

Formulation, Standardization, and Evaluation of Phytopharmaceutical Dosage Form with Anti-Infective Activity

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

by

Rahul Dhirajbhai Lalwani

229999901024

under supervision of

Dr. Kunjan Bodiwala

Associate Professor,

L. M. College of Pharmacy, Ahmedabad- 380009



GUJARAT TECHNOLOGICAL UNIVERSITY, AHMEDABAD

OCTOBER 2026

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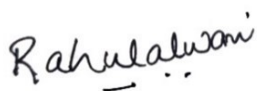
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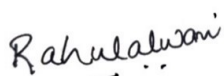
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Abstract

The current global health landscape is increasingly defined by the escalation of antimicrobial resistance, necessitating the transition from empirical traditional medicine to evidence-based, regulated phytopharmaceuticals. This research investigates the therapeutic potential of *Kigelia africana* fruit extract, addressing the critical gap between ethnobotanical utility and modern pharmaceutical standardization.

The study's methodological rigor is anchored to development of validated High-Performance Thin-Layer Chromatography (HPTLC) methods for simultaneous and precise quantification of four key markers: verbascoside, caffeic acid, ferulic acid, and coumaric acid. To optimize extraction efficiency, a 3² full factorial Design of Experiments (DOE) was implemented to analyze the synergistic effects of solvent composition and duration. The optimized extract, was rigorously screened via Headspace-Gas Chromatography (HS-GC) to ensure residual ethanol remained well below the minimum inhibitory concentration (MIC) for relevant pathogens. This ensures that the observed antimicrobial efficacy is intrinsic to the phytochemical profile rather than the solvent. This systematic approach transforms extraction from a simple preparation step into a strategic determinant of therapeutic quality.

The subsequent development of a topical gel utilized a Quality by Design (QbD) framework, systematically linking material attributes to Critical Quality Attributes (CQAs). The resulting dosage form demonstrated significant *in-vitro* activity against *Staphylococcus aureus* and *Escherichia coli*. Accelerated stability studies conducted under ICH Q1A(R2) guidelines confirmed the formulation's physical and chemical integrity, supporting a projected shelf life of three years.

This thesis offers a sophisticated scientific framework for the translational development of plant-derived anti-infective. By integrating advanced analytical validation with engineering-driven optimization, it provides a scalable, standardized pathway for botanical medicines to address the therapeutic void left by failing synthetic alternatives.

Keywords: *Kigelia africana*, Phytopharmaceutical, Anti-infective, topical gel, design of experiments, factorial design, extraction, verbascoside, caffeic acid, coumaric acid, ferulic acid

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“No matter how hard things are, you never give up, He always shows you the way.”, “Everything happens for its own good” are the two quotes from my parents which have and will always stay with me. This have been the foundation of my education. This journey has been joyful, enjoyable, overwhelming with rollercoaster of emotions, failures, tiny successes and a truly enriching experience.

It is an honour and privilege as I pen down this thesis as the final step towards a goal which never occurred to me ten years ago as I began my formal education as a pharmacist. As the college journey began, I did not what was research, how is it done, why is it performed, and all the whereabouts. All I knew was a pharmacist dispenses medicines from prescription and their therapeutic actions, combination of medicines to be given. Not an ounce more of knowledge I had it within me. The formative years in my bachelors paved the way for me to get into research, the know-hows, the linkage with all the subjects and how important it is to synchronize them to achieve one common goal- a better medicine. During my graduation, my heart was always belonged to pharmacology until pharmaceutical analysis swept me off my feet and immersed me into the universe of analysis. Though the soft corner for pharmacology still exists today, my head always jumps at the latter one. In my post-graduation, I had the privilege of joining one of the reputed and oldest colleges in the country-L. M. College of Pharmacy. This is where I met my guide, supervisor, mentor which also inspired me to get into research and how without analysis the chain of medicine discovery always falls short.

This is thesis is an abode to the Almighty my Guru, my mother-Geeta Lalwani, my father-Dhiraj Lalwani and my sister-Harshita. It has been a blessing in all ways to be their child who have empowered me from my childhood till date in taking independent

decisions. They have me shown the world through their eyes, to which the debts can never be paid. They both did not know what education lies beyond bachelors of pharmacy. Their innocence, trust, belief and conviction they've had in me has been my strength all the way. Next up is my loving younger sibling who has been the joy and fun in this journey. She has been the receiving end of the dejections, failures I have faced all these years, her smile and comfort have never let me to give things up. The goal was never mine; it was ours.

What do I say about my supervisor? I am short of words for him. We met during my post graduate studies. His love for core pharmaceutical analysis, teaching and the gem of a person he is what convinced me to choose him as my guide, mentor for the goal to be achieved. The clarity of thoughts, approaches to get a work done is the primary common thing between us. Though he almost convinced me to do part-time degree, I was never convinced fully. He has taught me good core Gujarati lines and words I am grateful besides pursuing this degree under him, we've played together in badminton and cricket. Though, he is a champion-player in table tennis, I could never learn the sport.

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always made me sit and got me out of the situations in which not all people do. They have been true leaders and gracious at every step of the way.

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Annexure VI List of conference presentations

CHAPTER-1

INTRODUCTION

1. Introduction

Antimicrobial resistance (AMR) has emerged as a critical global health crisis. Current estimates indicate that resistant infections already claim about 50,000 lives per year in Europe and the United States. Moreover, the economic and social burden is enormous: failure to control AMR could lead to an additional 10 million deaths annually by 2050, with cumulative global costs approaching 100 trillion USD. A recent analysis described AMR as a “silent pandemic,” estimating that 1.2 million deaths in 2019 were directly attributable to resistant bacterial infections worldwide, and projecting this could rise to ~20 million deaths per year by 2050 if unchecked. These figures underscore the urgency of new therapeutic strategies beyond conventional antibiotics.

The World Health Organization (WHO) has repeatedly warned that the post-antibiotic era is no longer a distant possibility but an imminent reality if urgent measures are not undertaken. Resistant bacterial infections already account for millions of deaths annually worldwide, with projections suggesting that by 2050 AMR could surpass cancer as the leading cause of mortality. The burden is not evenly distributed, with low- and middle-income countries carrying the greatest share due to high infection prevalence, limited access to newer drugs, and weaker health systems.

It has been estimated that over 40 million individuals worldwide suffer from bacterial and fungal infections, which are among the most common diseases to impact the human body, particularly the skin. More than 1.5 million different types of fungi exist, and about 100 are thought to cause serious human diseases. Numerous fungi can cause diseases such as invasive mycoses, allergies, and superficial infections, including *Candida albicans* (*C. albicans*) (yeast), *Aspergillus fumigatus* (*A. fumigatus*) (a filamentous fungus), *Cryptococcus neoformans* (*C. neoformans*), and *Trichoderma spp.* Drugs known as antifungal medicines are used to selectively treat a fungal infection while also eradicating fungal pathogens without

harming the host. Different antifungal substances operate in various ways. Some antifungal substances interact with the sterols or ergosterol found in fungal cell membranes. In contrast, other antifungal substances inhibit fungal enzymes such as cytochrome P₄₅₀ that are essential for the structure and function of ergosterol production. These antifungal medicines fall into several categories, including polyene antifungal drugs, azole antifungal drugs, allylamine antifungal drugs, and others. The pathogens *C. albicans* and *Aspergillus spp.* are the most frequently found. However, other fungi, such as *Candida spp.* that are not albicans, are playing a bigger role in systemic fungal infections. Numerous antifungal medications, such as azoles, allylamines, echinocandins, griseofulvin, and flucytosine, are therapeutically used to treat fungal infections. Drug resistance, in particular, has caused several issues and complications along the path to modern treatment. Compared with synthetically produced medications, phytochemicals from different plant species have shown promise as a source of medicine.

The golden era of antibiotic discovery, spanning the 1940s to the 1970s, provided a diverse arsenal of antibacterial agents. However, the pace of discovery has slowed dramatically in recent decades. Very few new classes of antibiotics have reached the clinic since the 1980s, and those that have are often modifications of existing scaffolds. Meanwhile, bacteria continue to evolve rapidly, rendering many of these agents ineffective. The mismatch between resistance development and antibiotic innovation has created a dangerous gap. WHO has described the antibiotic pipeline as “insufficient to address the challenge of AMR.”

Topical antibiotics, while essential in dermatology, are particularly susceptible to misuse. Over-the-counter availability, improper dosing, self-medication, and incomplete treatment courses contribute to sub-therapeutic exposure. This fosters the selection of resistant strains, particularly in community settings with limited monitoring. Compounding this problem is the widespread use of antibiotics in

agriculture and animal husbandry, which further drives resistance through environmental dissemination of resistant bacteria and genes. Collectively, these factors underscore the urgent need to diversify the therapeutic landscape beyond conventional antimicrobials.

1.1 The Need for Alternative Therapies

Recognizing that traditional antimicrobial discovery cannot outpace resistance has prompted a search for novel therapeutic strategies. Alternative approaches include bacteriophage therapy, antimicrobial peptides, immunotherapies, and drug repurposing. Among these, medicinal plants occupy a distinctive niche. Plants have co-evolved with microorganisms for millions of years, producing a diverse array of secondary metabolites as chemical defenses. These metabolites include alkaloids, terpenoids, phenolics, flavonoids, and glycosides, many of which exhibit potent antimicrobial properties.

Plant-derived therapeutics appeal for their chemical diversity and multi-targeted mechanisms. Unlike many conventional antibiotics that act on a single cellular target, phytochemicals often exert their effects through multiple pathways: disrupting membranes, inhibiting enzymes, scavenging free radicals, or modulating host immune responses. This polypharmacology not only enhances efficacy but also reduces the likelihood of rapid resistance development. Furthermore, botanical therapies often display synergistic effects when multiple compounds act in concert, producing activities greater than the sum of individual components. This complexity, once viewed as a limitation, is increasingly recognized as an asset in the fight against AMR.

Regulatory frameworks for botanicals vary by region but generally recognize both traditional use and evidence-based pathways. In the EU/UK, herbal products can obtain simplified Traditional Herbal Registration (THR) for minor indications or full Marketing Authorization for major claims. In the US, the FDA's Botanical Drug Guidance (first drafted in 2004 and finalized in 2006) provides a

pathway for botanical investigational new drug (IND) applications, requiring stringent phytochemical characterization, Good Manufacturing Practice (GMP) controls, and clinical trials akin to those for conventional drugs. Despite this complexity, four botanical drug products have received approval under these frameworks (e.g., sinecatechins for genital warts, crofelemer for HIV-associated diarrhea, birch triterpenes for epidermolysis bullosa, and pineapple-derived proteases for burn debridement). These examples show that botanical formulations with defined composition can meet regulatory requirements by combining modern analysis with traditional knowledge. The widespread use of botanicals as pharmaceuticals in developed nations is leading to their inclusion in industrialized countries' integrative healthcare systems, also known as complementary and alternative medicine systems. In both the medical and social communities, complementary and alternative medicine (CAM) is now accepted. Patients are using CAM (dietary/nutritional supplements, relaxation techniques, and various forms of social support) more frequently.

Given this backdrop, there is an urgent need to explore and rigorously evaluate plant-based antimicrobials.

1.2 Medicinal Plants as Pharmacological Resources

The therapeutic use of plants is as old as human civilization. Ayurveda, Traditional Chinese Medicine, and African ethno-medicine have relied heavily on botanicals to treat infectious and inflammatory diseases. Even in modern pharmacology, approximately 25–30% of approved drugs are either directly derived from plants or inspired by natural products. Quinine from *Cinchona* bark, artemisinin from *Artemisia annua*, and morphine from *Papaver somniferum* exemplify the enduring relevance of plant-based molecules.

In antimicrobial discovery, plants are a promising yet under-explored resource. Their secondary metabolites, shaped by evolutionary pressure, often have unique scaffolds and mechanisms not found in

synthetic libraries. Moreover, ethnopharmacological knowledge provides a rational starting point: plants traditionally used to treat infections are more likely to yield active compounds. Despite this, translating plant remedies into clinically approved drugs faces significant hurdles. These include variability in phytochemical content due to geography and harvest conditions, challenges in large-scale standardization, and the need for rigorous preclinical and clinical evaluation. Nonetheless, advances in analytical chemistry, biotechnology, and regulatory science are increasingly enabling the transformation of ethnobotanical insights into evidence-based phytopharmaceuticals. Medicinal plants provide new, potent chemical compounds needed to strengthen the immune system or support the body's defense against foreign substances. However, because of extensive evolutionary selection, natural products have a wider range of ring systems and are sterically more complex.

A wide spectrum of plant-derived bioactive compounds ranging from saponins to flavonoids has shown measurable antifungal activity in preliminary studies. These secondary metabolites constitute a prolific source of natural agents capable of neutralizing fungal strains in *in vitro* models. Extensive studies of tropical species have helped identify lead compounds with promising safety and efficacy. Lead compounds have significance in the drug development process, research on natural products and compounds derived from them have increased exponentially in recent years. These compounds are used either directly or as starting points for compound synthesis. Secondary metabolites are a good source of novel pharmaceutically significant chemicals. Plants are used as both food and medicine in many cultures. Over the years, traditional civilizations have prescribed pharmacologically active compounds from higher plants for a variety of ailments.

The chemical diversity of medicinal preparations in the plant kingdom makes it a rich source of new drug-development opportunities. Compared with synthetically generated medications, phytochemicals

from different plant species may be a better source of medicine, according to phytochemistry research. Over the past few years, researchers have designed and created several chemical structures with antifungal activity based on natural chemicals. As a result, the plant kingdom holds significant untapped potential that warrants thorough investigation. Drugs made from synthetic anti-inflammatories are hard to come by and have negative side effects. The integration of herbal-based remedies into modern medicine is supported by their multifaceted bioactive profiles, which provide potent remedial action against various ailments. Furthermore, these natural compounds often have reduced systemic toxicity, making them viable candidates for managing chronic conditions. Attributing to their good activity and relatively fewer adverse effects when compared to synthetic medications, herbal formulations have long drawn significant attention. Demand for herbal medicine is rising as more people recognize the benefits of natural drug products, including their lower risk of side effects, accessibility, cultural acceptance, and relative affordability.

1.3 *Kigelia africana*: Botanical profile

Kigelia africana (Lam.) Benth. (*K. africana*) (also known as *Kigelia pinnata*⁽¹⁾) Commonly known as the sausage tree, is a large deciduous tree in the Bignoniaceae family. Endemic to Africa, it is widely distributed across sub-Saharan regions (except for very arid zones). The tree typically grows along riverbanks and in moist woodlands. The common name ‘sausage tree’ derives from its distinctive heavy, cylindrical fruits, which can grow up to 1 m in length and resemble bundles of sausages or club-like pods. It is widely distributed in regions of South, West, and Central Africa, and South Asia: India, Pakistan, Bangladesh, and Sri Lanka⁽²⁾.

Morphologically, *Kigelia africana* presents as a tree with a squat trunk and scaly, light brown bark. It facilitates a broad, rounded canopy of leathery, deciduous foliage that can reach a lateral spread of 20 meters. The species is easily identified by its large, pendulous fruits (800 ×

120 mm) which hang from the branches on long, sturdy stalks, making them a defining characteristic for field identification and botanical study.⁽³⁾ (Figure 1-1)



Figure 1-1 *K. africana*

Beyond its horticultural value as an ornamental species in tropical climates, *K. africana* possesses an extensive profile in traditional medicine. The root bark is frequently used to manage venereal infections such as gonorrhea, while both the fruit and root systems are used to address postpartum hemorrhage. Furthermore, systemic conditions including malaria, renal dysfunction, and gastrointestinal distress specifically dysentery are treated using decoctions of the leaves and stem bark. The fruit serves a dual purpose, acting as a topical dressing for ulcerative lesions and an internal agent with purgative and aphrodisiac properties.

K. africana consists of a wide range of active ingredients like tannins, flavonoids, steroids, anthraquinones, phlobatannins, cardiac glycoside, terpenoids, saponins, elaidic acid, squalene, stearic acid, lapachol, palmitic acid, pinnatal, isopinnatal, kigelinol, isokigelinol, Beta-tocopherol, hentriacontane, kojic acid, norviburtinal, specioside, verminoside, and minecoside.⁽⁴⁻⁶⁾

Cumulative pharmacological investigations into *K. africana* reveal a diverse profile of therapeutic indices, ranging from anti-leprosy and anticancer potentials to acute antimalarial activity. Quantitative studies have further substantiated these traditional claims; for instance, ethanolic leaf extracts administered at a dosage of 450 mg/kg body weight demonstrated superior efficacy in reducing the ulcer index within aspirin-induced gastric lesion models. This underscores the plant's robust gastroprotective potential relative to other experimental groups.

The plant parts stem, bark, fruits, leaves have been used traditionally African region topically in wounds, as a part of treatment in sexually transmitted diseases and dysentery. The usage is found as remedy in healing property detrimental to eczema, psoriasis, plasmodia malaria, diabetes mellitus, and foot ulcers and used in curing rheumatism. *K. africana* fruits have uses in veterinary medicine in treatments of digestive disorders, leg edema, skin irritations and infections, mastitis, and brucellosis.

The most habitually used plant part are the fruits followed by its stem bark, roots and leaves in traditional medicine preparations. Fruits are usually sold in commercial markets, not freshly consumed because they are poisonous. Fruits along with bark assist in fermentation's brewing process enhancing the piquancy of traditional beers. The ripe fruit is very fibrous and used as fetishes in many African households. The fruit extracts are made into a dosage form of topical application.

The extracts of plant parts report a significant pharmacological action *in vitro* and *in vivo*⁽⁷⁾. Fruit extracts have been widely used and have demonstrated *in vivo* and *in vitro* activity.

The available literature (Figure 1-2) shows the extensive research as traditional medicinal plant. Its activities and uses are significant. This supports exploring the plant's potential as a phytopharmaceutical.

The plant is not listed in the ICMR Quality Standards of Indian Medicinal Plants books, which suggests that despite its availability in

the Indian region, it has not been researched, its pharmacological actions have not been extensively reported, and translational research has not been carried out.

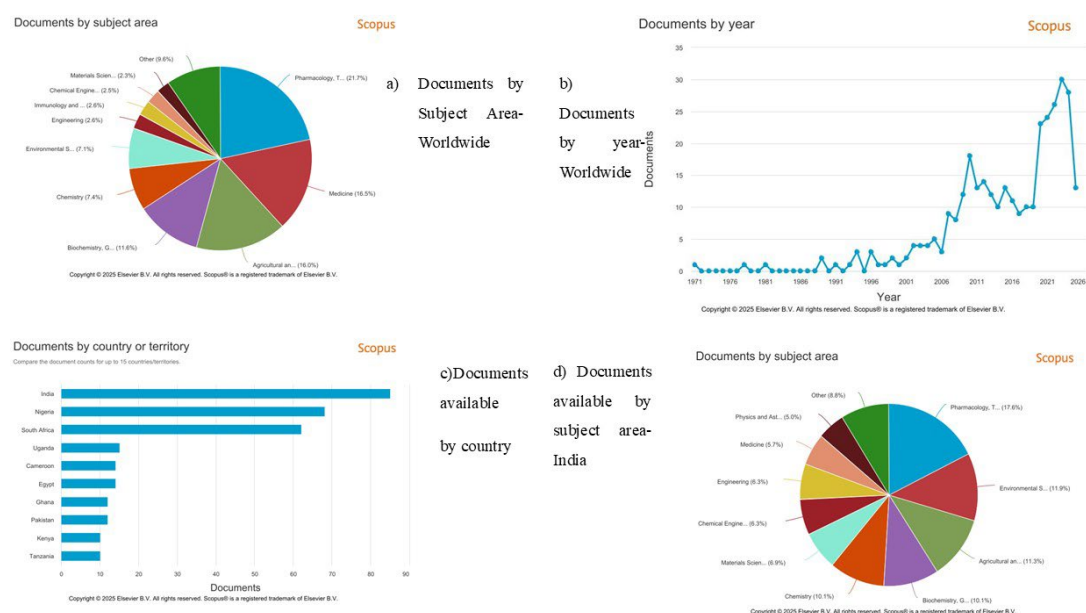


Figure 1-2 Research available on *K. africana*

While the pharmacological promise of *K. africana* is evident, translating this into a clinically viable formulation poses challenges. Extraction processes must be optimized to maximize yields of key active compounds while maintaining batch-to-batch reproducibility. Solvent composition, extraction time, temperature, and plant part used all influence the phytochemical profile. Statistical tools such as Design of Experiments (DOE) could be employed to optimize these parameters systematically.

1.4 Topical formulations

Systemic antibiotics are a blunt instrument. When you swallow a pill for a skin infection, the drug must survive the acidic environment of the stomach, pass through the intestinal wall, navigate the liver's metabolic filters, and enter the general circulation. By the time the drug reaches the skin capillaries, its concentration is often diluted to levels that barely tickle the pathogen. This sub-therapeutic dosing is a primary driver of AMR. It doesn't kill the bacteria; it merely introduces them to the enemy, allowing them to evolve defenses.

A topical dosage form, by contrast, acts as a precision strike. It delivers the active pharmaceutical ingredient (API) directly to the site of conflict, the epidermis and dermis. This approach bypasses the systemic tax and allows for a localized concentration that can be a thousand times higher than what is safely achievable through oral administration.

While topical delivery is logically superior, the vehicle, the base of the medication, matters as much as the drug itself. Traditionally, we have relied on ointments and creams.

- **Ointments** are greasy, occlusive, and composed mostly of hydrocarbons like petrolatum. While they are excellent for dry, chronic conditions like psoriasis, they are often counterproductive for infections. Their occlusive nature traps heat and moisture, which can actually encourage bacterial or fungal overgrowth in an active infection.
- **Creams** are emulsions of oil and water. They are more aesthetically pleasing than ointments but often require heavy surfactants and preservatives that can irritate already inflamed, infected skin.

In the context of treating an active infection, the gel, specifically, the aqueous hydrogel represents the pinnacle of topical design. A gel is a solid-to-semisolid system consisting of a network of cross-linked polymers (like carbomers or cellulose derivatives) that trap a high volume of liquid. Table 1-1 shows comparative features of topical dosage forms.

Table 1-1 Comparative features of topical dosage form

Feature	Ointment	Cream	Gel
Base	Hydrocarbon (Oil)	Emulsion (Oil + Water)	Aqueous/Hydro-alcoholic
Spreadability	Difficult/Sticky	Easy	Very Easy
Absorption	Poor (Stays on surface)	Moderate	Excellent/Rapid
Occlusion	High (Traps heat)	Moderate	Low (Breathable)
Patient Preference	Low (Greasy)	Moderate	High (Non-greasy/Cool)

The most forward-thinking application of gels involves integrating multi-constituent plant extracts. A gel is the perfect carrier for these botanical extracts. Because gels can be formulated with penetration enhancers like ethanol or propylene glycol, they can carry these complex plant molecules through the skin's barrier more effectively than a heavy cream ever could. This ensures the bacteria are hit with a lethal, multi-pronged chemical assault that leaves no survivors to develop resistance. ⁽⁸⁾

Most modern medical gels are built using carbomers, synthetic, high-molecular-weight polymers of acrylic acid. In their dry state, these molecules are tightly coiled. When dispersed in water, they begin to uncoil, but the real magic happens during neutralization. ⁽⁹⁾

Carbomers are acidic. When you add a base (like triethanolamine or sodium hydroxide), the acidic carboxyl groups (-COOH) along the polymer chain ionize into negatively charged carboxylate ions (-COO). These like charges repel each other, forcing the polymer chain to transition from a tight coil to an extended, rigid structure. This creates a three-dimensional fishnet that traps water and active antimicrobial agents.

Integrating plant extracts into the matrix is where the combat against AMR becomes sophisticated. Synthetic antibiotics often struggle to penetrate the biofilm; the protective fortress bacteria build around themselves. Plant extracts are often hydrophobic (oil-loving). In an old-fashioned cream, these oils would stay trapped in the oil phase. In a hydrogel, we can use micellar solubilization or nano-encapsulation.

The gel solves the two biggest hurdles in dermatology: bioavailability and compliance. By providing a cooling, non-greasy application that only releases its payload in response to the infection's own chemistry, we maximize the drug's impact while minimizing skin irritation.

This localized, high-concentration pulse of multi-targeted plant extracts ensures that even stubborn, resistance-resistant bacteria are overwhelmed before they can pass on resistance genes to the next generation. It turns the dosage form itself into an active participant in the healing process.

Ideal properties of a gel

1. **Physicochemical compatibility:** The vehicle must be pharmacologically inert, non-toxic, and demonstrate total compatibility with API and excipients.
2. **Pharmacological integrity:** The base must not interfere with the biological activity, release kinetics, or therapeutic efficacy of the incorporated drug.
3. **Storage and microbial stability:** The formulation must maintain its physical and chemical integrity throughout its intended shelf-life and remain strictly free from microbial contamination.
4. **Rheological performance:** The gel should exhibit superior thixotropic behavior and maintain its structural rheology to ensure consistent flow and application.
5. **Dermatological and aesthetic profile:** To maximize patient compliance, the base should be emollient, greaseless, and non-

staining, while remaining easily removable through water-washing.

6. **User ergonomics:** The delivery system must be designed for convenient handling and precise topical administration.
7. **Techno-economic feasibility:** The selection of components should support industrial scalability and cost-effective manufacturing without compromising quality standards.

CHAPTER-2

LITERATURE

REVIEW

2. Literature review

The phytochemical profile of *K. africana* is highly diverse, encompassing several classes of secondary metabolites with demonstrated biological activity. Iridoid glycosides such as specioside and verminoside are abundant in the fruit and bark. These compounds are noted for their anti-inflammatory and antioxidant effects, with verminoside in particular shown to suppress nitric oxide production and cytokine release in activated macrophages. Naphthoquinones such as kigelinone and lapachol are distinctive constituents that impart antimicrobial and cytotoxic properties.^(10, 11)

Lapachol derivatives have been investigated as anticancer agents due to their ability to induce apoptosis in tumor cells⁽¹²⁾. Phenolic acids, including caffeic, coumaric, and ferulic acids, contribute to the antioxidant and antimicrobial potential. These compounds can scavenge free radicals, inhibit microbial enzymes, and modulate inflammatory pathways. Flavonoids such as luteolin, quercetin, and kaempferol derivatives further enhance antioxidant capacity and may synergize with other constituents. Fatty acids (e.g., palmitic, oleic, linoleic acids), sterols (β -sitosterol, Stigmasterol), and triterpenes (Oleanolic acid) provide additional pharmacological versatility.^(13, 14)

Collectively, these compounds create a complex chemical milieu that supports the traditional use of *K. africana* for infections and inflammatory conditions. (Figure 2-1). Importantly, the consistent identification of certain marker molecules across studies provides a rational basis for standardization and quality control in phytopharmaceutical development.

