was calculated for each standard.

Linearity ^[10, 21]

Both gallic acid and rosmarinic acid standard was evaluated for linearity. Five various concentrations ranging from 5 to 30 $\mu\text{g/mL}$ of gallic acid and rosmarinic acid were injected into the HPLC system to produce a linear dynamic range for each. Using least squares regression, the slope, y-intercept, and correlation coefficient (r^2) were calculated to determine linearity.

RESULT AND DISCUSSION

LOD and LOQ ^[10]

The sensitivity of developed method was assessed by computing LOD and LOQ, which are impacted by the SD and slope of the calibration curve. The following formulae were used to calculate LOD and LOQ.

$$LOD = 3.3 \frac{\sigma}{S}$$

Quality assessment of *Terminalia chebula* and *Ocimum sanctum*:

The evaluation of the quality of herbal crude drugs is essential for determining the safety of their usage. All of the physicochemical parameters were found to be within the specified standard limits. The data is given in Table no. 1. *Terminalia chebula* and *Ocimum sanctum* preliminary phytochemical screening Secondary metabolites discovered comprise phenols, alkaloids, flavonoids, tannins, triterpenes, steroids, saponins, and proteins.

Table no. 1 Quality assessment of *Terminalia chebula* and *Ocimum sanctum*

Parameters	<i>Terminalia chebula</i>	<i>Ocimum sanctum</i>
Moisture content	2.58±0.289	0.75±0.215
Aqueous soluble extractive value	8.63±1.06	4.1±0.282
Alcohol soluble extractive value	13.2±0.616	8.7±0.163
Acid insoluble ash value	10.41±1.55	8.66±1.312
Total ash value	2.75±0.735	0.76±0.205
Water-soluble ash value	1.6±0.294	3.36±0.277

4773

Eur. Chem. Bull. 2022,11 (Regular Issue 12), 4771-4779

Chromatographic conditions

In the beginning, chromatographic tests were carried out by carefully reviewing the prior scientific reports on the chromatographic separation of gallic acid and rosmarinic acid that were available. The majority of previously published method i.e. for the use of acetonitrile, methanol, and other organic solvents. In order to simultaneous estimation of gallic acid and rosmarinic acid, we attempted to develop a perfect method using a systemic ratio of methanol and water as an aqueous phase with the best flow rate and wavelength.

QbD assisted RP-HPLC method development

The analytical target profile is a description of the different qualities that are unquestionably representative of technique performance and consequences. The selection of an analytical target is influenced by taking the technique objective into consideration. In the AQbD approach, defining ATP in relation to quality attributes is the first step. The objective of the proposed analytical method in the current investigation was to obtain a good separation for the measurement of gallic acid and rosmarinic acid with an acceptable analysis time and a modest tailing factor. CQAs were recognised as tailing factor (Not More Than 2) and theoretical plates based on the aforementioned Analytical target profile (Not less than 3000).

The rationale for choosing the tailing factor and theoretical plates as CQAs depends on the accuracy of each marker compound's quantitation. Tailing factor and theoretical plates have been chosen in order to achieve precise peak integration because quantitation accuracy declines as tailing factor increases. To accurately quantify the two chosen marker compounds, this is also crucial.

Method optimization by DoE

Two independent variables, notably mobile phase ratio (X1) and flow rate (X2), were varied at two different levels that were coded for low and high (-1 and +1, respectively), in accordance with the applied 22 complete factorial design. For the experiments, response factors such as the tailing factor and theoretical plates were used. Table no. 2 shows the selected experimental design variables resulting from four chromatographic experiments. The analytical method was further statistically improved by analysing numerous statistical parameters offered by Design-Expert Software, Version 13. Table no. 3 summarises the statistical data for the used design. The relationship between the independent variables (Mobile phase ratio (X1) and Flow rate (X2) and responses (Tailing factor of GA (R1), theoretical plates of GA (R2), Tailing factor of RA (R3), theoretical plates of RA (R4)) was represented by mathematical expressions in the form of polynomial equation

Table no. 2 Experimental design for screening of independent variables and responses

	Responses							
	Coded levels		Actual levels		Tailing factor GA	Theoretical plates GA	Tailing factor RA	Theoretical plates RA
Code	X ₁	X ₂	X ₁	X ₂	R ₁	R ₂	R ₃	R ₄
Trail 1	1	1	55:45	1.1	1.46	6263	1.48	6161
Trail 2	1	-1	55:45	1.0	1.21	6624	1.29	6641
Trail 3	-1	1	50:50	1.0	1.69	4798	1.62	4329
Trail 4	-1	1	50:50	1.1	1.47	5012	1.45	4976

X₁: Mobile phase ratio, X₂: Flow rat

Table no. 3 Summary of statistical parameters and polynomial equations.

