Unveiling therapeutic dimensions of *Butea monosperma*: Exploring Phytochemistry, Antioxidant, Anti-Urolithiasis and Anti-inflammatory Potentials.

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This is to certify that the dissertation report "Unveiling therapeutic dimensions of *Butea monosperma*: Exploring Phytochemistry, Antioxidant, Anti-Urolithiasis and Anti-inflammatory Potentials", is a bonafide work carried out by Ms Samiksha Satish Naik under my supervision in partial fulfilment of the requirements for the award of the degree of Master Science in Botany in the Department of Botany at the School of biological Sciences and Biotechnology, Goa University

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PREFACE

As I embarked on this journey of scientific inquiry and exploration, I was deeply motivated by the opportunity to contribute to the advancement of knowledge in the field of medicinal plants and natural remedies pertaining in regards to Kidney stone. This dissertation represents the culmination of months of rigorous research, experimentation, and analysis aimed at to understand the therapeutic potential of *Butea monosperma* flower extracts in fighting kidney stone disease, inflammation, and oxidative stress. The inspiration for this study stems from the pressing need for novel and effective treatments for kidney stone disease, a condition that afflicts millions worldwide and presents significant challenges to healthcare providers and patients alike. Modern therapies often fall short in addressing the multifaceted nature of the disease, highlighting the importance of exploring alternative approaches rooted in traditional knowledge and in nature's pharmacopeia.

In this preface, I extend my heartfelt gratitude to all those who have supported and guided me throughout this journey. My sincere appreciation goes to my research mentor, whose expertise and guidance has been invaluable in shaping the direction of this study. I am also indebted to my colleagues and peers for their collaboration, encouragement, and intellectual exchange, which has enriched my research experience immeasurably. I dedicate this dissertation to all those affected by kidney stone disease, with the hope that our collective efforts in scientific inquiry will pave the way for more effective treatments and improved quality of life for individuals fighting with this condition. As I present the findings of this study, I am humbled by the opportunity provided by Botany Discipline, School of Biological Sciences and Biotechnology to contribute to the broader scientific community and to make a meaningful difference in the lives of those in need. May this dissertation serve as a testament to the power of curiosity, perseverance, and collaboration in advancing our understanding of the natural world and harnessing its potential for the betterment of humankind.

-Ms. Samiksha Satish Naik

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-Miss. Samiksha Satish Naik

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ABBREVIATIONS USED

Entity	Abbrevation
CaOx	Calcium oxalate crystals
Aq extract	Aqueous extract
H_2O extract	Water extract
TPC	Total Phenol content
TFC	Total Flavonoids content
SEM	Scanning electron Microscope
HPTLC	High Profile Thin Layer chromatography
meOH	methanol
etOH	Ethanol
%	Percentage

Abstract

Kidney stone disease affects a substantial portion of the population, and conventional treatments often lack efficacy against its multifactorial nature. Inflammation and oxidative stress are key drivers of this condition, necessitating novel therapeutic approaches. *Butea monosperma*, known for its medicinal attributes, emerges as a promising avenue for addressing these challenges quantitatively. A diverse range of assays is employed to quantitatively assess the antioxidant capacities, anti-inflammatory, and anti-urolithiasis, of *Butea monosperma* flower extracts. These assays encompass aggregation and nucleation assays for calcium oxalate crystals, protein denaturation inhibition assays, DPPH assays for antioxidant potential, and preliminary phytochemical tests for phytochemical class identification.

Phytochemical analysis revealed the presence of phenols, flavonoids, alkaloids, steroids, triterpenoids, tannins, and saponins in the extracts. Furthermore, the aqueous extract with honey displayed the strongest antioxidant activity among the formulations tested, with IC_{50} values as low as 9.90 µg/mL in DPPH assays. Aqueous extract with honey exhibited the highest potency in inhibiting protein denaturation, achieving inhibition percentages exceeding 50%. Quantitative analysis reveals that the aqueous flower extract combined with honey demonstrates the most significant inhibition of calcium oxalate crystal aggregation and nucleation, with reductions of up to 70% observed in aggregation assays.

The quantitative findings highlight the promising therapeutic potential of *Butea monosperma* flower extracts, particularly in conjunction with honey. These extracts demonstrate quantifiable efficacy in preventing kidney stone formation, ameliorating inflammation, and mitigating oxidative stress. Further investigations are necessary to elucidate the precise mechanisms of action and optimize the clinical utility of these extracts.

CHAPTER 1: INTRODUCTION

CHAPTER I: INTRODUCTION

1.1 Background

Butea monosperma, a medium-sized deciduous tree indigenous to southern Asia, holds a significant position in traditional medicinal practices across the Indian subcontinent and Southeast Asia. Known by various names such as 'Flame of the Forest' and 'Palash' in English, 'Dhak' and 'Palash' in Hindi, and 'Tong-kwoaw' in Thailand, this species (Sindhia & Bairwa, 2010; Das & Smita, 2018) has not only adorned landscapes with its vibrant orange-red flowers but has also been revered for its medicinal properties throughout history (Sharma & Garg, 2009).

In recent years, scientific interest in *Butea monosperma* has intensified, driven by its diverse pharmacological potential and the pressing need for novel therapeutic interventions, particularly in addressing kidney-related ailments such as nephrolithiasis or kidney stones (Polina *et al*., 2020). The prevalence of kidney stones globally necessitates effective treatment options to alleviate the discomfort and complications associated with this condition.

The therapeutic significance of *Butea monosperma* lies in its rich phytochemical composition, which has been extensively explored in both traditional and modern medicinal contexts (Das & Smita, 2018). Extracts derived from various parts of the plant, including flowers, leaves, and bark, have demonstrated diverse bioactivities, making it a subject of interest for researchers investigating alternative treatments for kidney stones (Sharma *et al*., 2017).

Recent studies have shed light on the inhibitory properties of *Butea monosperma* extracts against the formation of calcium oxalate crystals, the primary constituents of kidney stones (Sharma *et al*., 2017). Specifically, certain fractions of these extracts have shown significant reductions in oxalate, calcium, creatinine, uric acid, and urea levels in serum and urine

samples, suggesting a potential role in preventing stone formation (Sharma *et al*., 2017). Additionally, *in vitro* studies have indicated the ability of *Butea monosperma* extracts to reduce the nucleation of calcium oxalate particles and dissolve urinary calculi, further highlighting its therapeutic promise in managing kidney stone formation (Mishra et al., 2022).

While other plant species like *Bauhinia variegata* have been investigated for their antilithiatic properties, *Butea monosperma* emerges as a distinctive candidate due to its documented efficacy and historical usage in traditional medicine (Mishra et al., 2022). Therefore, this dissertation aims to delve deeper into the phytochemistry and therapeutic potential of *Butea monosperma*, particularly focusing on its antioxidant, anti-urolithiasis, and anti-inflammatory properties, with the ultimate goal of contributing to the development of alternative treatments for kidney stones and related ailments.

1.1.1 History

In Unani and Ayurveda traditions, *Butea monosperma* holds a revered status, often regarded as a treasure bestowed by the gods and encompassing sacred groves. Legend has it that the tree incurred the wrath of Goddess Agnidev, who punished it for intruding upon the privacy of Lord Shiva and Goddess Parvati. As a result, *Butea monosperma* is imbued with spiritual significance, with its flowers offered to Goddess Kali through blood scarification rituals, while its leaves find utility in various Hindu rituals.

The tree's significance extends beyond spiritual realms, as its various parts serve practical and ceremonial purposes. Dry stems of *Butea monosperma* are utilized in sacred fires, symbolizing purity, and sanctity, while its wood is crafted into sacred utensils, reflecting its importance in religious ceremonies (Jhade *et al*., 2009). Furthermore, the leaves of *Butea*

monosperma are employed as wrappers for tobacco, adding a ceremonial touch to smoking rituals.

Beyond its ritualistic significance, *Butea monosperma* also contributes to livelihoods and traditional practices through its economic utility. The plant is valued for its resin and dyes, which are extracted for various purposes, while its soft yet durable wood is utilized as timber for construction and carpentry (Das & Smita, 2018). In rural areas, the leaves of *Butea monosperma* serve as practical substitutes for plates and cups, showcasing the plant's versatility and importance in everyday life (Jhade et al., 2009).

Additionally, the bark of *Butea monosperma* holds medicinal properties and is utilized in the treatment of various ailments, further cementing its status as a multifaceted resource deeply intertwined with cultural, spiritual, and practical aspects of life. In essence, *Butea monosperma* embodies a holistic connection between humans, nature, and spirituality, serving as a symbol of reverence and sustenance in Unani and Ayurvedic traditions.

1.1.2 Ethnobotanical Aspects of *Butea monosperma*

Ethnobotany is a multidisciplinary field that examines the intricate relationship between humans and plants, highlighting the diverse uses of plants for food, shelter, medicine, and economic purposes. Its primary objective is to understand the traditional knowledge associated with plants, including their medicinal and nutritional benefits, and to document and disseminate this ancient wisdom.

Traditional medicine, a cornerstone of ethnobotanical practices, encompasses a wide range of health practices, beliefs, and therapies rooted in plant, animal, and mineral-based remedies. These practices, passed down through generations, play a vital role in treating, diagnosing,

and preventing illnesses, both in humans and livestock, particularly in developing countries where access to modern healthcare services may be limited.

The utilization of medicinal plants, such as *Butea monosperma*, in traditional medicine offers several advantages, including lower costs compared to modern healthcare services and a cultural connection that resonates deeply with communities. *Butea monosperma* flowers, for example, are valued for their medicinal properties, serving as remedies for various ailments such as bowel issues, leprosy, gout, and eye diseases. The flowers, characterized by their bitter taste, act as astringents, aphrodisiacs, and expectorants. Extracts derived from the flowers are used to reduce spleen enlargement and alleviate swelling, often administered with milk and sugar to mitigate body heat and chronic diseases. Infusions made from soaking the flowers in water are consumed daily to treat conditions like leucorrhea, while a mixture of flower powder with honey serves as a remedy for kidney stone diseases.

Overall, the ethnobotanical exploration of plants like *Butea monosperma* not only sheds light on their therapeutic potential but also underscores the importance of traditional knowledge in addressing healthcare needs, preserving biodiversity, and promoting sustainable practices.

1.1.3. Classification and Morphology

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Fabales

Family : Fabaceae

Genus : *Butea*

Species : monosperma

1.1.4. Morphology of Flower *Butea monosperma*

Butea monosperma, commonly known as the Flame of the Forest, produces striking flowers with flamboyant colors, typically ranging from 2 to 4 inches in diameter, adorned with leaflets. These flowers are large and conspicuous, making them easily recognizable. The flowering season typically spans from February to the end of April. The corolla of the flower measures approximately 5 to 7 centimetres in length, contributing to its impressive appearance. The flowers are predominantly found on the upper regions of the tree, creating a stunning canopy that gives the tree its evocative name, "Flame of the Forest." Each flower exhibits a distinctive morphology, characterized by a recurved keel and wings shape, which are densely covered with fine hairs, or pubescence. These floral structures are uniform in size, contributing to the overall aesthetic appeal of the tree. The visual spectacle created by the abundant and vibrant flowers of *Butea monosperma* adds to its allure, attracting attention from afar and earning it a prominent place in the landscape. This captivating display serves not only as a testament to the tree's beauty but also as a source of inspiration and admiration for those who behold it (Singh & Srivastava 2022).

1.1.5. **Anti-urolithiasis Activity of *Butea monosperma* Flowers**

The anti-urolithiasis activity of *Butea monosperma* flower extracts has been investigated, focusing on their inhibitory effects on calcium oxalate crystal formation and aggregation. Microscopic observations have revealed the effectiveness of these extracts in hindering the nucleation and aggregation of calcium oxalate crystals.

Furthermore, other parts of the *Butea monosperma* tree, such as the bark and seeds, have demonstrated significant anti-urolithiasis activity. In vivo studies conducted on experimental models, including mice, have yielded promising results, indicating a reduction in the formation of calcium oxalate crystals. These findings suggest the potential of *Butea monosperma* as a therapeutic agent for kidney stone treatment. Further research may explore the incorporation of *Butea monosperma* extracts into medicinal preparations aimed at managing urolithiasis, offering a natural and potentially effective alternative to conventional treatments.

1.1.6. Anti-inflammatory Activity

Inflammation is a natural protective response triggered in injured tissues, involving a complex cascade of events such as enzyme activation, release of mediators, extravasation of fluids, cell migration, tissue breakdown, and repair processes. While inflammation serves a crucial role in healing, it often accompanies pain and results in increased vascular permeability, protein denaturation, and membrane alterations. Currently available drugs for inflammation, such as opioids and non-steroidal anti-inflammatory drugs (NSAIDs), are associated with side effects. Consequently, there is growing interest in exploring alternative treatments derived from traditional medicinal plants. These plants contain various bioactive compounds that have the potential to yield anti-inflammatory agents.

Research spanning the past two centuries has focused on investigating the biological activities of plants to identify compounds suitable for modern drug development. *Butea monosperma*, for instance, has demonstrated anti-inflammatory properties and analgesic effects attributed to bioactive compounds like flavonoids, steroids, glucosides, and other aromatic compounds.

Studies have shown that *Butea monosperma* exhibits both in vivo and in vitro activities against tumours, aids in wound healing, and possesses antihyperglycemic properties in addition to its anti-inflammatory activity. The plant's ability to block the activation of NF-κB, a key regulator of inflammation, is attributed to phytochemicals like butrin, isobutrin, and butein found in its flowers. These compounds have been observed to reduce the expression and production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 in macrophages stimulated by agents like phorbol 12-myristate 13-acetate and calcium ionophore A23187. The present study aims to provide a comprehensive understanding of the chemical and bioactive compounds present in medicinal plants, particularly focusing on the anti-inflammatory properties of *Butea monosperma* flowers. Through detailed scientific research, insights into the mechanisms of action and therapeutic potential of these compounds can be gained, paving the way for the development of novel anti-inflammatory agents. (Zahra et al., 2024)

1.1.7. Antioxidant property of flower *Butea monosperma*

Reactive oxygen species (ROS) are produced in the body because of both endogenous processes, such as normal aerobic respiration, and exogenous factors, including exposure to pollutants like organic solvents, ionizing radiation, and smoke. These species, including superoxide anion, hydrogen peroxide, nitric oxide, hypochlorite, hydroxyl radical, and lipid peroxide, can cause cellular damage to biomolecules such as nucleic acids, proteins, and enzymes. This damage contributes significantly to diseases such as urolithiasis, diabetes, cancer, inflammation, brain dysfunction, and the aging process. (Polachi *et al*., 2015)

Antioxidants play a crucial role in neutralizing free radicals. Natural antioxidants present in the body include superoxide dismutase, catalase, and glutathione. The 2,2-Diphenyl-1-

picrylhydrazyl (DPPH) assay is commonly used to assess the antioxidant properties of plant extracts. In this assay, the reaction between antioxidant molecules and the stable free radical DPPH causes a discoloration of the solution, indicating the scavenging of free radicals. (Lavhale & Mishra, 2007)

It is evident that the flower of *Butea monosperma* possesses significant potential in treating a variety of ailments, including those influenced by ROS. Research studies have been conducted to evaluate the antioxidant potential of *Butea monosperma* and other plant extracts, shedding light on their ability to combat oxidative stress and associated disorders.

1.1.8. Phytochemicals Studies

Phytochemicals are natural compounds found in plants, encompassing both primary and secondary metabolites pathways. They are present in various parts of plants, including flowers, bark, fruits, seeds, leaves, and roots. The quantity and composition of phytochemicals vary among plant species, with bioactive compounds such as flavonoids, steroids, glycosides, alkaloids, and terpenoids being commonly identified. *Butea monosperma* flowers are particularly rich in chemical constituents, including triterpenes, butein, butin, isobutein, corospsin, isocorospsin, sulphuron monospermeoside (butein 3e glucoside), isomonospermoside, chalcones, aurones, flavonoids (such as plasasitrin and prunctin), and steroids.

The total flavonoid content in *Butea monosperma* flowers is estimated using aluminum chloride, while the total phenol content is quantified to assess the phenolic content in the plant. Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) are employed to determine the presence of flavonoids, phenols, alkaloids, and steroids/sterols based on their respective Rf values. These chromatographic techniques utilize

specific mobile phases and stationary phases according to established principles. Research conducted by Badgujar *et al*. (2018) provides valuable insights into the diverse array of phytochemicals present in *Butea monosperma* flowers, highlighting their potential therapeutic applications and contributing to our understanding of the plant's medicinal properties.

1.1.9. Significance of Synergistic Effect of *Butea monosperma* Flower Extract and Honey

The synergistic effect of combining *Butea monosperma* flower extract with honey has shown promising results in inhibiting the formation of calcium oxalate crystals, both in their aggregate and nucleate forms. This inhibition of crystal formation is crucial in reducing the risk of kidney stone formation, highlighting the therapeutic potential of these natural remedies (Raj *et al*., 2024).

While individual studies have demonstrated the effectiveness of *Butea monosperma* in addressing kidney-related ailments, the specific mechanisms underlying its action, particularly in combating inflammation associated with nephrolithiasis, require further investigation. Although *Butea monosperma* possess broader medicinal properties, such as wound healing, management of obesity-related complications, and antimicrobial and antioxidant activities, are well-established, more research is needed to elucidate its role in mitigating kidney stone formation (Phatak & Hendre, 2015).

The examination of the synergistic efficacy of *Butea monosperma* flower extract and honey is expected to reveal a dose-dependent reduction in calcium oxalate crystallization, highlighting the cooperative potential of this combination therapy. Additionally, in vitro anti-inflammatory tests on *Butea monosperma* are anticipated to demonstrate significant inhibition of protein denaturation, indicating its potential in alleviating inflammation associated with urolithiasis.

Furthermore, the assessment of antioxidant potency in *Butea monosperma* flower extract is likely to unveil significant antioxidative capabilities, suggesting its ability to counteract oxidative stress associated with urolithiasis. Preliminary phytochemical profiling of *Butea monosperma* flower extract using chromatography and spectroscopic techniques is expected to provide insights into the identification of bioactive compounds, laying the groundwork for further investigations into their therapeutic potential in nephrolithiasis (Bensatal *et al*., 2020).

1.2. Aims and Objective

The study aims to delve deeper into the potential of *Butea monosperma* in managing kidney stones. By investigating its synergistic efficacy with honey in reducing calcium oxalate crystallization, evaluating its anti-inflammatory attributes relevant to nephrolithiasis, assessing its antioxidant potency, and conducting a comprehensive phytochemical profiling, this research seeks to uncover the therapeutic mechanisms and compounds within *Butea monosperma* that could offer effective strategies for managing kidney stone formation. Such advancements may pave the way for novel, natural-based interventions in addressing kidney-related ailments, offering valuable alternatives or complementary approaches to conventional treatments.

<u>Objectives</u>

1. Examine the Synergistic Efficacy of *Butea monosperma* Lam (Flower) Extract in Conjunction with Honey on Mitigating Calcium Oxalate Crystallization.
2. Evaluate the dose-dependent influence of the combined *Butea monosperma* flower extract and honey regimen, discerning their cooperative potential in abating the formation of calcium oxalate crystals.

3. Evaluate the Anti-Inflammatory Propensity of *Butea monosperma* Flower Extract to investigate the anti-inflammatory attributes of *Butea monosperma* flower extract, elucidating its therapeutic efficacy in ameliorating the inflammatory processes.
4. Assess the Antioxidant Potency of *Butea monosperma* Flower Extract to counteract oxidative stress by scavenging free radicals, potentially pivotal in managing urolithiasis.
5. Conduct Phytochemical Profiling of *Butea monosperma* Flower Extract to characterize the phytochemical constituents within *Butea monosperma* flower extract.

1.3. Hypothesis

The study aims to explore the potential of *Butea monosperma* in managing kidney stones by investigating its synergistic efficacy with honey in reducing calcium oxalate crystallization, evaluating its anti-inflammatory attributes relevant to nephrolithiasis, assessing its antioxidant potency, and conducting phytochemical profiling. Hypotheses include anticipating a synergistic effect of *Butea monosperma* flower extract and honey in reducing crystallization, dose-dependent reduction in crystal formation, significant anti-inflammatory and antioxidant properties of the extract, and identification of bioactive compounds contributing to its therapeutic effects.

1.4. Scope

This dissertation will comprehensively investigate the therapeutic potential of *Butea monosperma* in managing kidney stones. The scope includes studying its synergistic efficacy with honey in reducing calcium oxalate crystallization, evaluating its anti-inflammatory attributes relevant to nephrolithiasis, assessing its antioxidant potency, and conducting phytochemical profiling. The research will involve in vitro and in vivo experiments,

including assays for crystallization inhibition, anti-inflammatory activity, antioxidant capacity, and chromatographic analysis for phytochemical characterization. The findings will contribute to understanding the mechanisms underlying therapeutic effects of *Butea monosperma* on kidney stones and may offer insights into developing natural-based interventions for kidney-related ailments.

CHAPTER 2: REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

Polina *et al*. (2020) conducted a comprehensive study examining various extracts (ethyl acetate, chloroform, and methanol) obtained from *Butea monosperma* flowers. They investigated the extracts for their antimicrobial, antioxidant, and anticancer properties. Results indicated that the ethyl acetate extract exhibited the most potent antimicrobial effects against a range of bacteria and fungi, along with superior antioxidant activity and significant cytotoxicity against specific cancer cell lines. The methanol extract showed prominent antifungal activity and notable cytotoxicity against other cancer cell lines. These findings underscore the potential therapeutic applications of *B. monosperma* flowers due to their rich phytochemical profile.