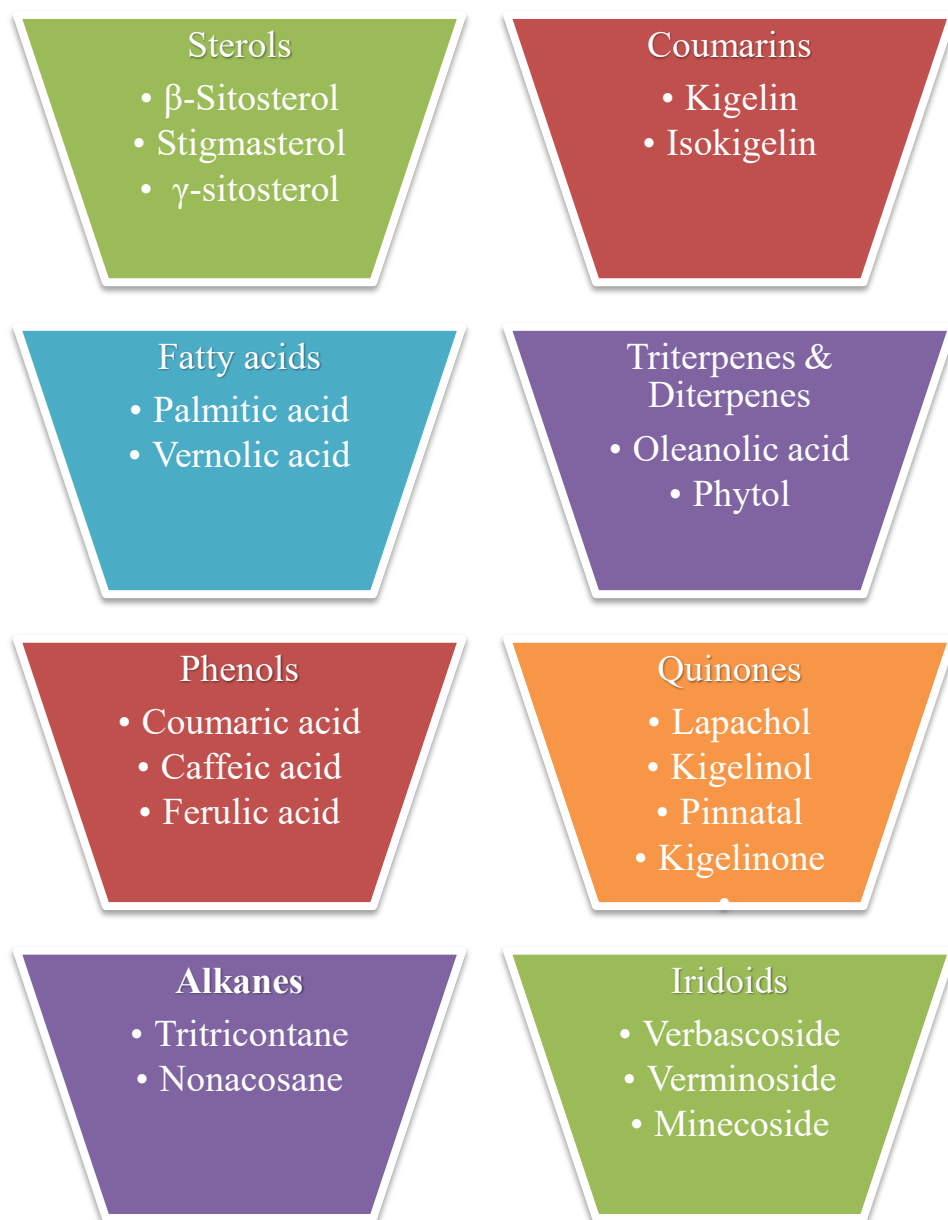


Figure 2-1 *K. africana* chemical constituents

2.1 Pharmacological activities

K. africana, the sausage tree, has garnered attention for its medicinal properties (used to treat various ailments, including skin infections, wounds, and inflammation) and diverse bioactive compounds. Research consistently shows that various parts of the plant are used to extract bioactive compounds, reflecting its diverse traditional applications. Studies have focused on extracts derived from the fruits, stem bark, leaves, and roots, each potentially yielding different phytochemical profiles and associated pharmacological activities

(Table 2-1). This multi-part use aligns with traditional practices, where specific plant sections were often used for distinct therapeutic purposes. ⁽¹⁵⁻¹⁸⁾

Table 2-1 Reported Pharmacological Activities

Compound Name	Plant Part Source	Primary Reported Pharmacological Activity
Kojic acid	Whole plant	Anti-candidal
Lapachol	Root	Anti-cancer (cytotoxic to HeLa cells)
Verminoside	Fruit	Anti-inflammatory (inhibits iNOS/NO release)
Verbascoside	Fruit	Anti-inflammatory
Palmitic acid	Fruit	Antibacterial
Nonanoic acid	Fruit	Antibacterial
8-heptadecenoic acid	Fruit	Antibacterial
Methyl ferulate	Fruit	Potential anti-cancer
Triterpenes	Leaves, Fruits	Various (6 novel compounds reported)
Lignans	Leaves, Fruits	Antioxidant, Anticancer (6 novel compounds reported)
4-(2,3dihydroxypropoxy)-3,5-dihydroxy-5-methylfuran-2-one	Various parts	Anti-plasmodial
Sucrose	Various parts	Anti-plasmodial
9,12-Octadecadienoic acid (Z, Z)	Fruit	Medicinal importance, therapeutic potential
13-Docosenoic acid, methyl ester (Z)	Fruit	Medicinal importance, therapeutic potential
ω -6 linoleic acid	Fruit	Anti-proliferative
1-O-deacetyl-2 α -methoxykhayanolide	Methanolic extract	Weak enzyme inhibition (AChE, BChE, LOX)

Anti-bacterial & Anti-fungal Activity

In vitro antibacterial and wound-healing activities of ethanolic fruit extract of *K. africana* were determined in albino rats ¹⁷. The *in vitro* antibacterial activity was tested against *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Salmonella typhi* (*S. typhi*). The wound-healing properties were evaluated using an excision wound-healing model with simple ointments containing extracts at varying

concentrations (3%, 5%, and 10% w/w). Results showed that ethanolic fruit extract was effective against all bacterial species, and *K. africana* ointments significantly increased wound contraction in 5 and 10 % w/w ointments. This study demonstrated phytoconstituents that promote wound healing and have potential antibacterial activity for human and veterinary use ⁽¹⁹⁾.

Antibacterial activity and antifungal activity of *K. africana* ethanolic fruit extract were tested against three pathogenic bacterial strains: *B. subtilis*, *S. aureus*, and *S. typhi*, and two fungal strains: *Epidermophyton floccusum* (*E. floccusum*) and *C. albicans*¹⁸. The study found that the ethanolic extract was effective against pathogenic bacterial species and showed antifungal activity against *C. albicans*, highlighting biologically active phytoconstituents in the fruit with potential use in the pharmaceutical industry. Additionally, this screening revealed its potential use against skin disorders.⁽²⁰⁾

Secondary metabolites from *Jacaranda Mimosifolia* (*J. mimosifolia*) and *K. africana* were isolated. The isolated compounds were tested for anti-candidal activity using the agar diffusion method and microbroth dilution technique against four *C. albicans* strains (ATCCL26, ATCC12C, ATCCP37039, and ATCCP37037). Methanolic stem bark extracts from both plants were extracted using silica gel column chromatography & elucidated by NMR and Mass spectrometry. Out of 17 compounds isolated in total from both plants, 3 compounds isolated from *K. africana* exhibited the highest anti-candidal activity. Kojic acid, isolated from *K. africana*, was likely the most active against the four *C. albicans* strains. ⁽¹⁸⁾

Anti-cancer activity

Twenty ethnobotanicals in Nigeria have been traditionally used for the management of breast cancer, which has been prominent in the State. ⁽¹⁰⁾ Of the 20 botanicals, one of them was *K. africana* fruit part was used in conjunction with *Capsicum frutescens* fruit which was prepared as decoction in water. The remedy was used as the patient

was fumigated over the preparation, exposing the breast to steam, and pus was seen to gush out. The purpose was to provide scientific validation of its anti-cancer potency.

Three extracts, namely methanolic, aqueous, and ethyl acetate, inhibited untreated cell growth of Colon carcinoma-2 (CaCo2) colorectal cancer cell line, inhibiting them by 37 to 55 %⁽²¹⁾. Additionally, methanolic extract inhibited HeLa cell proliferation. This showed anti-cancer activity of *K. africana* fruit extracts.

Experiments with *K. africana*, *Mimusops zeyheri* (*M. zeyheri*) and *Ximenia caffra* (*X. caffra*) oils strongly established the suppression of Caco-2 and human embryonic kidney-293 (HEK-293) cell proliferation.⁽²²⁾ All three tree oils suppressed both cell lines in a dose-dependent manner. Only *K. africana* oil suppressed cell proliferation, whereas *M. zeyheri* and *X. caffra* inhibited cell growth only at 80 mg/ml. The fatty acid profile of the three tree oils showed the highest percentage of α -linolenic acid, which has been reported to inhibit cell growth in Caco-2 cells.⁽²²⁾ This could explain the anti-proliferative effect of *K. africana* seed oil.

A naphthoquinone obtained from *K. africana*'s root extract was lapachol. We structurally modified lapachol to improve anticancer activity. We elucidated lapachol and its derivatives using mass spectrometry and nuclear magnetic resonance. To assess the therapeutic window and selective toxicity of the study compounds, we evaluated *in vitro* cytotoxicity against a panel of cell lines, including healthy human prostate, human cervical adenocarcinoma (HeLa), and murine fibroblasts, using the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The results showed that lapachol has significantly higher cytotoxic potency against HeLa cells than its structural derivatives, highlighting its potential as a targeted antiproliferative agent in this malignant model. The lapachol derivatives lost cytotoxic potency against cancer cells. Lapachol was found to be cytotoxic to both cancer and normal cell

lines. Hence, lapachol was reported as an anti-cancer agent, but its use should be monitored, as it is also cytotoxic to normal cells ⁽¹²⁾.

Anti-inflammatory activity & antioxidant activity

K. africana stem bark's ethanolic extract showed a dose dependent significant reduction in acetic acid induced mouse writhing. Anti-inflammatory activity was assessed using the carrageenan-induced paw edema method. The ethanolic extract showed dose-dependent significant inhibition between the 2nd and 5th hour (anti-inflammatory) and a dose-dependent significant inhibition in the number of writhes (analgesic activity). This showed that the ethanolic extract has significant analgesic and anti-inflammatory activity. The anti-inflammatory activity is likely due to inhibition of prostaglandin synthesis and analgesic activity mediated by peripheral inhibitory mechanisms⁽²³⁾.

The methanolic leaf extract of *K. africana* leaf and *Acacia hockii* De stem bark reported anti-inflammatory activity in Swiss albino mice by the carrageenan-induced hind paw method. The anti-inflammatory potential of *K. africana* leaf and *A. hockii* stem bark methanolic extracts was evidenced by the significant attenuation of hind paw edema in murine models. Specifically, the *K. africana* extract reduced the diameter of inflamed paws by 0.21% to 4.98%, while the *A. hockii* extract demonstrated a reduction ranging from 0.6% to 5.38%. These results showed a clear dose-dependent relationship, providing scientific validation for the ethnobotanical use of these species in managing inflammatory pathologies. The study scientifically supported the traditional usage of *K. africana* for its anti-inflammatory activity. ⁽²⁴⁾.

Phytochemical analysis of the polar extract from *K. africana* fruit revealed the presence of significant bioactive constituents, specifically the iridoid verminoside and the polyphenol verbascoside. *In vitro* pharmacological evaluations further showed that verminoside has potent anti-inflammatory activity, mediated by downregulation of

inducible nitric oxide synthase (iNOS) expression and subsequent inhibition of nitric oxide (NO) release. Verminoside did not affect cell viability, and it did not induce the release of pro-inflammatory mediators ⁽²⁵⁾.

The antioxidant property of methanolic leaf and stem bark extracts of *K. africana* & *Strophanthus hispidus* (*S. hispidus*) was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The results showed that leaf and stem bark have wound-healing properties, as assessed using the excision wound model and histopathological analysis of treated and untreated wound tissues. Both leaf and stem bark methanolic extracts had a significant influence on the rate of wound closure of 85.1 %. Phytoconstituents such as tannins, flavonoids, phenolic acids, and phenolic diterpenes play a major role in profuse angiogenesis, enhanced collagenation, and re-epithelialization (i.e., antioxidant and wound-healing effects). This justifies the medicinal uses of *K. africana* as an antioxidant & on wounds ⁽²⁶⁾.

K. africana fruit extract was prepared in methanol, water, and acetone and was subjected to antioxidant & anti-inflammatory in vitro assays. Antioxidant activity was performed using 2, 2'-Azino-bis (3-Ethylbenzthiazoline-6-sulfonic Acid (ABTS) radical scavenging assay. MTT cell viability was performed for cytotoxicity. Additionally, Enzyme-linked Immunosorbent Assay (ELISA) kits were used for a) 15-Lipoxygenase (LOX) and Cyclooxygenase (COX-2) enzyme and b) Nitric oxide (NO) inhibitory effect and measurement of pro-inflammatory cytokines: Interlukin-1 beta (IL-1 β), tumour necrosis factor alpha (TNF- α), and Interlukin-6 (IL-6) and the anti-inflammatory cytokine: Interlukin-10 (IL-10). The aqueous extract showed the highest cell viability and inhibited NO production. Acetone fruit extract strongly suppressed IL-1 β and IL-6. None of the three extracts inhibited IL-10. The acetone and aqueous fruit extracts had good antioxidant and anti-inflammatory activity ⁽²⁷⁾.

Antioxidant activities of extracts of *K. africana* (stem) and *Sterculia foetida* (stem) (*S. foetida*) were explored. Four solvents: n-hexane, ethyl acetate, methanol, and water were used for extraction. Antioxidant activity was determined using the DPPH method. The aqueous extract of *K. africana* (stem) and methanol extract of *S. foetida* (stem) showed prominent scavenging effect, i.e., antioxidant activity, in dose dependent manner which could be due to presence of flavonoids ⁽²⁸⁾.

The antioxidant properties of five medicinal plants were evaluated and reported for leaf and fruit extracts. *K. africana* was one of those five plants. DPPH free radical scavenging assay was used to evaluate antioxidant activity. Ethanolic leaf and fruit extract of *K. africana* was assessed through ELSIA kits for DPPH free radical assay. Leaf extract of *K. africana* had 59.66 % and fruit extract had 62.66 % of DPPH inhibition. Quercetin was used as a standard anti-oxidant which showed 93.66 % inhibition. Polyphenolic compounds are responsible for antioxidant activity which can be utilized as development of alternative anti-oxidants from natural sources ⁽²⁹⁾.

Other Activities

Anti-diabetic activities of extracts of *K. africana* (stem) and *S. foetida* (stem) extracted with four solvents: n-hexane, ethyl acetate, methanol and water separately. *In vitro* α -amylase inhibition method was performed calculating IC₅₀ values. Aqueous extract of *K. africana* (stem) & methanolic *S. foetida* (stem) showed significant inhibition of α -amylase which could be due to presence of number of flavonoids. This showed both plants as a potential source of anti-diabetic property ⁽²⁸⁾.

Anti-diabetic activity & hypolipidemic activities of *K. africana* fruit along with *Sorghum bicolor* (*S. bicolor*) stalk was experimented on alloxan-induced diabetic Wistar rats single extracts and in mixture ratio of (1:1). The hydroethanolic (2:8) extracts had a significant decrease in plasma glucose, triglyceride and low-density lipoprotein

(LDL)-cholesterol parameters, urea and creatinine parameters where a significant reduction was observed on comparison with control group. The extracts and their mixture exhibited anti-diabetic and hypolipidaemic activities due to presence of active phytoconstituents showcasing cardiovascular benefits of *K. africana* and *S. bicolor*⁽³⁰⁾.

K. africana whole fruit's different parts in aqueous extracts were reported on sperm parameters and testis in wistar mice. The extracts were prepared into whole fruit (AP) and fruit without peel (SP) two extracts separately. Both aqueous extracts enhanced spermatogenesis and did not affect the gonads. AP extract showed increase in sperm count efficiently whereas SP extract improved mobility and body shape of sperm significantly. Hence, naturotherapists use *K. africana* fruit for treatment of male infertility⁽³¹⁾.

The effects of ethanolic fruit extract of *K. africana* on development and maturation in immature rats of reproductive system and evaluation was conducted on reproductive system and on pubertal development. The results showcased *K. africana* extract in dose dependent manner showed anticipated puberty, increases body growth and sexual organ development due to stimulation of androgenic hormones by phytoconstituents present in extract. This supported scientifically traditional usage of *K. africana* in disorders of male reproductive system⁽³²⁾.

Experiment conducted on normal albino rats and reported activity of methanolic leaf and methanolic fruit extracts of *K. africana*. The study was aimed to monitor some biochemical parameters affected due to extracts. Oral administration of methanolic extracts of *K. africana* leaf and fruit lower serum lipid profile and increase High density lipoprotein (HDL)-cholesterol levels. Liver enzymatic levels of Aspartate transaminase (AST) and Alanine transaminase levels were found to be lower in normal rats suggestive of hepatoprotective activity. Hence, results suggested methanolic leaf and fruit extracts of *K. africana* can be utilized in maintenance and stabilizing normal

metabolic state⁽³³⁾.

Methanolic leaf extract of *K. africana* was investigated in normotensive wistar rats. The blood pressure and biochemical indices were monitored. *K. africana* was administered in dose dependent manner which resulted in significant reduction in serum activities of liver enzymes i.e. AST, Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP). A significant reduction in blood pressure was even observed in addition to reduction in total cholesterol, triglycerides, and LDL-C, very low-density lipoprotein cholesterol (VLDL-C) and coronary index. However, an increase in levels of HDL in comparison with control was observed. The methanolic extract even impacted superoxide dismutase activity, an anti-oxidant enzyme preventing generation of hydroxyl radicals and protect cellular constituents from oxidative damage. This suggested ethnobotanical usage of *K. africana* leaf as hypolipidaemic, hypotensive and as an antioxidant in diseases related to heart⁽³⁴⁾.

The metabolome modulatory effects of *K. africana* fruit extracts were studied in streptozocin (STZ)-induced diabetic rats investigating oxidative stress and hyperlipidaemic biomarkers in addition to evaluation of antidiabetic effects on 3T3 L1 adipocytes. The investigation showed *K. africana* fruit possessed pharmacological action of anti-diabetic on account of presence of anti-oxidants identified by modern time-of-flight mass spectrometer with gas chromatography, anti-hyperlipidaemic and has an ameliorative effect in maintenance and stability of biochemical markers. It is suggested the use against diabetes mellitus⁽³⁵⁾. Table 2-2 shows reported extracts containing various chemical constituents.

Table 2-2 Reported extracts containing various chemical constituents

Compound Name	Extract	Primary Reported Pharmacological Activity
Kigelianolide	Methanolic extract	Weak enzyme inhibition (AChE, BChE, LOX)
Deacetylkhayanolide E	Methanolic extract	Weak enzyme inhibition (AChE, BChE, LOX)
1-O-deacetyl-2 α -hydroxykhayanolide E	Methanolic extract	Weak enzyme inhibition (AChE, BChE, LOX)
Khayanolide B	Methanolic extract	Weak enzyme inhibition (AChE, BChE, LOX)

The use of *K. africana* ethanolic leaf extract as a hepato-renal protective agent was studied and reported⁽³⁶⁾. The study was conducted in adult male wistar rats where Carbon tetrachloride (CCL₄) model of toxicity was used. A significant decrease in serum liver enzymes (AST, ALT and ALP) was found in dose-dependent manner.

The usage of *K. africana* in treatment of malaria anti-plasmodial activity was evaluated on different morphological parts; leaf, root, stem bark, and fruit⁽³⁷⁾. *K. africana* & the semi-purified fractions using n-hexane, dichloromethane, ethyl acetate, butanol and methanol yielded two pure compounds: 4-(2, 3-dihydroxypropoxy)-3, 5-dihydroxy-5-methylfuran-2-one and sucrose using chromatographic and spectroscopic techniques. *K. africana*'s anti-plasmodial activity in order of fruit > root > leaf > stem bark.

Quantitative evaluation of anti-psoriatic activity of *K. africana* on various plant parts⁽³⁸⁾. The effect of methanol and hexane extracts of stem, bark, leaves and fruit on cell differentiation was evaluated in modified mouse tail test on albino mice. The topical application of extracts on induced orthokeratosis in male albino mice had significant effect on epidermal thickness. *K. africana* stem methanol extract exhibited highest anti-psoriatic activity with little irritation potential.

2.2 Analytical methods

This literature review explores the identification, isolation, and characterization of bioactive compounds from *K. africana*. Over the years, advances in chromatographic and spectroscopic techniques have enabled precise detection, quantification, and structural elucidation of such bioactive molecules.

These approaches facilitated the identification of a wide range of compounds such as phenolic acids, flavonoids, terpenoids, fatty acids, and limonoids. Bioassay-guided fractionation was further applied to evaluate antibacterial, anti-inflammatory, and enzyme inhibitory activities of plant extracts and their fractions.

A *K. africana* species with well-documented ethno-medicinal uses but still underexplored for its full phytochemical and pharmacological potential. Investigations revealed the presence of phytoconstituents including iridoids, flavonoids, fatty acids, and novel limonoids, several of which demonstrated biological activity against bacterial strains and key enzymes such as lipoxygenase and cholinesterases. Other plant sources, including *Lycopodium clavatum* (*L. clavatum*) and *S. bicolor*, were also studied for their bioactive constituents, thereby broadening the scope of phytochemical diversity considered.

This section integrates analytical chemistry, natural products isolation, and biological evaluation to highlight the therapeutic promise of phytoconstituents. The findings contribute to both the scientific understanding of these medicinal plants and the broader effort to identify novel biomolecules that may serve as leads for drug discovery.

Identification and estimation of bioactive ferulic acid in *L. clavatum* was carried out by high performance-thin layer chromatography (HPTLC) method⁽³⁹⁾. Ferulic acid was quantitatively estimated on silica gel 60F₂₅₄ HPTLC plates and scanned at 320 nm. The recovery was ranged in 95 % - 97%.

Analytical techniques were reported for determination of quercetin and its glycosides in different plants⁽⁴⁰⁾. The important aspect was to determine quercetin as an analyte in its conjugated forms or as the aglycone. Chromatography or electrophoresis separation helps in achieving it. Liquid chromatography with diode array detection (DAD) -MS or capillary electrophoresis (CE)-DAD-MS is one of the best appropriate techniques for separation, quantifications and identification.

A C₁₆ dilactone terpenoid was isolated from fungal endophytes of *K. africana* having activity against *P. aeruginosa*⁽⁴¹⁾. The bioactive fungal isolate was identified as *Neofusicoccum luteum* (ZF 52) based in genomic DNA sequencing and nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectrometry (HRMS), single crystal x-ray diffraction (XRD) were the techniques used. Extraction was performed using ethyl acetate which contained several bioactive molecules. Anti-*P.aeruginosa* activity was evaluated by disc diffusion assay. The ZF 52 was identified using various solvent systems developed over thin-layer chromatography (TLC) profile such as: chloroform, methanol and hexane: ethyl acetate. It was isolated by subjecting it to column chromatography and structure was determined using NMR spectroscopy and single crystal XRD analysis.

Active compounds were isolated from *K. africana* using ethyl acetate extract of fruits by bioassay-guided fractionation⁽⁴²⁾. Isolated compounds showed anti-bacterial activity against gram-negative & gram-positive bacteria. Palmitic acid, which as not reported in *K. africana* before, was found as a major compound in the mixture and it is known to have anti-bacterial activity. Structure elucidation of the active compounds was done using NMR and Gas Chromatography (GC)–Mass spectrometry (MS). The two other minor compounds found to be were nonanoic acid and 8-heptadecenoic acid.

A selective HPTLC method was developed for simultaneous estimation of juglone, quercetin, myricetin, rutin, caffeic acid, and

gallic acid⁽⁴³⁾. Separation was achieved on RP-18 F₂₅₄ TLC plate using solvent system of methanol: water: formic acid-acetic acid (48.8:46.4:2.2.4 v/v). The phenolic compounds were analyzed densitometrically at 254 nm. Linear regression data showed a higher correlation coefficient ($r^2 \geq 0.997$). The method can be used as quality control routine.

The phytochemical constituents of extracts evaluated of *K. africana* fruit and *S. bicolor* stalk⁽⁴⁴⁾. The hydroethanolic (2:8) extracts were tested by GC-MS. The major compounds were 9, 12 Octadecadienoic acid (Z, Z), and 13-Docosenoic acid, methyl ester (Z). The phytoconstituents found were of medicinal importance and had potential therapeutic properties which could be explored.

A rapid shotgun approach for characterization of *K. africana* fruit was explored⁽⁴⁵⁾. Rapid evaporative ionization mass spectrometry (REIMS) was used for investigating phytoconstituents and metabolic profile. Seventy-eight biomolecules were identified which possess drug-like behaviour. Flavonoids and iridoids, antioxidant molecules, were confirmed. Lipids were present as major components, such as ω -6 linoleic acid and ω -3 linolenic acid and are rich source of essential fatty acids involved in metabolic pathways which could open new ways in evaluation of therapeutic activity.

GC-MS analysis of *K. africana* leaf extract and fractions & its inhibitory role on 5-lipoxygenase was studied⁽⁴⁶⁾. The foliar biomass was extracted using 70% (v/v) ethanol and subsequently partitioned into n-hexane, ethyl acetate, and n-butanol fractions. LOX inhibition assays indicated that the crude hydroethanolic extract, aqueous fraction, and ethyl acetate fraction reached peak inhibitory potential at a concentration of 0.5 mg/mL. Conversely, the n-butanol and n-hexane fractions exhibited maximum efficacy at lower concentrations of 0.2 mg/mL and 0.4 mg/mL, respectively. Further characterization of the ethyl acetate fraction via GC-MS identified several bioactive phytoconstituents with documented anti-inflammatory properties,

corroborating the observed enzymatic inhibition.

Limonoids were isolated from ethyl acetate fraction soluble of methanolic extract of *K. africana*⁽⁴⁷⁾. Phytochemical investigation of the ethyl acetate-soluble fraction from *K. africana* methanolic extract led to the isolation of two novel limonoids: 1-O-deacetyl-2 α -methoxykhayanolide (1) and kigelianolide (2). In addition to these new compounds, three known constituents were identified: deacetylkhayanolide E (3), 1-O-deacetyl-2 α -hydroxykhayanolide E (4), and khayanolide B (5), further expanding the chemical profile of this species.

K. africana fruit extract was macerated in methanol and obtained six fractions (A-F)⁽⁴⁸⁾. Fraction C by radial chromatography was fractionated yielded 5 sub-fractions (C1-C5). Sub-fractions C3 & C4 were subjected to preparative thin layer chromatography and a derivative was obtained. This derivative was further analyzed in NMR and LC-MS spectral data and identified as methyl ferulate which could be examined further for its anti-cancer activity.

Phytochemical screening & phenolic content of methanolic extract of *K. africana* leaves was reported⁽⁴⁹⁾. Phytochemical analysis was done using TLC profile and phenolic content was determined by Folin-Ciocalteus reagent method. TLC technique was used to separate chemical compounds present using various solvent systems. Mobile phase of chloroform: acetone: formic acid (7.8: 1.65: 0.8) & petroleum ether: ethyl acetate (7:3) were used for development. Phenolic content revealed 3.53g of GA/100g of methanolic extract of *K. africana* (1 mg/ml) of plant extracts using Folin-Ciocalteus reagent.

K. africana was chemically investigated in methanol: dichloromethane (1:1 v/v) extract of leaves and fruits⁽¹⁷⁾. Triterpenes and lignans from *K. africana* were isolated which were elucidated by spectroscopic analysis. The extraction and isolation were carried using various solvent systems. Extraction was done by maceration, crude extract obtained was subjected to flash column chromatography

for leaf extract and column chromatography for fruit extract. The fractions were analyzed by TLC profile in hexane: ethyl acetate (7:3) and different compounds were obtained. Out of 10 compounds isolated and identified, 6 compounds were isolated for the first time from *K. africana*.

2.3 Formulation aspects

This section also explores the pharmaceutical applications of *K. africana* by developing topical formulations and investigating its potential in green nanotechnology. Medicinal plants provide structurally diverse bioactive compounds and serve as sustainable resources for designing novel drug delivery systems and antimicrobial agents.

We prepared and evaluated topical dosage forms, including creams and emulgels containing aqueous ethanolic extracts of *K. africana*, for antibacterial activity against *S. aureus* and *P. aeruginosa*. Comparative analysis showed that emulgels had superior release and diffusion profiles compared with creams, and formulations with higher extract concentrations showed significant *in vitro* activity. Stability studies further highlighted the suitability of specific emulgel and cream formulations as promising candidates for therapeutic applications.

In parallel, *K. africana* extracts were utilized for the green synthesis of copper, silver, and copper–silver bimetallic nanoparticles. We thoroughly characterized these nanoparticles using spectroscopic and microscopic techniques, including UV-Vis, FTIR, SEM/EDX, XRD, and TEM. We systematically evaluated their antimicrobial activity against bacterial and fungal pathogens, with notable inhibitory effects against *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *S. aureus*, *C. albicans*, *A. flavus*, and *A. niger*. The findings demonstrated the enhanced antimicrobial efficacy of these nanoparticles and emphasized their potential in biomedical and agricultural applications, such as nano-fungicides, nano-antimicrobials, and nano-fertilizers

Two topical dosage forms (cream and emulgel) with varying aquatic ethanolic extract (1- 10%) were formulated and evaluated for antibacterial activity against *S. aureus* and *P. aeruginosa*⁽⁵⁰⁾. Emulgels had higher *in vitro* activity, release rate, and diffusion rate than creams. Among the latter formulations, the 10 % extract cream exhibited significant *in vitro* activity, and the 7% and 10% extract emulgels showed significant *in vitro* activity. The effective formulation of one emulgel and aqueous cream exhibited stability, while others did not.

Synthesis of nanoparticles of *K. africana* fruit containing copper metal ions and investigation of its antimicrobial properties⁽⁵¹⁾. The ethanolic extract, which reduced copper ions and stabilized copper nanoparticles (CuNPs) via a green synthesis route, was quantitatively analyzed for the phytoconstituents present. The antimicrobial activity was tested against *S. typhi*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *Shigella* species. *P. aeruginosa* exhibited highest activity. Also, antifungal activity was carried out against *A. flavus* and *A. niger* and inhibited both strains considerably. This supports the applicability of CuNPs in different formulations such as nano-fungicides, nano-antimicrobials, and nano-fertilizers.

Silver monometallic (AgNPs) & copper-silver bimetallic (AgCuNPs) nanoparticles were synthesized using *K. africana* fruit extract via a green synthesis route and evaluated for antimicrobial activities.⁽⁵²⁾. The synthesized nanoparticles were characterized by UV-Visible spectrophotometer, FTIR, SEM/EDX, XRD, and TEM. Antibacterial activities were tested against *S. aureus*, *P. aeruginosa*, *E. coli*, & *K. pneumoniae*. The nanoparticles from *K. africana* fruit extract were more effective against bacterial strains.

These investigations highlight the versatility of *K. africana* not only as a source of phytoconstituents but also as a valuable agent for modern pharmaceutical formulations and nanotechnology-driven antimicrobial strategies.

CHAPTER-3

AIM

and

OBJECTIVES

3 Rationale

The urgency of AMR provides a compelling rationale for exploring plant-based antimicrobials. The global health community, led by WHO, has prioritized developing new antimicrobials and alternatives as part of the Global AMR Action Plan. Low- and middle-income countries, where the burden of infectious diseases is highest, stand to benefit significantly from affordable, locally sourced phytopharmaceuticals. *K. africana* integrated into traditional medicine is positioned to meet this need.

The scientific validation of *Kigelia*-based formulations has global implications. The plant represents a model for translating ethnobotanical knowledge into regulatory-grade therapeutics. Its development could set a precedent for other medicinal plants and foster innovation in phytopharmaceuticals. By combining traditional wisdom with modern analytical and regulatory science, *Kigelia* research contributes not only to combating AMR but also to diversifying the therapeutic arsenal available to humanity. Modern medicines for fungal and bacterial skin disorders have become resistant to prescribed anti-infective drugs. Traditional medicines have more favorable pharmacological actions, with fewer side effects, fewer adverse reactions, and no toxicological implications. Combining traditional medicines with modern synthetic chemicals into a new drug product, and establishing its safety, efficacy, and activity, carves out a new alternative medicine pathway.