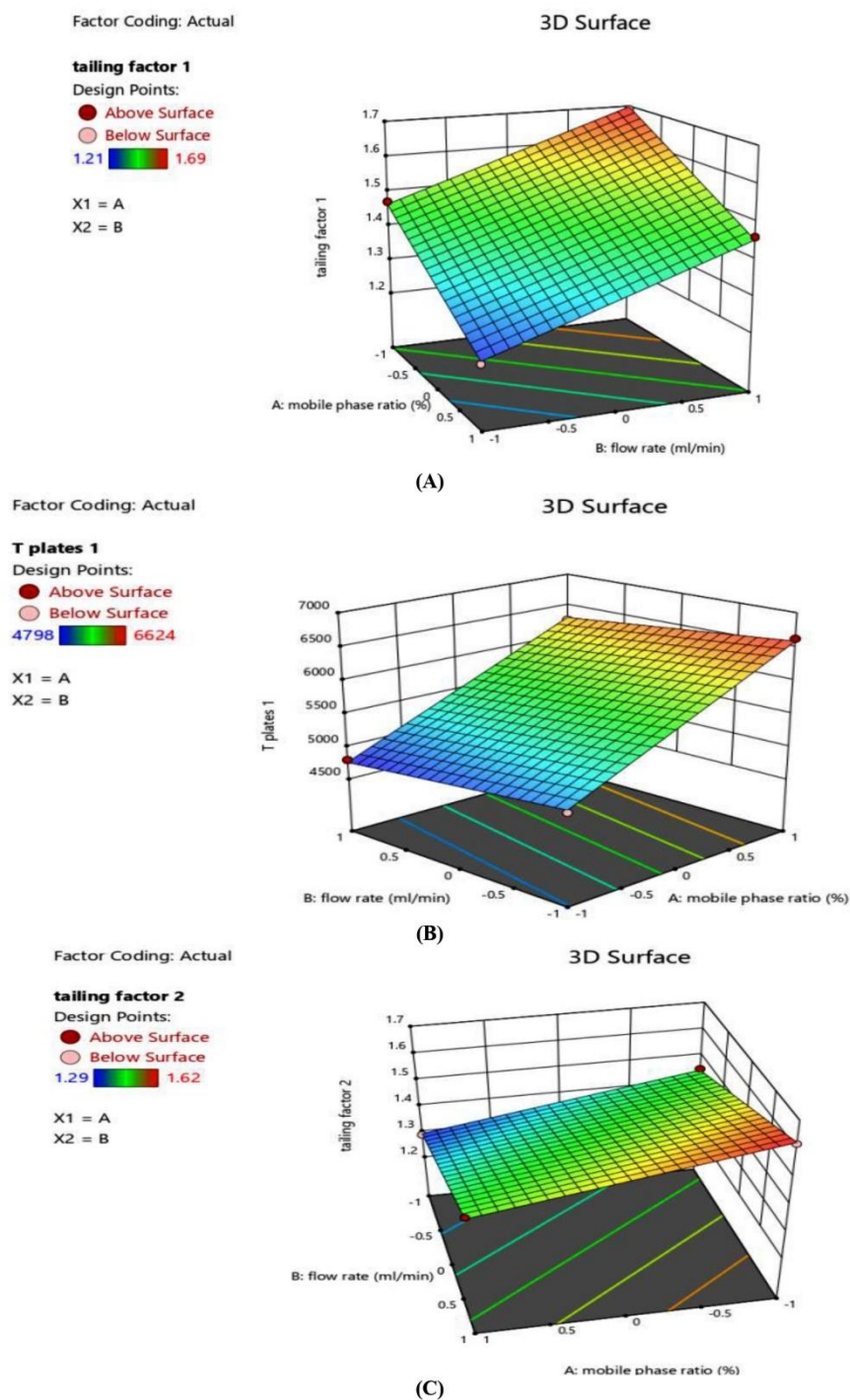
Response		p-value	Model significance	Polynomial equation
G A	R ₁	0.0441	Significance	+1.45-0.122*X ₁ +0.1175*X ₂
	R ₂	0.0469	Significance	+5674.2+769.2*X ₁ -143.7*X ₂
R A	R ₁	0.0426	Significance	+1.46-0.075*X ₁ +0.0900*X ₂
	R ₂	0.0454	Significance	+5526.7+874.25*X ₁ -281.75*X ₂

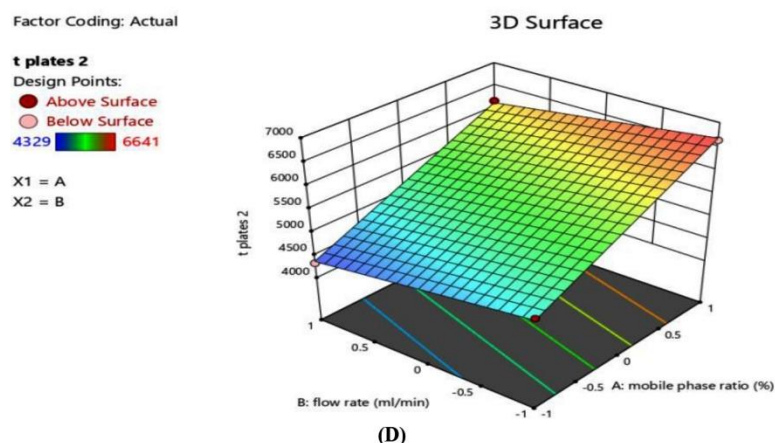
A positive coefficient sign signifies a synergistic impact, whereas a negative term signifies an antagonistic influence on the reaction. A higher coefficient indicates that the independent variable has a significant influence on the result. The Response Surface Plots were generated in order to graphically illustrate the each factor's impact on the responses

shown in Figure 1. The response surface plots demonstrate the connection between each dependent variable (CQA) and the independent variables. The Response Surface Figure showed that blue indicates a reduced tailing factor and red indicates a greater tailing factor in the case of response, R1 (Tailing factor GA), and R3 (Tailing factor RA).

Quality assessment and QbD approached HPLC method development and validation for simultaneous estimation of Gallic acid and Rosmarinic acid

Section A-Research paper





(D)

Figure 1. 3D response surface plots for dependent valuables, tailing factor of GA (A), theoretical plates of GA (B), tailing factor of RA (C), theoretical plates of R (D).

Establishment of MODR

Analytical Trial 2 (Methanol and Water Ratio 55:45) from the method operable Design incorporates various into the area of successful operating ranges and satisfies the ATP and CQA criteria for HPLC method depicted in Figure 2 The effectiveness of chromatographic conditions to produce

decreased tailing factor and peak width was a criterion for choosing the best run. Consequently, trial 2 with Methanol and Water (55:45) was selected as the most effective HPLC method and tabulated in Table no. 4 and The HPLC chromatogram for standard gallic acid and rosmarinic acid samples have been shown in Figure 3.

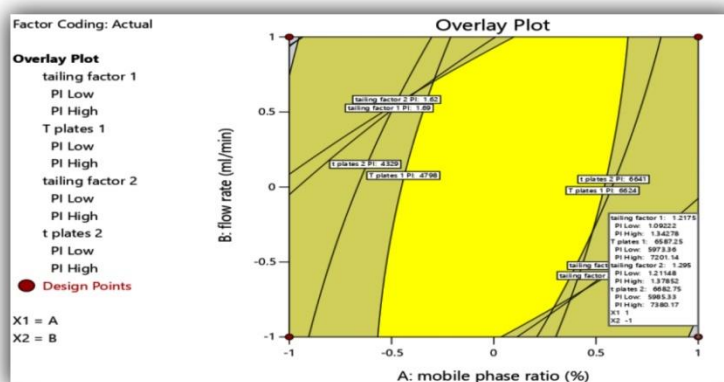


Figure 2. Overlay plot showing the design space with optimal analytical conditions.