In a study by Farooq *et al*. (2020), the hydro-ethanolic leaf extracts of *B. monosperma* were subjected to investigation for their antioxidant potential and enzyme inhibition properties. The researchers identified the 60% ethanolic fraction as particularly promising, demonstrating elevated levels of phenolic and flavonoid compounds. This fraction also displayed remarkable antioxidant activity and exhibited significant inhibition of α-glucosidase and α-amylase enzymes, suggesting its potential in managing diabetes and oxidative stress-related disorders.

Golandaz *et al*. (2020) delved into the evaluation of *B. monosperma* flower extract's effect on obesity induced by a high-fat diet in rats. Their findings suggested that the supplemented flower extract effectively countered weight gain parameters induced by the high-fat diet. These results imply that *B. monosperma* could potentially serve as an herbal remedy for addressing obesity-related complications.

More *et al*. (2014) focused on exploring the thermal wound healing properties of a gel containing B. monosperma flower extract. Their study demonstrated accelerated wound closure rates compared to untreated controls. The application of the gel formulation, particularly at a concentration of 1.5%, exhibited significantly enhanced wound healing effects, comparable to standard treatments.

Badgujar *et al*. (2018) investigated the phytochemical composition, antioxidative potential, and antiproliferative effects of *B. monosperma* leaf methanol extract. Their findings indicated the substantial potential of this extract and its fractions in treating tumours. Further research is suggested to isolate and identify the active compounds responsible for these therapeutic effects. Similarly, Talubmook and Budhakala (2012) focused on examining the antioxidant and antidiabetic activities of the *B. monosperma* flower extract. The study revealed moderate antioxidant activity and suggested a positive impact on reducing blood glucose levels, highlighting its potential in managing diabetes-related complications. In another study, Latkal *et al*. (2020) discussed the therapeutic effects of honey, attributing its activities to compounds like flavonoids, phenolic compounds, and vitamins. They highlighted its antioxidant, antibacterial, and anti-inflammatory properties, confirming its therapeutic potential in various health conditions.

Jerald *et al*. (2009) quantified rutin and assessed the in vitro antioxidant potential of B. monosperma flower extracts prepared using different methods. Their study revealed significant antioxidant activity, particularly in specific extraction methods, establishing the extract's potential as a natural antioxidant agent. Furthermore, Pattanayak *et al*. (2017) introduced a green synthesis approach for silver nanoparticle production using *B. monosperma* bark extract. These biologically synthesized nanoparticles displayed potent

antibacterial activity against various human bacteria and exhibited significant cytotoxic effects on human myeloid leukemia cell lines.

Polachi *et al*. (2015) explored the inhibitory effect of the n-butanol fraction of *B. monosperma* floral extracts against specific cancer cells. Additionally, they conducted in silico evaluations of active compounds targeting specific protein complexes. These studies collectively signify the diverse pharmacological potential of *B. monosperma* across various therapeutic applications, warranting further exploration and pharmaceutical development.

Sutariya and Saraf (2015) conducted a review encompassing recent in vivo and in vitro studies that indicated a wide array of properties associated with *Butea monosperma*. The plant exhibited anti-diabetic, anti-cancer, anti-inflammatory, anti-asthmatic, antioxidant, anti-convulsant, antimicrobial, antiviral, and hepatoprotective properties. The aerial part of the plant was found to contain an extensive range of phytochemicals, predominantly consisting of flavonoids, lactones, diterpenoids, diterpene glycosides, and phytosterols. Their review comprehensively discussed the mechanisms, traditional and folk medicinal uses, as well as the remarkable biological activities associated with *Butea monosperma*.

Mehta *et al*. (2011) investigated *Butea monosperma* roots and flowers using methanol extraction. Through extensive chromatographic separation and purification, four phytochemicals were isolated and their structures determined using spectroscopic techniques. The crude extract was then tested for antibacterial activity against both gram-negative and gram-positive bacteria, such as *Bacillus megatarium*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, using MIC protocols. Antifungal activity was evaluated against the same strains, comparing the results with the standard drug nystatin.

Karakas *et al*. (2017) analysed 19 different extracts and two fractions obtained from various parts of common daisy using different solvents and extraction methods. The study investigated the biological activities of these extracts and fractions through a series of bioassays, including cytotoxicity, disc diffusion assay, radical scavenging activity (DPPH), total phenolic content, oxygen radical absorbing capacity (ORAC), and cell-based assays using 2',7'-dichlorofluorescindiacetate (DCFH-DA). The results supported the potential therapeutic use of B. perennis in treating conditions such as wounds, rheumatism, inflammation, cancer, and eye diseases.

Malpani *et al*. (2012) explored the characterization, phytochemical determination, and antimicrobial activity of *Butea monosperma*. The antimicrobial activity of water and methanol extracts was studied against pathogenic bacteria using the cup plate agar well diffusion method. Their findings suggested the potential of this plant as a source of phytochemicals with antimicrobial properties, indicating its potential role in traditional medicine. In a study by Sant *et al*. (2014), the anthelmintic potential of water extracts from *Butea monosperma* flowers was evaluated against adult earthworms (*Pheretima posthuma*). Both the aqueous and alcoholic extracts exhibited significant anthelmintic activity, comparable to the standard drug Piperazine citrate, when prepared in saline water.

Rana and Avijit (2015) provided an overview of the extensive biological activities associated with *Butea monosperma*. The plant has been traditionally recognized for its medicinal properties, including astringent, bitter, aphrodisiac, antibacterial, and anthelmintic properties. It yielded red juice known as 'Butea gum' or 'Bengal kino' and exhibited a wide range of pharmacological effects, including antidiabetic, antimicrobial, wound healing, and antifungal activities. The flowers yielded a yellow colouring matter due to the presence of chalcones.

This detailed exploration highlighted the diverse potential of this plant in providing therapeutic benefits across various ailments.

In a study by Maharjan *et al*. (2011), methanolic extracts from *Butea monosperma* seeds, along with extracts from *Cissampelos pareira*, *Myrica esculenta*, and *Selinum tenuifolium*, underwent qualitative phytochemical analysis and antimicrobial assays. Their findings suggested the potential applicability of these plants in managing microbial infections based onthe observed properties in their extracts.

Mahanthesh *et al*. (2020) focused on utilizing an *in-silico* ADME tool, Swiss ADME, to profile the pharmacological and pharmacognostic aspects of *Butea monosperma* Lam. Their study paved the way for further investigations, urging researchers to delve into *in vitro* and *in vivo* studies to unravel the pharmacological foundations of traditional medicinal plants like *Cardiospermum halicacabum* and *Butea monosperma*.

Sudharameshwari *et al*. (2018) aimed to evaluate the combined effects of extracts for antioxidant and antimicrobial activities. Their study highlighted the significant inhibitory action of aqueous extracts against various microorganisms. Notably, the synergistic combination of *C. halicacabum* and *B. monosperma* extracts exhibited considerable antimicrobial potential, particularly against multi-drug-resistant pathogens.

Rao *et al*. (2019) conducted research centered around pharmacognostical standardization, formulation, and assessment of tablets integrated with methanolic extract from the stem bark of *Butea monosperma*, specifically targeting anti-cancer activity. Their investigation

suggested moderate anti-cancer properties in the methanolic extract, indicating its potential as an alternative or complementary agent to synthetic anti-cancer drugs.

Additionally, Aryal *et al*. (2021) investigated the antiurolithiatic activity of selected plant extracts, including *Achyranthes aspera*, *Lawsonia inermis*, *Ficus benghalensis*, *Raphnus sativus*, and *Macrotyloma uniflorum*. Their analysis of methanol extracts from these plants demonstrated significant anti-urolithiasis activity against calcium oxalate (CaOX) monohydrate crystals, suggesting potential therapeutic applications in renal stone disease treatment.

Sharma *et al*. (2017) found that the DCM (Dichloromethane) fraction exhibited significantly greater inhibitory potential compared to other fractions in their study. They induced urolithiasis in rats using ethylene glycol in drinking water for 28 days and then examined the curative effects of the mother extract and DCM fraction. Rats treated with the mother extract at a dose of 185 mg/kg and the DCM fraction at 7 mg/kg showed a substantial decrease in serum and urine markers such as oxalate, calcium, creatinine, uric acid, and urea. Histological examination revealed fewer calcium oxalate deposits and minimal kidney damage in rats treated with the mother extract and DCM fraction, supporting the traditional claims of this treatment.

Mishra *et al*. (2022) aimed to assess the antilithiatic activity and antioxidant potential of the ethanolic extract of *Bauhinia variegata* leaves. Their study confirmed the antilithiatic potential by screening various secondary metabolites, particularly triterpenoids, within the ethanolic extract. They evaluated the calcium oxalate dissolving ability of the plant extract and assessed its capability to dissolve urinary calculi in vitro. Overall, their findings verified

the antilithiatic and antioxidant capacities of the ethanolic extract obtained from *Bauhinia variegata* leaves.

Bensatal *et al*. (2020) conducted studies exploring the effects of numerous plants on various diseases, including urinary stones. Their study aimed to evaluate the in vitro anti-lithiasis activity of the saponin-rich fraction obtained from *Ziziphus lotus*. Qualitative analysis of secondary metabolites in the leaves of *Z. lotus* revealed the presence of polyphenols, saponins, flavonoids, alkaloids, and tannins. The saponin-rich fraction exhibited an inhibitory effect on the nucleation of CaOx particles, with a 55.23 ± 1.23% inhibition rate. Additionally, the weight of CaOx crystals decreased with increasing saponin concentration, and at 100% concentration, the extract demonstrated a maximum dissolution percentage of 91.95 ± 0.72%. This effect was attributed to the compounds' ability to chelate Ca^{2+} within this fraction, showcasing its potential for managing urinary stone conditions.

Zarin *et al*. (2020) conducted a study on the invitro anti-urolithiasis activity of various extracts of Musa sp. The observations regarding anti-urolithiasis studies via nucleation assay, aggregation assay, and microscopic observation resulted in Musa sp. showing potential in invitro anti-urolithiasis studies, as different plant extracts of *Musa pseudostem* inhibited calcium oxalate crystals.

Phatak & Hendre (2015) explored the anti-urolithiatic activities of *Kalanchoe pinnata* leaves extract using different in-vitro models. They investigated the inhibitory effect of the extract on in vitro crystallization through nucleation and aggregation assays. As a result, Kalanchoe pinnata showed significant inhibition of calcium oxalate crystals. Niharika *et al*. (2018) conducted a study evaluating the in vitro anti-urolithiasis activity of Gossypium. Both

ethanolic and aqueous extracts of the plant showed dissolution of calcium oxalate crystals, indicating potential for future medicinal applications in kidney stone disease treatment.

Raj *et al*. (2024) investigated the anti-urolithiasis effects of a polyherbal formulation prepared from Spinacia oleracea L. and Coriandrum sativum L. seeds. Their study assessed the ability of these plant extracts to reduce calcium oxalate crystals through nucleation and aggregation assays, with further microscopic evaluation. Egg semi-permeable membrane cystone was used as a standard positive control.

In a study by Lavhale & Mishra (2007), different extracts of *Butea monosperma* were examined for their antioxidant properties using the *in vitro* DPPH assay. The evaluation of various plant extract concentrations revealed antioxidant properties, suggesting potential therapeutic applications.

Padmanadhan & Jangle (2012) investigated the in vitro anti-inflammatory activity of a herbal formulated drug prepared from 80% alcoholic extracts of *Aloe vera* leaves, *Bacopa monnieri*, *Moringa oleifera* and *Zingiber officinale* rhizome. Their study found dose-dependent inhibition of protein denaturation and RBC membrane stabilization by the drug, indicating its potential for anti-inflammatory treatment.

Zahra *et al*. (2024) conducted a study to investigate the anti-inflammatory activity of *Butea monosperma* flower extract. Using an induced model, they assessed proinflammatory markers and performed detailed computerized analysis. Their research aimed to identify the chemical compounds and bioactive constituents of the medicinal plant. Results from *in vivo* evaluations suggested that the plant extract could effectively modulate inflammation and

provide pain relief. Overall, the study demonstrated the anti-inflammatory activity of *Butea monosperma* flower extract.

CHAPTER 4: MATERIALS AND METHODOLOGY

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Collection of Plant Material and Honey

The flowers of *Butea monosperma* were meticulously gathered in the month of May 2023 from the Taleigao Plateau region of Panaji, Goa. Authentication of the plant species was conducted by a certified botanist. Honey was sourced from local apiaries located in Dharbandora, Khandepar, Ponda, Goa. The honey obtained from these regions was utilized for subsequent experiments and analyses.

3.2 Preparation of Plant Extract

The freshly plucked flowers were subjected to a thorough cleansing under running tap water and subsequently air-dried in natural sunlight. After drying, the flowers were carefully sealed in airtight packaging and stored at room temperature (37°C) until further use. Once adequately dried, the flowers were finely ground into a powder under shaded conditions. This powdered plant material was then utilized for extraction processes employing two distinct methodologies: maceration and Soxhlet extraction (**Plate 3.1**).

3.3. Maceration Method

10 grams of the powdered plant material were combined with 100 ml of selected solvent options (such as ethanol, methanol, or water) and subjected to agitation on a shaker for 48 hours. Subsequently, the resultant solution was meticulously filtered using filter paper to eliminate any solid particulates. The extracted solution was then stored in a cold room at 4°C for further analysis and experimentation (**Plate 3.1**).

Plate 3.1 Preparation of *Butea monosperma* extracts for experimentation a. Drying flower *Butea monosperma* b. Grinded powder of flower *Butea monosperma* c. Soxhlet extraction method d. Maceration method e. Plant extract (methanolic extract, ethanolic extract, aqueous extract) f. Power of *Butea monosperma* flower extract (methanolic extract, ethanolic extract and aqueous extract) by Rotary evaporator g. Honey

3.4. Soxhlet Extraction

10 grams of the finely powdered plant material were densely packed into a muslin cloth and securely placed within the Soxhlet apparatus. Extraction was carried out for a period of 6 hours using a suitable solvent (such as methanol, ethanol, or water). Following extraction, the resulting extracts were carefully stored in a cold room at 4°C to maintain their integrity. The extracts obtained were further processed to obtain the powdered form using a rotary evaporator, which was then stored in the cold room at 4°C for subsequent utilization (**Plate 3.1**).

3.5. Honey Standardization

The honey underwent standardization procedures employing phytochemical tests to ensure quality and consistency. A standardized mixture was prepared using a 1:1 (v/v) ratio of honey to distilled water, with 1 mL of honey mixed thoroughly with an equal volume of distilled water. This standardized honey solution was utilized for various experimental analyses and assays (**Plate 3.1**).

3.6 Phytochemical Studies

3.6.1. Detection of Alkaloids (Evans, 1997)

a. Mayer's Test: To a few mL of the filtrate, a drop or two of Mayer's reagent were added by the side of the test tube. The formation of a white or creamy precipitate indicated a positive test.

Mayer's Reagent: Mercuric chloride (1.358g) was dissolved in 60 mL of water, and potassium iodide (5.0g) was dissolved in 10 mL of water. The two solutions were mixed and made up to 100 mL with water.

b. Wagner's Test: To a few mL of the filtrate, a few drops of Wagner's reagent were added by the side of the test tube. The presence of a reddish-brown precipitate confirmed a positive test.

Wagner's Reagent: Iodine (1.27g) and potassium iodide (2g) were dissolved in 5 mL of water and made up to 100 mL with distilled water.

c. Dragendorff's Test: To a few drops of the filtrate, 1 or 2 mL of Dragendorff's reagent were added. A prominent yellow precipitate indicated a positive test.

Dragendorff's Reagent: Bismuth carbonate (5.2g) and sodium iodide (4g) were boiled for a few minutes with 50 mL of glacial acetic acid. After 12 hours, the precipitated sodium acetate crystals were filtered off, and the clear, dead brown filtrate (4 mL) was mixed with 160 mL of ethyl acetate and 1 mL of water. The working solution was prepared by mixing 10 mL of the stock solution with 20 mL of acetic acid and made up to 100 mL with water.

3.6.2. Detection of Carbohydrates and Glycosides (Ramakrishna *et al*., 1994)

A. Fehling's Test: Boiling 1 mL of the filtrate with 1 mL each of Fehling solution A and B on a water bath resulted in a red precipitate, indicating the presence of sugar.

B. Barfoed's Test: Upon heating 1 mL of the filtrate with 1 mL of Barfoed's reagent on a boiling water bath for 2 minutes, a red precipitate indicated the presence of sugar.

Barfoed's Reagent: Copper acetate (30.5g) was dissolved in 1.8 mL of glacial acetic acid.

C. Benedict's Test: Addition of 0.5 mL of the filtrate to 0.5 mL of Benedict's reagent, followed by heating on a boiling water bath for 2 minutes, led to the formation of characteristic colored precipitates, indicating the presence of sugar.

3.6.3. Detection of Glycosides

A. Borntrager's Test (Evans, 1997): The filtrate hydrolysate (2 mL) was mixed with 3 mL of chloroform, and after separation, 10% ammonia solution was added to the chloroform layer. The presence of a pink color indicated the presence of glycosides.

3.6.4. Detection of Proteins and Amino Acids

A. Biuret Test (Gahan, 1984): Upon heating a ligand of 2 mL of the filtrate with one drop of 2% copper sulfate solution and 1 mL of ethanol (95%), followed by excess potassium hydroxide pellets, a pink coloration in the ethanolic layer indicated the presence of proteins.

B. Ninhydrin Test (Yasuma and Ichickawa, 1953): Addition of two drops of Ninhydrin solution to 2 mL of the aqueous filtrate resulted in a characteristic purple coloration, indicating the presence of amino acids.

3.6.5. Detection of Phytosterols (Fine, 1986)

A. Libermann-Burchard's Test: Dissolving the extract (50 mg) in 2 mL of acetic anhydride and then slowly adding one or two drops of concentrated sulfuric acid along the sides of the test tube led to an array of color changes, indicating the presence of phytosterols.

B. Saponification Test: Addition of a few drops of 0.5 N alcoholic potassium hydroxide solution to a small quantity of extract, along with a drop of

phenolphthalein, followed by heating on a water bath for 2 hours. The formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

3.6.7. Detection of Phenolic Compounds and Tannins

A. Ferric Chloride Test: Dissolving the extract (50 mg) in 5 mL of distilled water and then adding a few drops of neutral 5% ferric chloride solution resulted in a dark green coloration, indicating the presence of phenolic compounds.

B. Lead Acetate Test: Dissolving the extract (50 mg) in distilled water and then adding 3 mL of 10% lead acetate solution led to the formation of a bulky white precipitate, indicating the presence of phenolic compounds.

C. Alkaline Reagent Test: Treating an aqueous solution of the extract with 10% ammonium hydroxide solution resulted in yellow fluorescence, indicating the presence of flavonoids.

3.7. Estimation of Total Phenol Content

The total phenol content of *Butea monosperma* flowers was quantitatively estimated using the Folin Ciocalteu reagent, with slight modifications based on Ainsworth's method. Gallic acid was used as the reference standard at different concentrations (100, 200, 400, 600, 800, and 1000 µg/mL) to plot the calibration curve. A plant extract (1 mg/mL) was mixed with 5 mL of Folin Ciocalteu reagent (5%) and incubated for 15 minutes in darkness. The reaction was then neutralized with 5 mL of 7.5% w/v sodium carbonate solution in darkness, followed by an additional 45-minute incubation in the dark. The absorbance reading was measured at OD 760 nm or 765 nm using a UV/visible spectrophotometer. The total phenol content was estimated using the linear equation of the calibration curve obtained from gallic acid. The

total phenolic compound content in the plant extract was expressed as mg/g gallic acid equivalent (GAE) of dry extract.

The total phenol content was calculated using the formula:

$$TPC = C \times V \, M \quad TPC = MC \times V$$

Where:

- C = concentration of gallic acid
- V = volume of plant extract in mL
- M = dry weight of plant powder in grams

3.8. Determination of Total Flavonoid Content

The total flavonoid content was estimated using the Aluminium chloride colorimetric assay as described by Chen et al. (2002). The reaction mixture consisted of 1 mg/mL of plant extract and 4 mL of distilled water. To this mixture, 0.3 mL of 5% sodium nitrite solution was added and incubated for 5 minutes. Subsequently, 0.3 mL of 10% aluminium chloride was added and kept for an additional 5 minutes. Finally, 2 mL of 2M sodium hydroxide was added, and the total volume was made up to 10 mL. The absorbance reading was measured at OD 510 nm using a UV/visible spectrophotometer. Quercetin was used as the standard at varying concentrations (20, 40, 60, 80, and 100 µg/mL), and the linear equation obtained from the calibration curve was used for quantification.

The total flavonoid content was calculated using the formula:

$$TFC = C \times V \, M \quad TFC = MC \times V$$

Where:

- C = concentration of quercetin
- V = volume of plant extract in mL
- M = dry weight of plant powder in grams

3.9. Thin Layer Chromatography

In Thin Layer Chromatography (TLC), the TLC plates were marked with dimensions (10×10) and spots were marked from one end at 1.5 cm and another end for the solvent running spot at 1 cm. The plates were loaded with spots depending on the compounds being tested.

3.9.1. Test for Alkaloids

Four spots of plant extract were loaded onto the TLC plate. The plate was then developed in a solvent system consisting of Toluene: Ethyl acetate: Methanol: 25% ammonia in the ratio (30:30:15:1) v/v/v/v. After development, the plate was quickly dipped in Dragendorff's reagent for 3-5 seconds. Orange bands indicative of alkaloids were observed along the horizontal line, and photographs were taken under visible light, long UV, and short UV.