Developing standardized formulations and navigating stringent regulatory frameworks will be essential to translate this potential into clinically useful therapies.

Formulation presents additional hurdles: plant extracts can be unstable and degrade under light, heat, or oxidative conditions. Incorporating them into suitable dosage forms, such as topical

gels or creams, requires careful excipient selection to ensure stability, bioavailability, and patient acceptability. Standardization must extend to the final product, with validated methods to confirm marker content and biological activity post-formulation.

3.1 Aim of the work

To develop, standardize, & evaluate a phytopharmaceutical dosage form with anti-infective activity.

3.2 Objectives

1. To develop a suitable analytical method for estimation of selected markers.
2. To optimize the extraction process using design of experiments.
3. To evaluate the optimized extract for its chemical constituents and *in vitro* activity.
4. To develop a topical dosage form containing the optimized extract of *K. africana*.
5. To evaluate the optimized formulation for its physicochemical properties and *in vitro* activity.

CHAPTER- 4

MATERIALS

&

INSTRUEMENTS

4 Materials and instruments

Resources such as instruments, materials, and reagents used for the doctoral research work for method development, validation, analysis of herbal extracts, formulation, and evaluation. The materials and instruments used are in Table 4-1.

4.1 List of Instruments

Table 4-1 List of Instruments

Sr. No.	Instrument	Model No.	Manufacturer
1.	Linomat 5	-	CAMAG
2.	UV Cabinet 4	-	CAMAG
3.	TLC Scanner 4	-	CAMAG
4.	Microbalance	XPE-26	Mettler Toledo
5.	Analytical Balance	XPE-205	Mettler Toledo
6.	Water Purification System	DQ 5	Merck
7.	Brookfield Texture Analyzer	CT3-4500GM	Ametek
8.	Magnetic stirrer	2MLH	Remi Electronics Ltd.
9.	Overhead stirrer	RQT 127A/D	Remi Electronics Ltd.
10	Refrigerated centrifuge	Sorvall Legend X1	Thermo fischer Scientific
11	Stability Chamber	GMP-SC90L	Kesar Controls
12	pH meter	LMPH 10	Labman
13	HS- GC	8890	Agilent
14	Rheometer	MCR 102e	Antonpaar

4.2 List of Chemicals and reagents

The biomarkers (Verbascoside, Caffeic acid, Coumaric acid and Ferulic acid) were purchased from Yucca Enterprises, Mumbai, India. Reagents used for analytical experiments are in Table 4-2.

Table 4-2 List of Chemicals and reagents

Sr. No.	Reagent	Grade	Manufacturer
1.	Methanol	AR Grade	Fischer Scientific
2.	Toluene	AR Grade	Fischer Scientific
3.	Ethyl acetate	AR Grade	Fischer Scientific
4.	Chloroform	AR Grade	Fischer Scientific
5.	Formic acid	AR Grade	Fischer Scientific

The list of chemicals used in formulation development is listed in Table 4-3.

Table 4-3 List of chemicals used in formulation

Sr. No.	Chemical	Category	Supplier
1.	Carbopol 974	Polymer	Lubrizol
2.	Hydroxypropyl methylcellulose	Polymer	Loba Chemical Pvt. Ltd.
3.	Sodium Carboxyl methylcellulose	Polymer	Loba Chemical Pvt. Ltd.
4.	Methylcellulose	Polymer	Loba Chemical Pvt. Ltd.
5.	Methyl Paraben	Preservative	Loba Chemical Pvt. Ltd.
6.	Propyl Paraben	Preservative	Loba Chemical Pvt. Ltd.

Sr. No.	Chemical	Category	Supplier
7.	Propylene glycol	Solubilizing agent	Finar India Pvt. Ltd.
8.	Triethanolamine	pH adjusting agent	Finar India Pvt. Ltd.
9.	Acacia	Binder	Loba Chemical Pvt. Ltd.

CHAPTER-5

ANALYTICAL

METHODS

5 Analytical methods

For *K. africana*, four marker compounds are particularly significant: Verbascoside (VBS), Caffeic acid (CA), Coumaric acid (COA), and Ferulic acid (FA). VBS exhibits potent antioxidant, anti-inflammatory, and antimicrobial activities. Its well-defined chemical structure and ease of detection via analytical methods make it an ideal marker. CA, a hydroxycinnamic acid, has recognized antimicrobial and antioxidant effects and serves as a useful indicator of phenolic content. COA and FA, structurally related cinnamic acids, contribute to free radical scavenging, enzyme inhibition, and microbial suppression⁽⁵³⁻⁵⁸⁾.

Each of these compounds is a well-defined chemical entity with reproducible analytical signatures, enabling robust quality control during extract standardization and dosage-form development. Importantly, they are not merely convenient markers: they are pharmacologically active constituents that contribute directly to the antimicrobial, antioxidant, anti-inflammatory, and wound-healing activities. Their presence bridges ethnobotanical knowledge and modern pharmacology, providing a scientific rationale for tracking and quantifying these molecules. The selection of VBS, CA, COA, and FA as biomarkers for *K. africana* is both analytically pragmatic and pharmacologically justified.

From a regulatory perspective, standardization is a cornerstone of phytopharmaceutical development, ensuring reproducibility and quality, and marker compounds with clear pharmacological relevance are increasingly required as agencies demand demonstration of consistency between chemical profiles and biological effects. Together, these four molecules capture both the chemical diversity and therapeutic potential of *K. africana*.

VBS, as a phenylethanoid glycoside, reflects the complex

glycosidic profile characteristic of the plant that dictates its properties. VBS is formed from three main units: a caffeic acid moiety (a phenylpropanoid), a hydroxytyrosol moiety (a phenylethanoid), and a disaccharide composed of rhamnose and glucose. The caffeic acid and hydroxytyrosol are linked to the rhamnose and glucose units, respectively, through ester and ether bonds.

Phenolic acids have enormous structural diversity, but there are a few compounds that are chemically similar in nature. Examples include CA, COA, and FA. Structurally, these three phenolic acids are characterized by a core aromatic ring substituted with a prop-2-enoic acid moiety and a hydroxyl group. COA serves as the basic scaffold with a single hydroxyl substitution. CA is distinguished from COA by the presence of an additional hydroxyl group. At the same time, FA is differentiated from CA by the substitution of a methoxy group in place of the second hydroxyl moiety. The comprehensive chemical architectures of verbascoside (VBS), caffeic acid (CA), coumaric acid (COA), and ferulic acid (FA) are illustrated in Figure 5-1.

The comparative Table 5-1 summarizes the chemistry, pharmacology, ethnobotanical significance, and analytical advantages of these markers, highlighting why they are uniquely suited to guide the standardization and formulation of *Kigelia*-based phytopharmaceuticals.

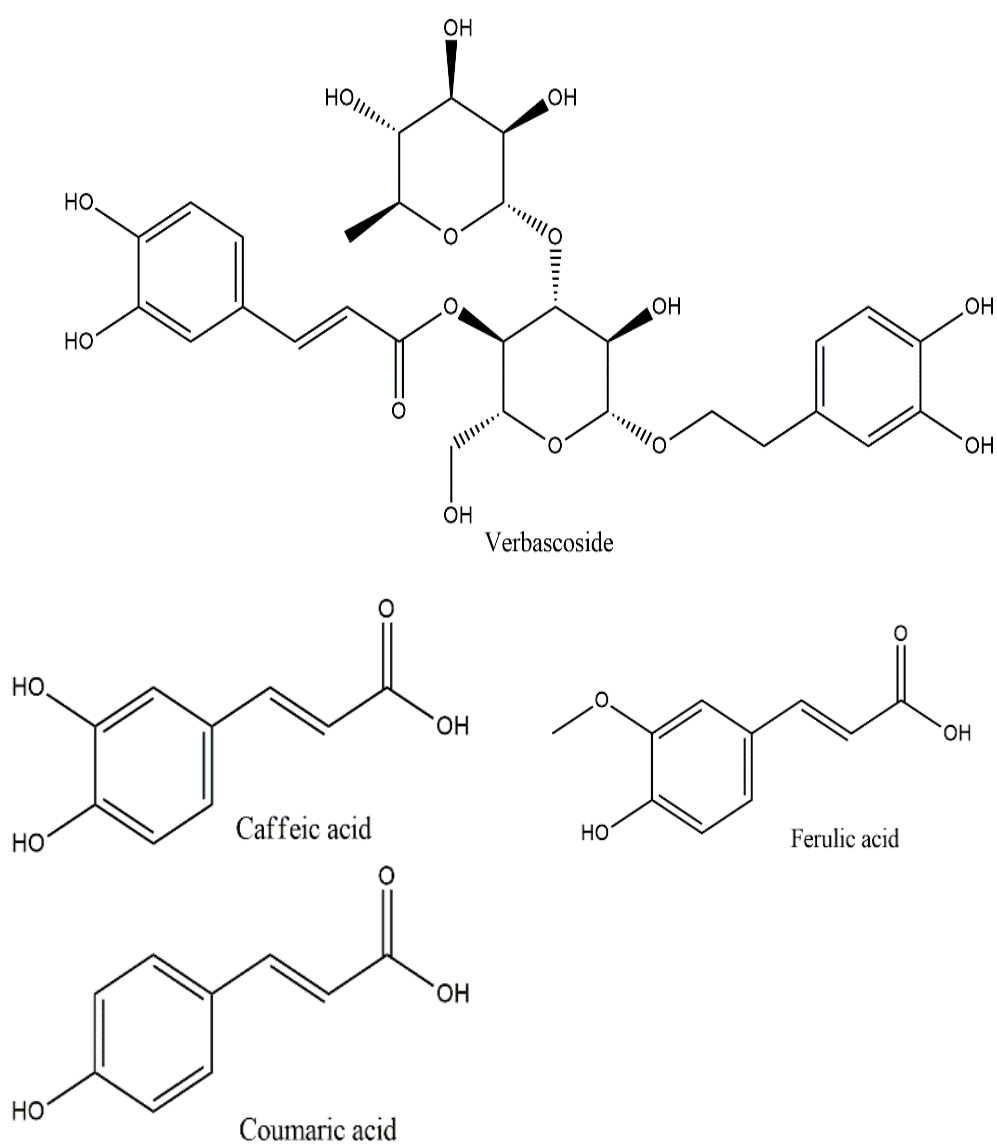


Figure 5-1 Markers in this study

Table 5-1 Markers summary

Compound	Chemistry	Pharmacology	Ethnobotany / Relevance	Analytical & Formulation Benefits
Verbascoside	Phenylethanoid glycoside; caffeic acid ester linked to hydroxytyrosol and sugars	Antioxidant, antimicrobial, anti-inflammatory, wound-healing	Occurs in plants used traditionally for skin disorders; abundant in <i>Kigelia</i>	Stable, strong UV absorbance; robust quantification (HPTLC/HPLC); reproducible across batches
Caffeic Acid	Hydroxycinnamic acid; catechol + acrylic acid moieties	Antimicrobial, antifungal, antioxidant, anti-inflammatory	Found in many ethnomedicinal plants for wound healing and infections	Sharp chromatographic peaks; high detectability; abundant and consistent in extracts
Coumaric Acid	Hydroxycinnamic acid isomer (<i>p</i> -coumaric dominant in <i>Kigelia</i>)	Antioxidant, antimicrobial, anti-inflammatory	Common in phenolic-rich plants traditionally used against infections	Predictable retention behavior; reproducible; useful quality control anchor
Ferulic Acid	Hydroxycinnamic acid with methoxy substitution (stable radical scavenger)	Antimicrobial, antioxidant, anti-inflammatory, wound-healing	Widely distributed in ethnomedicinal plants for skin & wound care	Stable, strong UV absorbance; robust quantification; complements other markers

Including multiple markers is essential for establishing the claim of a phytopharmaceutical and reflects the plant's multifaceted bioactivity. By ensuring that these compounds are consistently present at defined concentrations, researchers can link chemical profiles to pharmacological outcomes, thereby strengthening the case for regulatory approval.

In summary, using VBS, CA, COA, and FA as marker compounds provides both chemical assurance and therapeutic relevance for *K. africana*-based products. By anchoring extract standardization in molecules that are pharmacologically active, ethnobotanically significant, and analytically tractable, the development of a phytopharmaceutical dosage form is strengthened at every stage, from raw material validation to formulation, stability studies, and pre-clinical and clinical studies. This approach ensures reproducibility and regulatory compliance while establishing a mechanistic link between traditional use and modern scientific evaluation. As such, these markers form the cornerstone of transforming *K. africana* from an ethnomedicinal remedy into a rigorously defined candidate for contemporary phytopharmaceutical development.

5.1 Experimental work

5.1.1 Instruments and Equipment

Chromatographic analysis was conducted using a CAMAG HPTLC system (Muttenez, Switzerland), an integrated platform consisting of a Linomat 5 semi-automatic applicator, a TLC Scanner 4, and a dual-wavelength UV cabinet. Chromatographic development was performed in a twin-trough chamber, with all system operations and data processing managed via vision CATS software (Version 3.2). Precise sample deposition was achieved utilizing a specialized Hamilton application syringe (100 μ L capacity). All standards were weighed on a microbalance (Mettler Toledo) and an analytical balance (Mettler Toledo). An

electric water bath (CINTEX Industrial Corporation, Mumbai, India) was used for drying the extract.

5.1.2 Chemicals and materials

Reference standards of Verbascoside ($\geq 98\%$ w/w), Caffeic acid ($\geq 98\%$ w/w), Coumaric acid ($\geq 99\%$ w/w), and Ferulic acid ($\geq 99\%$ w/w) were purchased from Yucca Enterprises, Mumbai, India. Chloroform, ethyl acetate, formic acid, methanol, and toluene were of LR grade and used for HPTLC analyses, and ethanol of LR grade (Fisher Scientific, India) was used for extraction. Pre-coated aluminum-backed silica gel G60 F254 HPTLC plates (Sigma) were used for analyses, and Whatman filters (Type-1) were used for filtration.

5.1.4 Preparation of Solutions

Accurately weighed reference standard VBS (2 mg) was dissolved and diluted with methanol to 5 mL to obtain stock standard solution (400 $\mu\text{g/mL}$). A 1 mL aliquot of the primary stock solution was precisely transferred and diluted to a final volume of 10 mL with methanol. This dilution series yielded a working standard solution of VBS at 40 $\mu\text{g/mL}$.

Reference standards for CA, COA, and FA (10 mg each) were precisely weighed and individually reconstituted in methanol to a final volume of 10 mL, yielding primary stock solutions at 1000 $\mu\text{g/mL}$. To prepare the multicomponent working standard, specific aliquots of 0.4 mL (CA), 0.1 mL (COA), and 0.4 mL (FA) were harvested from their respective stocks, combined, and diluted to 10 mL. This procedure resulted in a calibrated working solution with final concentrations of 40 $\mu\text{g/mL}$ for CA, 10 $\mu\text{g/mL}$ for COA, and 40 $\mu\text{g/mL}$ for FA.

5.1.5 Preparation of extract and sample solution

A total of 14 *K. africana* fruits were harvested and sectioned into small pieces to facilitate drying. The material was sun-dried for 10 days, then dehydrated in a hot air oven at 45°C for 24 hours. The desiccated fruit pieces were then pulverized and passed through an 80# mesh sieve to ensure a uniform fine powder. For extraction, a 25 g portion of the powder was weighed, transferred to a thimble, and Soxhlet-extracted with 250 mL of absolute ethanol (100%) at 40°C for 36 hours.

The resulting ethanolic extract was concentrated and evaporated to dryness in a temperature-controlled electric water bath at 50°C, then filtered through Whatman No. 1 filter paper. For analytical preparation, 50 mg of the dried residue was precisely weighed and reconstituted in 4 mL of methanol. We facilitated dissolution by sonication for 10 minutes in a glass chamber, then adjusted the volume to 5 mL. This solution was filtered, and a 1 mL aliquot of the filtrate was diluted to 10 mL with methanol to yield a final working sample concentration of 1000 µg/mL. A 10 µL volume of this sample was applied to the HPTLC plate for the quantitative determination of marker constituents (% w/w).

5.2 HPTLC methods

The initial trials aimed to separate VBS, CA, COA and FA simultaneously. All reference and sample solutions were manually applied to aluminum-backed silica gel G60 F₂₅₄ HPTLC plates using capillaries. To separate the spots of these four markers, plates were developed using varying proportions of toluene, ethyl acetate, chloroform, and methanol as the mobile phase. To obtain resolved spots and reduce band diffusion, the mobile phases included glacial acetic acid and formic acid. A reported method (Method-1:Table 5-2) for VBS was adopted and verified for specificity, linearity, and precision. For CA, COA & FA, a newer method (Method -2) was developed and validated.

5.2.1 Verification of Method-1

The verification of Method-1 was carried out using the chromatographic conditions shown in Table 5-2.

Table 5-2 Reported method-1 chromatographic conditions

Parameter	Description
Mobile Phase	Toluene-ethyl acetate-methanol formic acid (3.5:5:1:1 v/v)
Plate dimensions	(10 × 10 cm ²)
Saturation time	40 minutes
Band length	8 mm
Migration distance	90 mm (from bottom of plate)
Detection wavelength	330 nm

5.2.1.1 Calibration curves

To establish the calibration range for VBS, specific aliquots of the working standard solution (40 µg/mL) were applied to the HPTLC plate in volumes of 5, 10, 15, 20, and 25 µL, corresponding to a mass range of 200, 400, 600, 800, and 1000 ng/band, respectively. After chromatographic development and densitometric analysis under optimized conditions, calibration curves were generated by correlating peak areas with corresponding concentrations.

5.2.1.2 Verification

The analytical performance of the developed HPTLC method was validated in accordance with the ICH Q2(R1) guidelines. The protocol focused on evaluating the method's reliability through the assessment of critical parameters, specifically linearity, precision, and specificity, to ensure its suitability for the quantitative analysis of the marker compounds.

Specificity

To verify method specificity, peak purity was assessed by scanning the resolved bands of the ethanolic sample extract at the peak start, apex, and end. The overlay of these spectra was evaluated to ensure that no co-eluting impurities interfered with the target marker signals, thereby confirming the homogeneity of the chromatographic zones.

Linearity

The linearity of the proposed method was evaluated by analyzing five distinct calibration levels (n=5) for VBS within the range of 200 to 1000 ng/band. We constructed calibration curves by correlating mean peak areas with their respective mass concentrations. To statistically validate linearity, we computed the linear regression equation, the coefficient of determination (R²), and the relative standard deviation (RSD) of the peak areas.

Precision

Methodological precision was comprehensively assessed through four parameters: repeatability of application, repeatability of scanning, and both intra-day and inter-day precision.

To evaluate application repeatability, six replicates of a 15 µL working standard solution were applied to the HPTLC plate. Following development and densitometric analysis, the RSD of the peak areas across the six tracks was calculated for VBS. Scanning repeatability was verified by scanning a single separated band of VBS six consecutive times without altering the

plate's position, from which the RSD of the peak area was determined.

Intra-day precision was established by analyzing VBS at three distinct concentration levels (200, 600, and 800 ng/band) at three separate intervals within a single day. Similarly, inter-day precision was evaluated by analyzing the same three concentrations across three consecutive days. In both cases, the RSD of the peak areas was computed for each level to confirm the assay's temporal stability and reproducibility.

5.2.2 Development and validation of method-2

The combined working standard solution containing CA (40µg/mL), COA (10µg/mL), and FA (40µg/mL), along with the ethanolic fruit extract, was initially applied to aluminum-backed silica gel 60 F₂₅₄ HPTLC plates using sealed capillaries for preliminary trials. A mobile phase consisting of chloroform: methanol: formic acid (9.5:0.5:0.1 v/v/v) (Table 5-3) was utilized to achieve optimal resolution of the three phenolic acids.

For wavelength optimization, 10µL of the combined working standard was applied using the Linomat 5 applicator. Post-development, the plate was dried and visualized at 254 nm to locate the chromatographic zones. The UV absorption spectra for the resolved bands of CA, COA, and FA were then recorded via densitometric scanning. Based on the resulting overlay spectra, we selected a detection wavelength of 330 nm for simultaneous quantification of these three markers in the *K. africana* extract.

5.2.2.1 Calibration curves

To establish the calibration ranges for the simultaneous quantification of CA, COA, and FA, specific volumes (5, 10, 15, 20, 25, and 30 µL) of the combined working standard solution were applied to the HPTLC plate. These applications corresponded to a mass range of 200- 1200 ng/band for both CA and FA, and 50- 300 ng/band for COA. After development and

densitometric analysis under the prescribed chromatographic conditions, we generated calibration curves for each marker by correlating peak areas with their respective mass concentrations.

Table 5-3 Method-2 Chromatographic conditions

Chromatographic conditions	Method-2
Stationary phase	Pre-coated aluminum-backed HPTLC plates with silica gel G60 F ₂₅₄ (10 × 10 cm ²)
Mobile phase	Chloroform–methanol–formic acid (9.5: 0.5: 0.1 v/v)
Saturation time	20 minutes
Migration distance	90 mm (from bottom of the plate)
Application conditions	
Band length	8 mm
Distance between tracks	13 mm
Dosage speed	150 nL/s
Scanning conditions	
Slit dimension	6 × 0.45 mm
Detection Wavelength	330 nm
Lamp	Deuterium
Measurement mode	Absorption
Scanning speed	20 mm/s
Spectral Scanning	200 to 450 nm
Integration conditions	
Baseline correction	Lowest slope with noise 0.05

Chromatographic conditions	Method-2
Peak detection sensitivity	0.1
Separation	1
Threshold	0.1

5.2.2.2 Validation

In accordance with the ICH Q2(R1) guidelines, the established HPTLC method underwent a rigorous validation protocol to confirm its analytical reliability. This comprehensive assessment evaluated several critical performance characteristics, including specificity, linearity and range, the limit of detection (LOD), and the limit of quantitation (LOQ). Additionally, the method's precision, accuracy, and robustness were systematically verified to ensure consistent, reproducible results for quantitative analysis of the targeted phenolic compounds.

Specificity

To verify the specificity of the analytical method, peak purity was evaluated by densitometrically scanning the resolved bands of the ethanolic sample extract. Scans were performed at three critical positions, the peak start, apex, and end, to assess the spectral homogeneity of the chromatographic zones. By comparing these spectra, the absence of co-eluting impurities was confirmed, ensuring that the detected signals for CA, COA, and FA were free from analytical interference.

Linearity

The linearity of the method was evaluated across six distinct concentration levels (n=5), spanning 200-1200 ng/band for CA and FA, and 50-300 ng/band for COA. Calibration curves were constructed by plotting the mean peak areas against their respective mass concentrations. Statistical parameters, including the linear regression equation, the coefficient of determination

(R^2), and the relative standard deviation (RSD) of peak areas, were used to assess fit. To evaluate the analytical sensitivity, the Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated using the following mathematical models: $LOD = (3.3 \sigma)/S$, $LOQ = (10 \sigma)/S$; where σ represents the response's standard deviation and S is the calibration curve's slope.

Precision

The precision of the developed HPTLC method was rigorously evaluated by assessing intraday precision, interday precision, and the repeatability of both sample application and densitometric scanning.

To verify application repeatability, six replicates of a 15 μ L working standard solution were applied to a single plate. After development under optimized conditions, we calculated the RSD of the peak areas for CA, COA, and FA across the six tracks. Scanning repeatability was assessed by performing six consecutive scans of the separated bands on all six tracks without repositioning the plate; the resulting RSD of the peak areas for each marker confirmed the instrument's measurement stability.

Intraday precision was established by analyzing three concentration levels for each marker CA and FA (200, 600, and 1200 ng/band) and COA (50, 150, and 300 ng/band) at three distinct intervals within the same day. Interday precision was determined similarly by analyzing the same three concentration levels across three consecutive days. For both parameters, we computed the RSD of the peak areas at each level to assess the method's temporal reproducibility and reliability.

Robustness

To evaluate method robustness, we introduced deliberate, minor variations in the optimized chromatographic parameters. We analyzed three concentration levels from the calibration curve while intentionally modifying the mobile phase volume (± 1 mL),

chamber saturation time (± 5 min), and detection wavelength (± 1 nm). The impact of these fluctuations was assessed by computing the RSD for both peak areas and R_F values, ensuring that the analytical performance remained stable despite small operational deviations.

5.3 Results and Discussion

The sieving process yielded a total of 1500 g of powdered material. The dried powder was brown. The ethanolic extract, prepared via solvent extraction, was reddish-brown in color and semi-solid in consistency. From 25 g of the dried fruit powder, 10 g of ethanolic extract was yielded.

Simultaneous separation of all four target biomarkers proved unsuccessful. This was primarily due to the high polarity of VBS, in contrast to the structural and chemical similarity among the remaining three markers. Preliminary chromatographic trials did not reveal a single set of conditions suitable for concurrent resolution of all four compounds.

A higher proportion of toluene and lower proportions of ethyl acetate and methanol enabled satisfactory separation of CA and FA with acceptable R_F values. However, under these conditions, the spots corresponding to COA, VBS, and CA overlapped, resulting in inadequate resolution. Based on these observations, it was concluded that two distinct chromatographic methods were required: Method 1 for estimating VBS, and Method 2 for simultaneously estimating CA, COA, and FA.

5.3.1 Verification of Method 1

Method-1 provided resolved and symmetric bands for VBS (R_F 0.15) (Figure 5-2).

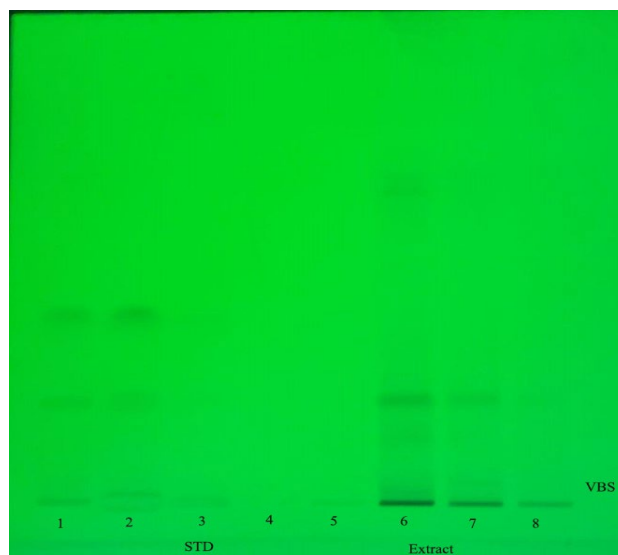


Figure 5-2 TLC photograph of VBS

The densitogram of the standard solution showing the separated peaks using chromatographic conditions (Figure 5-3). The R_f value of VBS was similar in the densitograms obtained from the standard and sample solutions.

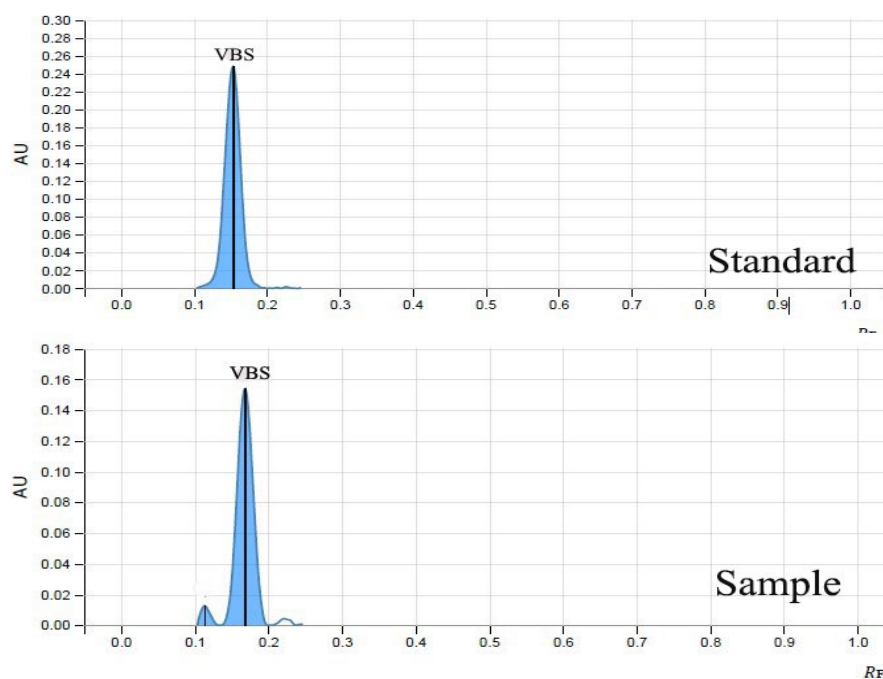


Figure 5-3 Densitogram of standard and sample VBS solution by method-I

Recorded absorption spectra of VBS from the standard solution were found to be in good correlation ($r^2 > 0.99$) with those

recorded from the sample solution (Figure 5-4). Peak purity of the sample solution is presented in Figure 5-5.