Table no. 4. Optimized Chromatographic Conditions.

Parameters	Chromatographic conditions
Stationary phase	ZORBAX C18 (250mm 4.6mm, 5m) column
Mobile phase	Methanol: Water
Mobile phase ratio	55:45
Flow rate	1 mL/min
Detection wavelength	298nm
Injection volume	20µL
Retention time of Gallic acid	2.6 min
Retention time of Rosmarinic acid	4.3 min

4776

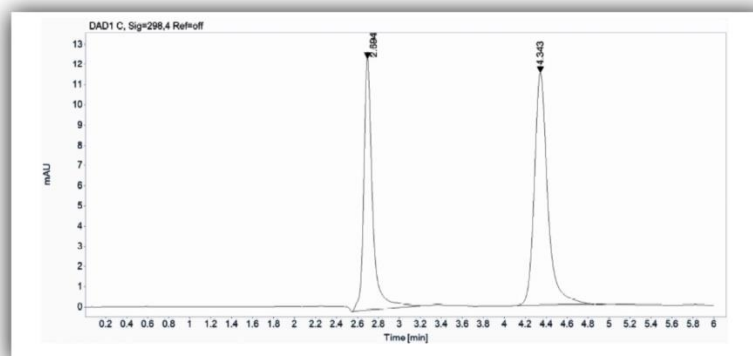


Figure 3. The HPLC chromatogram of simultaneous estimation of gallic acid and rosmarinic acid.

Method validation:

Table 5 provides a summary of the method validation data for the proposed RP-HPLC method. The % RSD of various parameters, including peak area, retention time, and tailing factor, was utilized to confirm the developed method's system suitability. The % RSD of peak area, retention time, and tailing factor were all within acceptable ranges. For the measured concentration ranges, the linear calibration curve for GA and RA was obtained. The LOD and LOQ were

determined using the data from the calibration curve's linear regression equation. The reproducibility and repeatability of an analytical method are the representations of its precision. The % RSD of intra-day and inter-day for gallic acid and rosmarinic acid reveals the developed method's high precision. The percentage recovery was calculated by analysing the produced sample at three distinct concentration levels, namely 50, 100, and 150%. As a conclusion, the observed results show that the developed method is reliable.

Table 5. Validation data of the proposed method.

Validation parameters	Gallic acid	Rosmarinic acid
System suitability		
Retention time Mean $\pm$ SD and %RSD	2.68 $\pm$ 0.0168 and 0.89%	4.27 $\pm$ 0.127 and 1.02%
Theoretical plates Mean $\pm$ SD and %RSD	6624 $\pm$ 0.0324 and 0.68%	6641 $\pm$ 0.0781 and 1.12%
Tailing factor Mean $\pm$ SD and %RSD	1.510 $\pm$ 0.0254 and 0.85%	1.358 $\pm$ 0.0874 and 0.65%
Linearity		
Linearity range μ g/mL	5-25	5-25
Correlation coefficient	0.9983	0.9984
LOD (μ g/mL)	0.146128	0.146509
LOQ (μ g/mL)	0.442813	0.443968
Precision		
Intra-day (% RSD)	1.84	1.31
Inter-day (% RSD)	1.55	1.12
Accuracy		
50% recovery	95.70182	96.44044
100% recovery	99.02062	98.86455
150% recovery	98.8373	99.3223

n=3

CONCLUSION:

The research study reveals the effective application of analytical QbD for the development of easy and reliable HPLC method for the simultaneous estimation of GA and RA.

The quality and safety of the crude sample were confirmed by the positive results of all the quality control tests used to evaluate the quality of the sample of crude herbs. Quite few more advantages of the newly developed method include reduced analytical time, successful separation in terms of well-defined peaks, and the employment of a simple mobile

4777

Eur. Chem. Bull. 2022,11 (Regular Issue 12), 4771-4779

phase combination. Through the use of QbD concepts and DoE tools, it was possible to determine the influencing factors that were thought to be crucial for achieving the ideal chromatographic conditions for the precise measurement of both markers. It can be stated that using the QbD approach culminated in the development of a more robust approach that can generate dependable, consistent, and high-quality data throughout the process while also reducing time. In addition, the method was validated in compliance with ICH requirements

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this research.

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4779

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RESEARCH ARTICLE

High-Performance Liquid Chromatography–Based Standardization of Stigmasterol in *Moringa oleifera*: Method Development and Validation Through Design of Experiment Approach

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ABSTRACT

The primary objective of this investigation was to develop and validate a robust reversed-phase high-performance liquid chromatography (RP-HPLC) method for quantifying stigmasterol in both *Moringa oleifera* powder and tablets. The analysis was conducted using a Box–Behnken design. Stigmasterol separation was achieved with a Phenomenex Luna C-18 column ($4.6 \times 150 \text{ mm}^2$, $5 \mu\text{m}$) and a mobile phase consisting of methanol and 0.1% formic acid in an 80:20% v/v ratio. The separation was performed at a flow rate of 1 mL/min, and stigmasterol was detected at a wavelength of 210 nm using a photodiode array detector. Method validation followed the guidelines set forth by the International Council for Harmonisation (ICH) Q2 (R1). This rigorous validation process evaluated critical parameters, including linearity, accuracy, system suitability, precision, and robustness. The results for each parameter were well within the acceptable range, confirming the reliability and efficacy of the established RP-HPLC method. The findings of this study highlight the potential of the RP-HPLC method as a dependable tool for the routine analysis of stigmasterol in herbal formulations.