3.9.2. Test for Flavonoids

Similar to the alkaloids test, four spots of plant extract were loaded onto the TLC plate and developed in a solvent system consisting of Ethyl acetate: Formic acid: Glacial acetic acid: Water in the ratio (10:0.5:0.5:3) v/v/v/v. After development, the plate was quickly dipped in 10% methanolic sulphuric acid for 3-5 seconds and then heated for 10 minutes. Red fluorescence indicative of flavonoids was observed along the horizontal line, and photographs were taken under visible light, long UV, and short UV.

3.9.3. Test for Sterols

Again, four spots of plant extract were loaded onto the TLC plate and developed in a solvent system consisting of chloroform: ethyl acetate (4:6) v/v. After development, the plate was quickly dipped in Anisealdehyde-sulphuric acid reagent for 3-5 seconds and then heated for

10 minutes. Purple maroon bands indicative of sterols were observed along the horizontal line, and photographs were taken under visible light, long UV, and short UV.

3.9.4. Test for Steroids

Similar to the sterols test, four spots of plant extract were loaded onto the TLC plate and developed in a solvent system consisting of n-butanol: methanol: water v/v/v. After development, the plate was quickly dipped in Anisealdehyde-sulphuric acid reagent for 3-5 seconds and then heated for 10 minutes. Purple maroon bands indicative of steroids were observed along the horizontal line, and photographs were taken under visible light, long UV, and short UV.

3.9.5. Test for Phenols

Four spots of plant extract were loaded onto the TLC plate and developed in a solvent system consisting of Cyclohexane: ethyl acetate: formic acid in the ratio (4:6:1). After development, the plate was quickly dipped in alcoholic ferric chloride for 3-5 seconds and then heated for 10 minutes. Dark blue zones indicative of phenols were observed along the horizontal line, and photographs were taken under visible light, long UV, and short UV.

3.10. HPTLC (High Performance Thin Layer Chromatography)

3.10.1. Test for Phenols

Three sample spots, including a standard (gallic acid), methanolic extract, and methanolic honey extract, were loaded onto the HPTLC plate. The plate was developed in a solvent system consisting of Tetrahydrofuran: toluene: formic acid: distilled water (16:8:2:1). After development, the plate was quickly dipped for 3-5 seconds in alcoholic ferric chloride. Dark

blue bands indicative of phenols were observed, and photographs were taken under visible light, long UV, and short UV.

3.10.2. Test for Flavonoids

Similar to the phenols test, sample spots were loaded onto the HPTLC plate, including a standard (quercetin), methanolic extract, and methanolic honey extract. The plate was developed in a solvent system consisting of Tetrahydrofuran: toluene: formic acid: distilled water (16:8:2:1) v/v/v/v. After development, the plate was quickly dipped for 3-5 seconds in 10% methanolic sulphuric acid. Fluorescent bands indicative of flavonoids were observed, and photographs were taken under visible light, long UV, and short UV.

3.11. Anti-Inflammatory Assay

3.11.1 Proteinase Inhibitory Assay

A proteinase inhibitory assay was performed using 0.06 mg trypsin and 1 ml of 20 mM Tris HCl buffer (pH 7.4), to which 0.02 ml of the extract sample and 0.980 ml of solvent (ethanol, methanol, or water) were added. The mixture was incubated at room temperature for 5 mins before adding 0.8% (v/v) Casein. After a further incubation period of 20 mins, the reaction was terminated by adding 70% perchloric acid. Following centrifugation, the absorbance of the supernatant was measured using a spectrophotometer at 210 nm against a phosphate buffer as a blank, which served as the control. The standard drug DMfo was used at varying concentrations (100, 200, 300, etc.), and the same protocol was followed. The percentage of protein denaturation was calculated using the formula:

% Inhibition of protein denaturation = (1 - A2/A1) × 100 Where: A1 = Absorbance at control A2 = Absorbance at extract

3.12. Antioxidant Studies

3.12.1 Determination of Antioxidant Activity By DPPH-Scavenging Assay

The antioxidant activity of the flower extract *Butea monosperma* was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. 4 mg of DPPH was diluted in 100 ml of methanol (4% w/v), and different concentrations (20, 40, 60, 80, and 100 µg/ml) of standard or extract solutions were prepared. The mixtures were then incubated at room temperature in the dark, and the decrease in absorbance was measured at OD 510 nm. Ascorbic acid was used as the positive control for plotting the standard graph. The percentage of free radical scavenging activity was calculated using the formula: % Free Radical Scavenging Activity = (Ae/Ac) / Ac × 100 Where: Ac = Absorbance at control Ae = Absorbance at extract. The concentration of the extract required for 50% radical scavenging activity (IC50) was calculated from the standard curve obtained from the respective concentrations.

3.13. Anti-urolithiasis Studies

3.13.1. Preparation of Calcium Oxalate Crystals by Homogeneous Precipitation Method

Calcium chloride dihydrate (4.41 g) was dissolved in distilled water, and sodium oxalate (4.02 g) dissolved in 2 N sulphuric acid was added separately to two beakers. The solutions were mixed to precipitate calcium oxalate crystals until excess sulphuric acid was removed. The resulting solution was washed with ammonia solution and distilled water, then dried in a hot air oven at 60°C for 4 hours.

3.13.2. Microscopic Observation under SEM

In this study, the surface morphology of calcium oxalate crystals was meticulously investigated utilizing a powerful tool known as a scanning electron microscope (SEM). SEM allows for high-resolution imaging of specimens by scanning them with a focused beam of electrons. This technique provides detailed insights into the structure, texture, and surface features of the materials under examination.

The calcium oxalate crystals, whose formation and characteristics were studied in previous sections, were subjected to SEM analysis to gain a deeper understanding of their microstructure. By magnifying the crystals several thousand times, SEM enabled researchers to visualize their surface morphology with remarkable clarity and detail.

3.13.3. Preparation of Semi-Permeable Membrane from Egg Shell

Egg shells were punctured at the apex with a glass rod, emptied, and thoroughly washed with distilled water. The shells were then decalcified in 2M HCl overnight. After decalcification, the membrane was washed with distilled water, treated with ammonia solution to remove traces of sulphuric acid, rinsed again with distilled water, and stored in a refrigerator at pH 7.4-7.5.

3.13.4. Evaluation of Anti-urolithiasis Activity by Titrimetric Method

Semi-permeable membranes prepared from egg shells were combined with 5 mg of calcium oxalate crystals and 1 mg/ml concentration of plant methanolic extract with honey (1:1 v/v) in a conical flask containing 0.1M Tris buffer. The flask was incubated at 37°C for two days. The contents were then transferred to separate test tubes, and 2 ml of 1N sulphuric acid was added. The resulting solution was titrated against 0.9494 standard $KMnO_4$ solution until a light pink colour was obtained. Triplicate readings were taken for accurate results, and the dissolution percentage was calculated.

3.13.5. Aggregation Assay

Calcium oxalate crystals in solution tend to aggregate, forming clusters. The inhibition of these aggregates by the plant extract was evaluated following the method described by Bewari *et al*. (2018). Calcium chloride and sodium oxalate (both at 50 mol/L) were mixed and heated to 60°C in a water bath overnight to produce CaOx crystal seeds. The solution was then centrifuged and evaporated at room temperature. The final concentration of CaOx crystals was prepared as 0.8 mg/mL and added to Tris buffer (0.05 mol/L Tris and 0.15 mol/L NaCl) at pH 6.5. Different concentrations of methanolic extract with honey (in a 1:1 ratio v/v) were added, and absorbance was measured at 620 nm at time intervals of 0, 30, 60, 90, and 120 mins. The percentage inhibition of calcium oxalate crystals was calculated using the formula:

$$\% \text{ Inhibition of calcium oxalate crystals} = [(\text{OD control} - \text{OD extract}) / \text{OD control}] \times 100$$

3.13.6. Nucleation Assay

The nucleation assay was conducted according to the method described by Hennequin et al. (1993) with some modifications. Sodium oxalate (3 mmol/L) and calcium chloride (0.5 mmol/L) were prepared in a buffer containing Tris (0.05 mmol/L) and NaCl (0.15 mmol/L) at pH 5.5. Calcium chloride (1.9 mL) was mixed with different concentrations (100, 200, 400, 600, 800, 1000 µg/ml) of the plant extract at 37°C, followed by the addition of sodium oxalate (1.9 ml). Spectrophotometric absorbance readings were measured at 0, 30, 60, 90, and

120 mins. The percentage inhibition of calcium oxalate crystals was calculated using the formula:

% Inhibition of calcium oxalate crystals = [(OD control - OD extract) / OD control] × 100

3.13.7. Microscopic Observation

Microscopic observation of calcium oxalate crystals using compound microscope was performed at 40x magnification using samples obtained from the nucleation assay. Samples containing different concentrations (negative control, positive control, 100, 200, 400, 600, 800, and 1000 µg/ml) of flower extract were examined under the microscope.

CHAPTER 4: RESULTS AND DISCUSSIONS

CHAPTER 4: RESULT AND DISCUSSION

4.1. Morphology and Utilization of *Butea Monosperma* as herbal medicine

In the morphology examination of *Butea monosperma*, particular attention was given to its flowers, which play a crucial role in traditional medicinal practices, especially in the treatment of kidney stones. *Butea monosperma*, commonly known as the flame-of-the-forest, is a deciduous tree renowned for its striking flowers, bark, seeds, and pods. The tree typically exhibits a medium to large-sized habit, boasting a spreading crown and reaching heights of 15 to 25 meters. Its bark is characterized by its rough, grayish-brown appearance, deeply furrowed to provide protection to the inner layers. The seeds, encapsulated within elongated, cylindrical pods, possess a woody texture and turn brown upon maturation. However, it is the vibrant red or orange flowers of *Butea monosperma* that hold significant medicinal value, particularly when used in combination with honey. Local practitioners in remote areas of Goa have long utilized the powdered form of *Butea monosperma* flowers, mixed with honey, to prepare traditional remedies for kidney stones. This practice emphasizes the indigenous knowledge and reliance on natural remedies within these communities, emphasizing the therapeutic potential of the plant's flowers when combined with honey for medicinal purposes (**Plate 4.1**).

4.1. Preliminary Phytochemical tests

Based on the preliminary phytochemical tests conducted on the flower extract of *Butea monosperma* and honey, several phytoconstituents were identified (**Plate 4.2, Table 4.1**). Alkaloids were detected in both honey and all extracts of *Butea monosperma*, as indicated by positive results in Mayer’s test and Wagner’s test. This suggests the presence of nitrogen-containing compounds with potential pharmacological activity.

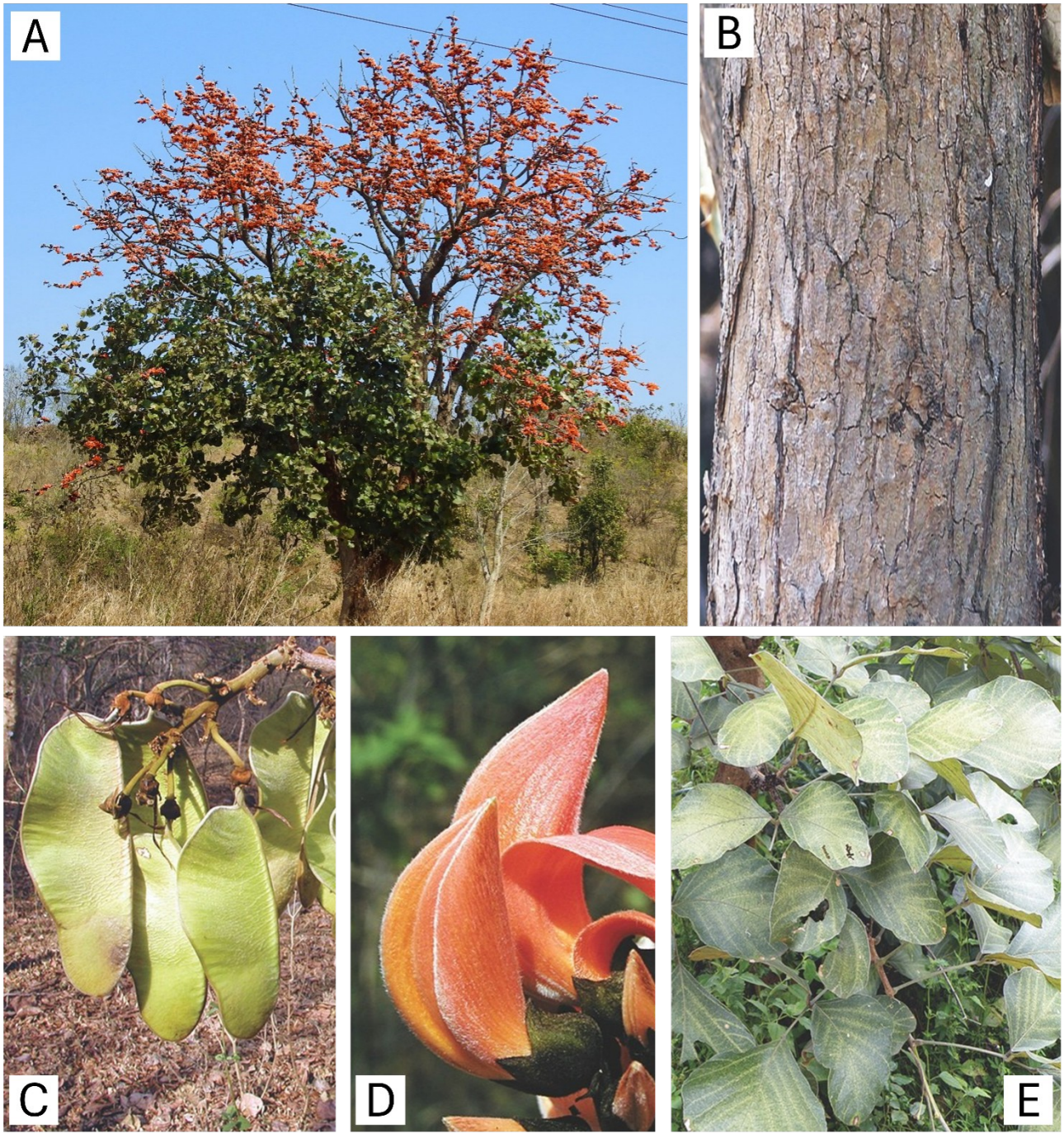


Plate 4.1: ***Butea monosperma tree*** **(A) Habit (B) Bark (C) Pods (D) Flower (E) Leaves**

Carbohydrates were found to be present in honey and all extracts, as evidenced by positive results in Barford, Fehling's, and Benedict's tests. This indicates the presence of sugars and related compounds in the samples. Glycosides were detected in honey and all extracts, as demonstrated by positive results in Borntrager's test. This suggests the presence of glycoside compounds that may have therapeutic effects.

Proteins and amino acids were identified in honey and all extracts, as indicated by positive results in the Biuret and Ninhydrin tests. This suggests the presence of proteinaceous compounds and free amino acids in the samples. Phenolic compounds were found in honey and all extracts, as shown by positive results in the Ferric chloride, Lead acetate, and Alkaline reagent tests. This indicates the presence of phenolic compounds, which are known for their antioxidant and anti-inflammatory properties. Fixed oils and fats were detected in honey and all extracts, as evidenced by positive results in the Saponification test. This suggests the presence of lipids in the samples, which may contribute to their therapeutic properties.

Overall, the presence of these phytoconstituents in both *Butea monosperma* flower extract and honey indicates their potential medicinal value, supporting their traditional use in the treatment of various ailments, including kidney stones. The combination of these phytoconstituents in honey with those from *Butea monosperma* flowers may synergistically enhance their therapeutic effects, making them promising candidates for further study and development into herbal remedies.

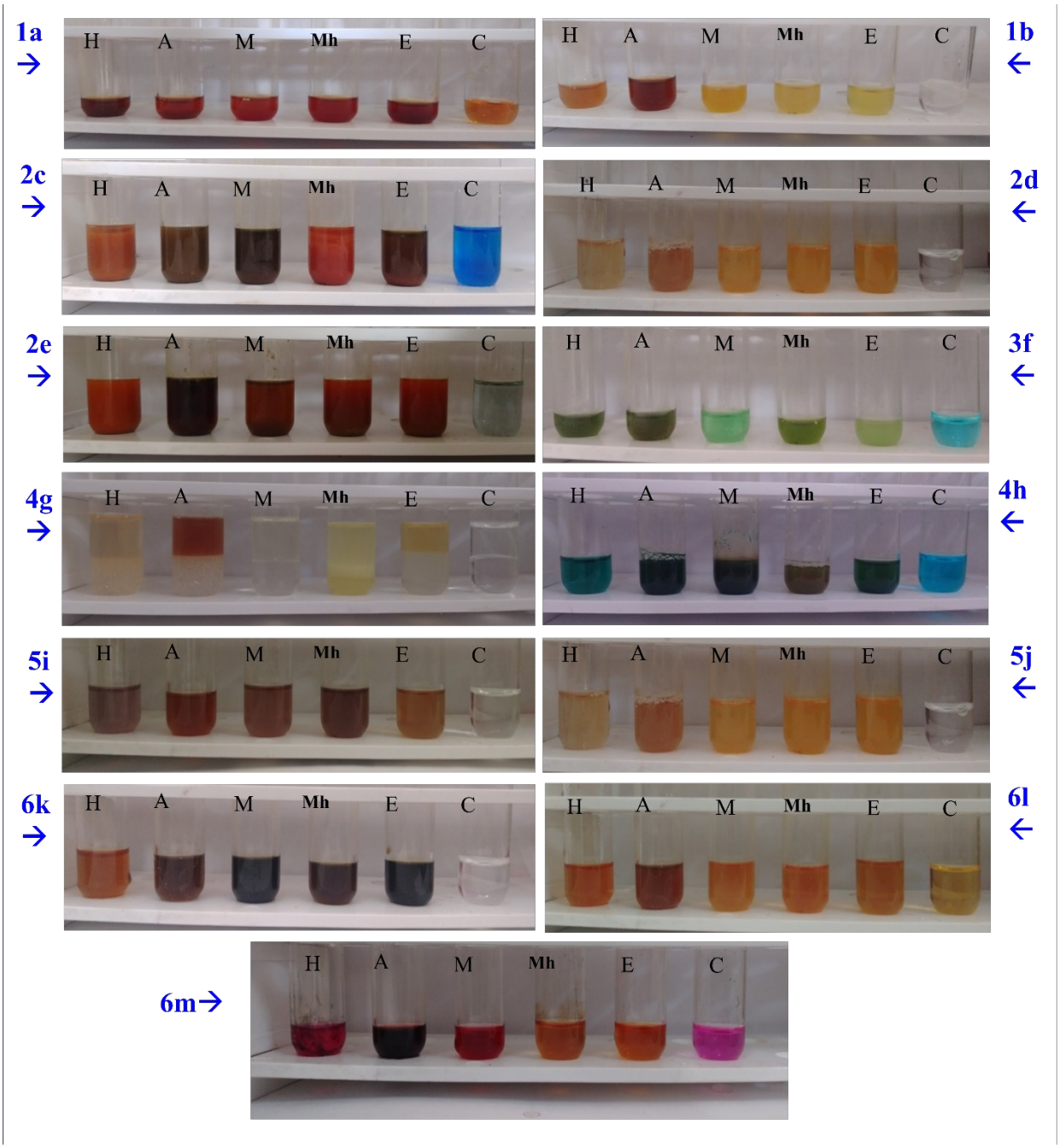


Plate 4.2. Phytochemical tests; 1) Test for alkaloids a. Mayer's test b. Wagner's test 2) Test for Carbohydrates c. Fehling's test d. Borford's test e. benedict test 3) Test for Glycosides f. Borntrager's tests 4. Test for protein g. Biuret test h. Nindyrin test 5. Test for Phytosterols i. Libermann- Burchard's test j. Sponification test 6. Test for phenol and tannins k. Ferric Chloride test l. Lead acetate test m. Alkaline reagent test.

Table no 4.1. Preliminary Phytochemical tests of *Butea monosperma* flower extract with and without honey

Phytochemical tests	Honey	Aqueous Extract	Methanolic (MeOH) extract	EtOH Extract	Aqueous+ Honey Extract
Test for Alkaloids					
1. Mayer's test	+	+	+	+	+
2. Wagner's test	+	+	+	+	+
Test for Carbohydrates					
1. Barford test	+	+	+	+	+
2. Fehling's test	+	+	+	+	+
3. Benedict's test	+	+	+	+	+
Test for Glycoside					
Borntrager's test	+	+	+	+	+
Test for Protein and Amino acids					
1. Biuret test	+	+	+	+	+
2. Ninhydrin test	+	+	+	+	+
Test for Phenols					
1. Ferric chloride test	+	+	+	+	+
2. **Lead acetate test**	+	+	+	+	+
3. **Alkaline reagent test**	+	+	+	+	+
Test for fixed Oil and Fats					
Saponification test	+	+	+	+	+

4.2. Determination of Total Phenol Content (TPC)

The estimation of total phenol content in different extracts of *Butea monosperma* flower, including aqueous, ethanolic, methanolic, and aqueous honey extracts, was conducted based on the absorbance readings at 760 nm and 765 nm. Standard gallic acid solutions at various concentrations were used to generate a calibration curve (**Figure 4.1**). The absorbance readings obtained from the standard solutions were as follows: at a concentration of 100 µg/ml, the absorbance was 1.04; at 200 µg/ml, it was 1.84; at 300 µg/ml, it was 2.67; at 500 µg/ml, it was 3.52; at 600 µg/ml, it was 4; and at 700 µg/ml, it remained at 4 (**Table 4.2; Plate 4.3**).