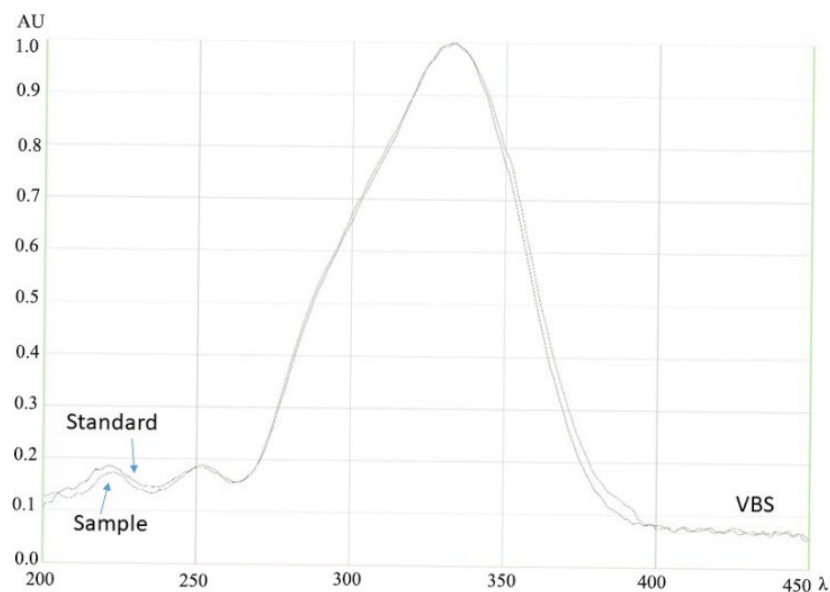


Figure 5-4 Overlay spectra of standard and sample VBS

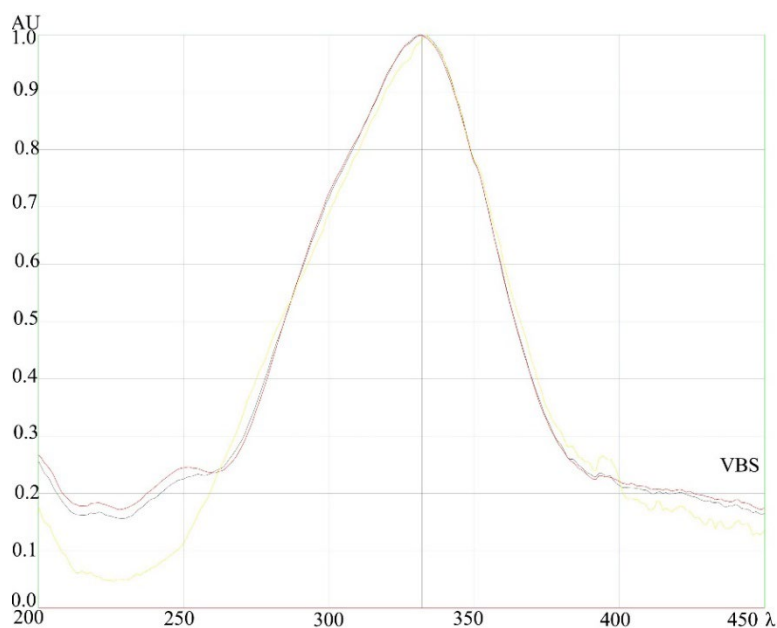


Figure 5-5 Peak purity of VBS

Peak areas corresponding to VBS were found to be linear with the concentration in the range of 200–1000 ng/band (Figure 5-6). The representative calibration curve (Figure 5-7), obtained from mean data (n=6) for 200 to 1000 ng/band, was linear with a good

coefficient of regression ($r^2 > 0.98$). The RSD of peak area at individual calibration-curve levels (n=5) for the marker was < 2%. Results prove that the method is linear in the respective range for VBS (Table 5-4).

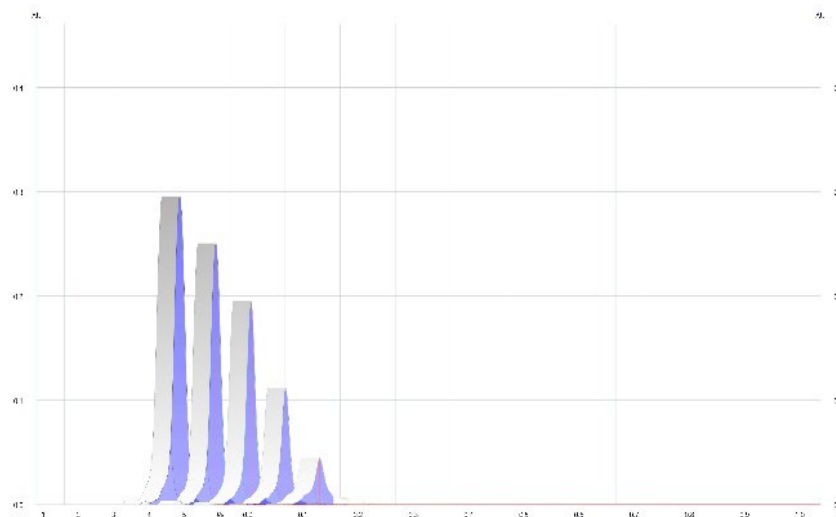


Figure 5-6 3-D Chromatogram view of VBS

Table 5-4 Linearity data for VBS

Range (ng/band)	VBS (40 µg/ml) Mean Area ± SD (n= 6)	RSD (%)
200	101.5 ± 0.0005	0.05
400	223.0 ± 0.0005	0.02
600	409.5 ± 0.00021	0.07
800	562.0 ± 0.00023	0.05
1000	704.5 ± 0.00023	0.04

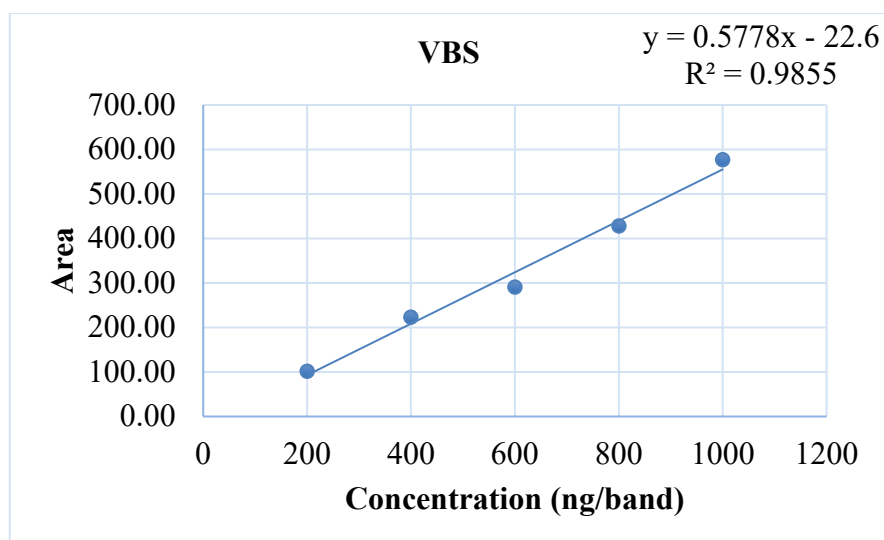


Figure 5-7 Calibration curve of VBS

The RSD of the measured peak areas for VBS during the repeatability (Table 5-5), intraday (Table 5-6), and interday (Table 5-7) precision studies was less than 2%, indicating that the method is precise. A summary of the results of verification of method-1 is presented in Table 5-8.

Table 5-5 Repeatability data for VBS

Repeatability of application			Repeatability of scanning	
Track	Concentration (ng/band)	Area (n=6)	Concentration (ng/band)	Area (n=6)
1	600	411	600	414
2	600	420		420
3	600	425		422
4	600	423		428
5	600	407		409
6	600	418		415
Mean area $\pm$ SD		417.33 $\pm$ 7.00	Mean area $\pm$ SD	418 $\pm$ 6.72
RSD (%)		1.65	RSD (%)	1.60

Table 5-6 Intra-day precision data for VBS

Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)
200	102.00 $\pm$ 1.01	0.98
600	411.33 $\pm$ 3.21	0.78
800	560.66 $\pm$ 5.13	0.91

Table 5-7 Inter-day precision data for VBS

Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)
200	104.33 $\pm$ 1.52	1.46
600	418.66 $\pm$ 5.85	1.39
800	561 $\pm$ 5.29	0.94

Table 5-8 Results of verification for method-1

Validation characteristics	Results
Linearity and range	
Range (ng/band)	200-1000
Regression equation	$y = 0.5778x - 22.6$
Coefficient of regression	0.98
RSD of peak area (%)	0.6–1.99
Precision (RSD, %)	
Repeatability of application	1.65
Repeatability of scanning	1.60
Intraday precision	0.78–0.98
Interday precision	0.94–1.46

5.3.2 Development and validation of method-2

Initial trials (Table 5-9) employed a mobile phase consisting of toluene, ethyl acetate, methanol, and formic acid in varying proportions. By increasing the proportion of toluene while simultaneously reducing the concentrations of ethyl acetate and methanol, the mobile phase composition was optimized to provide superior resolution. This modification ensured that CA and FA were consistently separated with R_F values within the ideal range for densitometric quantification.

However, in all three combinations, the COA spot remained merged with FA, preventing simultaneous resolution. Further attempts to increase methanol content beyond 0.5 mL resulted in a loss of resolution between COA and FA, indicating that methanol concentration was a limiting factor. To address this, toluene was replaced with chloroform and ethyl acetate was excluded from mobile phase entirely, with the goal of increasing the of CA and enhancing separation between COA and FA.

Subsequent trials using chloroform–methanol–formic acid still failed to resolve COA and FA adequately. Finally, a composition of chloroform–methanol–formic acid (9.5:0.5:0.1 v/v) successfully achieved complete separation among all three marker compounds. This optimized solvent system yielded distinct, well-resolved, and symmetrical chromatographic bands for CA (R_F 0.2), COA (R_F 0.45), and FA (R_F 0.55). Densitometric scanning of these separated zones revealed absorption maxima at 326 nm, 313 nm, and 324 nm for CA, COA, and FA, respectively. Consequently, a wavelength of 330 nm was selected as the optimal compromise for the simultaneous estimation of all three markers. The corresponding TLC chromatogram documents the effective separation achieved under these refined conditions (Figure 5-8).

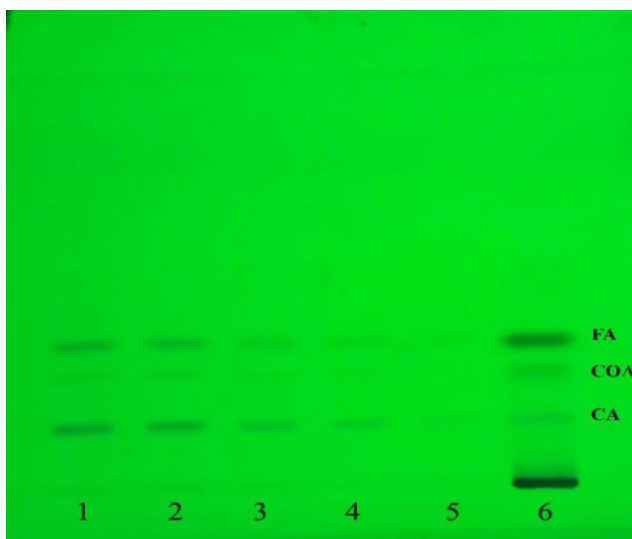


Figure 5-8 TLC photograph of CA, COA and FA

Table 5-9 Trials for development of method-2

Sr. No.	Mobile phase composition	Inference
1	Toluene–ethyl acetate–methanol–formic acid (2:6.5:1:0.1 v/v)	CA and FA were separated but COA spot was merged with FA
2	Toluene–ethyl acetate–methanol–formic acid (2:6.5:1.5:0.5 v/v)	CA and FA were separated but COA spot was merged with FA
3	Toluene–ethyl acetate–methanol–formic acid (4:4:1:1 v/v)	CA and FA were separated but COA spot was merged with FA
4	Chloroform–methanol–formic acid (5:5:0.1 v/v)	Resolution not achieved b/w COA and FA.
5	Chloroform–methanol–formic acid (6:4:0.1 v/v)	Resolution not achieved b/w COA and FA.
6	Chloroform–methanol–formic acid (4:6:0.1 v/v)	Resolution not achieved b/w COA and FA.
7	Chloroform–methanol–formic acid (9.5:0.5:0.1 v/v)	Separation was achieved among the three markers.

5.3.2.1 Validation

The peak areas corresponding to CA, COA, and FA demonstrated a linear relationship with concentration over the ranges of 200–1200 ng/band for CA, 50–300 ng/band for COA, and 200–1200 ng/band for FA (Figure 5-9 and Table 5-10 and Table 5-11). Calibration curves constructed from the mean of six replicates ($n=6$) for each analyte showed good linearity within their respective concentration ranges. The regression analysis yielded high correlation coefficients ($r^2 > 0.97$), confirming the linearity and suitability of the method for quantitative analysis (Figure 5-10). The RSD of peak area at individual levels of calibration curves ($n=6$) for these markers were $< 2\%$.

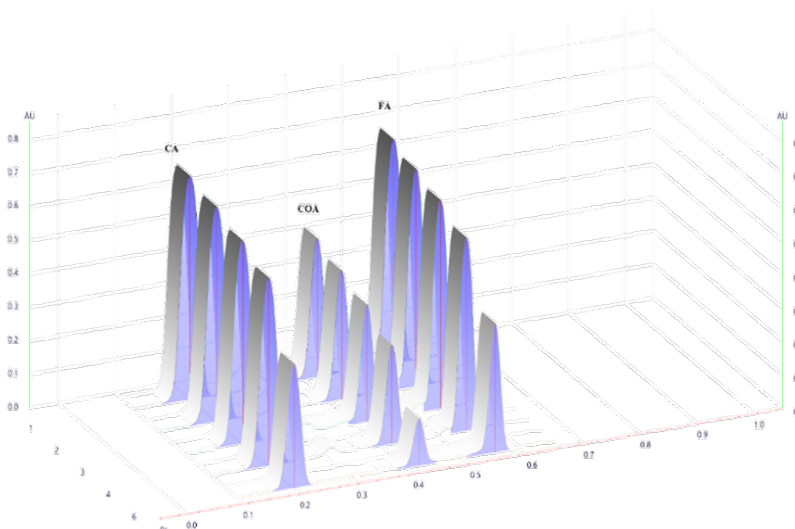


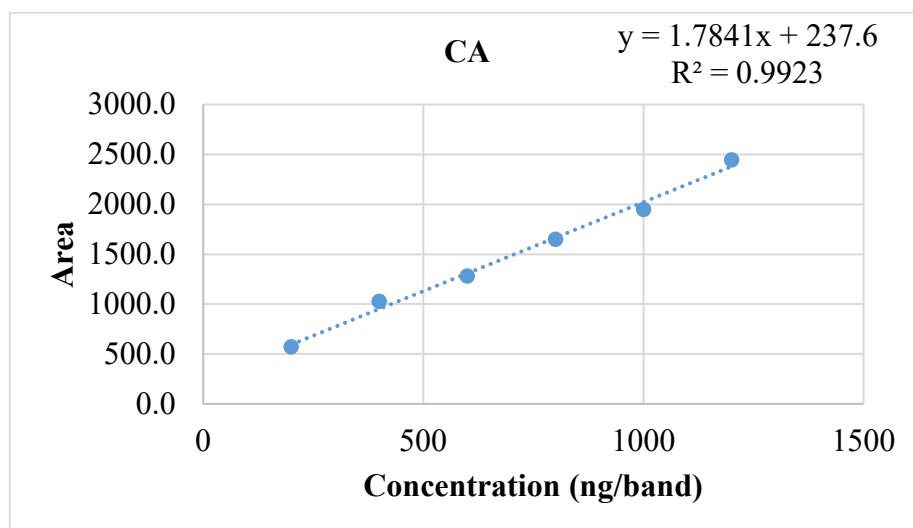
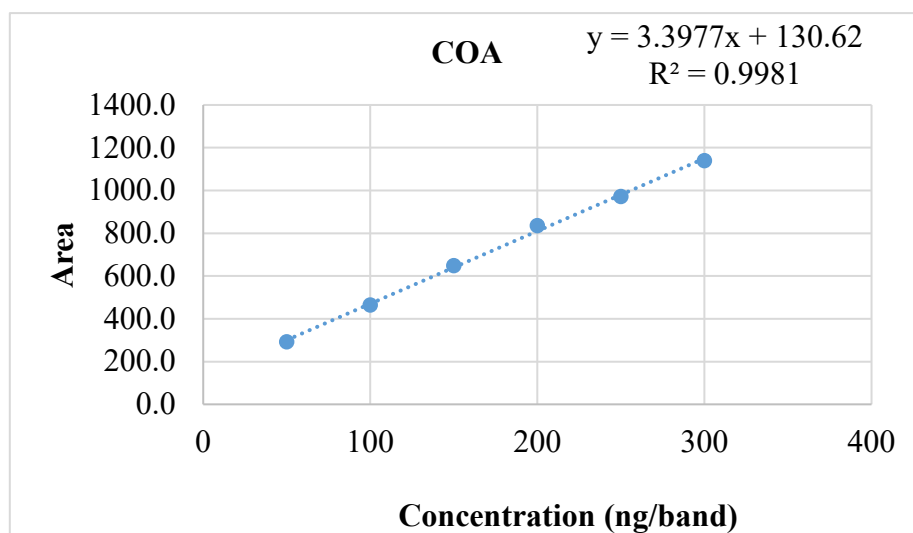
Figure 5-9 3-D chromatogram view of CA, COA and FA

Table 5-10 Linearity data for COA

Range (ng/band)	COA (10 µg/ml) Mean Area $\pm$ SD (n= 6)	RSD (%)
50	292.0 $\pm$ 5.01	1.71
100	464.8 $\pm$ 7.25	1.56
150	648.0 $\pm$ 9.83	1.51
200	835.2 $\pm$ 12.85	1.53
250	971.7 $\pm$ 10.23	1.05
300	1139.7 $\pm$ 16.66	1.46

Table 5-11 Linearity data for CA and FA

Range (ng/band)	CA (40 µg/ml) Mean Area ± SD (n= 6)	RSD (%)	FA (40 µg/ml) Mean Area ± SD (n= 6)	RSD (%)
200	571.0 ± 14.08	1.46	728.8 ± 7.80	1.07
400	1028.3 ± 16.71	1.62	1054.3 ± 13.01	1.23
600	1280.3 ± 16.08	1.25	1289.2 ± 17.49	1.35
800	1648.0 ± 31.04	1.88	1572.2 ± 17.54	1.11
1000	1947.7 ± 30.99	1.59	1934.0 ± 25.42	1.31
1200	2443.7 ± 31.23	1.27	2371.5 ± 37.30	1.57



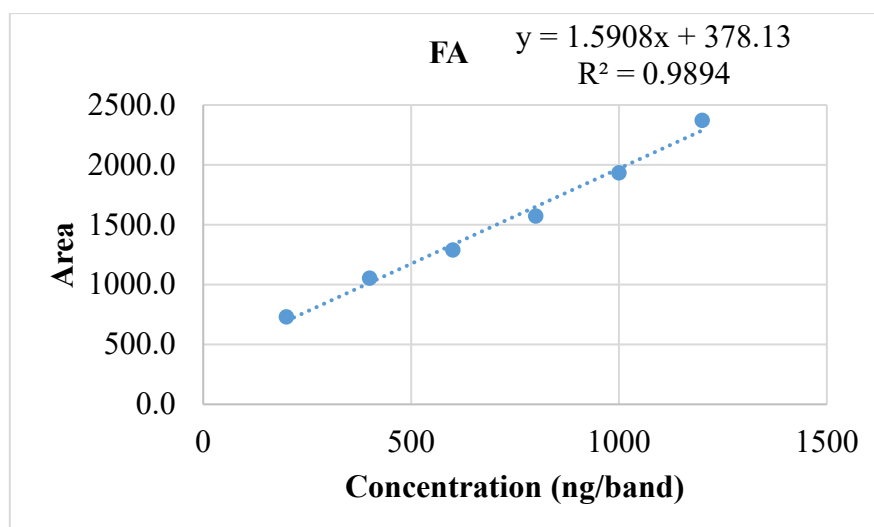


Figure 5-10 Calibration curve of CA, COA and FA

RSD of measured peak areas of repeatability study (Table 5-12, Table 5-13, Table 5-14), inter & intraday precision method-2 is precise (Table 5-15, Table 5-16, Table 5-17, Table 5-18).

Table 5-12 Repeatability data for COA

Repeatability of application			Repeatability of scanning	
Track	Concentration (ng/band)	Area (n=6)	Concentration (ng/band)	Area (n=6)
1	150	661	150	661
2	150	639		649
3	150	659		659
4	150	645		655
5	150	646		646
6	150	640		645
Mean area $\pm$ SD		648.33 $\pm$ 9.45	Mean area $\pm$ SD	652.5 $\pm$ 6.80
RSD (%)		1.46	RSD (%)	1.04

Table 5-13 Repeatability application data of CA and FA

Track	CA Concentration (ng/band)	Area (n=6)	FA Concentration (ng/band)	Area (n=6)
1	600	1262	600	1291
2	600	1299	600	1320
3	600	1270	600	1276
4	600	1278	600	1268
5	600	1318	600	1292
6	600	1272	600	1285
Mean area $\pm$ SD		1283.16 $\pm$ 21.15	Mean area $\pm$ SD	1288.66 $\pm$ 17.88
RSD (%)		1.65	RSD (%)	1.39

Table 5-14 Repeatability scanning data of CA and FA

CA Concentration (ng/band)	Area (n=6)	FA Concentration (ng/band)	Area (n=6)
600	1280	600	1301
	1315		1280
	1255		1276
	1275		1278
	1298		1299
	1292		1285
Mean area $\pm$ SD	1285.83 $\pm$ 20.68	Mean area $\pm$ SD	1286.50 $\pm$ 10.89
RSD (%)	1.60	RSD (%)	0.85

Table 5-15 Intraday precision data of COA

Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)
50	285.33 $\pm$ 2.51	0.88
150	550.00 $\pm$ 4.58	0.83
300	1146.33 $\pm$ 8.08	0.70

Table 5-16 Interday precision data of COA

Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)
50	287.66 $\pm$ 5.51	1.91
150	546.00 $\pm$ 7.54	1.38
300	1151.66 $\pm$ 13.57	1.17

Table 5-17 Intraday precision data of CA and FA

CA Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)	FA Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)
200	569.66 $\pm$ 6.11	1.07	200	740.00 $\pm$ 11.26	1.12
600	1281.67 $\pm$ 12.58	0.98	600	1286.00 $\pm$ 10.14	0.87
1200	2433.33 $\pm$ 8.14	0.49	1200	2371.33 $\pm$ 7.63	0.41

Table 5-18 Interday precision data of CA and FA

CA Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)	FA Concentration (ng/band)	Mean area $\pm$ SD (n=3)	RSD (%)
200	571.33 $\pm$ 8.50	1.48	200	740.00 $\pm$ 11.26	1.52
600	1283.67 $\pm$ 12.58	0.98	600	1286.00 $\pm$ 10.14	0.78
1200	1654.33 $\pm$ 8.14	0.49	1200	1583.33 $\pm$ 7.63	0.48

Respective R_F values of CA, COA and FA were found to be similar in the densitograms obtained from standard and sample solutions (Figure 5-11).

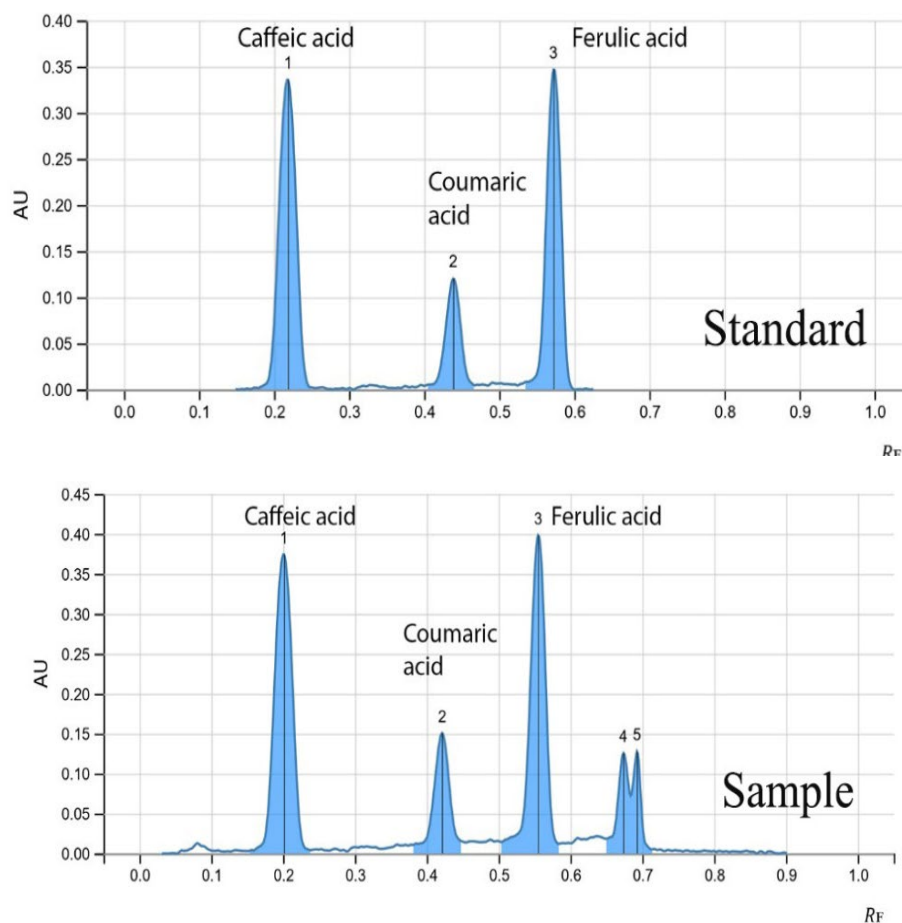


Figure 5-11 Densitogram of standard and sample solutions by method-2

Recorded absorption spectra of CA, COA and FA from standard solution were found to be in good correlation ($r^2 > 0.99$) with those recorded from sample solution (Figure 5-12). Peak purity of sample solution is presented in Figure 5-13.

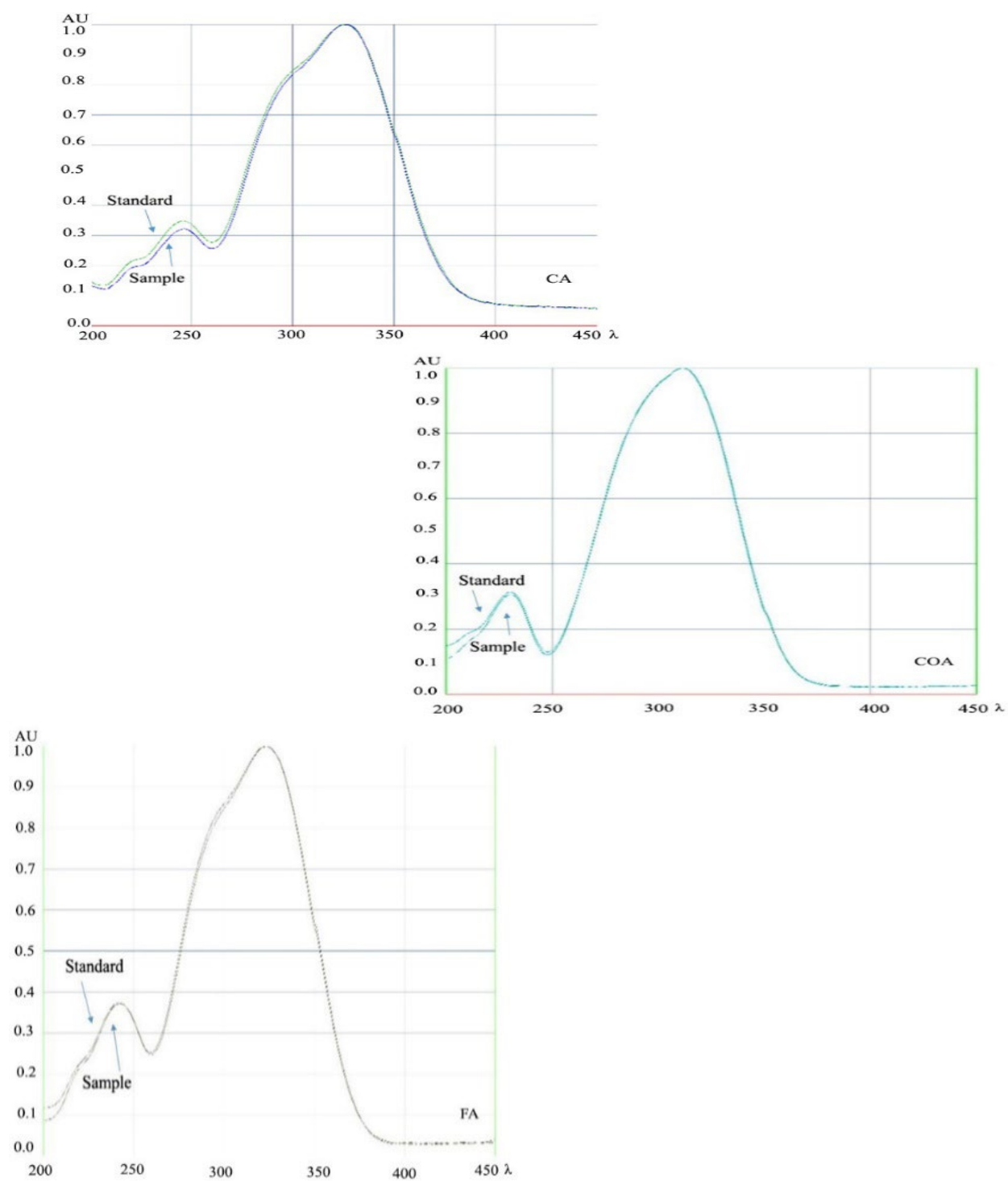


Figure 5-12 Overlay absorption spectra of standard and sample solutions; CA, COA and FA

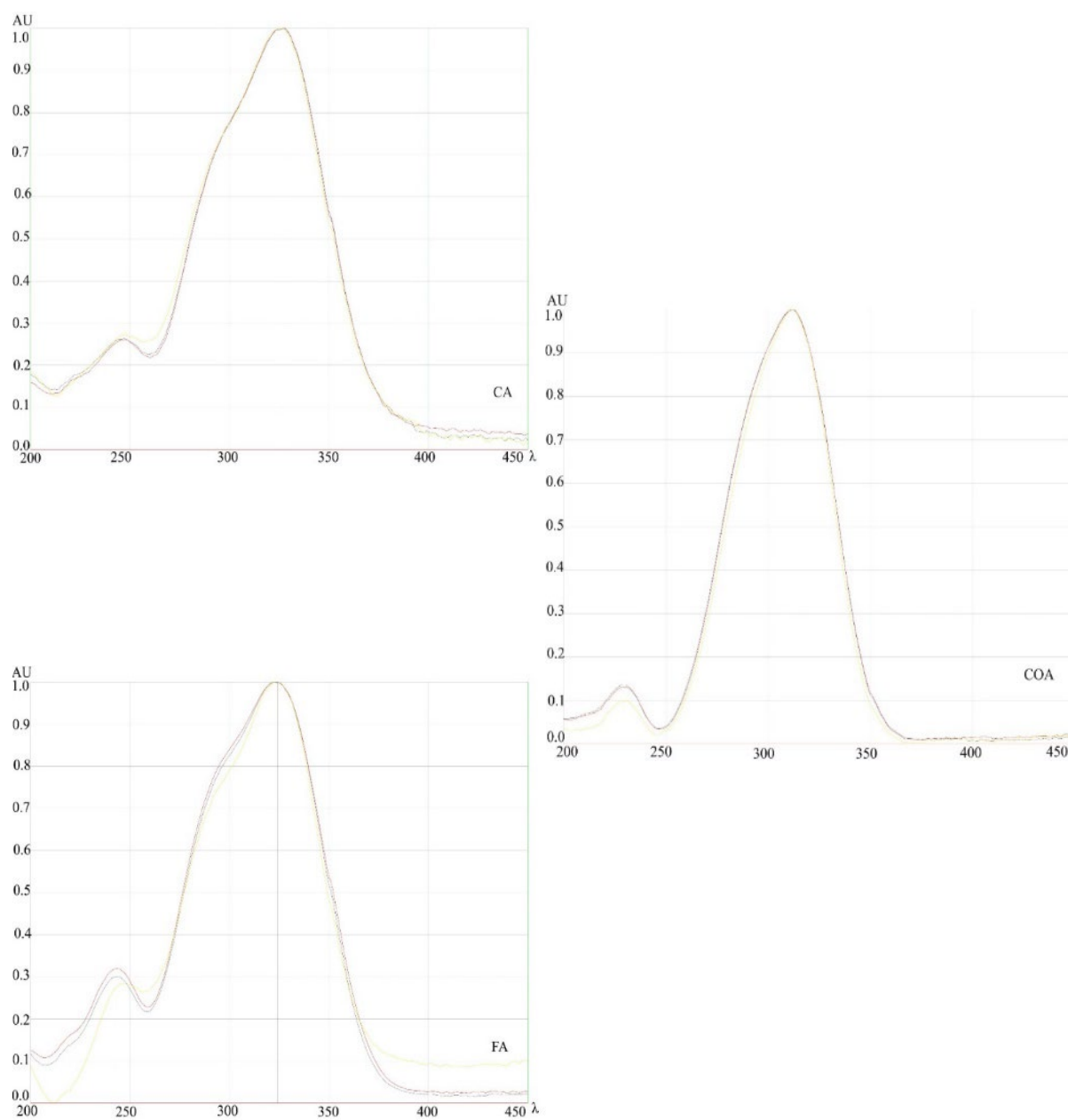


Figure 5-13 Peak purity of CA, COA and FA

RSD (< 2%) after making small deliberate changes in methods parameters in wavelength (± 1 nm), saturation time (± 5 min) and amount of mobile phase (± 1 mL) (Table 5-19).