1 | Introduction

Herbal medicines have been used for centuries as a primary source of healthcare across various cultures worldwide. Their popularity persists due to perceived effectiveness, fewer side effects, and cultural acceptance [1]. However, ensuring the consistency and quality of herbal medicines poses significant challenges due to the inherent variability in plant materials and extraction methods. Standardizing herbal phytoconstituents is crucial for guaranteeing their safety, efficacy, and reproducibility [2]. High-performance liquid chromatography (HPLC) has emerged as a powerful analytical technique for standardizing herbal medicines. HPLC offers precise identification and quantification of bioactive compounds present in complex herbal extracts [3]. By

separating individual phytoconstituents based on their chemical properties, HPLC enables the accurate measurement of active compounds, marker compounds, and potential contaminants. This analytical method allows for the development of quality control standards and regulatory guidelines to ensure consistency in herbal formulations [4, 5].

Stigmasterol (Figure 1), a phytosterol found abundantly in various plant sources, including *Moringa oleifera*, possesses notable therapeutic and nutritional properties [6]. *M. oleifera*, commonly known as the “drumstick tree” or “horseradish tree,” is renowned for its rich bioactive compounds with potential health benefits [7]. Stigmasterol, among these compounds, has garnered significant attention due to its hypocholesterolemic,

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1 of 9

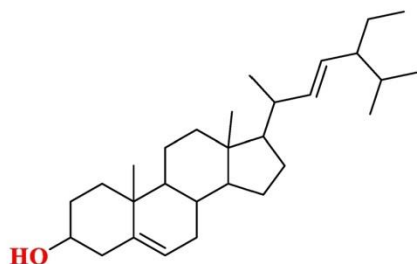


FIGURE 1 | Chemical structure of stigmasterol.

anti-inflammatory, antioxidant, and anticancer properties [8]. Estimating stigmasterol in *M. oleifera* is immensely significant in the pharmaceutical, nutraceutical, and food industries for elucidating its therapeutic potential and ensuring product quality [9–11]. HPLC emerges as a powerful analytical technique for precisely quantifying stigmasterol due to its sensitivity, selectivity, and efficiency in separating complex mixtures. HPLC methodology provides a robust platform for accurately determining stigmasterol content in *M. oleifera* extracts or products [12].

Numerous analytical techniques have been developed for estimating stigmasterol in various plant extracts and certain food materials. Sriraman et al. documented the presence of β -sitosterol and stigmasterol in the hydroalcoholic extract of *Bambusa bambos* using the reversed-phase (RP)-HPLC method [13]. They conducted compound separation through an isocratic mobile phase consisting of methanol and acetonitrile in a ratio of 90:10, with a flow rate of 1.0 mL/min, while maintaining the column at ambient temperature. In another study, Mukhtar et al. reported the determination of stigmasterol in the botanical extract of *Ficus deltoidea*. Separation was achieved using acetonitrile and acetic acid in water (75:25% v/v) in isocratic mode, monitored at 210 nm [14].

Additionally, Nachimuthu et al. reported the quantification of stigmasterol in *Dipteracanthus patulus* (Jacq.) Nees [15]. Meanwhile, Shah et al. documented the simultaneous estimation of phytosterols in *Cissus quadrangularis* [16]. Their proposed method utilized a Cosmosil C8 column (250 $\times$ 4.6 mm²) with a compatible Phenomenex C8 guard column, employing isocratic elution of acetonitrile and water (95:5 v/v) at 25°C. Although numerous researchers have reported single and simultaneous estimations of stigmasterol with other phytoconstituents in various plant extracts, none have specifically investigated the estimation of stigmasterol in *M. oleifera* plant extract. Therefore, the aim of this study was to develop and validate an HPLC analytical method for the concurrent estimation of stigmasterol in *M. oleifera* powder and prepare an in-house tablet formulation using a Design of Experiments (DoE) approach.

2 | Material and Methods

2.1 | Chemicals and Reagents

Stigmasterol (98% purity, as per the certificate of analysis) was obtained from Otto Chemicals Pvt. Ltd., Mumbai, India. HPLC-

grade methanol, acetonitrile, and formic acid were purchased from Hi-Media (Mumbai, India), respectively. Milli-Q water was collected from KLE College of Pharmacy, Belagavi, and the marketed formulation, *M. Oleifera* powder was obtained from a local pharmacy in Belagavi, Karnataka, India.

2.2 | Chromatography Instrument and Conditions

The analysis was performed using a Shimadzu Agilent 1220 Infinity II instrument (LC-20AD, Japan) featuring a quaternary pump with degasser (G7111A), auto-injector (G7129A), and PDA detector (G7115A). Data interpretation and analysis were conducted using the advanced OpenLab CDS software. Chromatographic separation and analysis were executed using a high-quality Phenomenex Luna C-18 analytical column with specifications of C-18(2) 100, an internal diameter of 150 mm, and a length of 4.6 mm, coupled with a particle size of 5 μ m. This column, manufactured by Phenomenex Inc. in Canada, USA, ensures precision and reliability in chromatographic analyses. For the stigmasterol analysis, an optimized mobile phase comprising methanol and 0.1% formic acid (80:20) was prepared and pumped through the column at a constant flow rate of 1 mL/min. Before use, the mobile phase was degassed using a PVDF filter membrane (0.45 μ m; Millex HV, Millipore, USA) with ultrasonic techniques to remove any potential gas bubbles that could interfere with the analysis. During sample analysis, a consistent injection volume of 10 μ L was maintained. The column temperature was set at 35°C, and detection was performed at a specific wavelength of 210 nm. This setup enabled accurate quantification and identification of stigmasterol within the samples under investigation.

2.2.1 | Preparation Stock Solution and Working Standard Solution

The primary stock solution of stigmasterol was prepared at a concentration of 1 mg/mL in methanol. Working standard samples were then prepared by diluting the stock solution with the mobile phase, resulting in concentrations ranging from 5 to 30 μ g/mL. Before conducting the HPLC analysis, all standard solutions were stored in securely sealed volumetric flasks at a temperature of 4°C [17, 18].