Using the calibration curve, the total phenol content in each extract of *Butea monosperma* flower was determined. The aqueous extract exhibited a total phenol content of 133 mg GAE/g of dry weight plant extract, while the ethanolic extract showed a higher content of 209.8 mg GAE/g. The methanolic extract demonstrated an even higher total phenol content of 252.2 mg GAE/g. Remarkably, the aqueous honey extract exhibited the highest total phenol content among all extracts, measuring at 536.2 mg GAE/g (**Table 4.3; Figure 4.3**).

These results suggest that the extraction method and the addition of honey significantly influence the total phenol content in *Butea monosperma* flower extracts. The higher phenolic content observed in the aqueous honey extract indicates the potential synergistic effects of honey in enhancing the phenolic content of the extract. Overall, these findings underscore the potential health benefits of *Butea monosperma* flower extracts, particularly when combined with honey, in providing antioxidant and therapeutic effects.

Table 4.2. Estimation of Total Phenol content (Standard Gallic acid at different concentration on absorbance at 760 nm ad 765 nm)

Concentration (µg/ml)	Absorbance at 760 and 765 nm
100	1.04
200	1.84
300	2.67
500	3.52
600	4
700	4

Figure 4.1: Standard Calibration curve using Gallic acid for Total Phenol content estimation

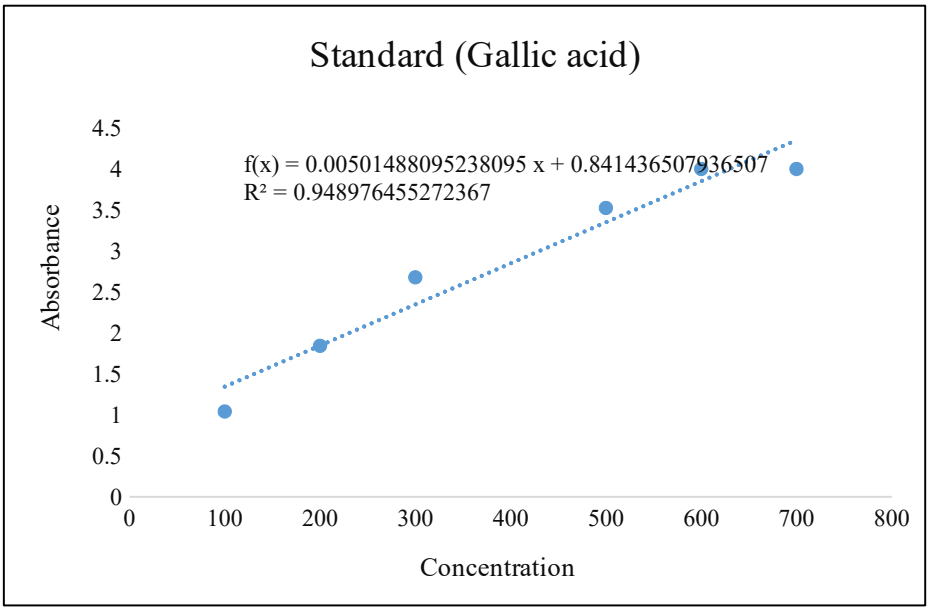


Table 4.3. Table showing Estimation of Total Phenol Content with different extract of flower *Butea monosperma* (Aqueous Extract, Ethanolic extract, methanolic extract and methanolic Honey extract on absorbance at OD 760 and 765 nm.

Concentration (µg/ml)	Total phenols (mg GAE/g of dry weight plant extract

Aqueous extract	133
Ethanolic extract	209.8
Methanolic extract	252.2
Aqueous honey extract	536.2

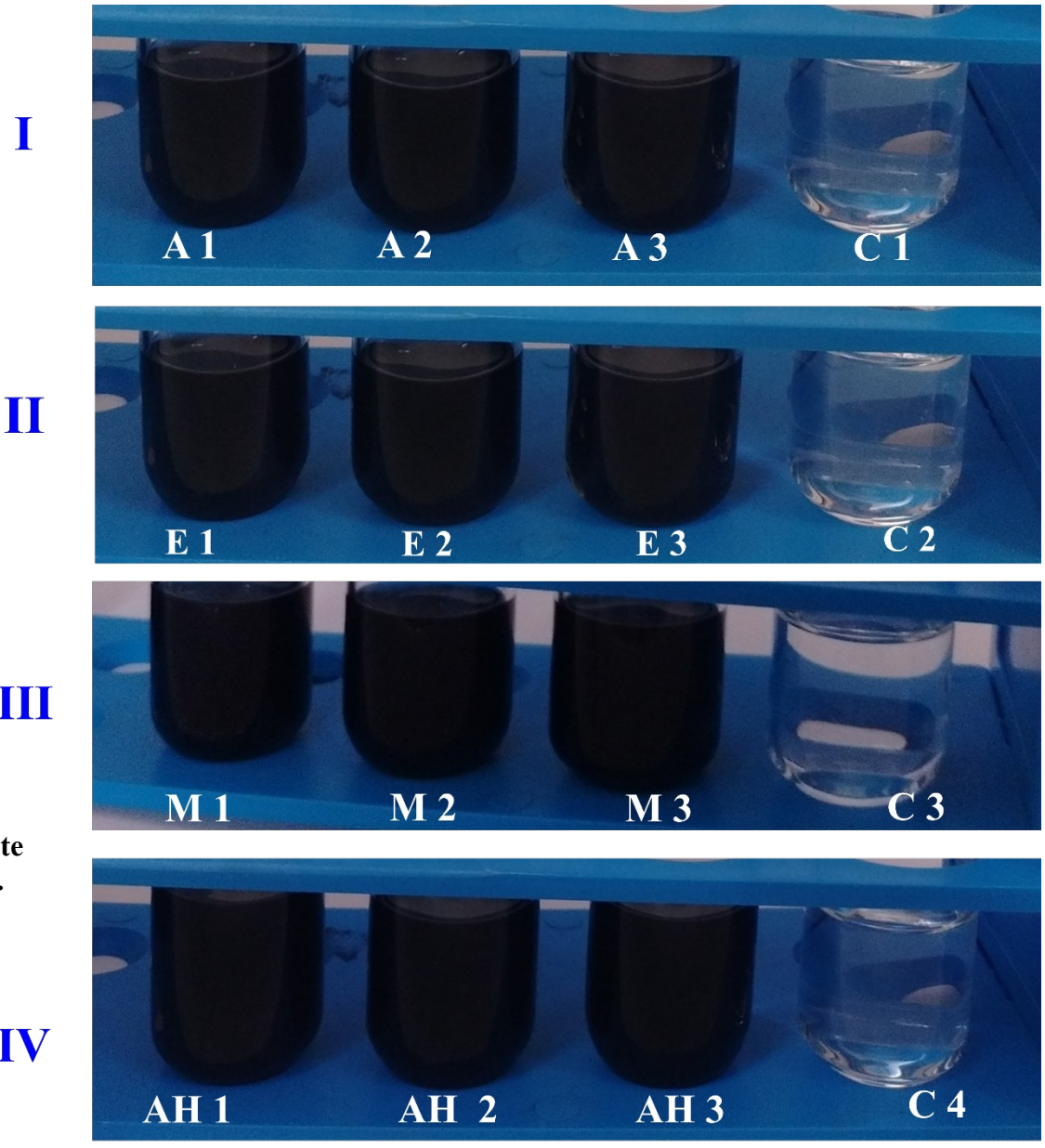
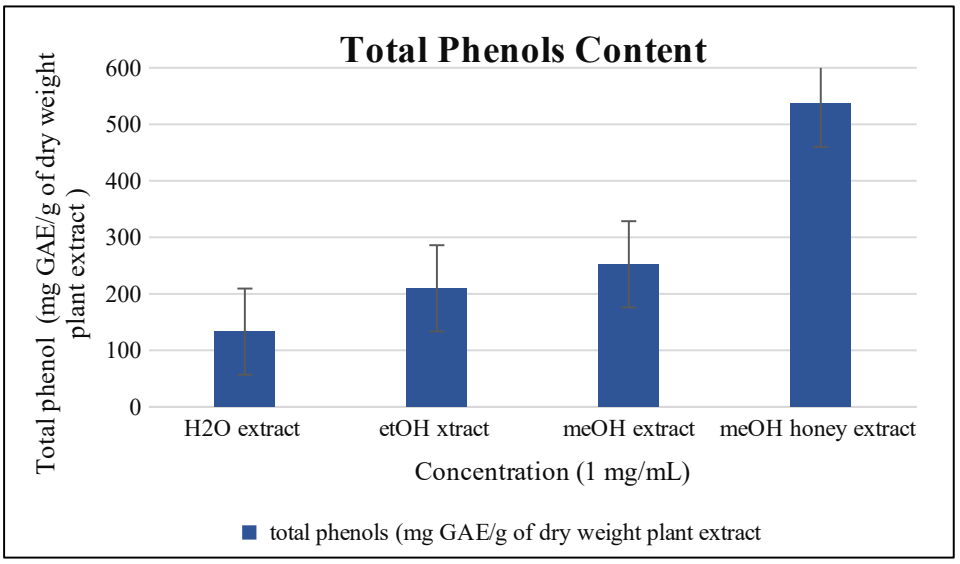


Plate 4.3.

Determination of Phenols; S) Standard Gallic acid I) Aqueous extract II) EtOH extract III) MeOH extract IV) Aqueous honey extract

Figure 4.2: Graph of Estimation of Total Phenol Content with different extract of flower *Butea monosperma* (Aqueous Extract, Ethanolic extract, methanolic extract and methanolic Honey extract on absorbance at OD 760 and 765 nm.



4.3. Total Flavonoid content

The determination of total flavonoid content in various extracts of *Butea monosperma* flower was conducted using a calibration curve generated from standard quercetin solutions at different concentrations and their corresponding absorbance readings at 510 nm (**Plate 4.4**). The absorbance readings obtained from the standard solutions were as follows: at a concentration of 100 µg/ml, the absorbance was 0.065; at 200 µg/ml, it was 0.123; at 400 µg/ml, it was 0.211; at 600 µg/ml, it was 0.296; at 800 µg/ml, it was 0.301; and at 1000 µg/ml, it was 0.328 (**Table 4.4; Table 4.5; Figure 4.2 and Figure 4.4**).

Using this calibration curve, the total flavonoid content in each extract of *Butea monosperma* flower was determined. The results indicated a similar trend to that observed for total phenol content, with the methanolic honey extract exhibiting the highest flavonoid content among all

extracts. These findings suggest that the addition of honey to the extraction process may enhance the flavonoid content of the extract, highlighting the potential synergistic effects of honey in augmenting the therapeutic properties of *Butea monosperma* flower extracts.

Table No 4.4: Determination of Total Flavonoids content (Standard Quercetin at different concentration on absorbance at 510 nm)

Standard Quercertin Concentration (µg/ml)	Absorbance at 510 nm
100	0.065
200	0.123
400	0.211
600	0.296
800	0.301
1000	0.328

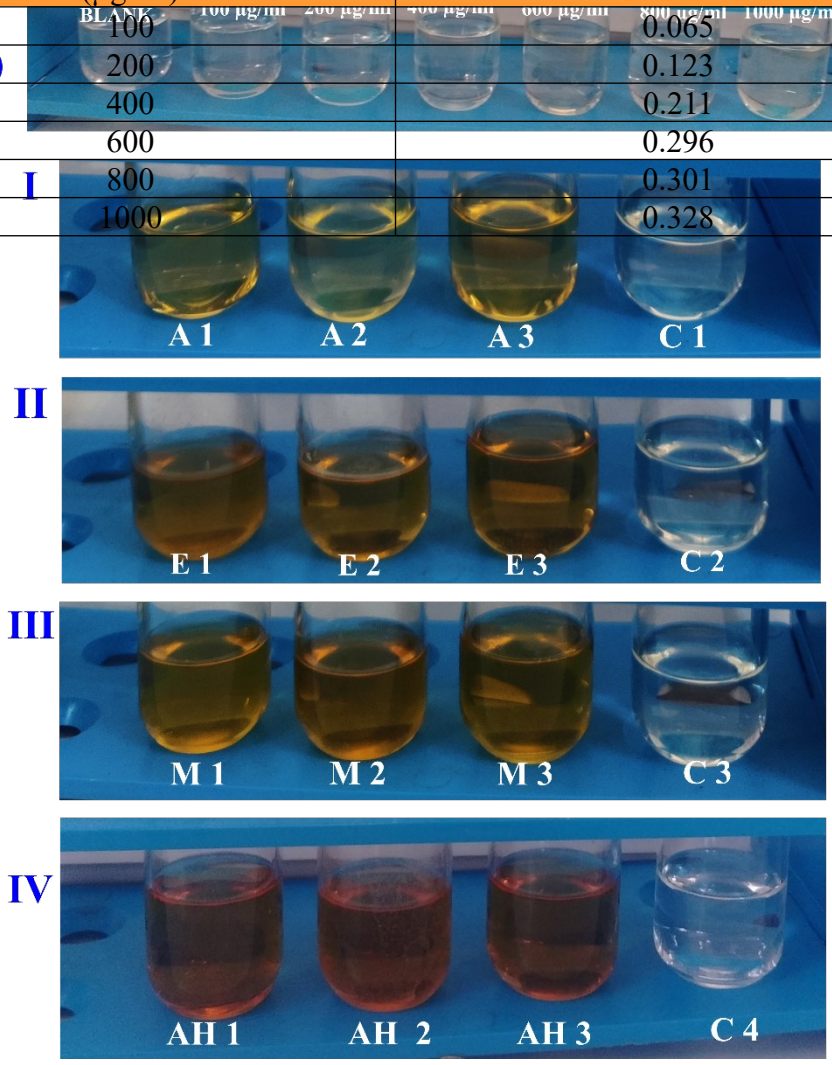


Plate 4.4. Determination of flavonoids; S) Standard Quercetin I) Aqueous extract II) EtOH extract III) MeOH extract IV) MeOH honey extract

Figure 4.3: Determination of Total Flavonoids; Calibration curve using Quercetin

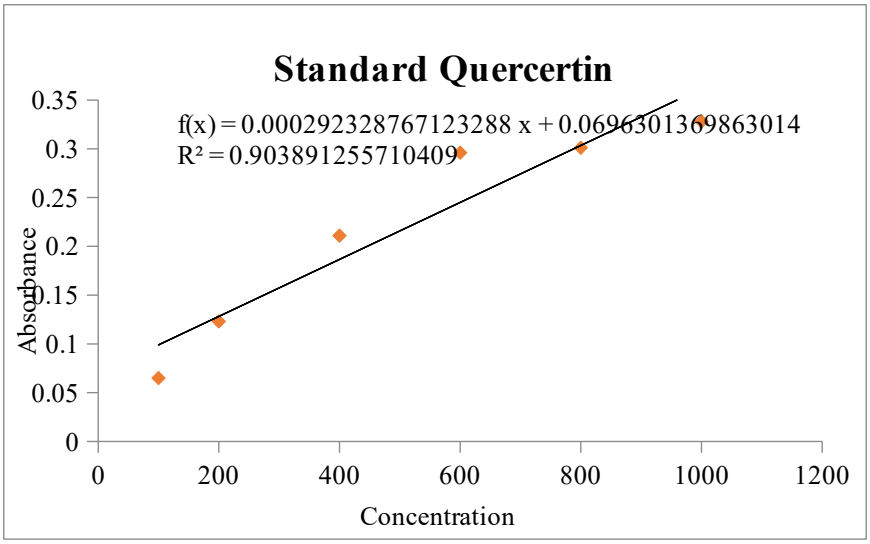
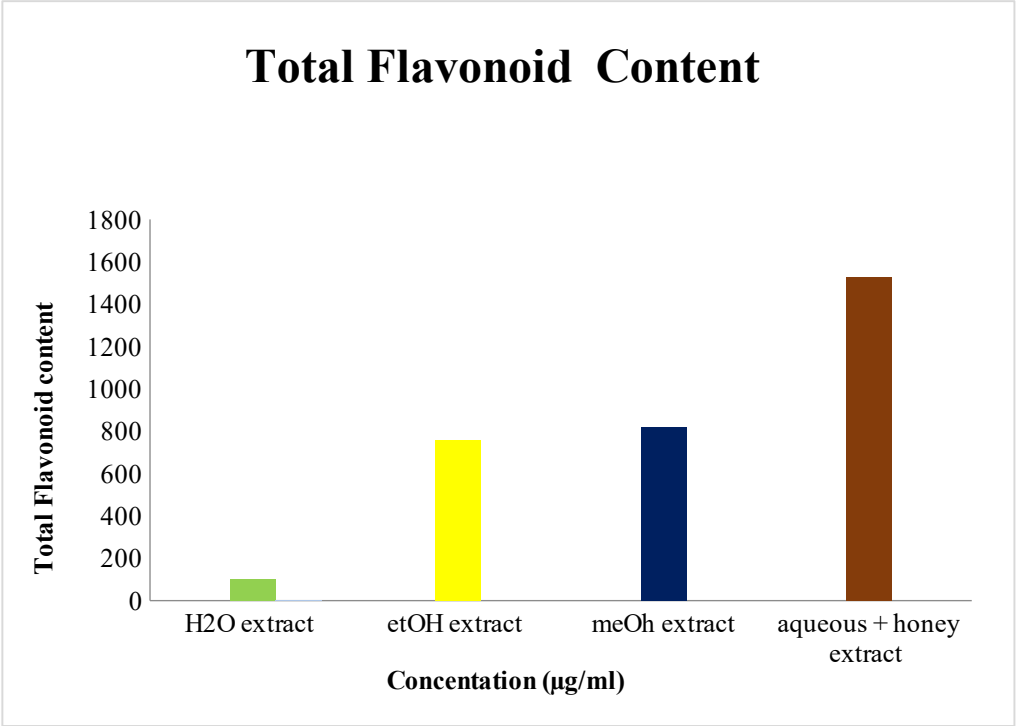


Table 4.5. Table showing Estimation of Total Flavonoid Content with different extract of flower *butea monosperma* (Aqueous Extract, Ethanolic extract, methanolic extract and methanolic Honey extract on absorbance at OD 510 nm.

Concentration Of Plant extract (µg/ml)	Total Flavonoid content (mg QE/g dry weight)
Aqueous extract	99.33
Ethanolic extract	756
Methanolic extract	821
Aqueous honey extract	1.528

Figure 4.4: Graph of Estimation of Total Flavanoid Content with different extract of flower *Butea monosperma* (Aqueous Extract, Ethanolic extract, methanolic extract and methanolic Honey extract on absorbance at OD 510 nm.



4.4. TLC and HPTLC analysis

The thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) analyses were conducted to identify the presence of phytoconstituents in different extracts of *Butea monosperma*. In the TLC analysis, the Rf values indicated the presence of various compounds, including flavonoids (Rf 0.99), alkaloids (Rf 0.56), phenols (Rf 0.81), steroids (Rf 0.88), and sterols (Rf 0.55). Notably, all extracts, including the ethanolic, methanolic, and aqueous extracts, as well as the aqueous extract with honey, exhibited positive results for the presence of alkaloids, flavonoids, steroids, sterols, and phenols (**Plate 4.5 to Plate 4.9; Table 4.6**).

Furthermore, the HPTLC analysis provided additional insights into the composition of the extracts (**Plate 4.10; Table 4.7**). The Rf values obtained for phenols were 0.77 for the methanolic extract and 0.78 for the aqueous extract with honey, while for flavonoids, the Rf

values were 0.99 for the methanolic extract and 0.98 for the aqueous extract with honey. These results further confirm the presence of phenolic compounds and flavonoids in the extracts.

Overall, the TLC and HPTLC analyses demonstrated the presence of a diverse range of phytoconstituents in the *Butea monosperma* extracts, highlighting their potential therapeutic properties. These findings support the traditional use of *Butea monosperma* in treating various diseases and ailments, emphasizing its significance in traditional medicine systems. Further studies focusing on isolating and characterizing these phytoconstituents could provide valuable insights into the pharmacological activities and potential therapeutic applications of *Butea monosperma*.