Table 5-19 Robustness data of COA, CA and FA

Method parameters	Variation	CA 600 ng/band		COA 150 ng/band		FA 600 ng/band	
		Peak area	R _F	Peak area	R _F	Peak area	R _F
Detection wavelength(± 1 nm) (n=3)	329 (-1)	1259	0.19	661	0.45	1291	0.55
	330 (0)	1305	0.20	639	0.46	1285	0.55
	331 (+1)	1266	0.18	659	0.46	1269	0.55
	Mean $\pm$ SD	1276.66 $\pm$ 24.78	0.17 $\pm$ 0.003	653 $\pm$ 12.16	0.46 $\pm$ 0.003	1281.66 $\pm$ 11.37	0.55 $\pm$ 0.003
	RSD (%)	1.94	1.78	1.86	0.76	0.88	0.55
Saturation Time (± 5 min) (n=3)	15 (-1)	1272	0.18	657	0.45	1291	0.55
	20 (0)	1285	0.17	654	0.46	1301	0.55
	25 (+1)	1275	0.18	651	0.46	1276	0.55
	Mean $\pm$ SD	1277.33 $\pm$ 6.80	0.18 $\pm$ 0.002	654 $\pm$ 3	0.46 $\pm$ 0.002	1289.33 $\pm$ 12.58	0.55 $\pm$ 0.004
	RSD (%)	0.53	1.46	0.46	0.49	0.97	0.85
Volume of Mobile Phase (± 1 mL) (n=3)	9 (-1)	1265	0.18	658	0.45	1305	0.55
	10 (0)	1285	0.17	654	0.46	1301	0.56
	11 (+1)	1275	0.18	651	0.46	1272	0.54
	Mean $\pm$ SD	1275 $\pm$ 10	0.18 $\pm$ 0.001	654.33 $\pm$ 3.51	0.45 $\pm$ 0.006	1292.66 $\pm$ 18.00	0.55 $\pm$ 0.006
	RSD (%)	0.78	0.84	0.54	1.32	1.39	1.20

LOD and LOQ for CA was found to be 32.57 and 98.71 ng/band, while 4.15 and 12.59 ng/band for COA and 44.41 and 134.57 ng/band for FA. Results suggested that the developed method is appropriately sensitive to estimate the CA, COA and FA in fruit extract. Summary of validation studies for method-2 is in Table 5-20.

Table 5-20 Results of validation studies for method-2

Validation characteristics		Results		
		CA	COA	FA
Range (ng/band)		200–1200	50–300	200–1200
Regression equation		$y = 1.7841x + 237.6$	$y = 3.3977x + 130.62$	$y = 1.5908x + 378.13$
Coefficient of regression		0.981	0.9908	0.9788
RSD of peak area (%)		0.6–1.99	0.56–1.79	1.22–1.51
Repeatability of application		1.65	1.46	1.39
Repeatability of scanning		1.60	1.04	0.85
Intraday precision		0.49–1.07	0.70–0.88	0.48–1.52
Interday precision		0.91–1.48	1.17–1.91	0.65–1.48
Detection limit (ng/band)		32.57	4.15	44.41
Quantitation limit (ng/band)		98.71	12.59	134.57
Accuracy (%)		100.4–102.8%	98.1–99.8%	97.9–99.1%
Detection wavelength (± 1 nm)	Peak area	1.94	1.86	0.88
	R _F	1.78	0.76	0.55
Saturation time (± 5 min)	Peak area	0.53	0.46	0.97
	R _F	1.46	0.49	0.85
Volume of mobile phase (± 1 mL)	Peak area	0.78	0.54	1.39
	R _F	0.84	1.32	1.20

5.4 Quantification of marker compounds

The validated Method-1 was utilized to quantify the VBS content in the ethanolic fruit extract of *K. africana*. Triplicate analysis (n=3) revealed a mean concentration of 1.39% w/w for VBS. Similarly, Method-2 was employed for the simultaneous determination of CA, COA, and FA. The fruit extract was found to contain 0.259% w/w of CA, 0.027% w/w of COA, and 0.182% w/w of FA (n=3). Representative densitograms illustrating the resolution and peak characteristics of the marker compound VBS are presented in Figure 5-3 and for CA, COA and FA is presented in Figure 5-11.

5.5 Conclusion

The primary advantage of these protocols is their reliance on HPTLC, which achieves the necessary sensitivity and specificity for quantitative analysis without the requirement for more complex or capital-intensive instrumentation. Furthermore, these validated methods provide a robust framework that can be adapted for the estimation of these specific marker compounds across various other plant species. Beyond quantification, the determination of these markers in *K. africana* facilitates comprehensive chromatographic fingerprinting, serving as a vital tool for the botanical authentication and quality control of the plant material.

CHAPTER-6

OPTIMIZATION

OF

EXTRACTION

PROCESS

6 Optimization of extraction process

Extracting bioactive compounds from plant materials involves navigating a complex interplay of factors that significantly influence the yield and quality of the final extract. The extraction process is a fundamental step in recovering and purifying bioactive compounds from herbal materials and directly affects the pharmacological efficacy, stability, and formulation of herbal products. Hence, extraction procedures must be designed to maximize yield while preserving chemical integrity and biological activity.

Design of Experiments (DOE) is a robust statistical and methodological framework that enables systematic elucidation of the principal effects and interactions of multiple independent variables on a given dependent response. This approach demonstrably minimizes the requisite experimental runs while maximizing the information yield, conserving valuable resources, accelerating discovery, and empowering the development of models that provide quantitative relationships between input parameters and desired outputs, facilitating precise process control and optimization.⁽⁵⁹⁻⁶¹⁾

In herbal extractions, DOE plays a crucial role in fine-tuning key parameters like solvent composition, temperature, extraction time, and solid-to-liquid ratio to enhance yield and bioactivity; strategic deployment of DOE is paramount for precisely calibrating critical parameters.⁽⁶²⁻⁶⁷⁾

Full factorial designs evaluate all possible combinations of factor levels, offering comprehensive insights into both main effects and interactions. This approach supports informed decision-making and process optimization, especially when the number of factors is relatively small (typically fewer than five). By enabling simultaneous analysis of multiple variables, such as solvent type, time, temperature, and plant-to-solvent ratio, this approach can

investigate their effects on extraction efficiency and bioactive properties simultaneously ⁽⁶⁷⁻⁶⁹⁾.

This chapter systematically investigates the critical parameters influencing the extraction of bioactive compounds from *K. africana* fruit, involving extraction optimization through a full factorial design, offering a systematic approach to understanding and improving the efficiency and effectiveness of extracting bioactive compounds, focusing specifically on how solvent selection and extraction duration directly impact extract yield, quality, and subsequent biological activity.⁽⁷⁰⁻⁷³⁾ .

6.1 Experimental work

6.1.1 DOE trials and generation of design space

Fourteen *K. africana* fruits were collected, cut into small pieces, and dried under direct sunlight. The dried material was then pulverized and passed through an 80# mesh sieve to obtain a uniform fine powder. A 25 g of the pulverized plant material was subjected to Soxhlet extraction utilizing 250 mL of the designated solvent. The process was maintained at a controlled temperature of 45°C to facilitate the exhaustive recovery of bioactive constituents while ensuring the thermal stability of the markers.

To optimize the extraction process, a 3² full factorial design with four responses (R1-concentration of VBS, R2-concentration of CA, R3-concentration of COA, R4-concentration of FA) was employed through Design-Expert® software which generated nine experimental runs and analyses was done in triplicates enabling systematic evaluation of the main and interactive effects of two critical process parameters: Factor A-solvent composition (% ethanol, three levels: 50%, 75% and 100%) and Factor B-extraction time (three levels: 18 h, 36 h and 54h). We performed these experimental runs using a Soxhlet extractor.

After extraction, the extracts were concentrated in a temperature-controlled water bath at 50 °C until the volume was reduced to

100 mL. A 5 mL aliquot was then filtered using Whatman filter paper. The resulting filtrate was analyzed to quantify four marker compounds: VBS (R1), CA (R2), COA (R3), and FA (R4). Quantification of VBS was performed using a validated analytical procedure (Method-1), whereas a distinct validated method (Method-2) was used to quantify CA, COA, and FA. Table 5-2 and Table 5-3 list the chromatographic conditions for both methods.

Experimental data, i.e., the quantified values of each marker calculated through post-analyses of the extracts, were fed into software, and four models were generated, one for each response. Analysis of variance (ANOVA) for each response was carried out; p-values were derived; equations in terms of coded and actual factors were obtained; and fit statistics (standard deviation, mean, CV %, R^2 , adjusted and predicted R^2 , Adequate precision) were obtained. The ANOVA model graphs (response surface graphs) for each response provided graphical representations of perturbation, interactions between variables, predicted vs. actual values, and contour plots visualizing the relationship between independent and dependent variables.

A common design space for all responses was generated graphically, in which each response criterion was set at certain limits based on the RSM graphs obtained to achieve a maximum concentration response for each; the combined overlay plot was then generated and validated.

6.1.2 Validation of the design model

Validation of the design model was carried out primarily by selecting four random points (flags) within the combined design space obtained from the overlay plot. We performed these four extraction trials and concentrated the extracts in a temperature-controlled water bath at 50 °C until the volume was reduced to 100 mL. A 5 mL aliquot was then filtered through Whatman filter

paper, and the resulting filtrate was analyzed to quantify four marker compounds. The concentrations of each marker (responses) calculated post-analyses were then compared to the predicted values generated by the models. Correlation analysis was performed.

6.1.3 Selection of optimized extraction process

From DOE trials and validation of the design space within the combined overlay plot, a design space point was selected as the optimized extraction point.

6.2 Results and Discussion

The complexity of herbal matrices necessitates a nuanced approach to solvent selection and process conditions. The solvent polarity must be carefully matched to the polarity of the target compounds to maximize extraction efficiency. Polar solvents, such as water, methanol, or ethanol, are typically chosen for the extraction of polar phytochemicals. In contrast, non-polar solvents, like hexane or dichloromethane, are more suitable for non-polar compounds.

Methanol is among the most commonly used solvents for *K. africana*, particularly for extracting material from stem bark, leaves, and fruits. Methanolic extracts have demonstrated diverse bioactivities. Ethanol has also been widely used, especially for fruit and leaf extractions. Ethanolic fruit extracts have shown antibacterial and wound-healing properties, while ethanolic leaf extracts have been investigated for their hepato-renal protective potential. Aqueous extracts, reflecting traditional decoction practices, are widely used to extract compounds from fruits, stems, and whole fruit preparations. Therefore, ethanol (%) was chosen as the solvent, with anti-infective activity as the target.

Extraction duration is a pivotal factor because it dictates the contact time between the solvent and the plant material. Sufficient extraction time is essential to allow complete

dissolution and diffusion of the target compounds from the plant matrix into the solvent. This extended contact period facilitates the comprehensive transfer of the desired bioactive substances, optimizing the extraction yield. Carefully controlling extraction time is crucial to ensure thorough recovery of the target analytes while preserving their structural integrity and bioactivity. Tailoring extraction duration to the specific plant material and solvent system is vital to developing efficient, effective extraction protocols.

Extraction duration is not merely a technical parameter; it is central to the reproducibility and therapeutic value of natural product formulations. Insufficient extraction time risks incomplete recovery of key phytochemicals that diffuse more slowly from the plant matrix. This under-extraction skews the phytochemical profile, yielding extracts with diminished or inconsistent bioactivity. Conversely, prolonged extraction increases compound release but, if not optimized, can increase solvent use, reduce efficiency, and, in some cases, degrade sensitive metabolites.

For pharmacological applications, these trade-offs underscore the importance of carefully calibrating extraction time. A standardized, evidence-based duration ensures that extracts consistently capture the full spectrum of bioactives in proportions reflective of the raw material. This reproducibility is critical for correlating biological effects with defined chemical markers, facilitating quality control, and enabling regulatory approval of herbal therapeutics. In this context, extraction time becomes not just an experimental variable but a determinant of clinical reliability and commercial scalability in natural product research.

6.2.1 DOE trials and generation of design space

The ethanolic extracts were reddish-brown and semi-solid. All the models were linear regression models without transformation. The process order for each response was quadratic to study how these variables influence extraction efficiency. The ethanolic extracts obtained through the experimental design were analyzed using validated methods: Method-1 for VBS and Method-2 for simultaneous quantification of CA, COA, and FA. Each run's quantified data for every marker (Table 6-1) was fed into Design-Expert software, and models were generated. The models generated established the quantitative relationship between the responses and the two factors.

Equations expressed in terms of coded factors serve as a predictive tool for estimating responses at specific factor levels. These coded equations are particularly valuable for identifying the relative influence of each variable, as the magnitude of the coefficients directly reflects their impact. In contrast, equations involving actual factors are unsuitable for comparing relative impact because the coefficients are scaled to each variable's units and the intercept is shifted from the center of the design space; they do not provide a normalized basis for factor comparison.

Table 6-1 Experimental runs with data

Run	Factor A Ethanol (%)	Factor B Time (hr.)	Response 1 VBS (µg/mL)	Response 2 CA (µg/mL)	Response 3 COA (µg/mL)	Response 4 FA (µg/mL)
1	100	54	91.8	87.9	7.54	84.1
2	100	36	150.8	282.1	29.31	197.21
3	100	36	151.2	281.9	29.46	197.53
4	75	18	126.1	136.9	8.19	166.9
5	50	18	128.4	130	31.89	127.2
6	100	54	91.3	87.4	7.85	83.87
7	50	54	43.9	0	2.16	0
8	50	54	43.01	0	2.24	0
9	50	54	43.1	0	2.38	0
10	50	18	130	129.4	32.69	127.45
11	75	18	126.7	137.8	8.32	166.54
12	75	36	146.9	240.1	8.11	156.2
13	75	18	126.9	138.2	8.71	166.1
14	100	18	126.8	172	28.1	213.9
15	75	54	77.8	43.6	0	52.8
16	100	54	90.9	87.1	7.93	82.9
17	50	36	145	187.9	31.95	122.1
18	50	36	145	188.6	32.09	121.8
19	75	54	78.4	44	0	53.4
20	100	18	125.8	171.4	28.56	241.27
21	100	18	127.8	171.2	28.69	214.5
22	100	36	149.8	282.44	29.59	197.1
23	50	18	127.4	130.5	32.8	127.01
24	75	36	146.2	240.8	8.47	155.8
25	75	54	78.2	43.93	0	53.07
26	50	36	145	188.1	32.16	122
27	75	36	146.8	241.2	8.89	155.2

VBS

The model generated by design-expert software suggests a quadratic relationship between the response and the factors. Details of the model fit summary are presented in Table 6-2. An F-value of 262.49 indicates that the model is statistically significant. There is only a 0.0001% probability that an F-value of this magnitude could occur due to experimental noise. Furthermore, P-values below 0.05 confirm that the individual model terms are significant, validating the strength of the relationship between the independent variables and the observed responses. In this case, factor A affects concentration levels, whereas factor B has a significant impact.

Final equations in terms of coded and actual factors are as follows:

Coded equation: $VBS = + 149.30 + 8.63*A - 28.19*B + 12.45*AB - 2.83*A^2 - 48.28*B^2$

Actual equation: $VBS = + 35.85 + 0.029*\text{Ethanol } (\%) + 7.08*\text{time}(\text{hr}) + 0.027*\text{Ethanol } (\%)*\text{Time } (\text{hr}) - 0.004*\text{Ethanol}^2 - 0.14*\text{time}^2$

Based on the quadratic model, response surface graphs (Figure 6-1) have been generated with the help of software. For VBS, factor B has a massive positive effect on its concentration level.

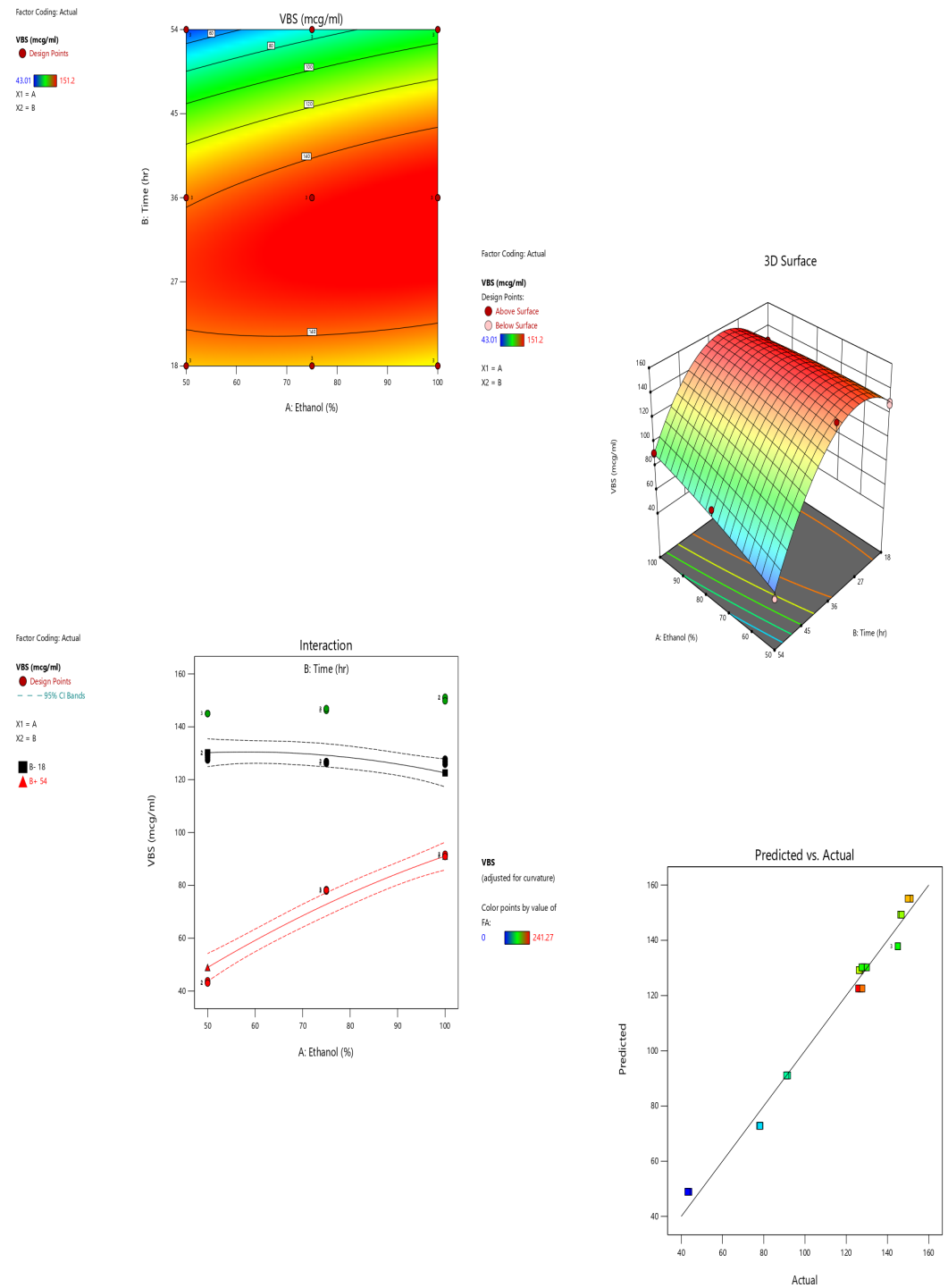


Figure 6-1 Response surface graphs of VBS

CA

The model generated by design-expert software suggests a quadratic model relationship between response and factors. Details of the model fit summary are presented in Table 6-2A model F-value of 661.03 underscores the model's high statistical significance, with a negligible 0.0001% probability that such a result is attributable to random noise. Consistent with standard statistical criteria, P-values less than 0.05 show that the model terms contribute significantly to the predicted response, confirming the robustness of the experimental design. In this case, factor A, factor B, and their interactions are significant in nature, and their interactions, along with the concentration, vary. Final equations in terms of coded and actual factors are as follows:

Coded equation: $CA = + 235.35 + 37.16*A - 51.30*B + 11.47*AB + 2.49A^2 - 141.94B^2$

Actual equation: $CA = - 250.00 - 0.02*\text{Ethanol } (\%) + 26.77*\text{time}(\text{hr}) + 0.02*\text{Ethanol } (\%) * \text{Time } (\text{hr}) + 0.003*\text{Ethanol}^2 - 0.43*\text{time}^2$

Based on the quadratic model, response surface graphs (Figure 6-2) have been generated with the help of software. For CA, all factors and their interactions positively affect its concentration level.



COA

The model generated by Design Expert software suggests a quadratic relationship between the response and the factors. Details of the model fit summary are presented in Table 6-2. The model's F-value of 42.18 confirms its statistical significance, with only a 0.0001% probability that an F-value of this magnitude resulted from experimental noise. P-values below the 0.05 threshold indicate that the associated model terms are significant, further validating the regression model's predictive capability and reliability. In this case, the complexity of factors and their interactions involved showcases variability in concentration levels.

Final equations in terms of coded and actual factors are as follows:

Coded equation: $COA = + 12.37 - 0.18*A - 9.88*B + 2.38*AB + 16.45A^2 - 10.11B^2$

Actual equation: $COA = + 154.53 - 4.14*Ethanol (\%) + 1.30*time(hr) + 0.005*Ethanol (\%)*Time (hr) + 0.02*Ethanol^2 - 0.03*time^2$

Based on the quadratic model, response surface graphs (Figure 6-3) have been generated with the help of software. For COA, all factors and their interactions are complex.

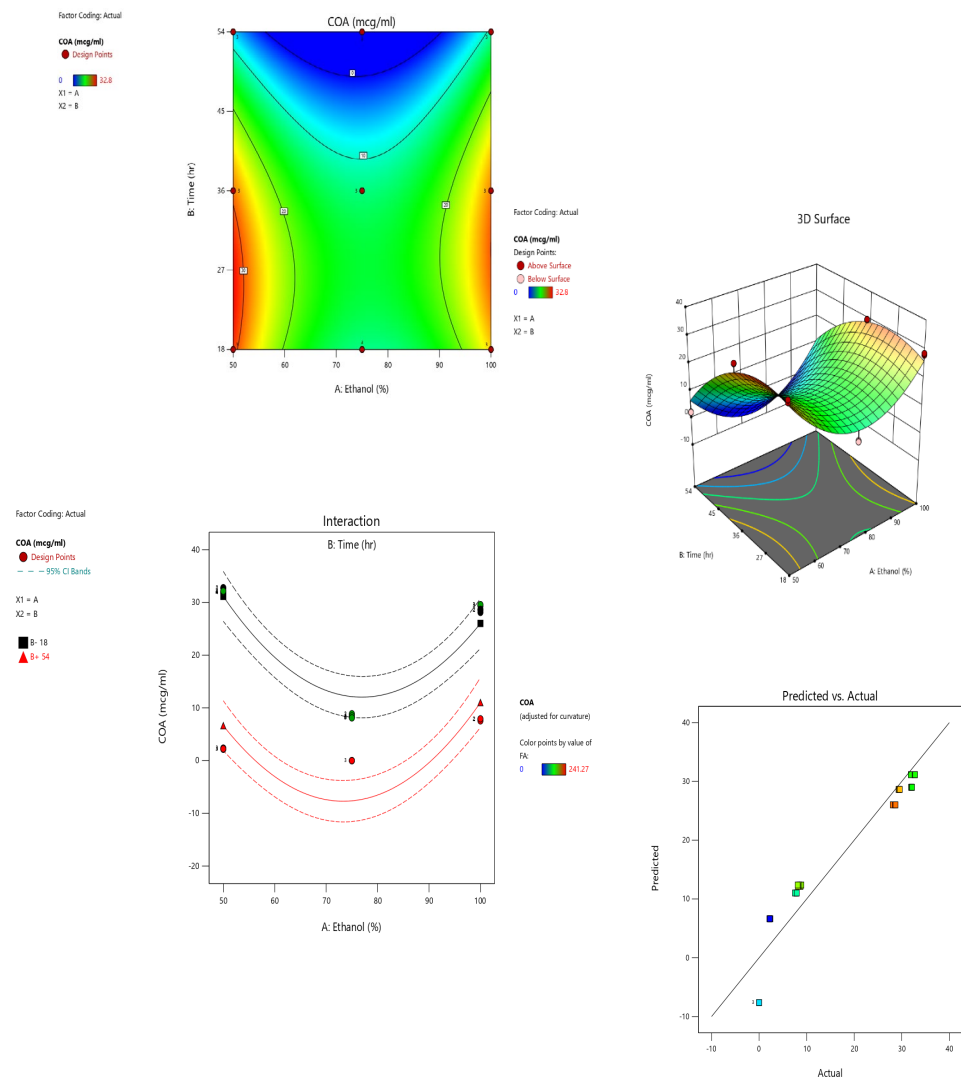


Figure 6-3 Response surface graphs of COA

FA

The model generated by Design Expert software suggests a quadratic relationship between the response and the factors. Details of the model fit summary are presented in Table 6-2. A model F-value of 441.88 indicates high statistical significance, with a 0.0001% probability that this value is due to noise. P-values below 0.05 further confirm that the model terms are significant, establishing a strong correlation between the variables and the experimental outcomes. In this case, factor A, factor B, and their interaction significantly affect concentration levels.

Final equations in terms of coded and actual factors are as follows:

Coded equation: $FA = + 158.03 + 42.49*A - 63.37*B - 3.10*AB + 0.44A^2 - 49.38B^2$

Actual equation: $FA = - 54.82 - + 1.84*\text{Ethanol } (\%) + 7.96*\text{time}(\text{hr}) - 0.006*\text{Ethanol } (\%) * \text{Time } (\text{hr}) + 0.0007*\text{Ethanol}^2 - 0.15*\text{time}^2$

Based on the quadratic model, response surface graphs (Figure 6-4) have been generated with the help of software.