2.3 | Preliminary Method Development Studies

Stigmasterol, being least soluble in aqueous phases, necessitated the use of organic solvents such as methanol and acetonitrile for dissolution. Initially, methanol proved to be the optimal solvent, as stigmasterol dissolved completely within it. Consequently, methanol was chosen as the mobile phase for chromatographic analysis. However, our initial attempts using methanol and water as diluents were unsuccessful, as no peaks were discernible in the chromatogram even after a 30-min analysis period. We then substituted water with ortho-phosphoric acid (OPA), which yielded observable peaks. Nonetheless, these peaks exhibited high tailing and a reduced number of theoretical plates, indicating suboptimal separation. In response, we replaced OPA with a mobile phase consisting of 0.1% formic acid in water, in a 70:30 ratio. This adjustment led to the appearance of stigmasterol peaks, albeit

TABLE 1 | Chromatographic factors considered within the Box–Behnken experimental design for the proposed high-performance liquid chromatography (HPLC) method.

Independent variables	Levels		
	Low (–1)	Medium (0)	High (+1)
X_1 = organic phase % (v/v)	70.0	80.0	90.0
X_2 = flow rate (mL/min)	0.8	1.0	1.2
X_3 = column oven temp (°C)	30	35	40
Dependent variables			
Y_1 = RT	Minimum	+++	
Y_2 = tailing factor	Minimum	+++	
Y_3 = theoretical plates	Maximum	+++	

Abbreviation: RT, retention time.

with slight tailing and increased retention times (RTs) exceeding 35 min. To achieve optimal separation and peak characteristics, we implemented a Quality by Design (QbD) approach, utilizing DoE methodologies. This systematic approach allows for the optimization of chromatographic conditions to ensure robust and reliable analytical results.

2.4 | Development of RP-HPLC Method Assisted by Box–Behnken Design (BBD)

The study employs response surface methodology (RSM) in conjunction with a comprehensive HPLC technique, considering both independent and dependent variables. The optimization procedure utilized a specialized design called BBD, featured in version 13.0 of DoE software. Compared to central composite design (CCD), BBD provides superior outcomes for the responses of independent variables, achieved with a reduced number of experimental runs. The investigation focuses on three independent variables: the percentage of organic phase (X_1), flow rate (X_2), and column temperature (X_3). The corresponding dependent variables are RT (Y_1), tailing factor (Y_2), and the number of theoretical plates (Y_3), as outlined in Table 1. During the optimization phase, the percentage of organic phase (X_1) was varied within the range of 70%, 80%, and 90%. The flow rate (X_2) was set at 0.8, 1, and 1.2 mL/min, whereas the column temperature (X_3) was maintained at 30°C, 35°C, and 40°C. Based on the responses obtained, a polynomial equation was formulated, and an exhaustive analysis of variance (ANOVA) was conducted to determine the optimal levels of X_1 , X_2 , and X_3 required to achieve the desired responses. The selected model was examined concerning the response variables: RT (Y_1), tailing factor (Y_2), and the number of theoretical plates (Y_3) [19–25].

2.5 | Sample Processing for *M. oleifera* Powder

An amount of 100-mg *M. oleifera* powder was accurately weighed and dissolved in 100 mL of methanol within a volumetric flask. The resulting solution underwent sonication for 20 min. After sonication, 1 mL was pipetted from the solution and diluted with the mobile phase to achieve a concentration of 10 µg/mL. Subsequently, the diluted solution underwent analysis for stig-

masterol content in the *M. oleifera* powder using the proposed HPLC method.

2.6 | Preparation of *M. oleifera* Extract

The *M. oleifera* plant, sourced locally from Belagavi, Karnataka, underwent rigorous authentication at the Central Research Facility of Shri B.M.K. Ayurveda Mahavidyalaya in Shahpur, Belagavi. Upon collection, the plant samples were cleaned, drained, cut, and subsequently dried to ensure optimal quality for further processing. The extraction of *M. oleifera*'s beneficial components was carried out using two conventional techniques: Soxhlet extraction and cold maceration. Initially, 50 g of powdered *M. oleifera* was macerated with 70% ethanol (v/v) for 24 h. Following this, the resulting marc underwent Soxhlet extraction with 95% ethanol (v/v). The extracts obtained from both techniques were combined, and the solvent was carefully evaporated using a rotary evaporator. The resultant extract was dried and stored in a closed container, shielded from light, to preserve its potency [26, 27].

2.7 | Preparation and Evaluation of *M. oleifera* Tablets

The tablets were prepared using the direct compression method. Initially, 350 mg of *M. oleifera* plant extract and excipients, including croscarmellose sodium (27.5 mg), microcrystalline cellulose (82.5 mg), lactose monohydrate (30 mg), talcum powder (6 mg), and magnesium stearate (4 mg), were accurately weighed for 20 tablets per formulation after passing through a mesh screen number 60. All excipients, except magnesium stearate and talcum powder, were mixed through proper trituration for 30 min in a mortar and pestle along with the extract. Subsequently, the previously sieved and accurately weighed magnesium stearate (lubricant) and talcum powder were added and further mixed for another 10 min. Following mixing, pre-formulation tests, such as angle of repose, loose bulk density, tapped bulk density, compressibility index, and Hausner's ratio, were conducted to assess the powder mixture. Subsequently, round and biconvex-shaped tablets, with an average weight of 500 mg, were compressed using a Rimek 10-station single-rotary tablet press with a die size of 12 mm. A total of 9 formulations, with 20 tablets per formulation,

were prepared. After tablet preparation, post-formulation parameters, such as friability, hardness, disintegration time, thickness, weight variation, and dissolution tests, were carried out to ensure the quality and integrity of the tablets [28, 29].

2.8 | Sample Processing for *M. oleifera* Tablets

A 500 mg tablet was dissolved in 250 mL of methanol and sonicated for 30 min to ensure complete dissolution. After sonication, 20 mL of the solution was extracted and diluted with the mobile phase to attain a final concentration of 10 µg/mL. The diluted solution was then analyzed for stigmasterol content in the *M. oleifera* tablets using the proposed HPLC method.

2.9 | Method Validation

HPLC method validation, in accordance with International Council for Harmonisation (ICH) guidelines, ensures the reliability and accuracy of analytical results. It encompasses parameters, such as linearity, limit of detection (LOD) and limit of quantification (LOQ), system suitability, precision, accuracy, and robustness. By validating the method, one can ensure its suitability for the intended purpose, providing confidence in the quality and integrity of analytical data generated using the HPLC technique [30, 31].