Table 4.6. Thin layer chromatography; Test for phenol, flavonoid alkaloids steroids and sterols

	Ethanolic extract	Methanolic extract	Methanolic honey extract	Aqueous extract
Test for Alkaloids (Rf 0.56)	+	+	+	-
Test for Flavonoid (Rf 0.99)	+	+	+	+
Test for Steroids (Rf 0.88)	+	+	+	+
Test for Sterols (Rf 0. 55)	+	+	+	+
Test for Phenols (Rf 0.81)	+	+	+	+

Table 4.7. HPTLC analysis for Flavonoids and Phenols

Plant extract	Test for phenol Rf	Test for flavonoid Rf
Methanolic extract	**0.77**	**0.98**
Methanolic honey extract	**0.87**	**0.9**

Plate 4.5. TLC analysis for Alkaloids (Lane 1: ethanol extract; Lane 2: Methanol extract, Lane 3: Aqueous extract with Honey and 4: Aqueous extract of *Butea monosperma*)

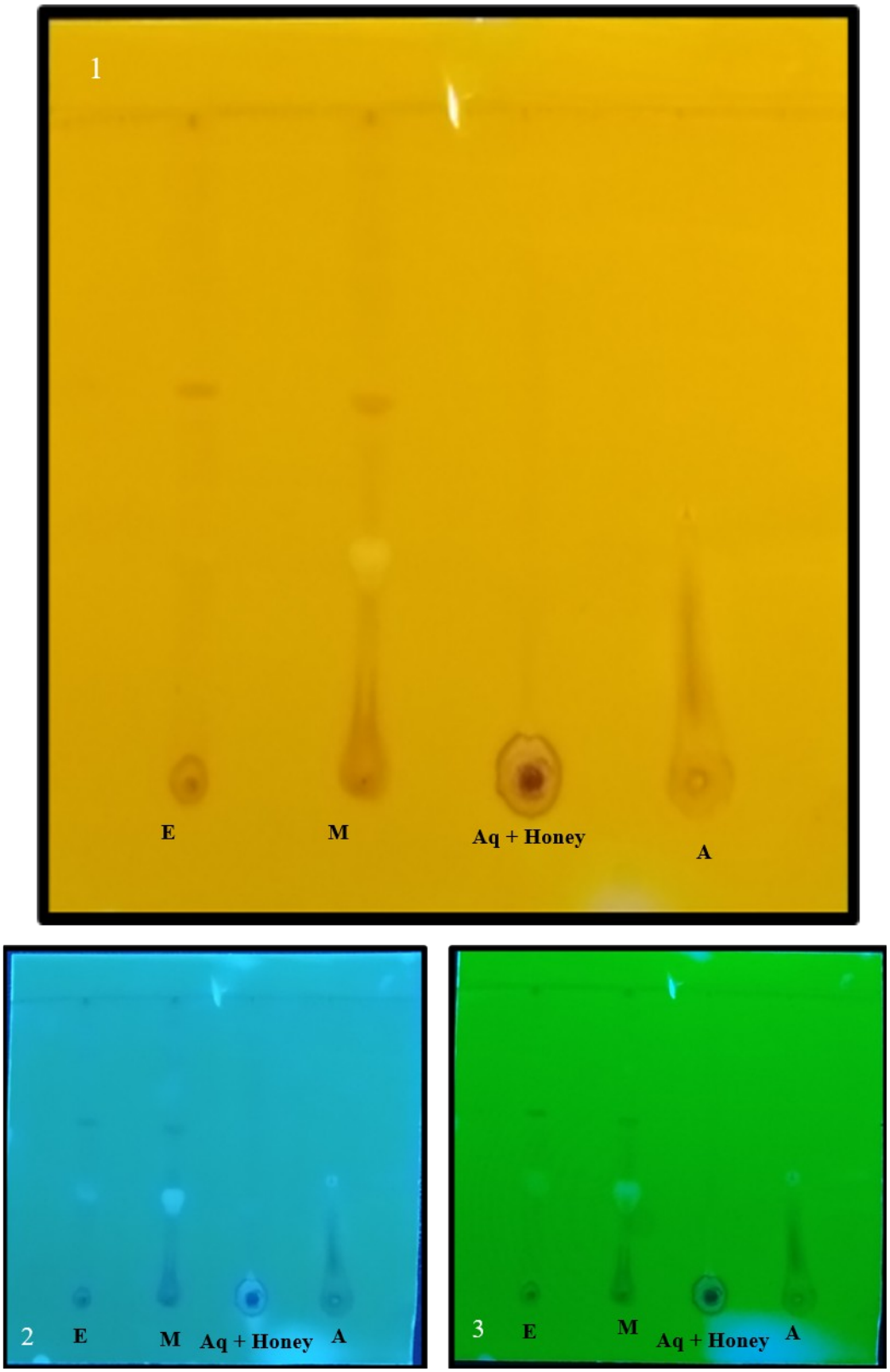


Plate 4.6. TLC analysis for Flavonoids (Lane 1: ethanol extract; Lane 2: Methanol extract, Lane 3: Aqueous extract with Honey and 4: Aqueous extract of *Butea monosperma*)

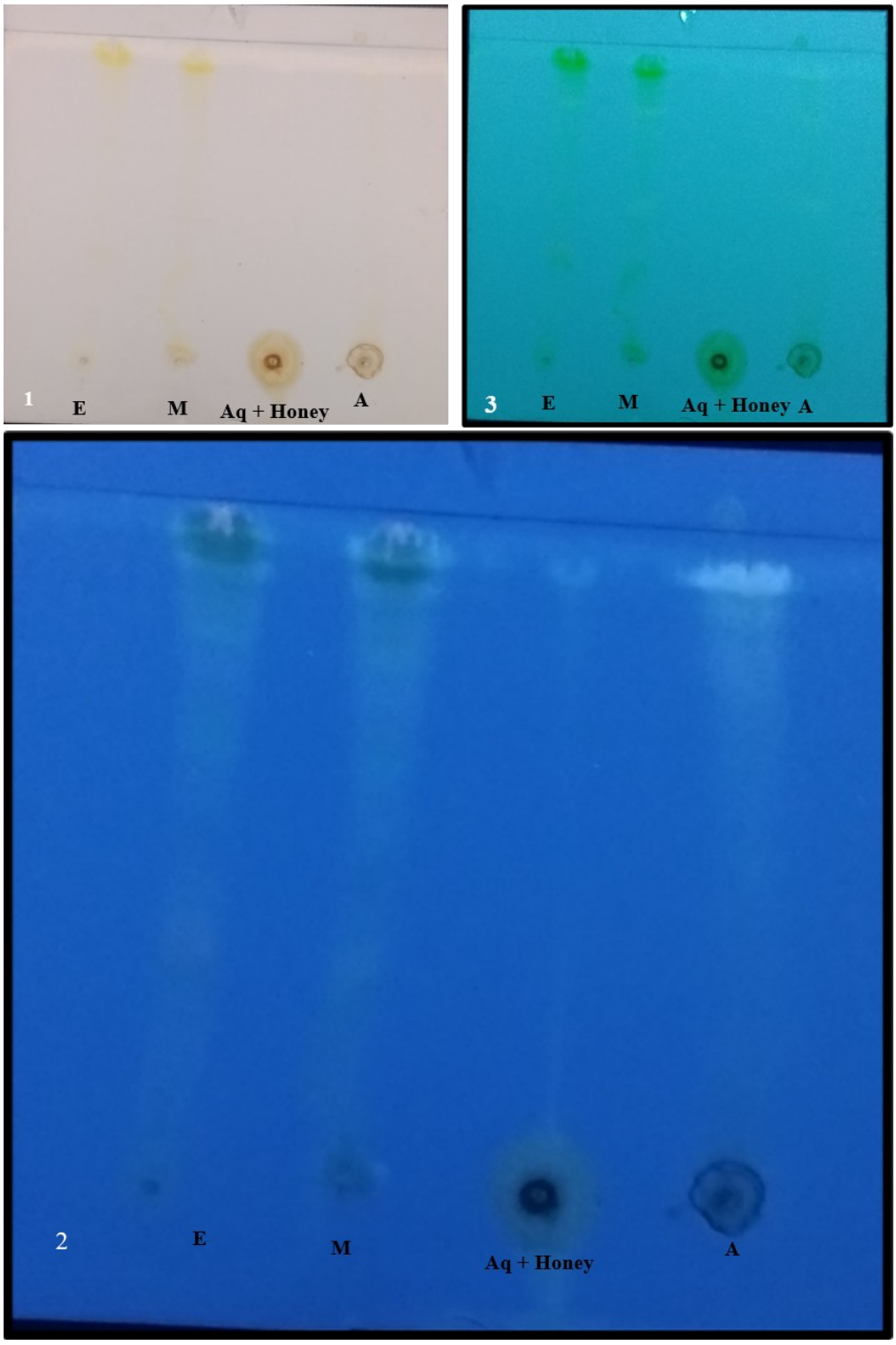


Plate 4.7. TLC analysis for Steroids (Lane 1: ethanol extract; Lane 2: Methanol extract, Lane 3: Aqueous extract with Honey and 4: Aqueous extract of *Butea monosperma*)

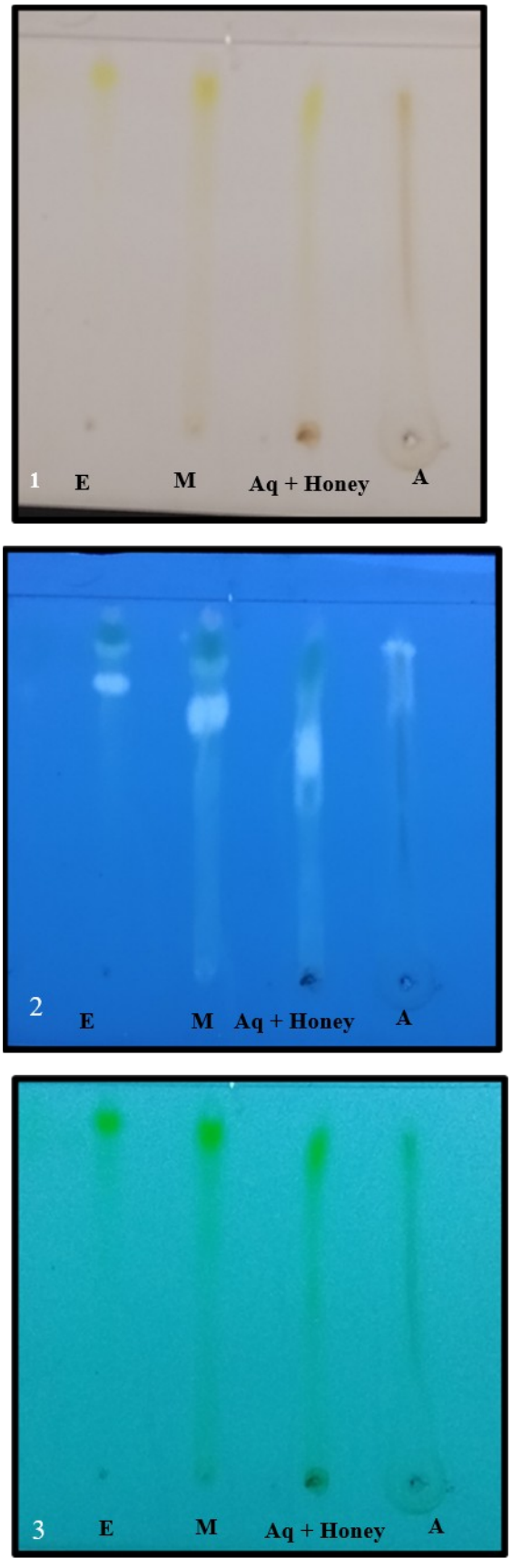


Plate 4.8. TLC analysis for Sterols (Lane 1: ethanol extract; Lane 2: Methanol extract, Lane 3: Aqueous extract with Honey and 4: Aqueous extract of *Butea monosperma*)

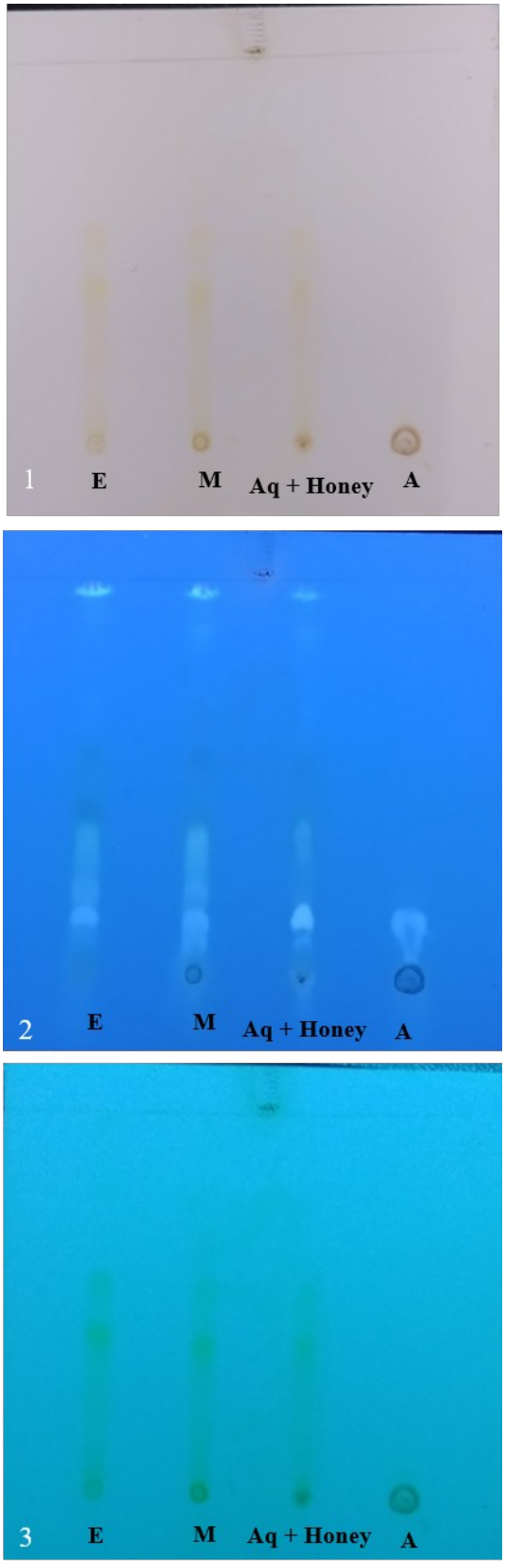
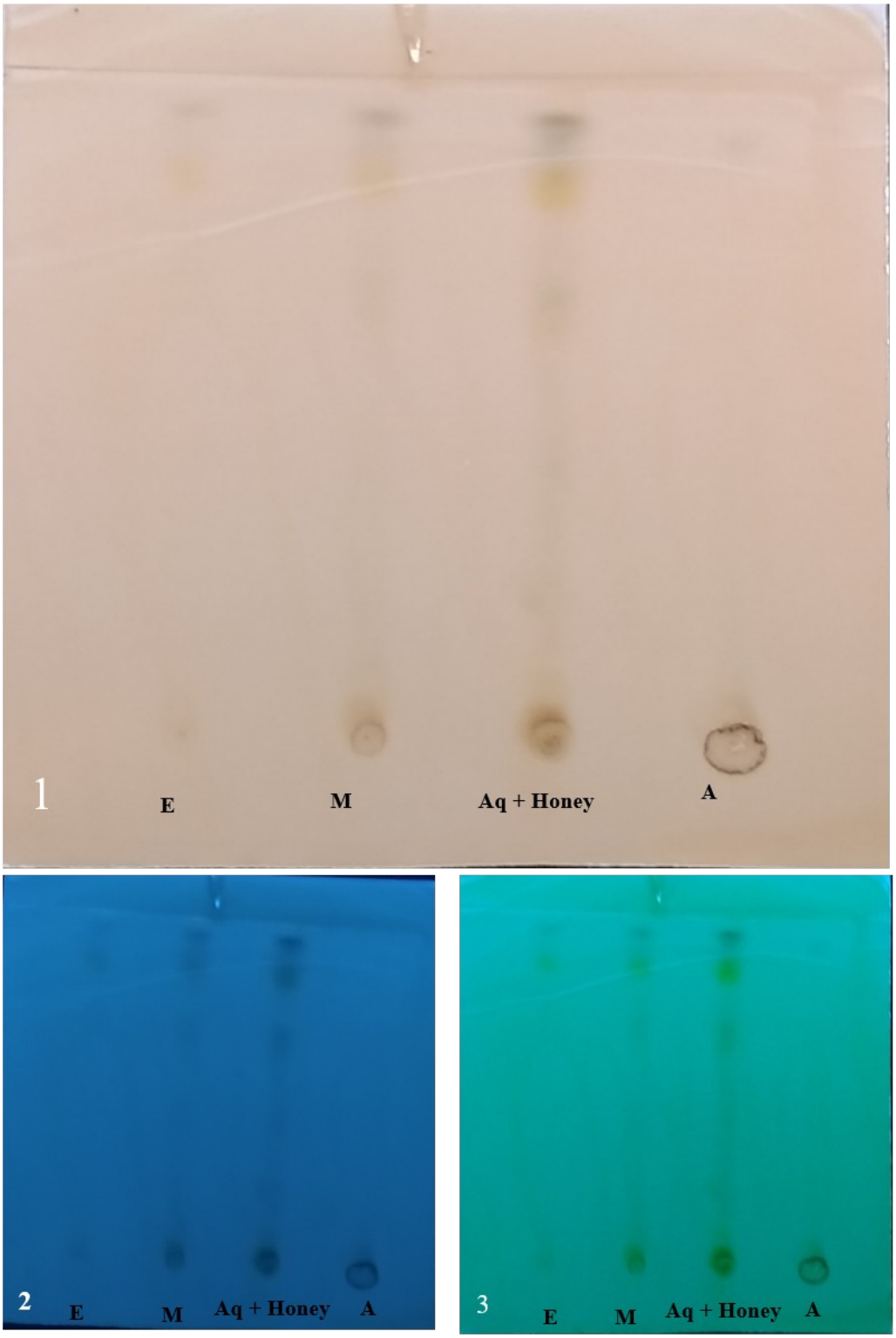


Plate 4.9. TLC analysis for Phenols (Lane 1: ethanol extract; Lane 2: Methanol extract, Lane 3: Aqueous extract with Honey and 4: Aqueous extract of *Butea monosperma*)



4.5. Anti-oxidant property activity of *Butea monosperma* extracts along with Aqueous Flower Extract of *B. monosperma* combined with honey

In this study, the antioxidant properties of the flower extract were evaluated using the DPPH assay, a method based on the scavenging activity of antioxidants against the stable free radical DPPH. Results indicated that the aqueous extract with honey at 100 µg/mL exhibited the highest antioxidant properties, with a scavenging activity of 61.6%, followed by the methanolic extract (57.64%), ethanolic extract (57.31%), and aqueous extract (54.76%) studied at different concentrations (control, 20, 40, 60, 80, 100 mg/mL). These antioxidant properties play a significant role in scavenging free radicals, contributing to the dissolution of calcium oxalate crystals. This synergistic effect of honey and aqueous extract demonstrates efficacy comparable to the standard Ascorbic acid. **Table 4.8** presents the percent scavenging activity exhibited by the aqueous extract with honey, ethanol extract, and methanol extract of *Butea monosperma*.

Additionally, **Table 4.9** displays the IC_{50} values obtained from the DPPH assay, representing the concentration of the extract required to inhibit 50% of the DPPH radicals. The IC_{50} values were found to be 101.44µg/mL for the aqueous *Butea monosperma* extract, 25.8 mg/mL for the ethanol extract of *Butea monosperma*, 51.57 µg/mL for the methanol extract, and 9.90µg/mL for the aqueous extract with honey. Surprisingly, the IC50 value for aqueous extract with honey was much lesser than that of L-ascorbic acid used as standard in the study. These results further confirm the potent antioxidant activity of the aqueous extract with honey, highlighting its potential for therapeutic applications (**Plate 4.10; Table 4.8 and Table 4.9; Figure 4.5**).

A lower IC50 value indicates higher antioxidant potency, with the aqueous extract with honey exhibiting the lowest IC50 value of 9.90 µg/mL. This signifies that smaller concentrations of

the aqueous extract with honey are needed to achieve significant scavenging activity, further reinforcing its potent antioxidant efficacy (**Figure 4.6**).

These antioxidant capabilities are pivotal in neutralizing free radicals, thereby contributing to the dissolution of calcium oxalate crystals, a process crucial in mitigating kidney stone formation. The synergistic effect observed between honey and the aqueous extract underscores their efficacy, demonstrating performance akin to the standard Ascorbic acid.

Table 4.8: DPPH assay percent scavenging activity exhibited by Aqueous extract, ethanol extract, methanol extract of *Butea monosperma* along with aqueous extract with honey.

	Concentration (µg/mL)				
Samples	**20**	**40**	**60**	**80**	**100**
Standard (Ascorbic acid)	48.01±2.04	66.36±2.84	81.98±2.11	92.93±1.12	95.69±1.05
Aqueous	51.33±0.09	52.37±0.16	52.85±0.01	53.89±0.09	54.76±0.19
Ethanol	53.58±0.83	54.05±0.09	54.76±0.19	56.39±0.09	57.31±0.09
Methanol	45.51±0.57	51.50±0.09	53.07±0.09	54.98±0.33	57.64±1.95
Aqueous extract +Honey	51.50±0.09	57.64±0.09	58.02±0.25	59.05±0.16	61.61±0.19

Table 4.9. IC_{50} value obtained from DPPH assay

Samples	**IC_{50} µg/mL**
Standard	***14.76***
Aqueous extract	***101.44***
Ethanol extract	***51.57***
Methanol extract	***41.67***
Aqueous extract + honey	***9.90***

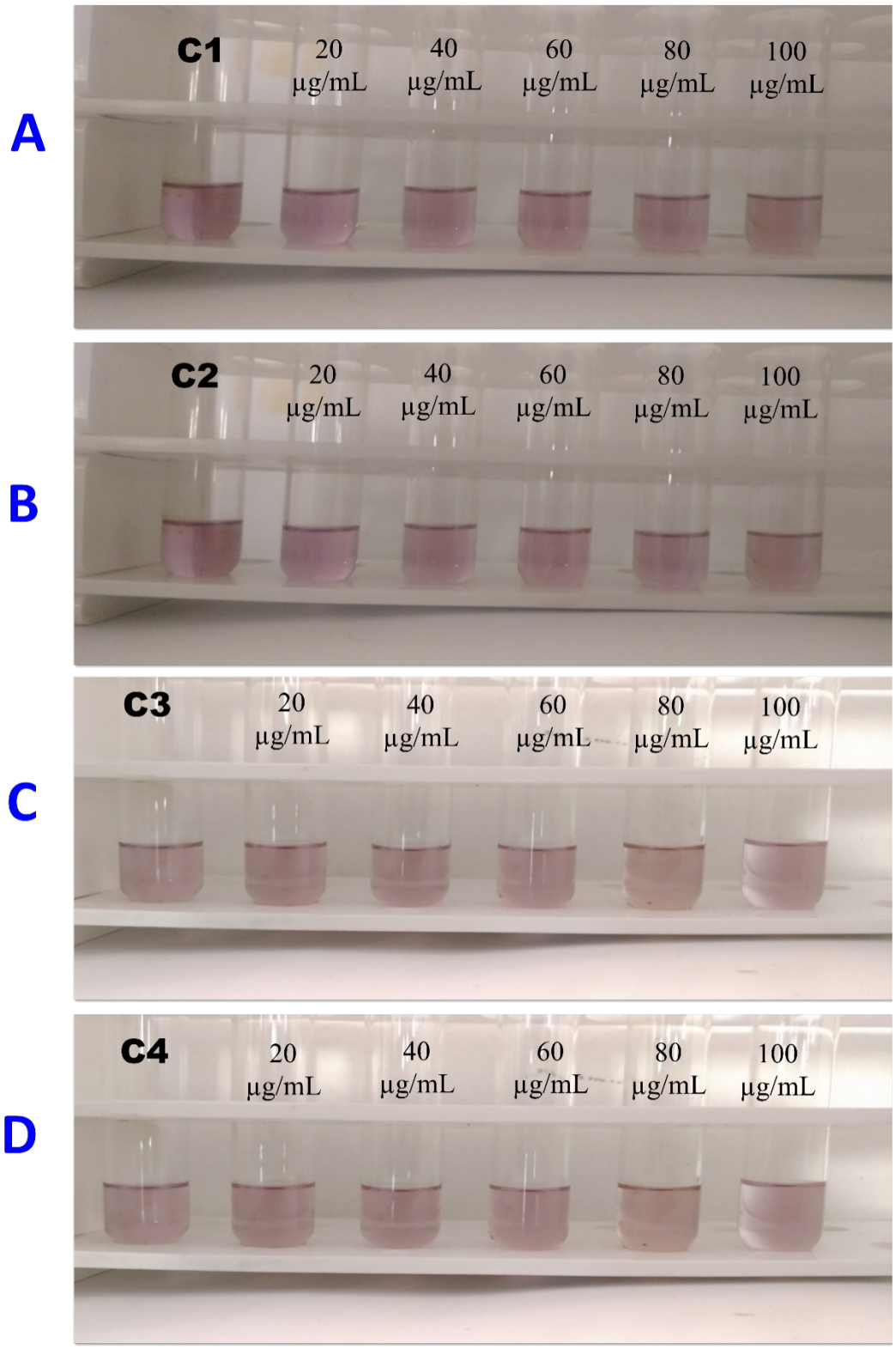


Plate 4.10. DPPH scavenging activity assay A) Aqueous extract B) EtOH extract C) MeOH Extract D) Aqueous extract of *Butea monosperma* with honey.