Chapter-6 Optimization of Extraction Process

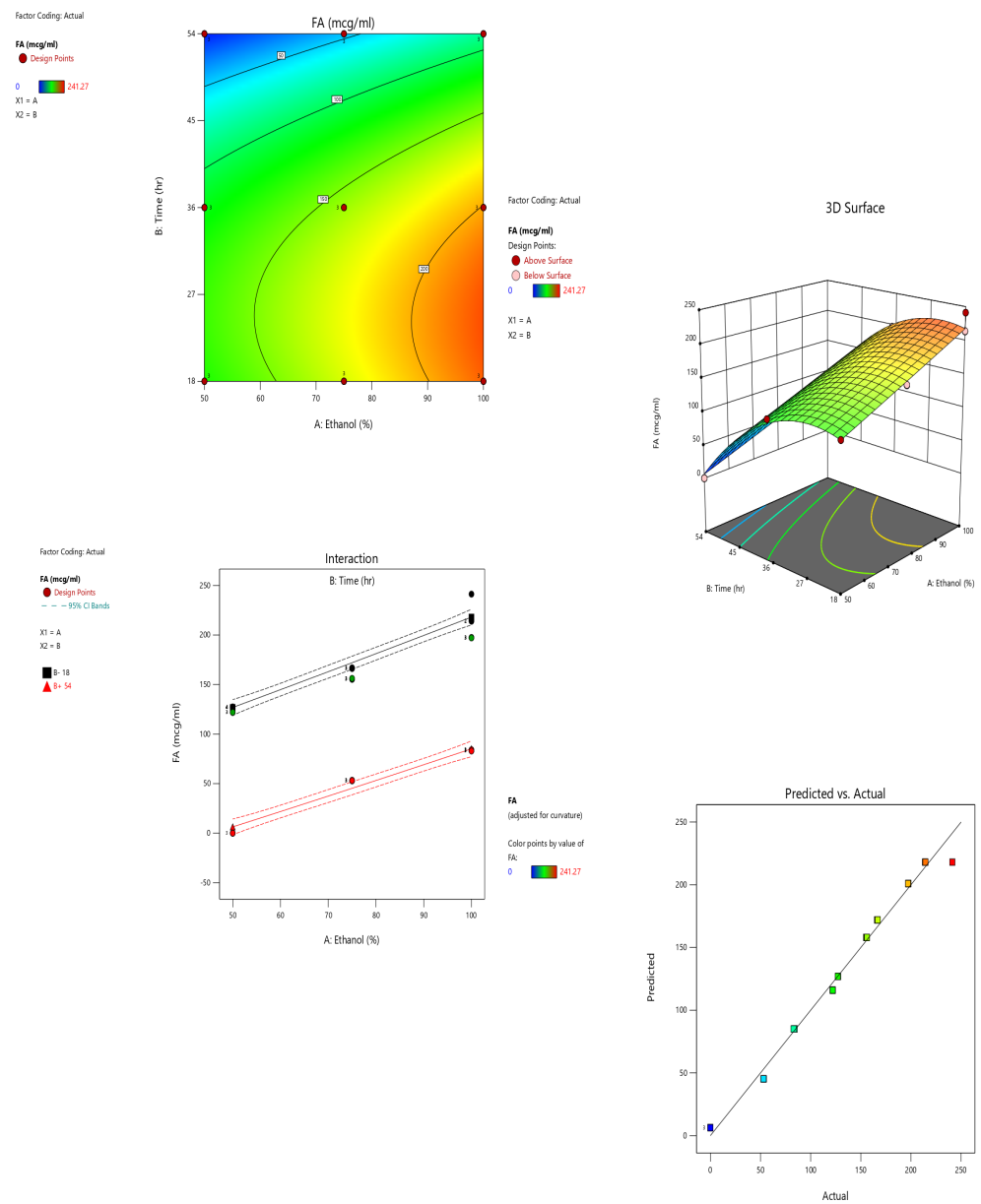


Figure 6-4 Response surface graphs of FA

Table 6-2 ANOVA model summary of all responses

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model Summary for VBS						
Model	31544.85	5	6308.97	262.49	< 0.0001	Significant
A-Ethanol	1341.45	1	1341.45	55.81	< 0.0001	
B-Time	14308.12	1	14308.12	595.31	< 0.0001	
AB	1859.78	1	1859.78	77.38	< 0.0001	
A ²	48.15	1	48.15	2.00	0.1716	
B ²	13987.36	1	13987.16	581.96	< 0.0001	
Pure Error	8.19	18	0.4548			
Cor Total	32049.58	26				
Model Summary for CA						
Model	1.947E+05	5	38947.93	661.03	< 0.0001	Significant
A-Ethanol	24860.01	1	24860.04	421.93	< 0.0001	
B-Time	47377.60	1	47377.60	804.10	< 0.0001	
AB	1580.1	1	1580.1	26.82	< 0.0001	
A ²	37.30	1	34.30	0.6331	0.4351	
B ²	1.209E+05	1	1.209E+05	2051.68	< 0.0001	
Pure Error	3.29	18	0.1826			
Cor Total	1.960E+05	26				

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model Summary for COA						
Model	4062.00	5	812.40	42.18	< 0.0001	Significant
A-Ethanol	0.6160	1	0.6160	0.0320	0.8598	
B-Time	1757.26	1	1757.26	91.23	0.1223	
AB	68.02	1	68.02	3.53	0.0742	
A ²	1622.63	1	1622.63	84.24	0.4351	
B ²	613.47	1	613.47	31.85	< 0.0001	
Pure Error	1.31	18	0.0727			
Cor Total	4466.50	26				
Model Summary for FA						
Model	1.195E+05	5	23907.42	441.88	< 0.0001	Significant
A-Ethanol	32947.20	1	32947.20	600.64	< 0.0001	
B-Time	72292.50	1	72292.50	1336.17	< 0.0001	
AB	114.95	1	114.95	2.12	0.1597	
A ²	1.16	1	1.16	0.0215	0.8849	
B ²	14631.29	1	14631.29	27043	< 0.0001	
Pure Error	490.77	18	27.26			
Cor Total	1.20E+05	26				

Optimization of the design was done by setting lower and upper limits for each marker. From the Table 6-1 It is evident that each marker's concentration had a variable relationship with the factors. Hence, every marker's limits criteria set were different i.e. VBS: 100–160 $\mu\text{g/mL}$ (0.92–0.148% w/w), CA: 200–282.4 $\mu\text{g/mL}$ (0.19–0.26 % w/w), COA: 2.16–32.8 $\mu\text{g/mL}$ (0.019–0.29 % w/w), FA: 180–241.27 $\mu\text{g/mL}$ (0.16–0.22 % w/w) which gave an overlay plot (Figure 6-5).

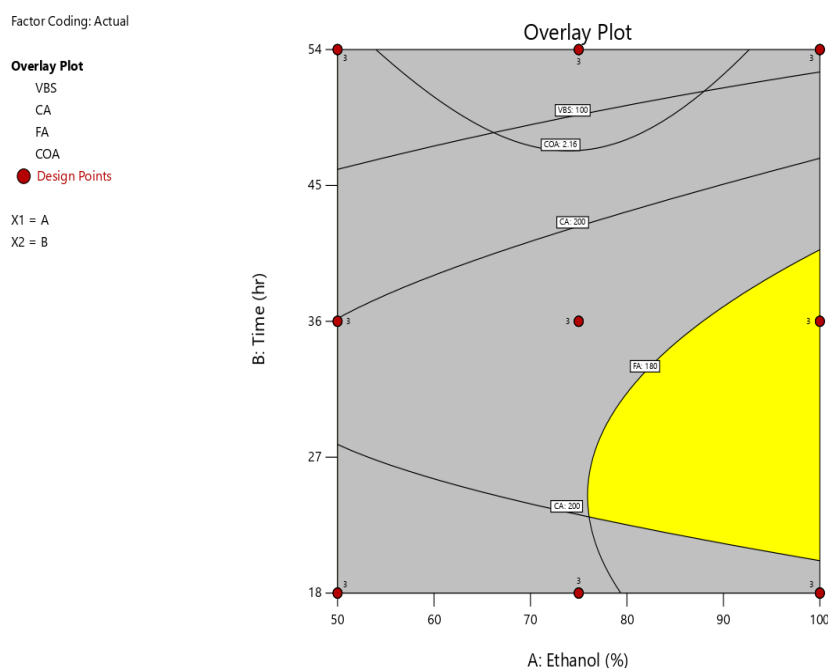


Figure 6-5 Overlay plot of the design

6.2.2 Validation of the design model

Analyses for the quantification of four marker compounds: VBS (R1), CA (R2), COA (R3), and FA (R4) using the HPTLC methods, as per the validation of the design model, were carried out at the four random points (flags) selected within the overlay plot. The concentrations of each marker (response) calculated post-analysis were then compared with the model-predicted values. Correlation coefficients were measured between the predicted values and actual (result) values, as shown in Table 6-3. Correlation (> 0.99) shows the design is highly accurate and reliable for all the models generated.

Table 6-3 Design validation with data

Ethanol (%)	Time (hrs)		VBS (µg/mL)	CA (µg/mL)	FA (µg/mL)	COA (µg/mL)	Correlation
80	18	Predicted	128.6	150.9	181.2	12.2	0.992
		Actual	124.8	148.1	178.6	13.2	
60	20	Predicted	137.6	157.4	148.7	20.6	0.991
		Actual	132.7	154.3	145.4	21.8	
90	30	Predicted	154.9	257.1	200.2	19.9	0.991
		Actual	130.5	236.8	197.7	21.4	
95	24	Predicted	146.7	237.1	214.3	23.8	0.997
		Actual	140.2	234.6	210.1	25.7	
98	38	Predicted	151.2	262.4	186.5	24.7	0.991
		Actual	146.4	259.7	183.4	25.6	

6.2.3 Selection of the optimized extraction process

The validated optimized extract (ethanol 90 % and time 30 hrs) was selected as the optimized extraction point and used as a raw material for topical formulation. This point was almost the center of combined overlay plot of all the responses. This would also provide any room of error, if any, would exist in quantifying the chosen markers from the optimized extract. It lays the foundation for choosing the raw material of the topical phytopharmaceutical dosage form.

6.3 Conclusion

In summary, the optimization extraction process for herbal materials is not merely a preparative technique but critical determinant of product quality, efficacy, and sustainability. An optimized extraction protocol tailored to the specific phytochemical profile of the plant material is essential for producing high-value herbal formulations. The optimization of extraction protocols for herbal materials, exemplified by *K. africana*, transcends mere preparative technique; it is a decisive factor in the quality, therapeutic efficacy, and sustainability of the final herbal products. Here a 3² full factorial design was successfully studied, a powerful statistical tool, to systematically dissect the individual and synergistic effects of ethanol

concentration and extraction time on the yields of four critical marker compounds: VBS, CA, COA, and FA.

Our findings reveal a nuanced relationship between the investigated factors and the concentration of each marker. For instance, extraction time significantly enhanced VBS concentration, whereas CA and FA concentrations were positively influenced by both independent variables and their interaction. Conversely, complex interaction was observed on COA yield. This differential responsiveness underscores the complex chemical diversity of *K. africana*'s metabolome and the imperative for precise parameter control during extraction.

The robust validation of the predictive models, evidenced by correlation coefficients exceeding 0.99 between predicted and actual values, attests to the high accuracy and reliability of this experimental design approach. Such predictive capability is invaluable for pharmaceutical and natural product development, particularly given the inherent sensitivity of phytochemicals within complex herbal matrices to processing conditions. The application of DOE minimizes the experimental burden while maximizing the generation of actionable insights, thereby enabling the precise tuning of extraction processes to augment yield, enhance bioactivity, and improve overall operational efficiency.

In conclusion, this provides a rigorous, data-driven methodology for establishing optimized extraction parameters for *K. africana* fruits and an optimized extract. This approach is highly transferable and scalable to other botanicals, offering a foundational framework for developing high-value herbal formulations where chemical integrity and biological activity are paramount. This statistically informed, forward-thinking strategy for natural product extraction is critical for advancing the discovery and development of plant-derived therapeutics.

CHAPTER-7

EVALUATION

OF

OPTIMIZED

EXTRACT

7 Evaluation of optimized extract

The optimized extract was evaluated on three parameters that form a crucial basis for topical formulation as a raw material. The three parameters are the ethanolic content of the extract, *in Vitro* activity of the extract, and Assay of the extract.

Alcohols are low-molecular-weight solvents produced chemically and by microbial fermentation.⁽⁷⁴⁻⁷⁸⁾ Ethanol, the principal alcohol in clinical antiseptics and disinfectants, rapidly partitions into lipid bilayers and, by disrupting hydrophobic interactions and solvent structure, produces membrane perturbation and protein denaturation that compromise cellular integrity and key metabolic enzymes. Compared with water, ethanol is less polar. Although complete sterilization cannot be achieved by ethanol at any concentration, maximal antimicrobial activity occurs at concentrations of 80–90%. Hence, determining the ethanolic content of the extract was crucial, as it would be used directly as a raw material in topical formulation and as evidence to claim anti-infective activity. Therefore, ethanolic content of the optimized extract was estimated via headspace gas chromatography (HS–GC).

Secondarily, the *in vitro* activity of the optimized extract was carried out against *E. coli* and *S. aureus* species.⁽⁷⁹⁾

Lastly, the assay of the optimized extract evaluation benefits in determining the individual marker concentrations present, which is also a crucial parameter of a raw material evaluation.

7.1 Experimental work

7.1.1 Ethanolic content of optimized extract

The optimized extract was dried in a temperature-controlled electric water bath at 45 °C until a constant weight and a semi-solid mass were obtained. The dried optimized extract was stored in an Eppendorf tube, and its ethanolic content was evaluated at Vaibhav Analytical Services, Ahmedabad, via Headspace Gas Chromatography (HS-GC).

HS–GC analysis was carried out using an Agilent 8890 gas chromatograph equipped with a flame ionization detector. 1000 μ L of the optimized extract sample solution was injected into SPB 624 (30 m $\times$ 0.25 mm) d_f 1.40 μ m. The HS–GC chromatographic conditions are presented in Table 7-1.

Table 7-1 HS–GC chromatographic conditions

Column	SPB-624 (30 m $\times$ 0.25 mm) d_f 1.40 μ m
Carrier gas	Nitrogen
Diluent	Water
SPL/Injection port temperature	250°C
Column flow	0.5 mL/min
Detector temperature	260°C
Column temperature	80°C iso
Injection volume	1000
Split ratio	1:1
Run time	10 minutes

7.1.2 *In vitro* activity of the optimized extract

This study used two bacterial stains, *S. aureus* and *E. coli*, as test micro-organisms. The test organisms were from stock culture, plated on blood agar medium to obtain isolated colonies. Individual colonies of each species were emulsified in sterile phosphate-buffered saline (pH 7.2) to a turbidity of 0.5 McFarland standard (0.5×10^8 organisms/mL). Bacteria were grown and maintained on Mueller-Hinton agar plates at 35 °C until use. We then stored them under aerobic conditions. We evaluated the optimized extract against *E. coli* and *S. aureus* at Arihant Hospital, Unit of Dixon Healthcare Pvt. Ltd., Belgaum, Karnataka. We used the well diffusion test methodology, with

gentamicin (standard), a control (sterile water), and a blank (ethanol).

The procedure for the well-diffusion test is:

The optimized extract was considered 100% when preparing dilutions for the diffusion assay. The test organisms *E. coli* and *S. aureus* were revived from stock culture by plating on blood agar medium to obtain isolated colonies. Individual colonies of each species were emulsified in sterile phosphate-buffered saline (pH 7.2) to a turbidity of 0.5 McFarland standard (1×10^6 organisms/mL).

The optimized extract (test compound) was subjected to two-fold dilution in a 48-well tissue culture plate. We selected five wells for each compound. Add 200 μ L of BHI (Brain Heart Infusion) broth to each well. Add 200 μ L of stock solution (100%) to the first well, mix well, and transfer 200 μ L to the second well. Repeat this procedure up to the fifth well, then discard 200 μ L.

The drug concentrations after this dilution in the five wells were 50%, 25%, 12.5%, 6.25%, and 3.13%, respectively. The bacterial suspension prepared as mentioned earlier was plated onto sterile plates using cotton swabs. Mueller-Hinton agar was used to determine the antimicrobial activity of the test compounds. A sterile cotton swab was dipped into the BHI broth containing the organism suspension and was inoculated onto the surface of the respective medium. Then, five 8mm wells were cut into each agar plate using a plastic template. Into each well, 75 μ L of different drug dilutions were inoculated and appropriately labeled. The plates were incubated at 37°C for 24 hours. The zone of inhibition, if any, was noted around each test compound and measured with a scale.

7.1.3 Assay of the optimized extract

The dried optimized extract, obtained via the aforementioned procedure, was accurately weighed (1 g) and subsequently dissolved in a minimal volume of methanol. This solution was transferred to a 10-mL volumetric flask and diluted to volume with the same solvent to achieve a standardized concentration for further analysis. The resulting solution was filtered using a Whatman filter (Type 1). The filtrate obtained was used to quantify VBS and COA in the dried optimized extract. An aliquot of 0.5mL from the filtrate was diluted up to 10mL with methanol in a volumetric flask. This solution was used to quantify CA and FA in the dried optimized extract. HTPLC method -1 was used to quantify VBS, and method -2 was used to quantify CA, COA, and FA.

7.2 Results and Discussion

7.2.1 Ethanolic content of optimized extract

HS–GC is predominantly used to determine the volatile contents present in a sample. Because ethanol is volatile, HS–GC was used by default to estimate it in the dried optimized extract. The ethanolic content of the optimized extract analyzed by HS–GC was found to be below the minimum inhibitory concentration (MIC) limits reported in the literature review. Ethanol's MIC against *E. coli* and *S. aureus* is reported to be 8.75 %. The optimized extract's ethanolic content was calculated and found to be 6.8 % (Table 7-2).

Table 7-2 Ethanolic content results

Sample	RT (min)	Area	Ethanol (%)
Ethanol (Standard)	4.386	2617.96	100
Optimized extract	4.390	34877.59	6.8

Chapter- 7 Evaluation of optimized extract

The chromatograms of the blank, ethanol as a standard, and the optimized extract's ethanolic content are displayed in Figure 7-1.

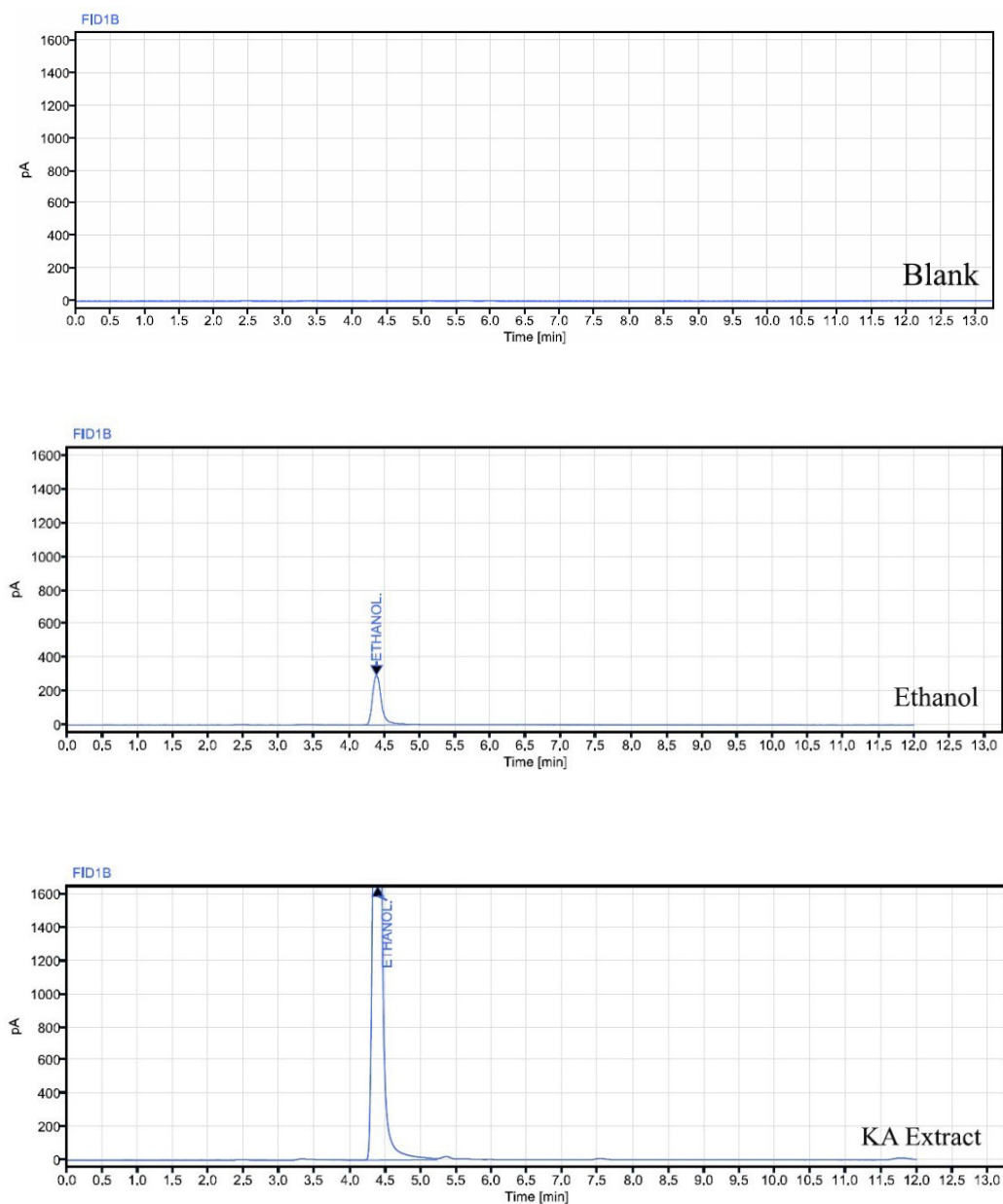


Figure 7-1 HS–GC chromatograms

The ethanolic content was calculated to be 6.8%, which is below the reported MIC values of ethanol against *E. coli* and *S. aureus*. However, to verify this reported claim, the optimized extract and an ethanolic solution (6.8%) were tested against *E. coli* and *S. aureus*.

7.2.2 *In vitro* activity of the optimized extract

In vitro activity results of the optimized extract and ethanol (6.8%) as a control against *E. coli* and *S. aureus* showed that the optimized extract was effective against both species. The study used gentamicin as a standard and blank (sterile water) as a control.

The results (Table 7-3) display the effectiveness of the optimized extract against both species. Zones of inhibition against both species were on par with the standard and ethanol as controls; the reported literature claim was verified that ethanol's concentration at the reported MIC can be the concentration required to inhibit growth. Moreover, this study ensures that the activity of the optimized extract against both species is due to the extract's rich presence of phytochemicals, not ethanol. The *in vitro* activity plate photographs are displayed in Figure 7-2.

Table 7-3 In vitro activity of the optimized extract

Sample	ZOI (mm) of <i>E. coli</i>	ZOI (mm) of <i>S. aureus</i>
Blank 1 (Ethanol 6.8 %)	R	R
Blank 2 (Sterile water)	R	R
Optimized extract	17	15
Gentamicin (Standard)	16	17

R indicates Resistance with no Zone of Inhibition.

Chapter- 7 Evaluation of optimized extract

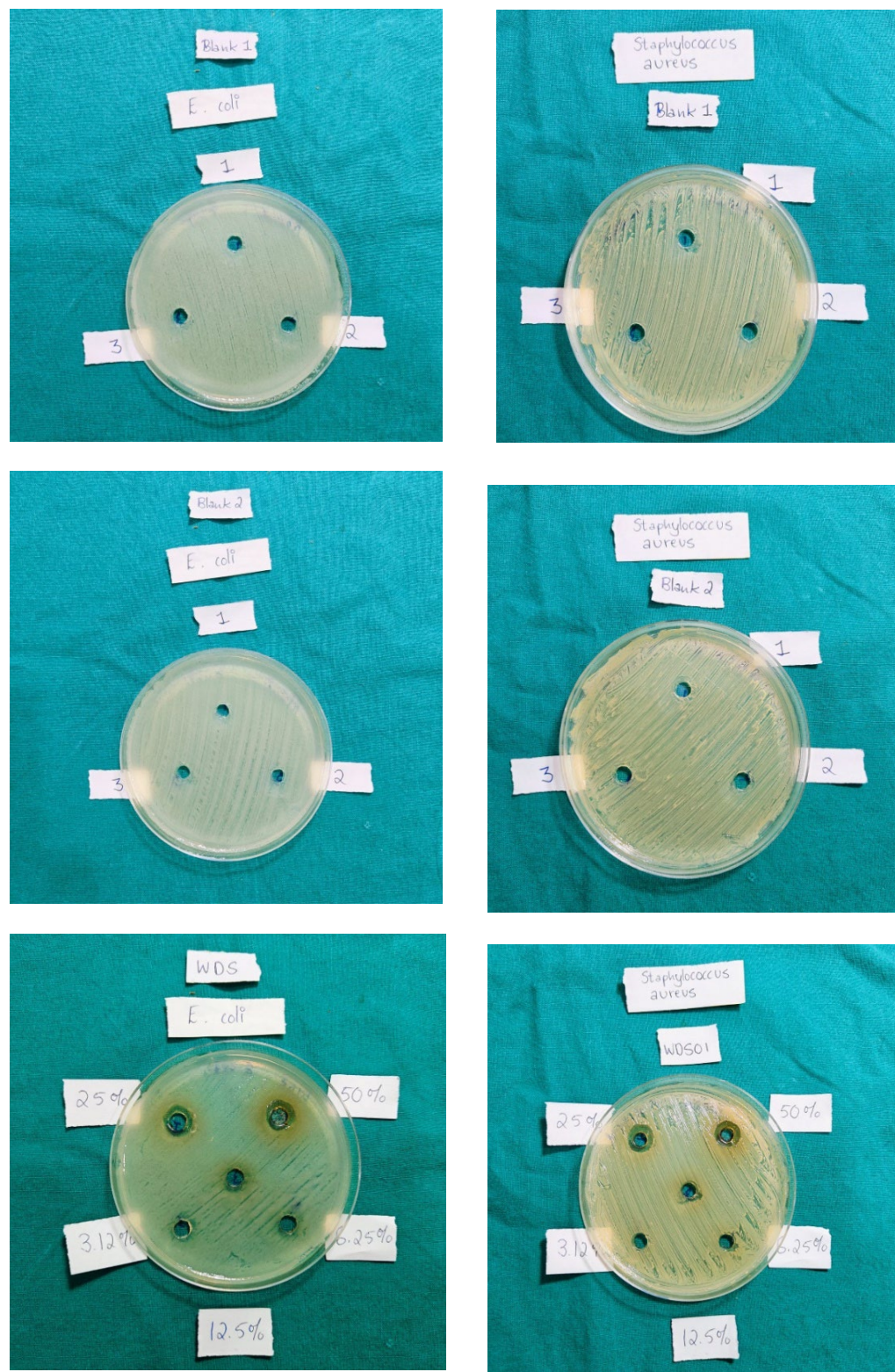


Figure 7-2 In vitro activity plate photographs

7.2.3 Assay of the optimized extract

Method-1 was used to dry the optimized extract and determine the VBS content. The dried optimized extract contained 130.5 µcg/mL (0.12% w/w) VBS. Method-2 was applied to the dried optimized extract to determine the CA, COA, and FA contents. The dried optimized extract contained 236.8 µg/mL (0.22% w/w) CA, 21.4 µg/mL (0.19% w/w) COA, and 197.7 µg/mL (0.182% w/w) FA. The results obtained are within the ranges set for each biomarker in the previous chapter.

7.3 Conclusion

The evaluation of the optimized extract highlights three important aspects crucial for its use in formulation. Primarily, the ethanolic content of the optimized extract was found to be less than the MIC, explaining its in vitro activity against bacterial species due to the presence of biomarkers. Second, the assay results for the optimized extract confirm that the selected point is appropriate for further evaluation, strengthening the model and design. Lastly, evaluating the optimized extract bridges the gap between the essential characteristics required for the extract as a raw material and those required for the topical dosage form. The optimized extract can now be used as a raw material to develop, evaluate, and standardize the phytopharmaceutical topical dosage form.

CHAPTER-8

FORMULATION

DEVELOPMENT

8.0 Formulation development

Developing topical gels involves multiple product- and process-related variables, each of which can significantly affect the safety, efficacy, and overall quality of the final formulation. To address these complexities, the pharmaceutical industry increasingly relies on the Quality by Design (QbD) framework.^(80, 81) QbD is a systematic, science-based, risk-oriented approach to pharmaceutical development that prioritizes upfront definition of objectives and a thorough understanding of both products and processes. By integrating quality into the design phase rather than relying solely on end-product testing, this framework ensures more robust manufacturing and consistent therapeutic performance. Its central principle is that product quality should not be tested into the final product but built into the design from the earliest stages of development.

The International Council for Harmonization (ICH) supports the adoption of QbD and incorporated it into the ICH Q8 guideline.⁽⁸²⁾ Within this framework, product development is guided by a clear understanding of how formulation and process variables influence the quality attributes of the final dosage form. Unlike the conventional one-variable-at-a-time strategy, QbD uses a holistic, statistical approach that allows multiple variables to be studied simultaneously and their interactions to be understood. This not only improves efficiency but also strengthens the robustness of pharmaceutical products.

A fundamental component of QbD implementation is defining the Quality Target Product Profile (QTPP), which delineates the essential quality characteristics required to ensure the safety, efficacy, and patient acceptability of the final product⁽⁸³⁾. After establishing the QTPP, identify Critical Quality Attributes (CQAs). These CQAs include measurable physical, chemical, biological, or microbiological properties that must be maintained within predefined limits to ensure the desired product quality.