2.9.1 | Linearity

To assess the linearity of the developed method, various concentrations of stigmasterol stock solution ranging from 5 to 30 µg/mL were prepared using mobile phase (methanol: 0.1% formic acid). Six replicate determinations ($n = 6$) were conducted for each test concentration. The resulting peak areas were plotted against the corresponding test concentrations, and statistical analysis was performed using the least squares method. Linearity was evaluated by determining the regression coefficient (R^2) and the y-intercept of the linear regression plot of the response versus concentration.

2.9.2 | LOD and LOQ

The LOD and LOQ for the HPLC technique were determined using the standard deviation method. The following equations were employed to calculate these limits:

$$\text{LOD} = 3.3 \times \frac{\text{SD}}{\text{S}} \quad (1)$$

$$\text{LOQ} = 10 \times \frac{\text{SD}}{\text{S}} \quad (2)$$

2.9.3 | System Suitability

System suitability parameters in the HPLC method validation confirm the proper functioning of the chromatographic system and ensure reliable analytical results. Peak area, theoretical plates, and tailing factor for stigmasterol were determined by

running the samples six times to verify the chromatographic system's suitability.

2.9.4 | Precision

The precision parameter in HPLC method validation assesses the reproducibility and repeatability of results. Intra-day (Repeatability): The standard solution was prepared in triplicates, with concentrations of 10, 15, and 20 µg/mL. The analysis was conducted at three distinct time intervals within the same day. Inter-day (Intermediate): The standard solution was prepared in triplicates, with concentrations of 10, 15, and 20 µg/mL. The analysis was carried out over 3 consecutive days, and the samples were stored in well-closed containers in a dark place.

2.9.5 | Accuracy

Accuracy was evaluated by applying the proposed method to analyze placebos containing predetermined quantities of stigmasterol across the linear range of the method. These quantities represented 50%, 100%, and 150% of the nominal levels. Percentage recovery (%), indicating accuracy, was calculated for standard stigmasterol, *M. oleifera* powder, and prepared tablet samples.

2.9.6 | Robustness

The robustness of the developed method was evaluated by introducing minor variations in chromatographic conditions, including changes in mobile phase volume (78:22% and 82:18%), flow rate (0.8 and 1.2 mL/min), and detection wavelength (208 and 212 nm).

3 | Results and Discussion

3.1 | Development of RP-HPLC Method Assisted by BBD

The BBD was employed to optimize chromatographic conditions, with the volume of the organic phase, flow rate, and column temperature selected as independent variables. The dependent variables, including RT, tailing factor, and theoretical plates, were designated as responses. Design of Expert software version 13.0 was utilized to interpret the acquired results. A total of 15 runs were generated by the software, including 3 center points, as listed in Table 2. ANOVA is used in software to analyze experimental data and assess the significance of factors and their interactions on the response variables. The statistical data of the applied design are summarized in Tables S1–S3. Polynomial equations are employed to establish the relationship between independent variables (factors) and response variables. Positive values indicate synergistic effects, whereas negative values suggest antagonistic effects on the model. Strong correlations among all equations ensure the attainment of desired optimal values for the selected responses (Table 3).

TABLE 2 | Experimental runs, variables, and measured responses using the response surface method (Box–Behnken) for the optimization study.

Run	Factor 1 A: Organic Phase %	Factor 2 B: Flow rate mL/min	Factor 3 C: Column Temp (C)	Response 1 RT (Min)	Response 2 TF	Response 3 TP
1	80	0.8	30	17.6	1.1	7253
2	70	0.8	35	22.5	1	6587
3	90	0.8	35	13.4	1.39	7265
4	90	1	40	12.8	1.43	7298
5	80	1	35	15.04	1.22	8764
6	70	1	40	20.9	1.02	6642
7	80	0.8	40	16.8	1.05	7569
8	80	1.2	30	14.21	1.3	7542
9	80	1	35	15.02	1.21	8762
10	70	1.2	35	19.1	1.09	6720
11	70	1	30	21.2	1.08	6652
12	90	1	30	13	1.41	7165
13	90	1.2	35	12.1	1.44	7192
14	80	1.2	40	14	1.31	7432
15	80	1	35	14.98	1.23	8738

Abbreviation: RT, retention time.

TABLE 3 | Regression equation coefficients (polynomial equations) of selected variables with 95% CI.

Sr. no.	Variables	Constant	A	B	C	AB	AC	BC	A ²	B ²	C ²
1	Retention time (min)	15.01	−4.05	−1.36	−0.188	0.525	0.025	0.147	1.54	0.219	0.419
	(95% CI)	±1.54	±1.51	±1.18	±0.76	±0.24	±0.020	±0.088	±0.30	±0.20	±0.094
2	Tailing factor	1.22	0.185	0.075	−0.010	−0.010	0.020	0.015	0.027	−0.017	−0.012
	(95% CI)	±0.095	±0.014	±0.036	±0.0065	±0.005	±0.003	±0.004	±0.023	±0.004	±0.016
3	Theoretical plates (N/m)	8754.6	289.8	26.5	41.1	−51.5	35.7	−106.5	−1161.7	−651.9	−653.71
	(95% CI)	±305.53	±13.93	±3.45	±4.66	±5.24	±4.14	±8.95	±83.45	±12.62	±12.90

The response surface plots for Y_1 (RT) show that increasing the organic phase decreases RT, whereas increasing flow rate increases RT. Column temperature has minimal impact, with a slight decrease in RT as temperature rises (Figure 2a). For Y_2 (tailing factor), increasing both the organic phase concentration and flow rate escalates the tailing factor, indicating a direct correlation (Figure 2b). Column temperature has no discernible effect on the tailing factor. For Y_3 (theoretical plates), increasing the organic phase concentration and flow rate up to 80% v/v and 1 mL/min, respectively, increases the number of theoretical plates, suggesting better separation efficiency. However, beyond these levels, the number of theoretical plates declines (Figure 2c). Column temperature has minimal effect on Y_3 , remaining relatively consistent across variations. Using Design-Expert software version 13.0 by Stat-Ease, Inc., optimal values for the parameters were identified: 80% v/v organic phase, 1 mL/min flow rate, and 35°C column temperature. These predictions closely matched experimental results, particularly in Run 9, with a low prediction error of 5%. Under these optimal conditions, excellent chromatographic separation of the drug was achieved (Figure 3).