Figure 4.5: Graph showing Percent scavenging activity (A) Standard Calibration curve for Ascorbic acid and (B) % Scavenging activity of Aqueous extract, ethanol extract, methanol extract of *Butea monosperma* along with aqueous extract with Honey

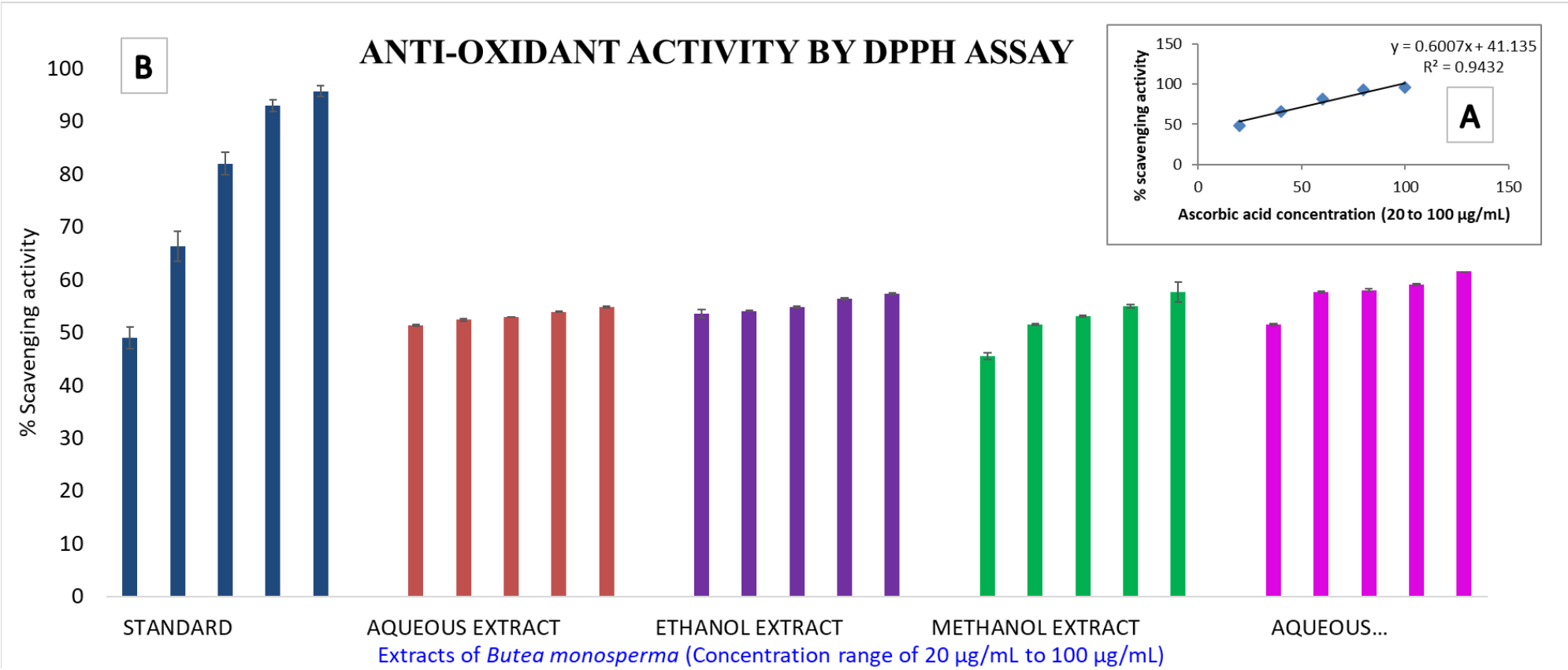
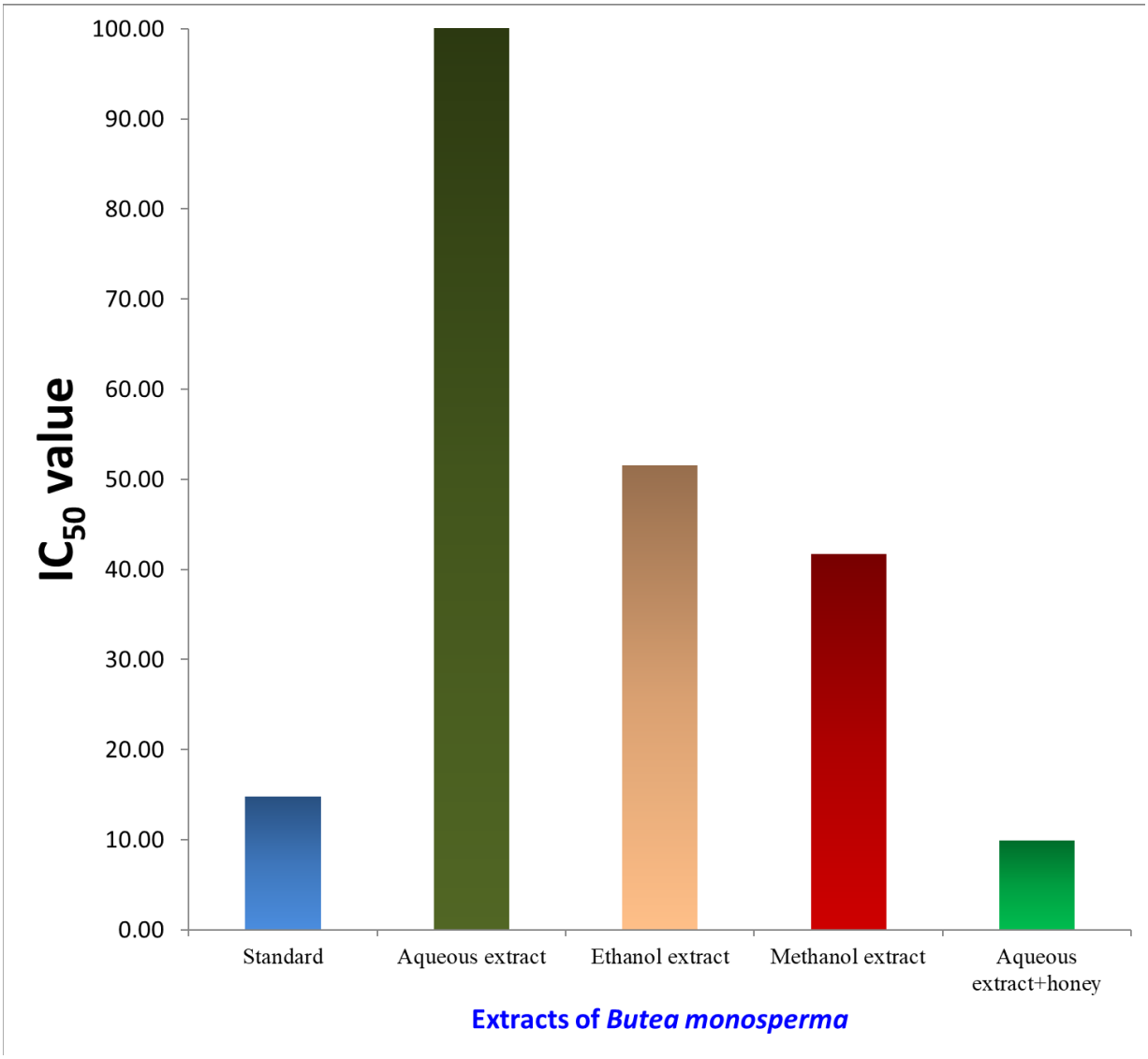


Figure 4.6: Graph showing IC_{50} values of Aqueous extract, ethanol extract, methanol extract of *Butea monosperma* along with aqueous extract with honey



4.6. Screening Anti-inflammatory activity of *Butea monosperma* extracts along with Aqueous Flower Extract of *B. monosperma* combined with honey

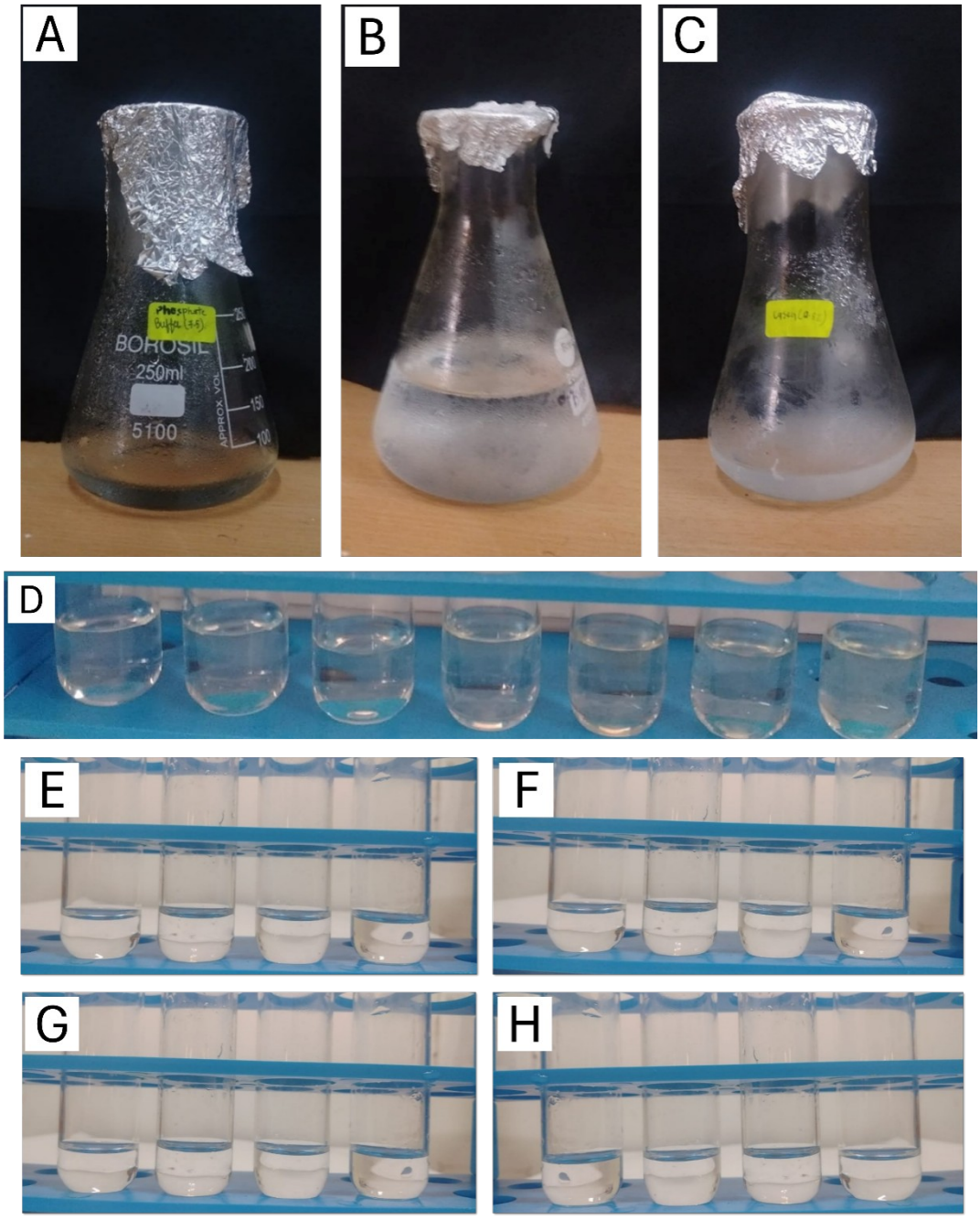
Kidney stone disease often triggers inflammation in the kidneys due to protein denaturation and subsequent inflammatory responses in the excretory system. Understanding the need for effective anti-inflammatory treatments, this study delved into assessing the anti-inflammatory potential of various extracts derived from the *Butea monosperma* flower.

The methodology employed a protein inhibitory assay to evaluate the ability of *Butea monosperma* extracts to inhibit protein denaturation, a key aspect of inflammatory processes. The standard anti-inflammatory drug Diclofenac Sodium (DMFO) served as a reference point for comparison (**Table 4.10**).

Surprisingly, the investigation revealed notable variations in anti-inflammatory efficacy among the different extracts. While the ethanolic, methanolic, and pure aqueous extracts displayed varying degrees of inhibition, the most remarkable results were observed with the aqueous extract combined with honey (**Plate 4.11; Figure 4.7**).

Intriguingly, the aqueous extract, when combined with honey in a 1:1 ratio, exhibited the highest anti-inflammatory activity, with a remarkable percentage inhibition of protein denaturation at 49.5%. This surpassed the efficacy of the methanolic extract (29.9%), ethanolic extract (17.4%), and pure aqueous extract (14.9%) (**Table 4.11 and Figure 4.8**).

These findings highlight the potential therapeutic efficacy of the aqueous extract combined with honey from the *Butea monosperma* flower in alleviating inflammation associated with



kidney stone disease. The synergistic action of the aqueous extract and honey presents a promising avenue for further research and the development of novel anti-inflammatory treatments.

Plate 4.11. Anti-inflammatory assay showing (A) Phosphate buffer (pH 7.5), (B) Trypsin (0.06mg/ml) in Tris HCl buffer, (C) Casein (0.8% v/v), (D) Series control, (E) 1mg/ml flower aqueous honey extract of *Butea monosperma*, (F) 1 mg/ml ethanolic extract, (G) 1mg/ml methanolic extract, and (H) 1 mg/ml Aqueous extract.

Table 4.10: Anti-inflammatory assay: Proteinase inhibitory Assay of Standard DMFO at different concentration at 210 nm absorbance

Standard Drug dose (µg/ml)	Percentage of Inhibition in Protein Denaturation

	(%)
100	15.70 ± 0.0045
200	16.44 ± 0.89
400	20.59 ± 0.05
600	24.52 ± 0.02
800	30.16 ± 0.43
1000	34.62 ± 0.034

Figure 4.7: Standard calibration curve for anti-inflammatory activity using Standard Diclofenac Sodium (DMFO)

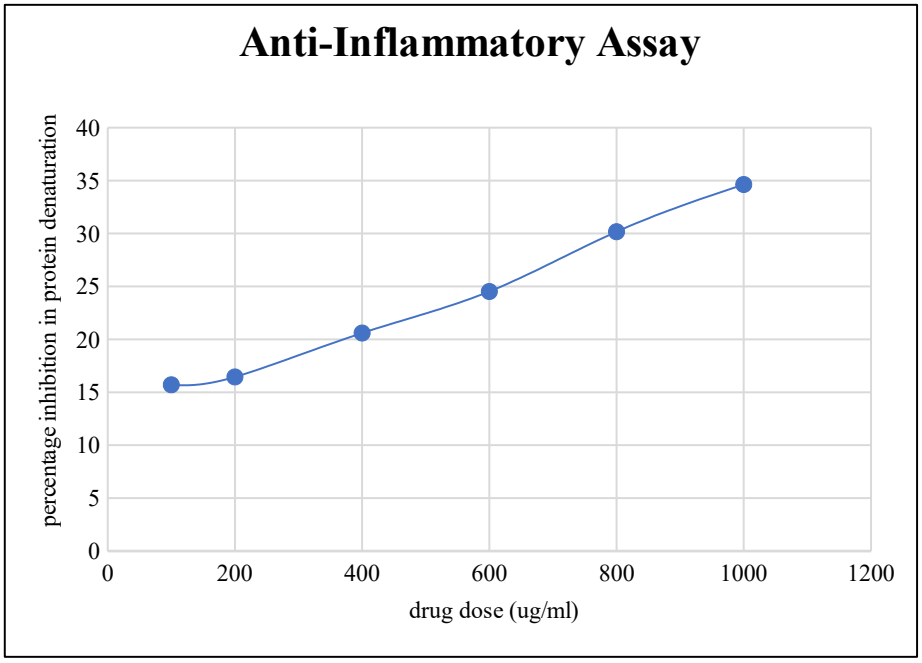
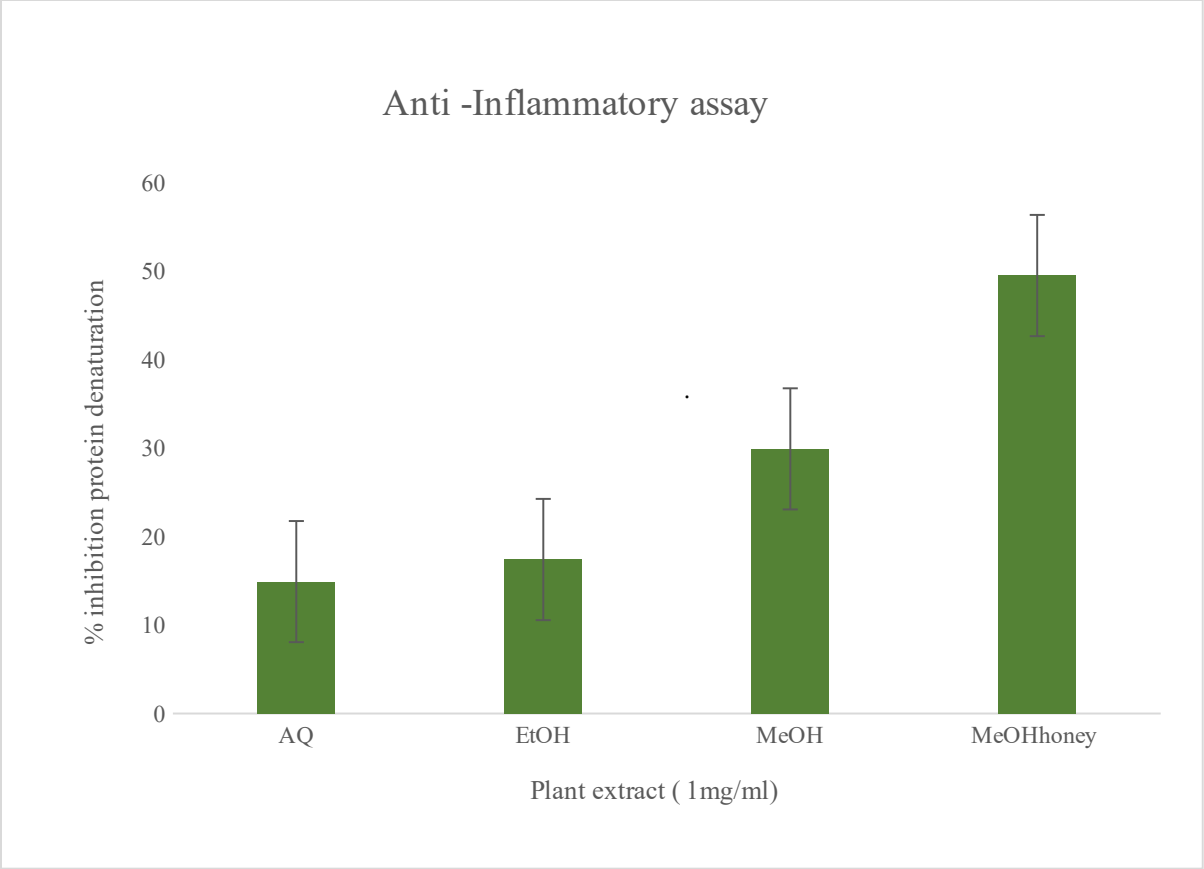


Table 4.11. Anti-inflammatory Activity: Protease inhibitory Assay of different extracts of *Butea monosperma* flower (Aqueous Extract, Ethanolic extract, Methanolic extract and Aqueous extract of *B. monosperma* with Honey at OD 210 nm.