CQAs are influenced by both the properties of the active pharmaceutical ingredient (API) and excipients, as well as by manufacturing processes and intermediates. Careful identification and control of CQAs are therefore essential to guarantee consistent product performance.

To link the QTPP with the CQAs, the framework requires systematic evaluation of Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs). CMAs describe raw material properties that can affect product quality, while CPPs refer to the key process variables that must be controlled to achieve the desired outcome. Establishing these relationships provides a clear scientific rationale for designing and optimizing formulations.

Risk assessment plays a central role in this process. It provides a structured method for ranking and prioritizing the potential impact of different material attributes and process parameters on product quality. A commonly used tool is the Ishikawa (or fishbone) diagram, which visually maps possible cause–effect relationships between CMAs, CPPs, and CQAs. This qualitative approach helps identify critical factors requiring tighter control and guides the design of experiments (DoE) to explore their influence quantitatively. Alongside risk assessment and DoE, process analytical technology (PAT) offers real-time monitoring and control, further supporting consistent manufacturing.⁽⁸⁴⁾

The ultimate aim of QbD is to reduce variability, improve process understanding, and ensure that the final pharmaceutical product meets regulatory standards and patient needs. Statistical optimization and modelling within this framework not only streamline development but also provide a transparent justification of quality assurance to regulatory bodies.

By systematically identifying the QTPP, CQAs, CMAs, and CPPs, and applying risk assessment, the study sought to establish a well-optimized formulation with consistent quality attributes.

This methodology ensures that the final product achieves the desired therapeutic outcomes while maintaining regulatory compliance.

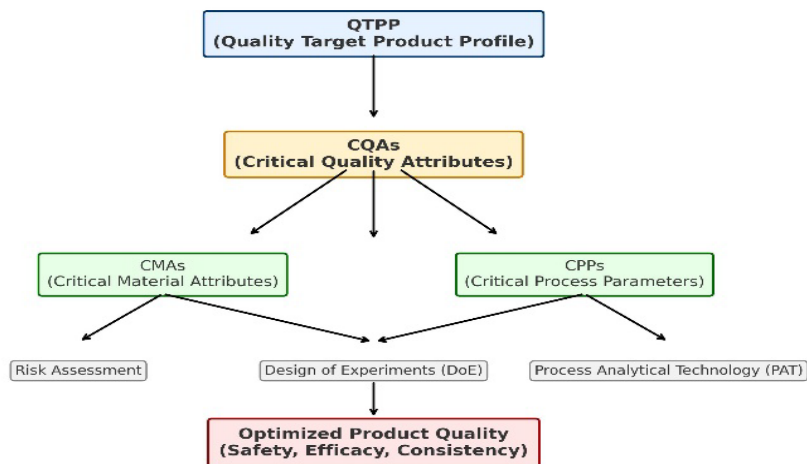


Figure 8-1 Formulation development by QbD

8.1 Experimental work

8.1.1 Materials and methods

Table 8-1 List of chemicals used in formulation

Sr. No.	Chemical	Category	Supplier
1.	Carbopol 974	Polymer	Lubrizol
2.	Hydroxypropyl methylcellulose (HPMC)	Polymer	Loba Chemical Pvt. Ltd.
3.	Sodium Carboxyl methylcellulose (SCMC)	Polymer	Loba Chemical Pvt. Ltd.
4.	Methylcellulose (MC)	Polymer	Loba Chemical Pvt. Ltd.
5.	Methyl Paraben	Preservative	Loba Chemical Pvt. Ltd.
6.	Propyl Paraben	Preservative	Loba Chemical Pvt. Ltd.
7.	Propylene glycol	Solubilizing agent	Finar India Pvt. Ltd.
8.	Triethanolamine	pH adjusting agent	Finar India Pvt. Ltd.
9.	Acacia	Binder	Loba Chemical Pvt. Ltd.

8.1.2 Defining Quality Target Product Profile (QTPP) and Critical Quality Attributes (CQAs)

The Quality Target Product Profile (QTPP) serves as the foundational design objective for pharmaceutical development. Essential QTPP considerations include the intended clinical application, route of administration, and specific delivery systems or dosage forms. It also encompasses required dosage strengths and the selection of appropriate container-closure systems. Furthermore, the QTPP must define therapeutic moiety release profiles and attributes that influence the pharmacokinetic characteristics of the specific dosage form. Finally, it establishes rigorous drug product quality criteria such as sterility, purity, stability, and dissolution profiles required for the intended commercial product. Table 8-4 QTPP for topical gel Summarizes QTPP for topical gel.

A Critical Quality Attribute (CQA) is defined as a physical, chemical, biological, or microbiological property that must remain within an appropriate limit, range, or distribution to guarantee the intended product quality. These attributes are typically associated with drug substances, excipients, intermediates, and the final drug product. Potential CQAs, derived from the QTPP or existing scientific knowledge, guide product and process development. Quality Risk Management (QRM) is subsequently employed to prioritize these attributes for further evaluation. Ultimately, an iterative cycle of risk assessment and empirical experimentation identifies relevant CQAs and determines the extent to which variability in these attributes affects overall drug product quality. Table 8-5 Provides CQAs for topical gel and their justification.

8.1.3 Preparation and procedure of preliminary batches for

Selection of polymer

Preliminary batches (Table 8-2) were prepared to extract the gelling polymer agent for selection. Different polymers such as Carbopol, Sodium Carboxymethylcellulose (Sodium CMC), Hydroxypropyl Methylcellulose (HPMC), and Methylcellulose (MC) of different grades were tried in different concentrations, and different excipients were tried and tested for making the gel. The prepared herbal gel was evaluated for basic physical properties, demonstrating good spreadability, optimum pH, good washability, grittiness, and homogeneity.

Table 8-2 Selection of polymer from preliminary batches

Category	Excipient	B1	B2	B3	B4	B5	B6	B7	B8
Active Ingredient	Extract (%)	5	5	5	5	5	5	5	5
Polymer	Carbopol 974P (%)	1	1.5	2	-	-	-	-	-
	HPMC (%)	-	-	-	5	10	-	-	-
	Sodium CMC (%)	-	-	-	-	-	5	-	-
	MC (%)	-	-	-	-	-	-	5	10
Preservatives	Methyl Paraben (%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Propyl Paraben (%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
pH adjusting agent	Triethanolamine	Qs	Qs	Qs	Qs	Qs	Qs	Qs	Qs
Solvent	Water (mL)	Up to 100 mL	Up to 100 mL	Up to 100 mL	Up to 100 mL	Up to 100 mL	Up to 100 mL	Up to 100 mL	Up to 100 mL

8.1.3.1 Procedure for preparation of gels

Procedure for preparation of Carbopol gel

Accurately weighed quantity of Carbopol 974P was dispersed in one-third portion of water at 40 °C with constant stirring using a magnetic stirrer. Dissolve the extract in another one-third portion of water and add it slowly to the gel base; add the remaining one-third preservative solution with constant mixing. Use triethanolamine to adjust the pH to 6.8, then stir carefully and

slowly until the gel forms.

Procedure for preparation of HPMC gel

An accurately weighed quantity of HPMC was dispersed in one-third of the hot water heated at 80 °C, with constant stirring using a magnetic stirrer, until a thin, hazy dispersion formed. The second one-third portion of pre-heated water solution containing methyl and propyl paraben was added to the gel base and left overnight. The next day, the remaining one-third portion of water containing extract was added to the gel base and mixed until a homogeneous gel formed, then left overnight to swell.

Procedure for preparation of Sodium CMC gel

Accurately weighed sodium CMC was dispersed in one-third portion of water at 50 °C with constant stirring using a magnetic stirrer for 30 minutes. The extract was dissolved in the second one-third portion of water and added to the gel base. Add the remaining one-third of the pre-heated, dissolved methyl and propyl paraben solution to the gel mixture and mix until thoroughly combined, then keep overnight to swell.

Procedure for preparation of Methylcellulose gel

Accurately weighed MC was dispersed in one-third of the water at 50 °C with constant stirring using a magnetic stirrer for 30 minutes. The extract was dissolved in the second one-third portion of water and added to the gel base. The remaining one-third portion of the pre-heated, dissolved methyl and propyl paraben solution was added to the gel mixture and mixed until all contents were thoroughly combined, then kept overnight to swell.

8.1.4 Risk assessment linking material attributes and process parameters to CQAs

Risk assessment is a rigorous, science-based component of Quality Risk Management (as delineated in ICH Q9) that helps identify material attributes and process parameters that may impact product CQAs. This process begins in the early stages of pharmaceutical development and is refined iteratively as process knowledge and empirical data increase. By utilizing established risk assessment tools, researchers can systematically rank parameters, including manufacturing processes, equipment specifications, and input material characteristics, to prioritize those most likely to influence the final product quality based on a synthesis of prior knowledge and preliminary experimental results.

The risk evaluations were completed to determine the CMAs and/or CPPs from the polymer selected from preliminary batches that affect the CQAs of the pharmaceutical drug delivery. A preliminary study identified CMAs and CPPs using a comprehensive Risk Evaluation Matrix (REM). This qualitative risk assessment involved assigning low, medium, or high-risk levels to each material characteristic and process variable, facilitating a structured evaluation of their potential impact on the product's quality profile as detailed in the study parameters. Table 8-8.

REM was used to assess the impact of every material characteristic and process variable on topical gel CQAs. Carbopol batches were made with varying percentages of carbopol, binder, solubilizing agent, and extract, and were examined for good spreadability, optimum pH, appropriate viscosity, stability, and good washability; REM was then revised. The optimized formulation batch obtained after the revised REM was evaluated.

Table 8-3 Raw material attributes

Material	Attributes
Extract	Concentration of markers
	Ethanol content of extract (%)
Polymer (Carbopol)	Grade
	Concentration (%)
Preservatives	Strength
Purified Water	Conductance
pH adjusting agent (Triethanolamine)	pH maintenance

8.1.5 Evaluation parameters for optimized gel

Physical characteristics

Organoleptic evaluation of the gel was carried out on appearance, grittiness, homogeneity, and fragrance.

The gel pH was determined using a calibrated digital pH meter. Specifically, 1 g of the gel was dispersed in 10 mL of distilled water and allowed to equilibrate for 2 hours; the pH of the resulting dispersion was then measured to ensure compatibility with the site of application.

Brookfield Rheometer CT3 texture analyzer 4500 was used to determine adhesiveness and cohesiveness of the optimized gel formulation.

Viscosity

Gel viscosity was measured by determining the rheological properties of the formulation using a controlled-stress rheometer (Anton Paar MCR 102e) equipped with a plate-plate geometry (25mm diameter and 500 μm gap); the sample was transferred to the rheometer plate, and viscosity was measured. Viscosity was measured at a shear rate of 100 s^{-1} at 25 °C.

Spreadability

Spreadability denotes the extent to which the gel readily glides over the skin or affected tissue upon application. This attribute is a critical determinant of the formulation's therapeutic efficacy, as it governs the uniformity of the medicinal dose. The spreading value is expressed in seconds and represents the time required for two glass slides to separate under a defined weight. In this procedure, approximately 1 g of the gel is sandwiched between two slides; a specified weight is then applied to the upper slide, and the time taken for the slides to separate is recorded. A shorter separation time indicates better spreadability and easier application.

It was calculated using the formula: $S = M \times L/T$.

Where S = spreadability; L = length traveled by the glass slide; M = weight; and T = time (in seconds) it took to separate the slides.

In-vitro activity

We tested the in vitro activity of the optimized gel against *E. coli* and *S. aureus* using a well-diffusion method.

Assay

The gel assay was determined using HPTLC methods for markers. A gel equivalent to 1 g of extract was taken, extracted with 10 mL ethanol three times, and centrifuged in a cooling centrifuge at 10,000 rpm for 30 minutes. The supernatant was collected and concentrated in a temperature-controlled water bath to approximately 5 mL. The final volume was used to estimate marker concentrations using HPTLC methods as described in Chapter 4. The basis of the fixing values of the four actives was determined from the results of the assay of the optimized extract used as raw material in formulation. The resulting assay values gave us the concentration values for all four actives, which were then tested in vitro against the species. The in vitro results showed

it was effective against both species; therefore, based on the concentrations found in the optimized extract, we assigned the assay values.

Stability studies

The stability of the gel formulation was evaluated in a stability chamber under accelerated storage conditions of 40 ± 2 °C and $75 \pm 5\%$ relative humidity, in accordance with ICH Q1A (R2) guidelines. This study assessed the formulation's long-term stability and predicted its shelf life. The gel was subjected to these accelerated conditions for six months, with sampling and analysis at 0, 3, and 6 months using HPTLC methods. Evaluation parameters included physical stability (pH, viscosity, adhesiveness, and cohesiveness) and chemical stability, determined by assay of the active constituents.

8.2 Results and Discussion.

In QbD-based development of topical gels, the process begins with defining the Quality Target Product Profile (QTPP). The QTPP summarizes the intended product characteristics, including safety, efficacy, route of administration, dosage form, and patient acceptability. Establishing the QTPP at the outset provides a scientific foundation for identifying critical attributes. It ensures that subsequent formulation and process design target an optimized, reliable topical drug delivery system. Table 8-4 Showcases the QTPP for the topical gel. Table 8-5 Shows CQAs with their justification.

Table 8-4 QTPP for topical gel

Sr. no.	QTPP		Target	Justification
1	Dosage form		Gel	Topical site of action
2	Amount in jar		30 g	—
3	Route of administration		Topical	Infections occur in the top layers of the skin; local action is achievable.
4	Dosage strength		10 %	Based on <i>in vitro</i> studies
5	pH		6.8-7.2	Compatible with skin
6	Therapeutic use		Anti-microbial	Anti-infective activity
7	Physical attributes	Color		Color, odor, appearance, and spreadability are not considered critical, as these are not directly linked to the efficacy and safety of the dosage form.
		Odor	No unpleasant odour	
		Appearance	Transparent gel	
		Spreadability	Acceptable spreadability for application	
8	Assay of the optimized extract		VBS: NLT 10 µg/mL	The drug content, or assay, is a fundamental quality parameter that ensures the formulation remains within established potency limits to guarantee therapeutic efficacy and patient safety. Material attributes and process parameters can affect the formulation assay.
			COA: NLT 0.21 µg/mL	
			CA: NLT 20 µg/mL	
			FA: NLT 18 µg/mL	
9	Container closure system		Round jar	Container closure system
10	Stability		Should be stable for 6 months under accelerated conditions	As per ICH Q1A(R2)

Table 8-5 CQAs for topical gel and their justification

Quality Attributes	Target	Is this CQA?		Justification
Physical Attributes	Color	Acceptable to the patient	No	While color, shape, and physical appearance are not directly linked to safety or efficacy and are thus not classified as critical quality attributes, they are maintained as target specifications to ensure patient acceptability and product identification.
	Odor	No unpleasant odor	No	
	Appearance	Acceptable to the patient	No	
Assay	VBS: NLT 10 µg/mL	Yes		The assay is a critical measure of the formulation's safety, efficacy, and overall performance, as it is directly affected by variations in material attributes and process parameters.
	COA: NLT 0.21 µg/mL			
	CA: NLT 20 µg/mL			
	FA: NLT 18 µg/mL			
Stability	Should be stable for 6 months under accelerated conditions	Yes		The gel should be stable, as instability affects patient compliance and physical and chemical stability and efficacy.
Viscosity	Should have adequate viscosity as a gel.	Yes		Measuring viscosity is crucial for controlling the texture, stability, and application properties of gels.

8.2.1 Preliminary batches for selection of polymer

We preliminarily evaluated batches B1-B7 for pH, spreadability, grittiness, washability, and homogeneity. Batches B1-B3 had good appearance, consistency, and spreadability. Batches B4-B5 had poor consistency, variable viscosity, and unacceptable

spreadability. Batches B6-B8 had Poor consistency, variable viscosity, and unacceptable spreadability. Polymers other than carbopol did not meet the ideal physical quality required for a gel; after 7 days on the benchtop, the gels liquefied and phase separation was observed. However, gels prepared with carbopol met the preliminary requirements for a gel; therefore, carbopol was chosen as the polymer, and further risk assessment was carried out. (Table 8-6)

Table 8-6 Results for preliminary batches for selection of polymer

Parameter	B1	B2	B3	B4	B5	B6	B7	B8
pH	7.01	7.12	7.2	6.9	6.8	7.4		7.08
Spreadability	6.0	6.3	7.1	7.4	7.8	7.2	7.1	7.2
Grittiness	+	+	+	+	+	+	+	+
Wash-ability	Good	Good	Good	Poor	Very Poor	Poor	Poor	Very poor
Homogeneity	Good	Good	Good	Good	Good	Good	Good	Good

8.2.2 Raw material attributes: linking CMAs with CQAs and REM

CQAs derived from the QTPP of the formulation have been linked with material attributes of the gel; their justification has been provided in Table 8-7 and the Risk evaluation matrix (REM) has been provided in Table 8-8.

Table 8-7 Linking CMAs with CQAs

Raw material attributes	Drug Product CQAs	Justification
Concentration of markers	Assay	Assay is affected by marker concentration. Hence, it is a high risk. But it can be controlled.
	Viscosity	Marker concentration does not affect the product's viscosity.
Ethanol content of extract (%)	Assay	Ethanol content does not affect the product's assay or viscosity.
	Viscosity	
Carbopol grade	Assay	Carbopol grade does not affect the product assay. The risk is low.
	Viscosity	Carbopol grade affects product viscosity. Through an RLD, the viscosity target would be achieved. The risk is high.

Raw material attributes	Drug Product CQAs	Justification
Carbopol (%)	Assay	Carbopol concentration does not affect the product assay. The risk is low.
	Viscosity	Carbopol concentration affects product viscosity. The risk is high but will be controlled through optimization trials.
Preservative strength	Assay	Preservatives do not impact assay or product viscosity. The risk is low.
	Viscosity	

Table 8-8 Preliminary Risk assessment

Drug Product CQAs	Raw material attributes				
	Extract		Polymer		
	Concentration of markers	Ethanol content (%)	Carbopol grade	Carbopol (%)	Preservatives strength
Assay	High	Medium	Medium	Medium	Low
Viscosity	Low	Low	High	High	Low
Low	This level represents a broadly acceptable risk, indicating that the parameters are sufficiently controlled and no further investigation or mitigation is required.				
Medium	The identified risk is considered acceptable; however, further investigation may be warranted to implement additional controls and further minimize potential variability.				
High	The risk is unacceptable and requires thorough investigation and robust mitigation strategies to reduce it to a permissible level.				

The high REM risks impacting the drug product CQAs required further investigation to reduce the risk. The polymer (Carbopol) grade and its concentration significantly affect the viscosity of the drug product. Hence, the Carbopol grade and other excipients were kept constant, and key trials were carried out to assess the impact on viscosity and other QTPPs. For RMAs affecting Assay (i.e., marker concentration), as discussed in detail in the previous chapter, the assay of the optimized extract was evaluated, and marker concentrations were controlled and fell under low risk. From the batches made for polymer selection, a few gels

liquefied, which led to adding a binder agent to hold the extract and cross-link the gel; grittiness was also observed in almost all batches made for polymer selection. Hence, a solubilizing agent was added to improve mixing of the extract with other excipients. Therefore, acacia was used as a binder, and propylene glycol was used as a solubilizing agent. Table 8-9 shows key trials of gels made with and without a binder and a solubilizing agent.

Table 8-9 Carbopol key trials

Sr. no.	Grade	Conc. (% w/v)	Extract	Binder	Solubilizing agent	Wash ability	Inference
1	974P	2	5	No	No	No	Excessive consistency & phase separation were observed; gritty and non-spreadable.
2	974P	1.5	5	No	No	No	Excessive consistency & phase separation were observed; gritty and non-spreadable.
3	974P	1	5	Yes	Yes	Yes	Adequate consistency, non-gritty and spreadable.
4	974P	0.5	5	No	No	No	Inadequate consistency, gritty & spreadable
5	974P	0.5	5	Yes	Yes	Yes	Inadequate

Sr. no.	Grade	Conc. (% w/v)	Extract	Binder	Solubilizing agent	Wash ability	Inference
							consistency & adhesive, not so good spreadability.
6	974P	1	10	Yes	Yes	Yes	Adequate consistency, non-gritty & good spreadability.
7	974P	1.5	5	Yes	Yes	No	Excessive consistency, non-gritty and non-spreadable.
8	974P	2	10	Yes	Yes	No	Excessive consistency, non-gritty and non-spreadable.

From Carbopol's key trials, trial no. 6 involving Carbopol grade 974P (1% w/v) with 10% extract showed adequate viscosity, was non-gritty, and had good spreadability. However, trial no. 3 also had similar results; the only thing that was different between the two was the extract's concentration. The former trial showed better in vitro activity than the latter; therefore, the former was chosen as the final optimized formulation. Table 8-10 Shows revised risk assessment.

Table 8-10 Risk assessment: revised

Drug Product CQAs	Raw material attributes				
	Extract		Polymer		
	Concentration of markers	Ethanol content (%)	Carbopol grade	Carbopol concentration	Preservatives strength
Assay	Low	Low	Low	Low	Low
Viscosity	Low	Low	Low	Low	Low

Table 8-11 shows the optimized formulation table.

Table 8-11 Formulation table for gel

Sr. No.	Ingredient	Strength	Usage
1)	Extract	10 %	Active Ingredient
2)	Carbopol (974)	1 %	Gelling agent
3)	Acacia	1 %	Binder
4)	Propylene glycol	5 %	Solubilizing agent
5)	Preservatives a) Methyl Paraben b) Propyl Paraben	0.1 % 0.1 %	Storage
6)	Water	q.s. up to 100 mL	Solvent
7)	Triethanolamine	q.S	pH balancer

8.2.3 Evaluation parameters for optimized gel

The optimized gel made according to the formulation table as in Table 8-11 was evaluated.

The physical characteristics of the gel were appropriate: the optimized gel had a distinct appearance and characteristic aroma, was non-gritty, and showed excellent homogeneity; the measured pH was 7.01, which is compatible with the skin. Adhesiveness and Cohesiveness, measured by a Brookfield viscometer texture analyzer, were 0.94 mJ and 0.86, respectively. The optimized gel formulation showed a spreadability of 6.3, indicating good spreadability.

Rheometry measured the viscosity of the optimized gel formulation at 40.619 cP. A viscosity of 40.619 cP suggests the gel has a semi-fluid consistency, allowing easy application to the skin. It is neither too thick nor too runny, ensuring good adherence without excessive dripping. This viscosity level supports smooth spreading and even distribution of herbal active ingredients. Viscoelasticity is more desirable than viscosity alone in the formulation. This is because the former captures the substance's ability to exhibit both viscous (liquid-like) and elastic (solid-like) characteristics. This dual nature improves performance by maintaining formulation stability, preventing sedimentation during storage, and bridging the gap between a thick, protective layer and a smooth, elegant application that enhances patient compliance.

The formulation assay was carried out using HPLC methods as discussed in Chapter 4. The ranges described for each marker in the extract also apply to the gel formulation, as the extract is a raw material for the gel formulation. Formulation assay results are displayed in Table 8-12.

Table 8-12 Formulation assay: Results

Sr. no.	Marker	Concentration (µg/mL)	Range (µg/mL)	% w/w	Range (% w/w)
1	VBS	12.16	10-16.00	0.0113	0.092–0.0148
2	COA	2.38	0.21–3.28	0.0188	0.0019–0.029
3	CA	23.99	20–28.24	0.0198	0.019–0.026
4	FA	23.99	18–24.12	0.0220	0.016–0.022

Accelerated stability studies were performed at (40 ± 2 °C) and humidity (75 ± 5 %) conditions over 6 months. Gels were kept in a round transparent plastic jar covered with aluminum foil in a stability chamber. Physical evaluation showed the gel was intact, with no phase separation observed. Table 8-11 lists the other parameters evaluated. The accelerated stability study showed negligible changes in the gel's performance characteristics during the study period. Analysis of the stability samples across different

parameters indicated that the gel is stable at high temperature and humidity. Table 8-13 shows the stability study results.

Table 8-13 Accelerated Stability data

Sr. No.	Test	Results		
		0 month	3 months	6 months
1	Organoleptic evaluation	Has a good appearance and is non-gritty.	Has a good appearance and is non-gritty.	Has a good appearance and is non-gritty
2	Colour	Brown	Brown	Brown
3	Weight variation	-	1%	1.5 %
4	pH	7.05	6.99	6.96
5	Viscosity (cP)	40.169	39.872	39.303
6	Adhesiveness (mJ)	0.94	0.93	0.93
7	Cohesiveness	0.86	0.85	0.83
8	Spreadability	6.3	6.2	6.1
9	Homogeneity	Excellent	Excellent	Excellent
10	Phase separation	No	No	No
11	Assay			
	VBS (µg/mL)	12.16	11.99	11.87
	COA (µg/mL)	2.385	2.35	2.32
	CA (µg/mL)	24.99	24.65	24.40
	FA (µg/mL)	23.99	23.66	23.42

Calculation of shelf life:

1) VBS

VBS follows zero-order kinetics, meaning the degradation rate constant is independent of concentration. Hence, through the integrated rate law:

$$C = C_0 - k_0t$$

Where,

C = Final concentration

C_0 = Initial concentration

k = rate constant $\mu\text{g mL}^{-1} \text{ day}^{-1}$

t = time (days)

The shelf life calculated for VBS to reach its lower limit is 1340 days (3.6 years). (Figure 8-2)

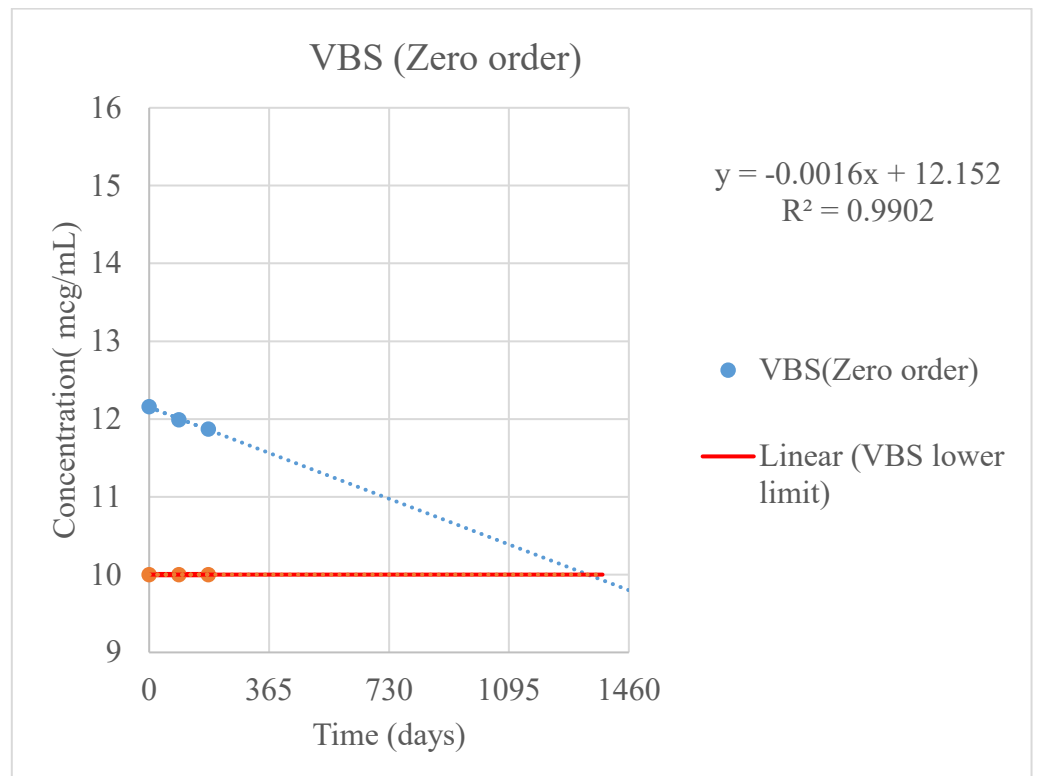


Figure 8-2 Shelf life: VBS

2) COA

COA follows zero-order kinetics, meaning the degradation rate constant is independent of concentration. Hence, through the integrated rate law:

$$C = C_0 - k_0t$$

Where,

C = Final concentration

C₀ = Initial concentration

k = rate constant $\mu\text{g mL}^{-1} \text{ day}^{-1}$

t = time (days)

The shelf life calculated for COA to reach its lower limit is 7592 days/ 20.8 years. (Figure 8-3)