3.2 | Recovery Assay in *M. oleifera* Powder

The proposed method was successfully employed to determine stigmasterol in *M. oleifera* powder, with six replicate measurements conducted. Results outlined in Table 4 exhibit outstanding recoveries spanning from 96.1% to 98.6%. Quantitative estimation revealed distinct peaks for stigmasterol, emphasizing the method's exceptional sensitivity, accuracy, and precision. This reaffirms the reliability of the obtained results, as vividly depicted in Figure 3.

3.3 | Evaluation of Prepared *M. oleifera* Tablets

Tablets were formulated utilizing the direct compression method, each containing 500 mg. These tablets underwent comprehensive evaluation for both pre- and post-compression parameters. Results revealed superior attributes, particularly notable for their exceptional hardness, friability, and disintegration time. Furthermore, the prepared blend underwent assessment, encompassing

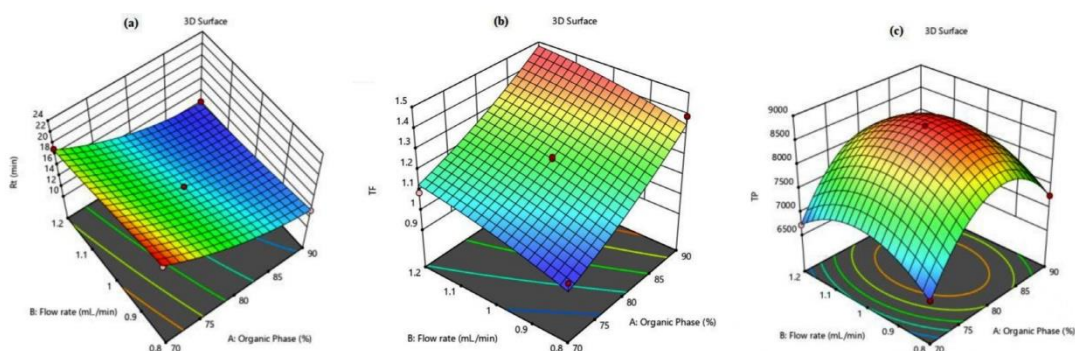


FIGURE 2 | (a) Response surface plot of response Y_1 (retention time), (b) Y_2 (tailing factor), and (c) Y_3 (theoretical plates).

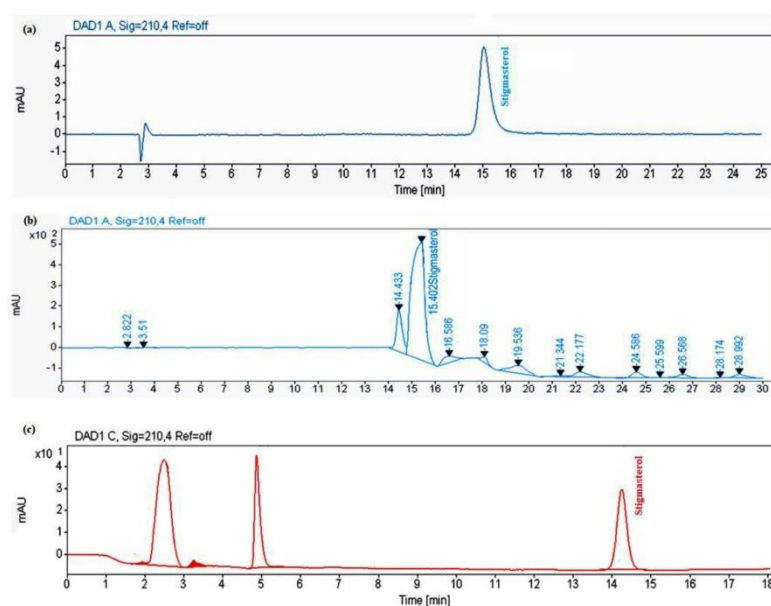


FIGURE 3 | HPLC chromatogram of (a) standard stigmasterol (10 µg/mL), (b) *Moringa oleifera* powder (10 µg/mL), and (c) *M. oleifera* tablet (10 µg/mL). HPLC, high-performance liquid chromatography.

TABLE 4 | Recovery assay of stigmasterol in *Moringa oleifera* powder and prepared tablets.

Sample	Excess samples added to analytes (%)	Spiked (µg/mL)	Found (µg/mL)	Recovery (%)
Powder	50	15	14.79 ± 0.21	98.6
	100	20	19.22 ± 0.43	96.1
	150	25	24.34 ± 0.03	97.3
Tablets	50	15	13.82 ± 0.12	87.2
	100	20	16.2 ± 0.05	81.1
	150	25	20.5 ± 0.23	82.2

parameters, such as angle of repose, tapped density, loose bulk density, compressibility index, and Hausner's ratio. The obtained results fell well within the accepted ranges, indicating the formulation's quality and consistency. Specifically, the tablets exhibited hardness ranging from 3.2 ± 0.02 to 4.8 ± 0.21 kg/cm², thickness between 6.7 ± 0.08 and 7.1 ± 0.18 mm, friability spanning from $0.65\% \pm 0.02\%$ to $0.92\% \pm 0.21\%$, and disintegration time varying from 11.46 ± 0.05 to 13.2 ± 0.2 min. The selected optimized formulation underwent a drug release study to assess the release profile of stigmasterol. The study involved measurements at various time intervals: 0.5, 1–8, 12, and 24 h. After 24 h, the results revealed that 76.42% of stigmasterol, a cumulative drug, was released from the formulation (Figure S1). Additionally, the weight variation test yielded results ranging from 499.1 ± 0.25 to 503 ± 0.06 mg, further attesting to the tablets' uniformity and compliance with standards.

3.4 | Recovery Assay in Prepared *M. oleifera* Tablets

The method proposed was effectively utilized to ascertain the presence of stigmasterol in prepared *M. oleifera* tablets through six replicate measurements. As depicted in Table 4, the results showed favorable recoveries ranging from 81.1% to 87.2%. Quantitative analysis showcased clear peaks indicative of stigmasterol, underlining the method's remarkable sensitivity, accuracy, and precision. This reaffirms the reliability of the obtained results, as vividly illustrated in Figure 3.