Plant extract (mg/ml)	Percentage of protein denaturation
Aqueous extract	14.9 ± 0.46
Ethanolic extract	17.4 ±5.67
Methanolic extract	29.9 ± 0.67
Aqueous Extract + honey (1:1)	49.5 ± 0.006

Figure 4.8: Protease inhibitory assay, depicting the inhibition of protein denaturation by different extracts of *Butea monosperma* flower. The extracts include Aqueous Extract, Ethanolic extract, methanolic extract, and Aqueous Honey extract. The absorbance values are recorded at OD 510 nm, indicating the extent of inhibition achieved by each extract.



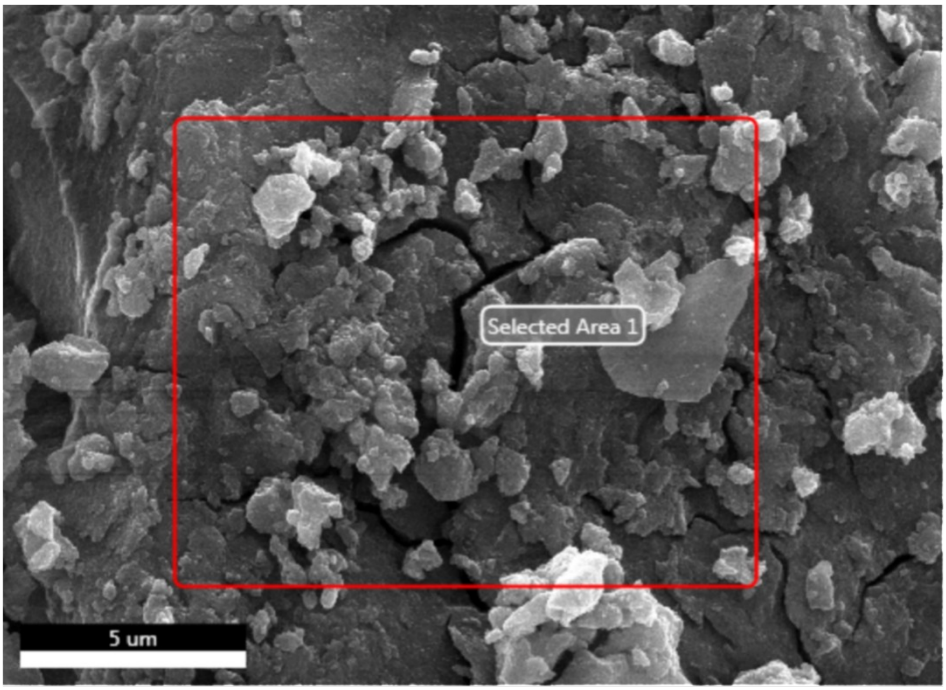
4.7. Evaluation of Anti-urolithiasis Activity of Synergistic Effect of Aqueous Flower Extract of *Butea monosperma* and Honey (1:1 v/v)

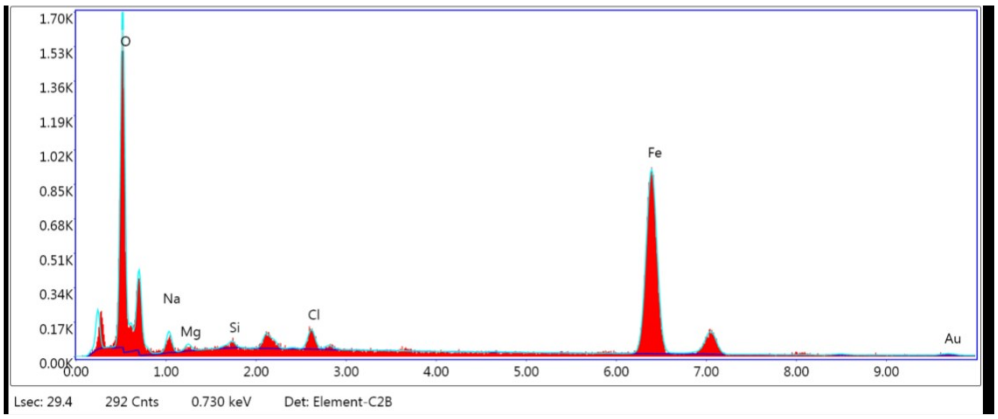
4.5.1. Preparation of Calcium Oxalate Crystals by Homogeneous Precipitation Method:

Calcium oxalate crystals were successfully prepared using the homogeneous precipitation method. The dissolution of calcium chloride dihydrate and sodium oxalate in their respective solvents facilitated the formation of calcium oxalate crystals upon mixing. The excess sulphuric acid was carefully removed, and the resulting solution was subjected to thorough washing with ammonia solution and distilled water. Subsequently, the solution was dried in a hot air oven at 60°C for 4 hours, leading to the formation of well-defined calcium oxalate crystals.

4.5.2. Characterisation of CaOx crystals by microscopic observations under SEM: The surface morphology of the calcium oxalate crystals was meticulously examined using a scanning electron microscope (SEM). SEM analysis revealed the elemental composition of the crystals, providing valuable insights into their structural characteristics. The elemental analysis indicated the presence of oxygen (O), sodium (Na), magnesium (Mg), silicon (Si), chlorine (Cl), iron (Fe), and gold (Au) in varying proportions. Oxygen was found to be the predominant element, constituting 28.9% of the crystal composition, followed by iron at 57.8%. These findings contribute to a comprehensive understanding of the chemical composition and structure of calcium oxalate crystals, elucidating their potential role in various biological processes and pathological conditions (**Plate 4.12**).

Plate 4.12. SEM image showing calcium oxalate crystals with a scale bar of 10 µm, acquired at an accelerating voltage of 10 kV and a magnification of 5000x. Elemental analysis revealed the presence of oxygen (O), sodium (Na), magnesium (Mg), silicon (Si), chlorine (Cl), and iron (Fe).





eZAF Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
O K	28.9	56.4	372.7	7.4	0.1599	1.1739	0.4723	1.0000
NaK	5.1	7.0	24.6	18.7	0.0098	1.0716	0.1791	1.0003
MgK	0.9	1.2	8.2	30.0	0.0027	1.0915	0.2675	1.0007
SiK	0.6	0.6	9.7	29.5	0.0032	1.0768	0.5135	1.0026
ClK	1.9	1.7	33.6	17.0	0.0153	1.0064	0.7845	1.0109
FeK	57.8	32.3	465.3	2.5	0.5352	0.9172	0.9994	1.0111
AuL	4.8	0.8	5.4	59.3	0.0303	0.6212	1.0182	0.9871

4.5.1. Evaluation of Anti-urolithiasis Activity by Titrimetric Method

Semi-permeable membranes prepared from egg shells were combined with 5 mg of calcium oxalate crystals and a 1 mg/mL concentration of plant methanolic extract with honey (1:1

v/v) in a conical flask containing 0.1M Tris buffer. The flask underwent incubation at 37°C for two days. The contents were then transferred to separate test tubes, and 2 ml of 1N sulphuric acid was added. The resulting solution underwent titration against 0.9494 standard KMnO4 solution until a light pink colour was observed. Triplicate readings were obtained for accuracy, and the dissolution percentage was calculated (**Plate 4.13; Plate 4.14 and Table 4.12;**).

The anti-urolithiasis study evaluated the synergistic effect of *Butea monosperma* flower extract and honey, demonstrating concentration and time-dependent effects. Utilizing the titrimetric method, the solution containing 1 mg/ml of the Aqueous extract of *Butea monosperma* and honey extract exhibited the highest dissolution percentage of calcium oxalate crystal, reaching approximately 65%. This finding highlights the potential efficacy of the extract in reducing the formation or growth of calcium oxalate crystals associated with urolithiasis. Further analysis and discussion are warranted to elucidate the therapeutic implications of these results.

Table 4.12. The dissolution percentage calculated from Titrimetric method for Anti-urolithiasis studies.

Concentration	% dissolution of Calcium Oxalate crystals
1mg /mL Aqueous extract combined with honey	65 ± 0.057

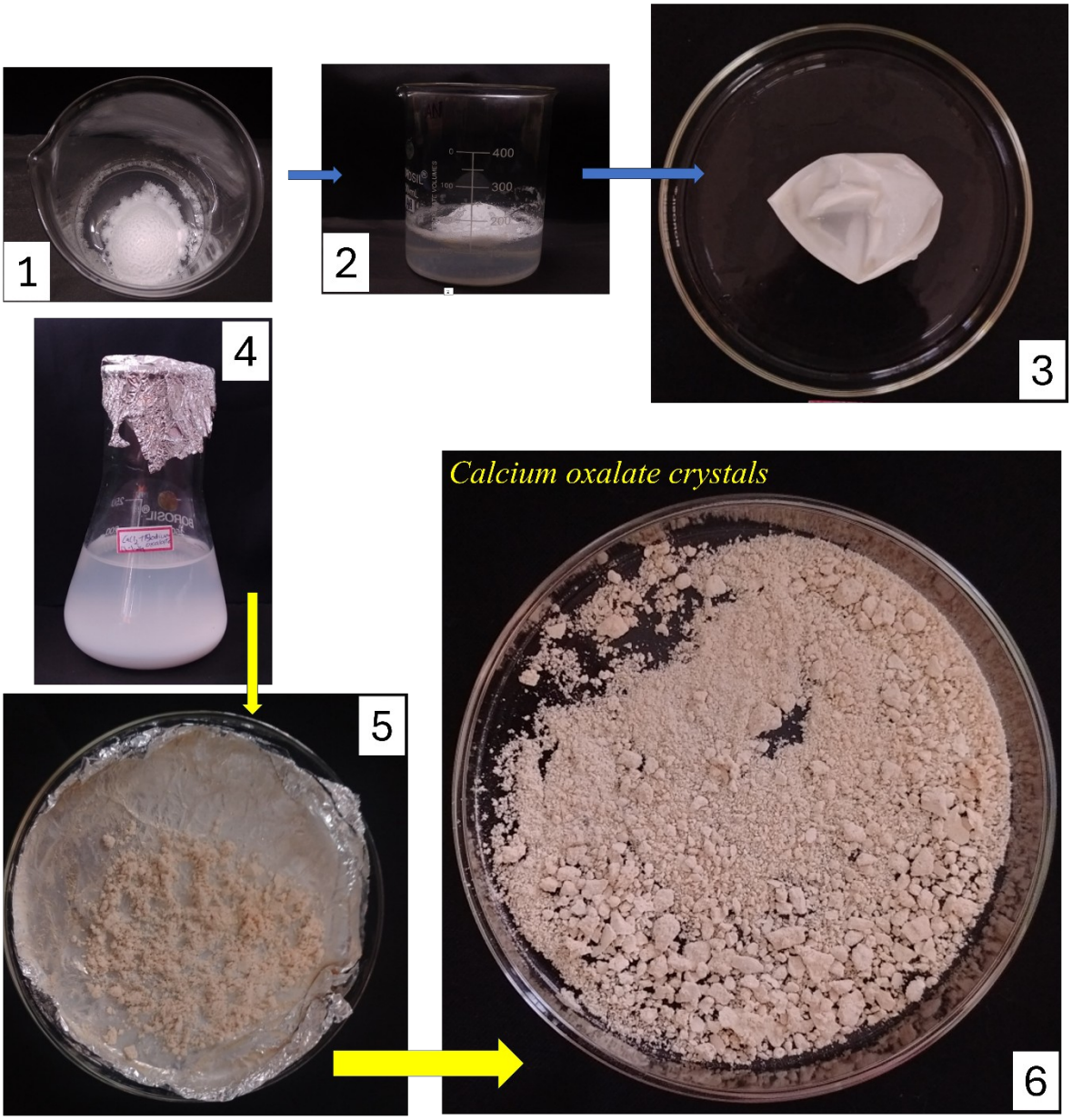
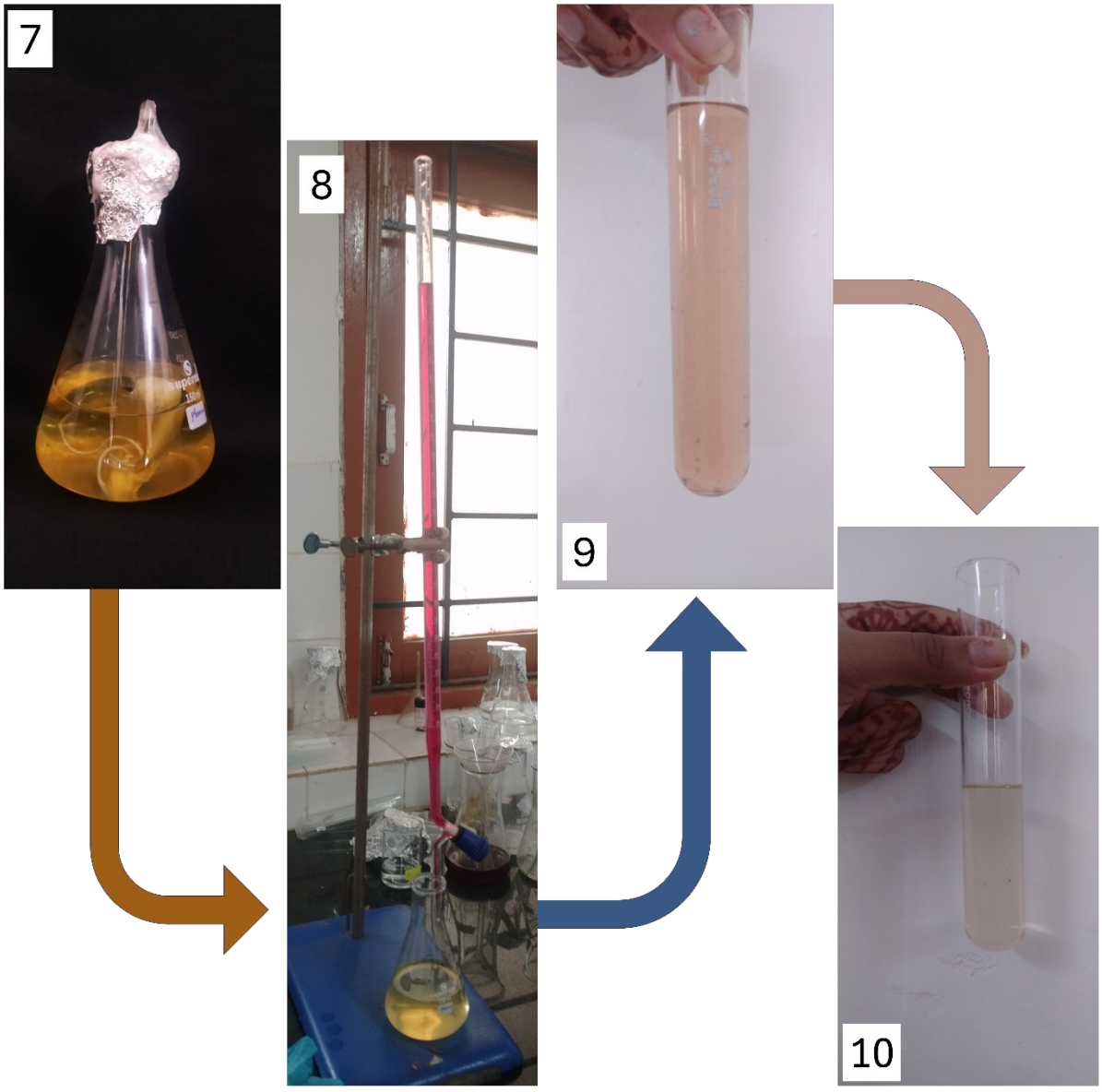


Plate 4.13. Sequential steps (1-5) in the evaluation of anti-urolithiasis activity by the titrimetric method: 1. Decalcification of egg shells overnight, 2. Preparation of semi-permeable membranes from egg shells, 3. Creation of calcium oxalate crystals using 50 mmol/l sodium oxalate and calcium chloride dihydrate, 4. Drying of crystals at 37°C, and 5. Preparation of calcium oxalate crystals.

Plate 4.14. Sequential steps (6-10) in the evaluation of anti-urolithiasis activity by the titrimetric method: 7. Titration against 0.9494 N KMnO4; 8. Addition of 2 mL of 2N H_2SO_4; 9. Observation of pink colour change; 10. Confirmation of pink colour change.



4.5.2. Aggregation assay for evaluation of Anti-urolithiasis Activity of Aqueous Flower Extract and Honey Combination from *Butea Monosperma*

Plate 4.15 and **Table 4.13** presents the results of the aggregation assay, indicating the percentage of inhibition of calcium oxalate (CaOx) crystals at different concentrations of *Butea monosperma* aqueous flower extract combined with honey over various time intervals. The negative control showed no inhibition throughout the experiment, while the positive control (cystone) exhibited inhibition percentages ranging from 38.5% at 0 minutes to 57.7% at 120 minutes.

At a concentration of 100 µg/ml, the inhibition percentages increased from 5.9% at 0 minutes to 17.8% at 120 minutes. Similarly, concentrations of 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml demonstrated increasing inhibition percentages over time. Notably, the highest inhibition was observed at 1000 µg/ml, reaching 75.8% at 120 minutes (**Plate 4.15**).

The aggregation assay revealed that the synergistic effect of honey and aqueous flower extract of *Butea monosperma* led to a significant reduction in the aggregation of CaOx crystals. Particularly, the maximum reduction was observed at 1000 µg/ml concentration, surpassing the inhibition achieved by the positive control cystone.

This time-dependent study further highlighted the effectiveness of the aqueous flower extract and honey combination in increasing the dissolution of CaOx crystals over the course of 120 minutes. The percentage of dissolution at 120 minutes was notably higher for the *Butea monosperma* and honey extract compared to the standard cystone.

These results suggest that the aqueous flower extract and honey (1:1 v/v) possess potent anti-urolithiasis activity, inhibiting the formation and aggregation of calcium oxalate crystals. The observed effects support the potential therapeutic application of *Butea monosperma* flower extract combined with honey in the management of urolithiasis.

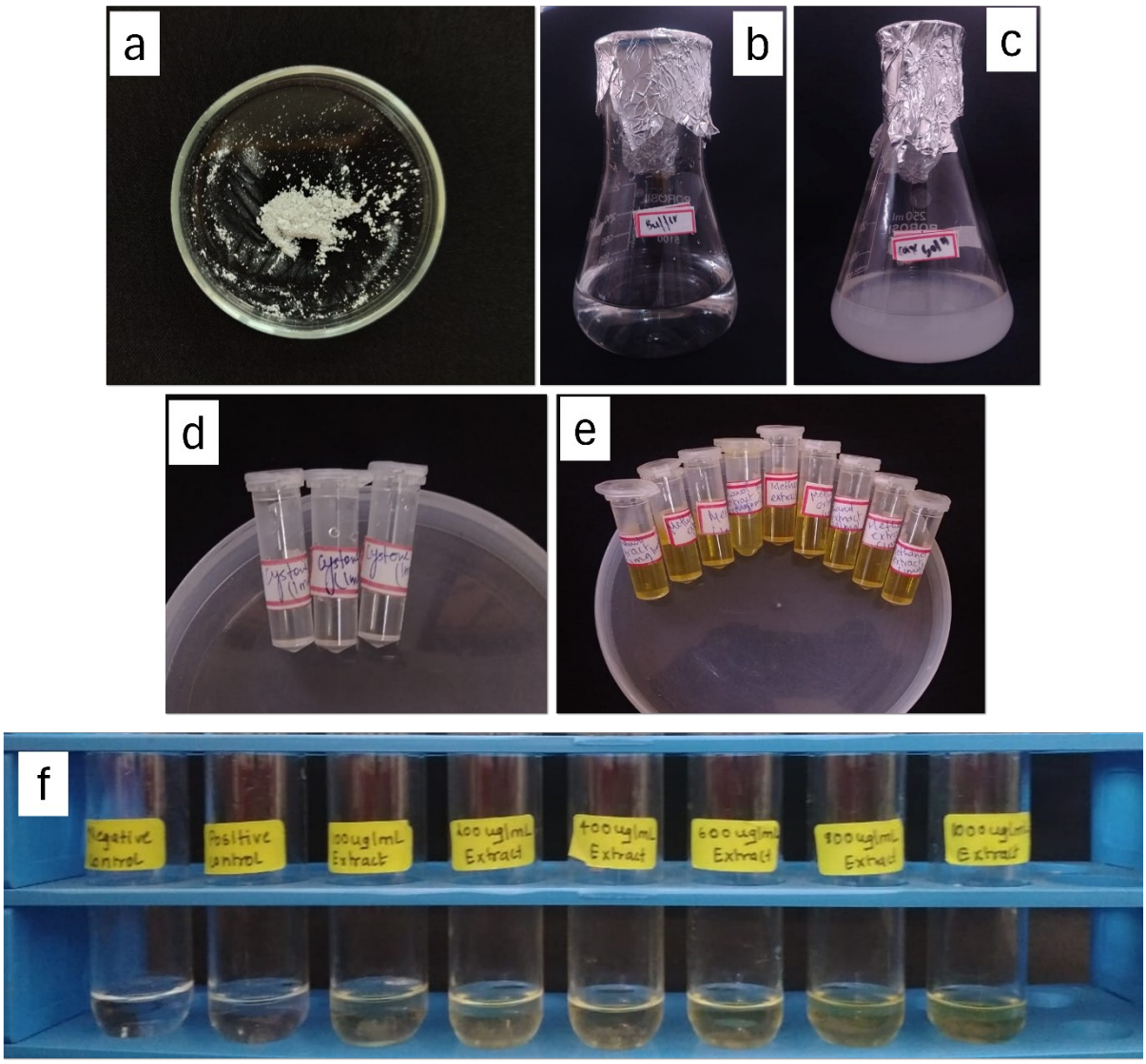


Plate 4.15. Aggregation Assay showing (a) the preparation of calcium oxalate crystals from a solution containing 50 mmol/L, (b) utilizing Tris HCl buffer, with (c) a concentration of 80 mg in 100 ml Tris HCl buffer. The experiment includes (d) 1 mg/ml Cystone solution (e), the addition of 1 mg/ml flower aqueous and honey extract of *Butea*

***monosperma* (1:1 v/v), alongside and (f) a series of plant extract concentrations ranging from 100 µg/ml to 1000 µg/ml.**

Table No. 4.13: Aggregation Assay - Inhibition of Calcium Oxalate Crystals at Different Concentrations of Aqueous Flower Extract of *Butea Monosperma* Over 30-Minute Intervals

Concentration	Percentage of inhibition of CaOx Crystals at				
	0 min	30 mins	60 mins	90 mins	120 mins
Negative control	-	-	-	-	-
Positive control (Cystone)	38.5±1.59	40.76 ±0.006	43.55 ± 0.008	49.6 ± 0.007	57.7 ± 0.01
100 µg/ml	5.9 ± 0.005	7.8 ± 0.006	8.3 ± 0.98	13.7 ± 0.45	17.8 ± 0.89
200 µg/ml	16 ± 0.67	17.65 ± 0.06	19.2 ± 9.80	25.5 ± 0.089	32 ± 0.76
400 µg/ml	22.7 ± 0.079	24.32 ± 0.97	26 ± 0.56	32.1 ± 4.6	39.2 ± 4.99
600 µg/ml	26.3 ± 0.006	28.5 ± 0.43	31.89 ± 0.988	48.78 ± 0.005	53.6 ± 0.07
800 µg/ml	38 ± 0.085	41.23 ± 0.65	44.3 ± 6.8	56.64 ± 0.054	61.98 ± 0.01
1000 µg/ml	50 ± 0.65	54.1 ± 6.78	58.32 ± 9.07	65.23 ± 9.88	75.8 ± 5.44

4.5.2. Nucleation assay for evaluation of Anti-urolithiasis Activity of Aqueous Flower Extract and Honey Combination from *Butea Monosperma*

The nucleation assay revealed the inhibitory effect of *Butea monosperma* flower aqueous extract and honey on calcium oxalate (CaOx) crystals, demonstrating a concentration-dependent increase in inhibition. The maximum inhibition was observed at a concentration of 1000 µg/mL of methanolic flower extract with honey (1:1 v/v), achieving 49.5% inhibition, surpassing the positive control (cystone) which resulted in 28% reduction of crystals. Moreover, the plant extract exhibited a higher dissolution percentage of CaOx crystals over time, with the maximum dissolution percentage observed at 1000 µg/mL concentration and honey at 120 minutes, yielding a dissolution percentage of 63%, comparable to that of

cystone. These findings underscore the effectiveness of *Butea monosperma* flower aqueous extract in combination with honey in inhibiting CaOx crystal formation (**Plate 4.16; Table 4.14; Figure 4.9**).