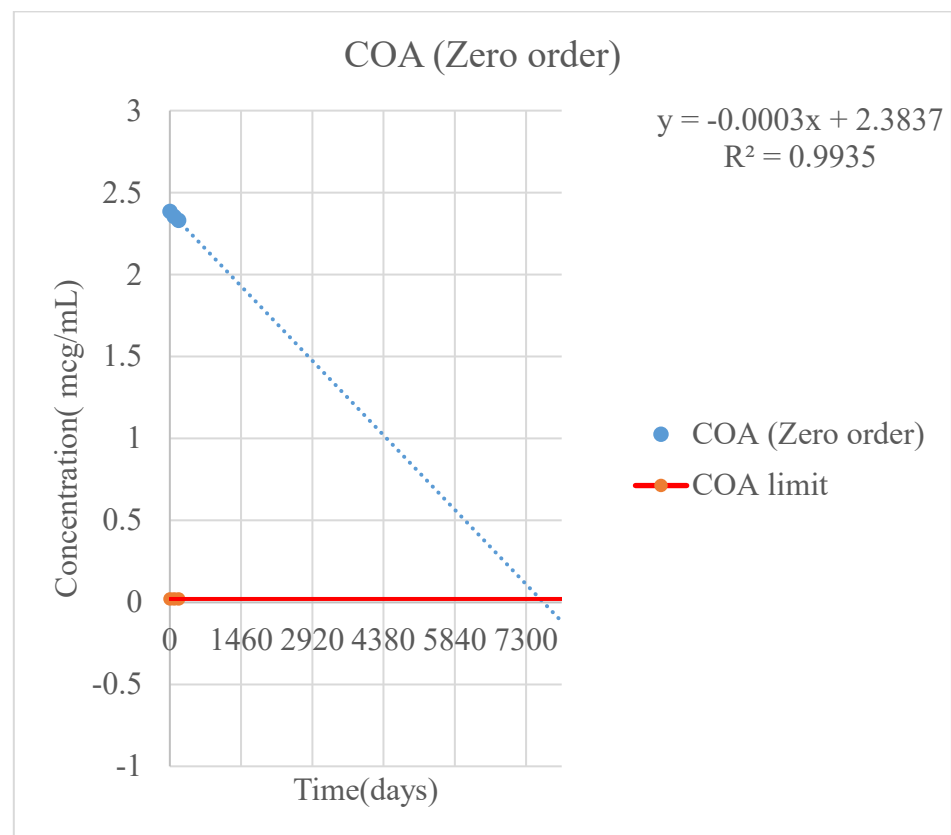


Figure 8-3 Shelf life: COA

3) CA

CA follows zero-order kinetics, meaning the degradation rate constant is independent of concentration. Hence, through the integrated rate law:

$$C = C_0 - k_0t$$

Where,

C = Final concentration

C₀ = Initial concentration

k = rate constant $\mu\text{g mL}^{-1} \text{ day}^{-1}$

t = time (days)

The shelf life calculated for CA to reach its lower limit is 1529 days (4.19 years). (Figure 8-4)

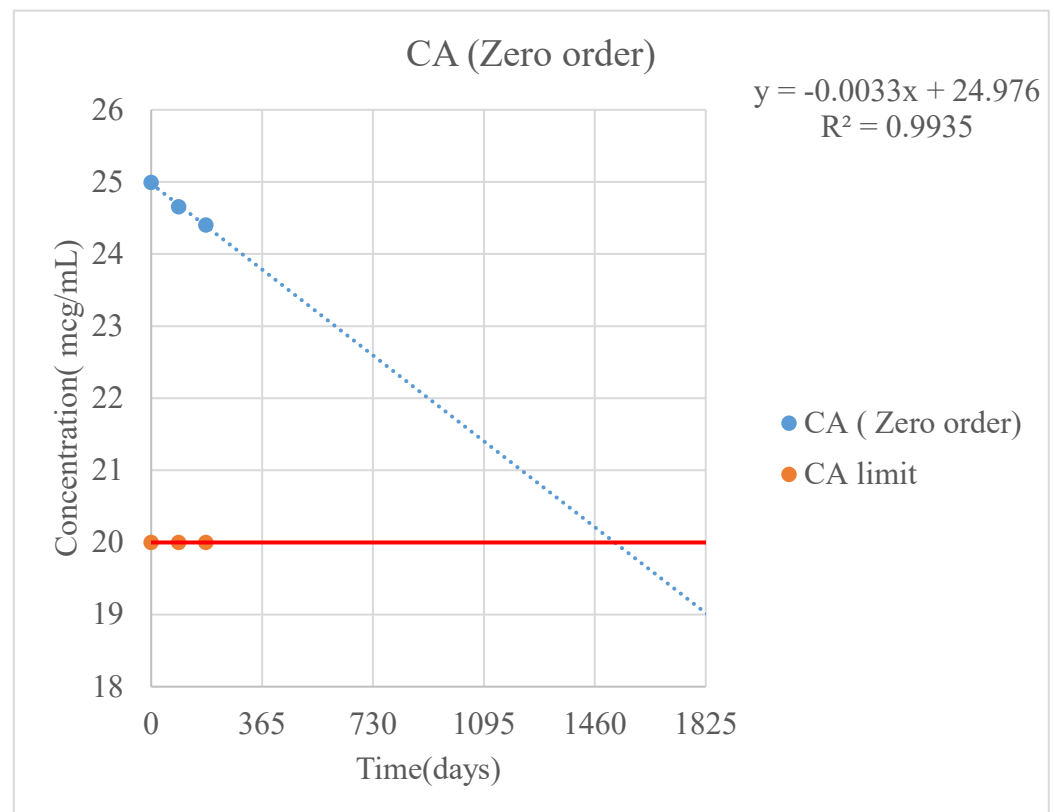


Figure 8-4 Shelf life: CA

4) FA

FA follows zero-order kinetics, which means the degradation rate constant is independent of concentration. Hence, through the integrated rate law:

$$C = C_0 - k_0t$$

Where,

C = Final concentration

C_0 = Initial concentration

k = rate constant $\mu\text{g mL}^{-1} \text{ day}^{-1}$

t = time (days)

The shelf life calculated for FA to reach its lower limit is 1912 days (5.23 years). (Figure 8-5)

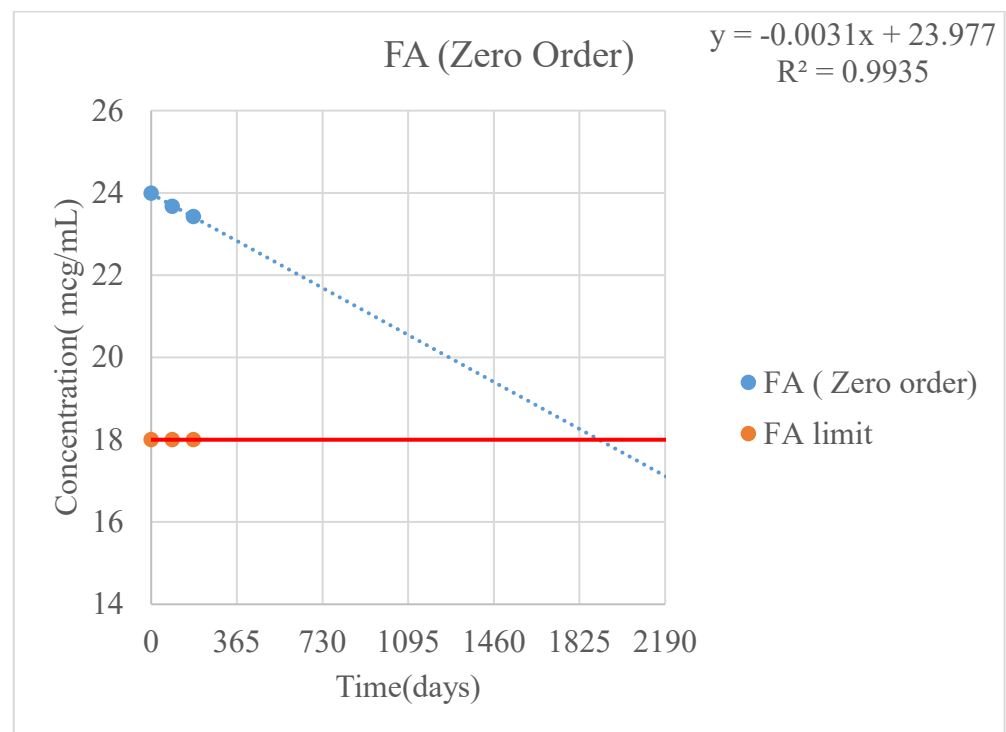


Figure 8-5 Shelf life: FA

8.3 Conclusion

The development of topical drug delivery systems, particularly those incorporating complex bioactive extracts, necessitates a transition from traditional trial-and-error methodologies to a science-based, risk-oriented framework. This research addresses these complexities by applying the Quality by Design (QbD) framework to the formulation of a standardized phytopharmaceutical gel. Unlike conventional approaches that rely on end-product testing, the QbD strategy prioritizes an upfront understanding of product and process variables to build quality into the design from the earliest stages. This systematic evolution is supported by the International Council for Harmonisation (ICH) guidelines, specifically ICH Q8 and Q9, which advocate for a holistic and statistical approach to pharmaceutical development.

The foundation of this formulation process began with the definition of a Quality Target Product Profile (QTPP), which delineated the essential quality characteristics required to ensure the safety, efficacy, and patient acceptability of the final product. From this profile, Critical Quality Attributes (CQAs) were identified, representing the measurable physical and chemical properties such as assay, viscosity, and stability that must remain within predefined limits. These attributes were systematically linked to Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs) through a rigorous Risk Evaluation Matrix (REM). By identifying high-risk parameters early, such as polymer concentration and marker potency, the study was able to focus optimization efforts where they would have the greatest impact on product performance.

Through a series of preliminary batches, various gelling agents were evaluated to find the most robust matrix for the herbal extract. While polymers like HPMC and Sodium CMC exhibited poor consistency and phase separation, Carbopol 974P emerged

as the optimal gelling agent due to its superior spreadability and homogeneity. The formulation was further refined by incorporating 1% Acacia as a binder to promote cross-linking and 5% Propylene glycol as a solubilizing agent to eliminate the grittiness observed in initial trials. The resulting gel, containing a 10% extract concentration at a skin-compatible pH of 7.01, demonstrated excellent physical characteristics, including an appropriate semi-fluid viscosity of 40.619 cP was the experimentally measured viscosity of the optimized formulation and was associated with the desired application properties. The resulting gel was tested against both the species (*S. aureus* and *E. coli*) and it displayed effectiveness against both the species.

The robustness of this design was confirmed through extensive stability studies conducted under accelerated conditions (40 ± 2 °C and $75 \pm 5\%$ RH) in accordance with ICH Q1A (R2) guidelines. Over a six-month period, the gel maintained its physical integrity, with no observed phase separation or significant changes in pH and viscosity. Chemical stability was verified using validated HPTLC methods to monitor the degradation of key markers, including Verbascoside (VBS), Coumaric acid (COA), Caffeic Acid (CA), and Ferulic Acid (FA). The degradation kinetics of VBS, COA, CA and FA were evaluated using the respective concentration-time data. The predicted time to reach the predefined lower acceptance limit was 3.67 years for VBS, 20.80 years for COA, 4.19 years for CA and 5.23 years for FA. VBS exhibited the shortest predicted chemical stability period among the investigated markers. These findings provide supportive evidence for the chemical stability of the formulation; however, the predicted marker-based degradation period should not be considered, in isolation, as definitive evidence of the finished-product shelf-life. The overall shelf-life should be established by considering the stability of all relevant critical quality attributes under appropriate long-term and

accelerated storage conditions.

In conclusion, this chapter establishes a new benchmark for the formulation of bioactive extracts. The resulting gel is a standardized, clinically potent, and commercially viable product, representing a substantial advancement in Pharmaceutical Quality Assurance and topical drug delivery systems.

CHAPTER-9

SUMMARY

&

CONCLUSION

9.0 Summary & Conclusion

Drug resistance has produced a slew of problems & challenges on the road to modern medicine. Phytopharmaceuticals derived from plant species have demonstrated potential as a medicinal resource when compared to synthetically produced drugs. One of the most prevalent illnesses affecting human species, especially the skin, is a fungal infection, which is thought to affect over 40 million people globally. The need to Phytopharmaceutical(s) in specific dosage forms which have used as traditional medicinal plant to be inculcated in modern day treatments. Phytopharmaceuticals are of botanical origin, they are defined under D&C act, and have to meet the standards of new drug approval. They have sufficient reliability ensuring patient safety and minimum side effects, adverse reactions and below par toxicological implications. Hence, regulatory agencies around the globe have been publishing guidelines for industrial standards for botanical origin. This makes it crystal clear that an alternative medicine pathway is underway wherein traditional medicines being used in specific regions can be linked to scientific knowledge and be commercialized.

This research marks a significant advancement in the systematic development of phytopharmaceutical topical deliveries by successfully transitioning from empirical formulation to a rigorous Quality by Design (QbD) framework, this research addresses the critical global priority of Antimicrobial Resistance (AMR) through the creation of a high-performance, stable, and industrially viable herbal gel.

A significant contribution of this work lies in the unprecedented standardization of a complex herbal matrix. Utilizing validated HPTLC methodologies, this research achieved simultaneous stabilization and quantification of four critical bioactive markers: Verbascoside (VBS), Caffeic Acid (COA), Cinnamic Acid (CA),

and Ferulic Acid (FA). The scientific soundness of the formulation is further validated by its physiological compatibility (pH 7.01) and its potent in-vitro antimicrobial efficacy against *E. coli* and *S. aureus*, proving its therapeutic readiness.

Optimization of extraction process via a factorial design benefit in quantifying the presence of markers. The optimization extraction process for herbal materials is not merely a preparative technique but critical determinant of product quality, efficacy, and sustainability. An optimized extraction protocol tailored to the specific phytochemical profile of the plant material is essential for producing high-value herbal formulations. The optimization of extraction protocols for herbal materials, exemplified by *K. africana*, transcends mere preparative technique; it is a decisive factor in the quality, therapeutic efficacy, and sustainability of the final herbal products. A 3² full factorial design was successfully studied, a powerful statistical tool, to systematically dissect the individual and synergistic effects of ethanol concentration and extraction time on the yields of four critical marker compounds: VBS, CA, COA, and FA.

The findings revealed a nuanced relationship between the investigated factors and the concentration of each marker. For instance, extraction time significantly enhanced VBS concentration, whereas CA and FA concentrations were positively influenced by both independent variables and their interaction. Conversely, complex interaction was observed on COA yield. This differential responsiveness underscores the complex chemical diversity of *K. africana*'s metabolome and the imperative for precise parameter control during extraction.

The robust validation of the predictive models, evidenced by correlation coefficients exceeding 0.99 between predicted and actual values, attests to the high accuracy and reliability of this experimental design approach. Such predictive capability is invaluable for pharmaceutical and natural product development,

particularly given the inherent sensitivity of phytochemicals within complex herbal matrices to processing conditions. The application of DOE minimizes the experimental burden while maximizing the generation of actionable insights, thereby enabling the precise tuning of extraction processes to augment yield, enhance bioactivity, and improve overall operational efficiency.

This provides a rigorous, data-driven methodology for establishing optimized extraction parameters for *K. africana* fruits and an optimized extract. This approach is highly transferable and scalable to other botanicals, offering a foundational framework for developing high-value herbal formulations where chemical integrity and biological activity are paramount. This statistically informed, forward-thinking strategy for natural product extraction is critical for advancing the discovery and development of plant-derived therapeutics.

The evaluation of the optimized extract showcases three important aspects which are crucial and essential for the usage of extract in formulation. Primarily, the ethanolic content of optimized extract found to be lesser than MIC explains the in-vitro activity against bacterial species due to the presence of the biomarkers. Secondly, the results of assay of the optimized extract confirms the point selected for further evaluation as appropriate enhancing the ruggedness of the model and design. Lastly, evaluation of the optimized extract bridges the gap between essential characteristics required for extract as a raw material and the topical dosage form.

This research demonstrates a fundamental transition in the development of topical phytopharmaceuticals, replacing empirical trial-and-error with the science-led QbD methodology. By defining a rigorous Quality Target Product Profile (QTPP) and executing a systematic Risk Evaluation Matrix (REM), the

study successfully optimized a Carbopol 974P-based delivery system. The strategic inclusion of 1% Acacia and 5% Propylene glycol effectively resolved initial grittiness and phase separation, yielding a homogenous formulation with a skin-compatible pH. These parameters establish a robust baseline for ensuring uniform drug distribution and high patient acceptability.

Long-term performance and structural robustness were confirmed through accelerated stability testing adhering to ICH Q1A (R2) guidelines. Throughout the six-month assessment, the gel maintained exceptional physical and chemical integrity, showing no signs of phase separation or significant fluctuations in performance metrics. Quantitative HPTLC monitoring of key markers indicated zero-order degradation kinetics and supported shelf-life up to 3 years. These results validate the formulation's durability and guarantee that therapeutic potency remains intact under diverse environmental conditions.

In conclusion, this work provides a standardized antimicrobial therapy that harmonizes traditional herbal wisdom with contemporary pharmaceutical rigor. From industrial and regulatory perspectives, the implementation of ICH Q8/Q9 protocols and Process Analytical Technology (PAT) creates a scalable manufacturing model that significantly reduces batch variability. By elevating complex bioactive extracts into commercially viable, high-quality topical products, this research establishes a definitive benchmark for pharmaceutical quality assurance and ensures reliable clinical outcomes. This integrative approach ultimately enhances patient confidence in natural medicine by applying the highest standards of modern drug development.

9.1 Contribution of Research Work

9.1.1 Contribution to Socio-Economic Relevance and Impact

The most significant contribution of this work lies in translating a traditionally derived botanical intervention into a scientifically standardized and pharmaceutically developable topical antimicrobial product. The study establishes a QbD-based approach for the systematic extraction, standardization and formulation of bioactive plant constituents, thereby providing a scientific foundation for the development of an affordable, reproducible and potentially scalable topical therapy for bacterial and fungal infections. This approach contributes to the broader objective of reducing the therapeutic burden associated with antimicrobial resistance while supporting the regulated development of plant-based medicines.

A major strength of the work is that the extraction process was not developed through conventional trial-and-error experimentation. Critical extraction variables were systematically investigated and optimized using a three-level factorial design within the QbD framework. Extraction yield and recovery of the selected bioactive markers, namely Verbascoside, Coumaric acid, Caffeic acid and Ferulic acid, were experimentally quantified, optimized and standardized. Their selection was further supported by the observed in-vitro antimicrobial activity, providing a biological basis for their use in extract standardization.

Collectively, the optimized extraction, standardized bioactive-marker profile and QbD-based formulation development provide a technically feasible foundation for scale-up and industrial manufacture. This establishes a translational pathway from botanical raw material to a standardized pharmaceutical dosage form while supporting consistency, quality and reproducibility of the final product.

9.1.2 Regulatory & Technological Contribution and Industrial Excellence

From a regulatory and technological perspective, this research provides a systematic framework for the development of a standardized phytopharmaceutical in accordance with modern pharmaceutical quality principles. The integration of Quality by Design, design-of-experiments, risk-based assessment, analytical standardization and stability evaluation establishes a science-based approach for controlling variability in both extraction and formulation processes.

The industrial relevance of the work is supported by the experimental optimization of the extraction process, including critical process variables, extraction yield and recovery of the selected bioactive markers. The resulting process provides a defined and reproducible basis for scale-up of the standardized botanical extract. Importantly, industrial manufacture would involve scale-up of the standardized extract rather than the requirement for individual isolation of each marker compound. The QbD-derived understanding of process variables and their influence on critical quality attributes further facilitates rational process transfer and scale-up.

The formulation development similarly demonstrates technological readiness through systematic optimization of critical material attributes and process parameters. The optimized formulation exhibited acceptable physical characteristics and maintained its integrity during accelerated stability testing. Chemical stability was assessed through validated HPTLC methods by monitoring Verbascoside, Coumaric acid, Caffeic acid and Ferulic acid, with kinetic modelling providing quantitative estimates of their degradation behaviour.

The regulatory significance of the work lies in establishing measurable quality attributes and analytical markers for a complex botanical product, thereby addressing one of the major

challenges in phytopharmaceutical development: converting inherent variability of plant-derived materials into a controlled and reproducible pharmaceutical product. The integration of traditional phytomedicine with contemporary pharmaceutical development principles therefore provides a scientifically justified pathway towards regulated commercialization.

Overall, the study establishes the technical feasibility and QbD-based process foundation for industrial-scale extraction and formulation of the standardized botanical product. While pilot- and commercial-scale validation would constitute the subsequent stage of development, the present work provides the experimental process understanding, standardization strategy and quality framework required to support such scale-up.

CHAPTER-10

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86. Harshilkumar Jani (2022) Development and Evaluation of Adhesive Gel of Tranexamic Acid for Dental Use. M. Pharm Thesis. Gujarat Technological University.

Annexures

Annexure I *K. africana* authentication letter

Annexure II Fellowship letter

Annexure III Patent application

Annexure IV Summary of compliance report (DPC-I to VI)

Annexure V List of Publications

Annexure VI List of conference presentations

Annexure I-K. *africana* authentication letter

DEPARTMENT OF BOTANY, BIOINFORMATICS & CLIMATE CHANGE IMPACTS
MANAGEMENT



SCHOOL OF SCIENCE
GUJARAT UNIVERSITY
AHMEDABAD – 380009.
Phone No. 26302578

Dr. Archana Mankad
email: aumankad@gujaratuniversity.ac.in
Professor and Head
Ref: GU/BOT/2023

Date: 28/07/2023

Certificate

It is hereby certified that the Herbarium specimen prepared by Rahul Dhirajbhai Lalwani

Sr.No.	Family	Botanical name	Authentication number
1	Bignoniaceae	<i>Kigelia Africana</i> (Lam.) Benth. (<i>Kigelia pinnata</i>)	GU/BOT/B/K1


Dr. Hitesh A. Solanki
Professor
Department of Botany,
Bioinformatics
& Climate Change Impact Management
School of Sciences
Gujarat University, Ahmedabad-380009

Annexure II-Fellowship letter

The SHODH (ScHeme of Developing High Quality Research) Fellowship

was awarded by the Education Department, Government of Gujarat. The aim of this fellowship is to increase orientation amongst the students and to develop high quality research.

The fellowship included stipend for two years and annual contingency.

Tenure: October 2023 to September 2025



Education Department, Government of Gujarat

SHODH - ScHeme of Developing High Quality Research

Managed by: Knowledge Consortium of Gujarat

Pragna Puram Campus, Opp. PRL, Nr. L.D.College of Engineering, Ahmedabad-380015
Mo. No.:9979200152



E-mail: shodhsupport-kcg@gujgov.edu.in

Website: <https://shodh.guj.nic.in/>

KCG/SHODH/2023-24

Print Date :25-10-2023 2:17 PM

AWARD LETTER

To,
Lalwani Rahul Dhirajbhai
Pharmacy
GUJARAT TECHNOLOGICAL
UNIVERSITY



Sub: Award Letter of Approved beneficiary under SHODH Scheme

Ref No : 202201379

Dear Student,

We congratulate you for the award of SHODH Fellowship Scheme. On the basis of your submission of Undertaking, Attestations, and Evaluation done by Scrutiny Committee, now this makes it a formal offer of award under SHODH. The details of fellowship are given below: -

Name of Supervisor/Guide	Dr Kunjan Bodiwala
Date of Joining to Ph.D.	17/01/2023
Ph.D. Registration Number	229999901024
Ph.D. Research Title	Formulation Standardization and Evaluation of Phytopharmaceutical with Antiinfective activity
Tenure of Fellowship	October 2023 to September 2025
Stipend Amount (Monthly)	Rs. 15,000=00 for 2 Years
Contingency Amount (Yearly)	Rs. 20,000=00 for 2 Years
Total Payable Amount for 2 Years	Rs. 4,00,000=00

Annexure III-Patent Application

Patent Status	Provisional	Complete	Published
Patent No	202521069561		
Filing date	July 21, 2025		
Title	A topical gel formulation of <i>Kigelia Pinnata</i>		

Controller General of Patents, Designs
& Trade Marks



सत्यमेव जयते

G.A.R.6
[See Rule 22(1)]
RECEIPT



Docket No 46967

Date/Time 2025/07/21 18:35:00

SANJAY KUMAR MAGANBHAI
PATEL 627-Gala Empire, Drive In
Road, Thaltej, Ahmedabad,
Gujarat 380054 Email:
patent@excelonip.com

Sr. No.	App. Number	Ref. No./Application No.	Amount Paid	C.B.R. No.	Form Name	Fee Payment	Remarks
1	202521069561	TEMP/E-1/77516/2025-MUM	1600	26150	FORM 1	Full	A TOPICAL GEL FORMULATION OF KIGELIA PINNATA
2	E-106/3663/2025/MUM	202521069561	0	-1	FORM28	Full	

Annexure IV-Summary of DPC compliance report

DPC	Date	Comments
I	July 18, 2023	Add 'dosage form in title. Drying of the fruits should be compared with air drying. Student needs to get detailed idea about formulation development needs. Detailed methodology/protocol should be worked out.
II	December 30, 2023	Identify target strains of microorganism and their source. Prepare a proper protocol for antimicrobial study and follow it. Identify standard control to be used and its concentration for activity. Extend the experimental design for the extraction process. Student should create a roadmap for the remaining work.
III	July 19, 2024	The dose of extract should be finalized before beginning formulation. Concentration of standard used for activity should be evaluated against the dose of extract to be used. For model validation, conditions outside the design space should be taken up as well.
IV	December 23, 2024	State the stability conditions to be carried out for formulation. Finalize the evaluation parameters of the formulation. Specificity of the formulation should be done via analytical method. The research/review paper under communication/being written should be mentioned in the presentation.
V	May 30, 2025	Assay of the formulation. Optimization of sample preparation technique. State the research publication stages appropriately with all details. Elaborate recent published work, if available, in literature.
VI	November 3, 2025	The committee members expressed satisfaction of the completion of the project and appreciated the outcome and it's contribution in totality.

Annexure V-List of Publications

Publication-1

Type of Publication: Research

Title:

Journal: Natural Product Research

ISSN/ISBN No.: 1478-6427

Indexing: Scopus

Published on April 10, 2024

NATURAL PRODUCT RESEARCH
<https://doi.org/10.1080/14786419.2024.2340762>



Quality control methods for fruit extracts of *Kigelia africana* using high performance thin layer chromatography

Kunjan B. Bodiwala^a, Nancy Banker^a, Rahul Lalwani^{a,b}, Mamta B. Shah^c and Anuradha K. Gajjar^a

^aDepartment of Pharmaceutical Chemistry and Quality Assurance, L. M. College of Pharmacy, Ahmedabad, India; ^bGujarat Technological University, Ahmedabad, India; ^cDepartment of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad, India

ABSTRACT

Kigelia africana is a tree native to Africa but also found in eastern and southern parts of India with reported anti-bacterial, anti-inflammatory, and immunomodulatory activities. Verbascoside, caffeic acid and ferulic acid are important markers for the quality control of the plant. Two different HPTLC methods were developed and validated; method – 1 for estimation of verbascoside and caffeic acid while method – 2 for estimation of caffeic acid and ferulic acid. Developed methods were applied to the methanolic fruit extract to determine the quantities of markers. Both methods were found to be linear, specific, precise, accurate, sensitive and robust. Results indicated that both methods can be used for quantitative determination of verbascoside, caffeic acid and ferulic acid in fruit extract. The developed methods may be utilised as a part of the quality control and standardisation for the raw material and extracts of *Kigelia africana* and can also aid to chromatographic fingerprinting of the plant.

ARTICLE HISTORY

Received 29 September 2023
Accepted 1 April 2024

KEYWORDS

Kigelia africana; HPTLC; verbascoside; caffeic acid; ferulic acid

Publication-2

Type of Publication: Research

Title: High-performance thin-layer chromatography method for the quantitative assessment of caffeic acid, coumaric acid and ferulic acid in ethanol extracts of *Kigelia africana* fruits

Journal: Journal of Planar Chromatography

ISSN/ISBN No.: 0933-4173

Indexing: Scopus

Published on July 14, 2025

JPC – Journal of Planar Chromatography – Modern TLC
<https://doi.org/10.1007/s00764-025-00368-6>

ORIGINAL RESEARCH PAPER



High-performance thin-layer chromatography method for the quantitative assessment of caffeic acid, coumaric acid, and ferulic acid in ethanol extracts of *Kigelia africana* fruits

Rahul Lalwani^{1,2} · Kunjan Bodiwala¹ · Anuradha Gajjar¹

Received: 8 January 2025 / Accepted: 1 July 2025
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Abstract

Kigelia africana, a tree indigenous to Africa and also found in eastern and southern India, has demonstrated antibacterial, anti-inflammatory, and immunomodulatory properties. This study aimed to quantify the levels of phenolic compounds—specifically caffeic acid (CA), coumaric acid (COA), and ferulic acid (FA)—in the ethanolic fruit extract of *K. africana* using a high-performance thin-layer chromatography (HPTLC) method. The method was developed and validated following the International Council for Harmonisation (ICH) Q2(R1) guidelines. The validation results indicated that the method is linear (for CA: 200–1200 ng/band, for COA: 50–300 ng/band, and for FA: 200–1200 ng/band), specific, precise, and accurate, with recoveries ranging from 98% to 102%. It exhibited high sensitivity with a low limit of detection and quantification, and was robust with relative standard deviations (RSD) < 2% for precision and peak area. The HPTLC method successfully quantified the levels of CA, COA, and FA in the ethanolic extract of *K. africana*, demonstrating its effectiveness and reliability for the analysis of these bioactive compounds.

Other publications

- 1) Gajjar, A., Gohel, N., Lalwani, R. *et al.* Instrumental thin-layer chromatography method for the simultaneous estimation of gabapentin and nortriptyline hydrochloride in tablet dosage form. *JPC* (2025).
<https://doi.org/10.1007/s00764-025-00376-6>
- 2) Jani H, Ranch K, Lalwani R, Patel Y, Bodiwala K, Gajjar A. Sensitive HPLC Method to Support Pre-formulation Studies and to Determine Critical Quality Attributes of Therapeutic Contact Lenses Containing Dorzolamide Hydrochloride. *Current Pharmaceutical Analysis*. 2024 Nov;20(9):994-1004.
<http://dx.doi.org/10.2174/0115734129341673241106101546>

Annexure VI-List of Conference Presentations

1) Category: Oral Presentation

Conference: 27th Convention of Indian Society of Pharmacognosy and International Conference

Organized by: Jamia Hamdard, Delhi

Title: DOE based optimization of extraction process from fruits of *Kigelia africana*

2) Category: Poster Presentation

Conference: International Conference on Traditional Medicine & Phytopharmaceuticals & International Congress of Society for Ethnopharmacology

Organized by: CSIR-IIIM, Jammu

Date: February 16th- 18th, 2024

Title: Extraction of biomarkers from fruits of *Kigelia Africana*: Optimization with DOE