3.5 | Method Validation

A linear correlation was established through the construction of a calibration curve, wherein the average peak area of the analyte, stigmasterol, was plotted against their respective concentrations spanning from 5 to 30 µg/mL. The linear regression equation derived from this curve facilitated the determination of both the LOD and LOQ. Additionally, to confirm the suitability of the developed method, the % relative standard deviation (% RSD) of various parameters, including peak area, RT, theoretical plates, and tailing factor, was assessed. % RSD values for peak area, RT, tailing factor, and theoretical plates were all found to be less than 2%, signifying the acceptability of the method. The analytical process' robustness was evaluated by making slight adjustments to HPLC parameters, such as wavelength, column oven temperature, and mobile phase ratio. Results indicated that % RSD for both peak area and RT of the analyte remained comfortably within the acceptable threshold of $\leq 2\%$. Furthermore, assessments of intra- and inter-day precisions revealed the high precision of the developed method. The degree of accuracy, gauged through percentage recovery, showcased a strong level of precision at concentrations of 50%, 100%, and 150%. These findings collectively underscore the reliability and robustness of the newly developed method, as outlined in Table 5.

4 | Conclusion

The study underscores the pivotal role of HPLC in ensuring the quality and consistency of herbal medicines, particularly

TABLE 5 | Summary of validation parameters.

Sr. no.	Validation parameters	Stigmasterol
1	Linearity	
	Linearity range (µg/mL)	5–30
	Slope	6.2857x
	Intercept	2.4333
2	Correlation-coefficient	0.9994
	LOD (µg/mL)	1.52
	LOQ (µg/mL)	4.592
4	System suitability	
	Peak area (% RSD)	1.2
	Retention time (% RSD)	0.32
	Theoretical plates (% RSD)	0.69
	Tailing factor (% RSD)	1.31
3	Precision	
	Intra-day (% RSD)	0.85
	Inter-day (% RSD)	0.98
4	Robustness	
	Mobile phase ratio (% RSD)	0.85
	Change in the wavelength (% RSD)	1.74
	Column oven temp (% RSD)	1.34
5	Accuracy	
	50% recovery	96.72
	100% recovery	97.68
	150% recovery	99.01

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; % RSD, % relative standard deviation.

focusing on the estimation of stigmasterol in *M. oleifera*. Through method development and validation, the proposed HPLC method demonstrated exceptional sensitivity, accuracy, and precision in quantifying stigmasterol in both *M. oleifera* powder and prepared tablets. The BBD facilitated the optimization of chromatographic conditions, revealing significant influences of organic phase concentration and flow rate on response variables, such as RT, tailing factor, and theoretical plates. The developed method exhibited robustness and reliability, as evidenced by low % RSD values for key parameters and high recovery rates across various concentrations. Moreover, the method's accuracy was confirmed through percentage recovery assays, highlighting its suitability for pharmaceutical and nutraceutical applications. Overall, this study underscores the importance of rigorous method development and validation in ensuring the quality and efficacy of herbal formulations, paving the way for standardized approaches in herbal medicine research and production.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Research data are not shared.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

ANNEXURE – III

PRESENTATIONS







SOCIETY OF PHARMACOGNOSY
(Formerly Known as Indian Society of Pharmacognosy)

&

SHARADCHANDRA PAWAR COLLEGE OF PHARMACY
At- Dumberwadi, Post - Khamundi, Maharashtra, India-410504. Website: www.spcop.in

Organizes

**26th National Convention of Society of Pharmacognosy &
International Conference on NEW VISTAS OF HERBAL AND NATURAL PRODUCTS: INDIAN CONTEXT**
2nd & 3rd April 2023

CERTIFICATE

This is to certify that Ms./Mr. Mahantesh Kunchanur has participated in the above Convention held at SGMSPM's Sharadchandra Pawar College of Pharmacy, Dumberwadi, Otur, Pune, Maharashtra, India as

☐ Delegate ☐ Chairperson ☐ Resource Person ☒ Oral Presenter ☐ Poster Presenter ☐ Member LOC



Dr. Navin Sheth
Vice Chancellor GTU
President Society of Pharmacognosy



Dr. Umesh Patil
General Secretary, ISP
Society of Pharmacognosy



Dr. Machindra Chavan
LOC, Organizing Secretary
26th ISP





Dr. Ganesh Dama
LOC, Chairman
26th ISP

	
CERTIFICATE	
<i>This is to certify that</i>	
<u>Mr/Ms/Mrs/Dr/Prof. Mahantesh Kunchanur</u>	
<u>Asso. Professor, KLE College of Pharmacy Nipani - 591237</u>	
has participated at the International Conference on Pharmacovigilance, Pharmaceutical Care and Biomedical Research, Organized by Sarada Vilas College of Pharmacy, Mysuru in association with Association of Community Pharmacists of India (ACPI), South India Branch, held on 24th and 25th January 2023.	
 Dr. Ananth Nayak Nagappa President ACPI	 Dr. Hanumanthachar Joshi Convener
 Dr. Charan C. S Organizing Secretary	 Dr. Jinesh Nagavi Jt. Organizing Secretary

ANNEXURE – IV

NPTEL CERTIFICATE

	NPTEL-AICTE Faculty Development Programme (Funded by the MoE, Govt. of India)			This certificate is awarded to MAHANTESH KUNCHANUR for successfully completing the course Health Research Fundamentals with a consolidated score of 62 %	 Prof. Andrew Thangaraj NPTEL Coordinator IIT Madras		(Jul-Sep 2023)	Roll No: NPTEL23HS93S34630378	Duration of NPTEL course : 8 Weeks	The candidate has studied the above course through MOOCs mode, has submitted online assignments and passed proctored exams. This certificate is therefore acceptable for promotions under CAS as per AICTE notifications dated 24th July 2018, similar to other refresher / orientation courses. F.No. AICTE / RIFD / FDP through MOOCs / 2017-18
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