Microscopic observations corroborated these findings, revealing a decrease in both the size and number of calcium oxalate crystals with increasing concentration of aqueous honey extract. Additionally, CaOx crystals stained with alizarin red stain under a microscope at 40x magnification displayed a hexagonal shape, with the highest concentration (1 mg/mL) resulting in a significant reduction in crystal size (**Plate 4.17**).

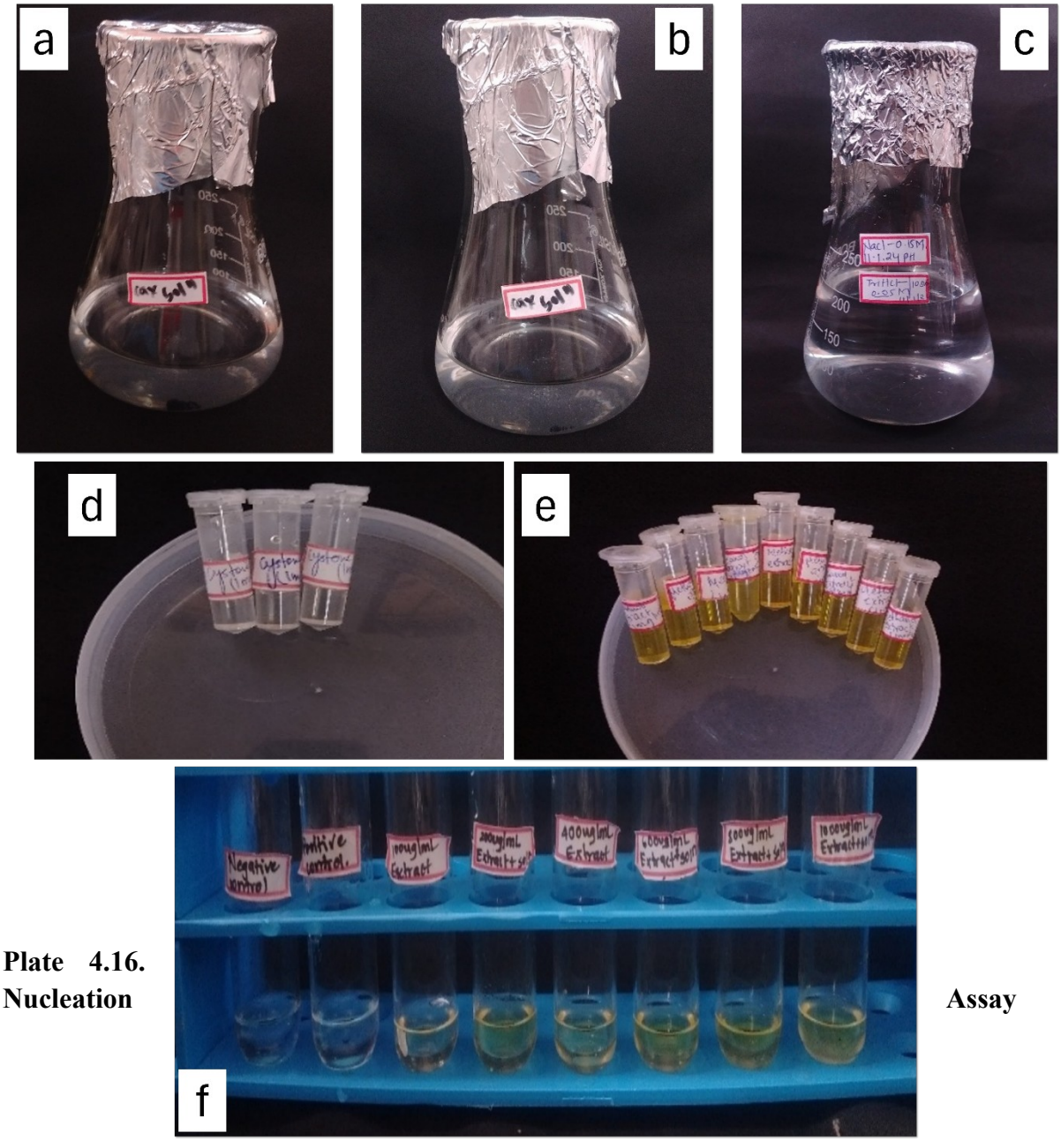


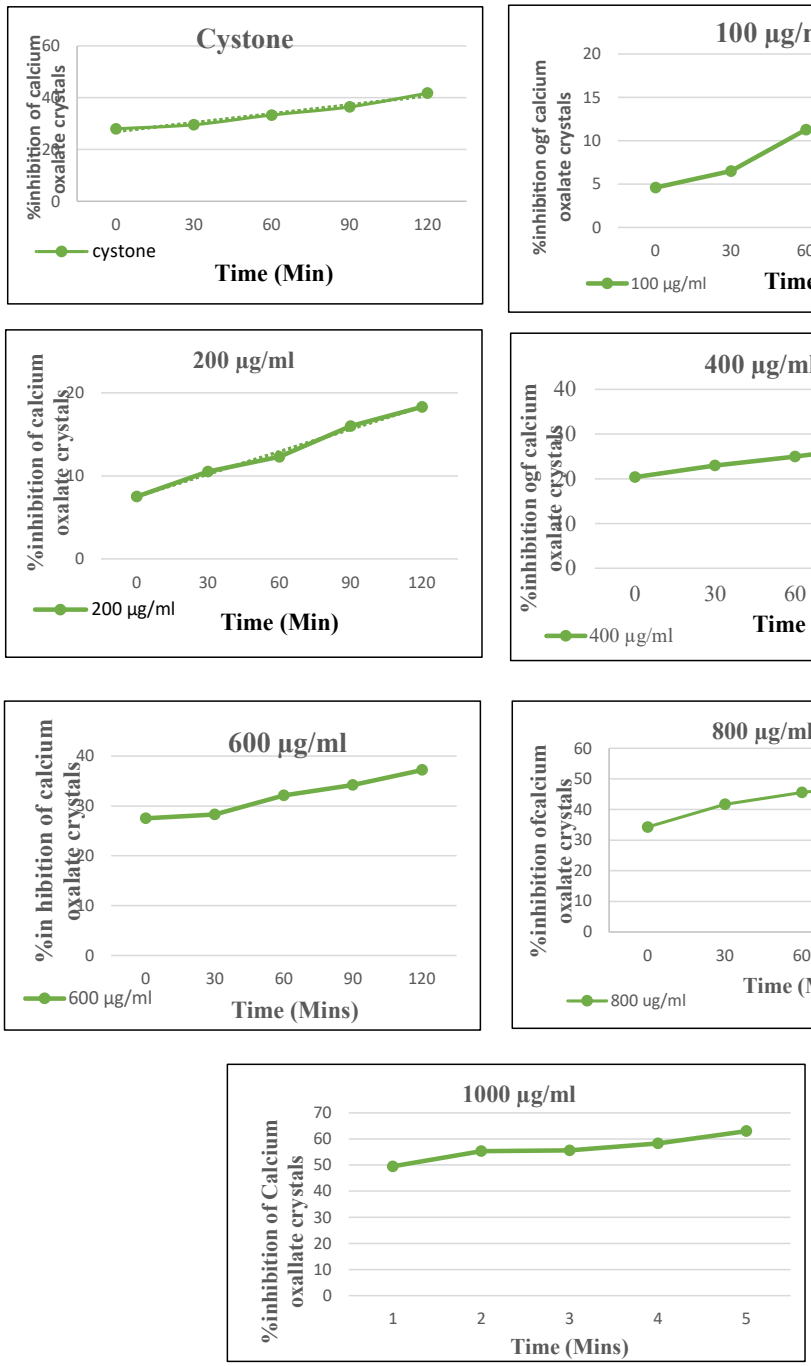
Plate 4.16. Nucleation Assay

depicting (a) sodium oxalate solution, (b) calcium dihydride chloride solution, (c) Tris HCl buffer, (d) 1 mg/ml cystone solution, (e) 1 mg/ml plant extract (aqueous extract and honey 1:1 v/v), and (f) a series of concentrations for the Nucleation Assay (including Negative control, positive control, 100 µg/ml, 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml).

Table No. 4.14: Nucleation assay - Inhibition of Calcium Oxalate Crystals at Different Concentrations of Aqueous Flower Extract of *Butea Monosperma* Over 30-Minute Intervals

Concentration	Percentage inhibition of calcium oxalate crystals at				
	0 mins	30 mins	60 mins	90 mins	120 mins
Negative control	-	-	-	-	-
Positive control (Cystone)	28 ±0.5	29.56 ± 0.23	33.32 ± 0.005	36.45 ± 0.004	41.76 ± 0.006
100 µg/ml	4.6 ± 0.006	6.5 ± 0.006	11.3 ± 6.7	12.3 ± 4.3	16.9 ± 7.66
200 µg/ml	7.5 ± 4.33	10.5 ± 5.88	12.3 ± 3.9	16 ± 2.5	18.3 ± 0.004
400 µg/ml	20.36 ± 6.09	23 ± 5.89	24.96 ± 4.00	27.3 ± 2.11	31.5 ± 8.007
600 µg/ml	27.5 ± 5.33	28.3 ± 0.089	32.1 ± 0.005	34.2 ± 0.78	37.2 ±0.87
800 µg/ml	34.3 ± 6.44	41.7 ± 0.56	45.6 ± 4.77	48.3 ± 2.11	50.1 ± 0.006
1000 µg/ml	49.5 ± 0.004	55.3 ± 0.004	55.6 ± 0.56	58.3 ± 0.006	63 ± 0.986

Figure 4.9: Graph of Nucleation assay showing the inhibition of CaOx crystals on different concentration over 30 mins interval of time.



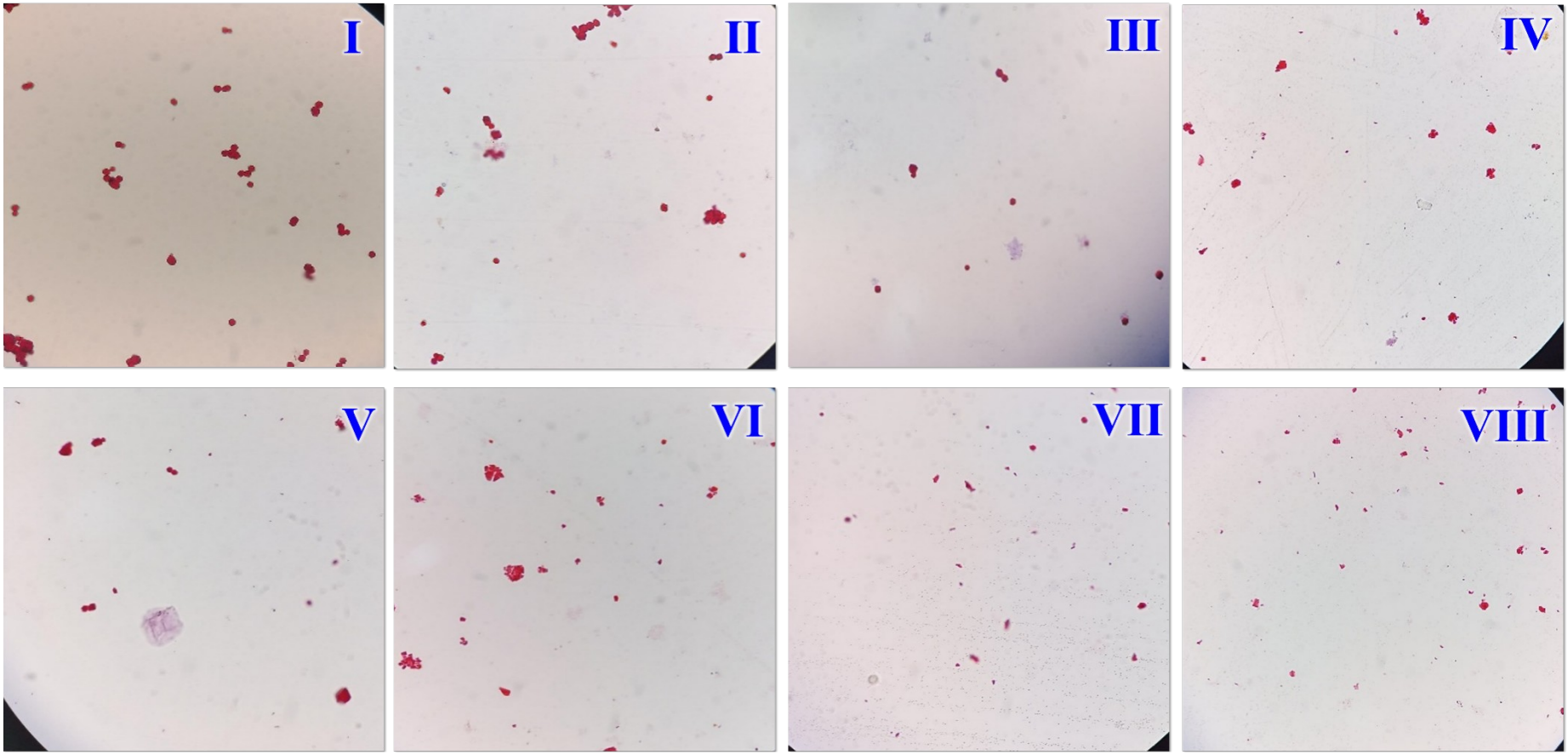


Plate 4.17. Microscopic observation of Ca Ox crystals under 40x scale I. Negative control II. Positive control (cystone 1mg/ml) III. 100µg/ml IV.200 µg/ml V. 400 µg/ml VI. 600 µg/ml VII. 800 µg/ml VIII. 1000 µg/ml

CHAPTER 5: CONCLUSION

Kidney stone disease remains a significant health concern globally, necessitating comprehensive research efforts to identify effective preventive and therapeutic strategies. This dissertation aimed to investigate the potential of *Butea monosperma* flower extracts, particularly in combination with honey, for combating kidney stone formation through various pharmacological assays.

The investigation began by exploring the phytochemical constituents present in *Butea monosperma* extracts, revealing the presence of alkaloids, carbohydrates, glycosides, proteins, phenols, and fixed oils/fats. Further analysis through thin-layer chromatography and high-performance thin-layer chromatography provided insights into the specific phytoconstituents contributing to the observed pharmacological activities. The antioxidant properties of the extracts were examined through DPPH assays, revealing remarkable scavenging activity, particularly in the aqueous extract with honey. The low IC_{50} value obtained for this formulation indicates its potent antioxidant efficacy, suggesting its potential application in oxidative stress-related conditions. Additionally, the anti-inflammatory activity of the extracts was assessed using proteinase inhibitory assays, highlighting the potent anti-inflammatory potential of the methanolic extract with honey. This finding underscores the therapeutic promise of *Butea monosperma* extracts in alleviating inflammation associated with kidney stone disease.

Further, the anti-urolithiasis activity of the extracts was evaluated through titrimetric and aggregation assays, demonstrating significant inhibition of calcium oxalate crystal formation and aggregation. Notably, the aqueous extract with honey emerged as the most effective formulation, exhibiting superior dissolution and inhibition properties compared to other extracts.

Overall, the findings from this dissertation underscore the promising therapeutic potential of *Butea monosperma* flower extracts, especially when combined with honey, in mitigating kidney stone formation and associated inflammatory and oxidative stress pathways. Future research should focus on elucidating the underlying mechanisms of action and conducting clinical trials to validate the efficacy and safety of these herbal formulations in managing kidney stone disease. By leveraging the natural synergies between botanical extracts and honey, novel therapeutic approaches may be developed to address the growing burden of kidney stone disease and improve patient outcomes.

SUMMARY

This dissertation investigated the potential of *Butea monosperma* flower extracts, particularly in combination with honey, for combating kidney stone disease through various pharmacological assays. The study aimed to elucidate the therapeutic efficacy of these herbal formulations in inhibiting calcium oxalate crystal formation, alleviating inflammation, and scavenging free radicals associated with oxidative stress.

Data analysis revealed significant findings across multiple assays:

1. **Phytochemical analysis:** Preliminary phytochemical tests indicated the presence of phenols, flavonoids, alkaloids, steroids, triterpenoids, tannins, and saponins in the *Butea monosperma* flower extracts, suggesting a rich phytochemical composition contributing to their therapeutic potential.

    i. ***Total Phenolic Content:*** Estimation of total phenolic content using gallic acid as a standard demonstrated varying concentrations across different extracts. The aqueous extract with honey exhibited the highest phenolic content, followed by the ethanolic and methanolic extracts. This suggests that honey enhances the phenolic profile of *Butea monosperma* extracts, potentially augmenting their antioxidant and anti-inflammatory properties.

    ii. ***Total Flavonoid Content*****:** Similar to phenolic content, the total flavonoid content varied among extracts, with the aqueous extract with honey showing the highest concentration. Flavonoids are known for their antioxidant and anti-inflammatory effects, further supporting the therapeutic potential of *Butea monosperma* extracts in mitigating kidney stone disease.

    iii. ***Thin Layer Chromatography (TLC) and High-Performance Thin Layer Chromatography (HPTLC):*** Thin layer chromatography analysis revealed the presence of phytoconstituents such as alkaloids, flavonoids, steroids, and phenols in *Butea monosperma* extracts. HPTLC analysis further characterized these phytoconstituents, highlighting their diverse chemical profiles and potential therapeutic roles.

2. **Antioxidant Activity:** DPPH assays demonstrated remarkable scavenging activity, particularly in the aqueous extract with honey, with a scavenging activity of 26% compared to other extracts. The IC_{50} value, representing the concentration required to inhibit 50% of DPPH radicals, was lowest for the aqueous extract with honey at 9.90 µg/mL, indicating its potent antioxidant efficacy.
3. **Anti-inflammatory Activity:** Proteinase inhibitory assays revealed the anti-inflammatory potential of the methanolic extract with honey, exhibiting a percentage inhibition of protein denaturation of 49.5% compared to other extracts.
4. **Anti-urolithiasis Activity:** Titrimetric and aggregation assays demonstrated the potent inhibitory effects of *Butea monosperma* extracts on calcium oxalate crystal formation and aggregation. The aqueous extract with honey exhibited the highest dissolution percentage, with a maximum inhibition of 65% observed at a concentration of 1 mg/mL.

The synergistic effect of honey in combination with *Butea monosperma* extracts was consistently evident across all assays, emphasizing its importance in enhancing the therapeutic properties of the herbal formulations. This synergism may be attributed to the presence of bioactive compounds in honey, such as flavonoids and phenolic acids, which complement the phytoconstituents present in *Butea monosperma* extracts.

Overall, the findings from this dissertation highlight the promising therapeutic potential of *Butea monosperma* flower extracts, particularly when combined with honey, in mitigating kidney stone formation and associated inflammatory and oxidative stress pathways. Further research and clinical trials are necessary to validate these findings and develop novel therapeutic interventions for kidney stone disease management.

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