DEVELOPMENT AND EVALUATION OF ORAL POLYHERBAL FORMULATION FOR LITHOLYTIC ACTIVITY IN ETHYLENE GLYCOL INDUCED UROLITHIASIS IN WISTAR RATS

A

Thesis

Submitted for the Award of the Ph.D. degree of

PACIFIC ACADEMY OF HIGHER EDUCATION AND RESEARCH UNIVERSITY

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2024

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Place: Pune

ACKNOWLEDGEMENT

I express my warmest gratitude to honorable **Dr. Hemant Kothari,** Dean, Pacific University Udaipur, Rajasthan for giving admission to Ph.D. Programme in university.

I express my sincere feelings of gratitude to my research guide **Dr. Rajesh Khathuriya,** Professor**,** Pacific University Udaipur, Rajasthan and my Co-guide **Dr. Suresh L. Jadhav** Principal, Vishal Institute of Pharmaceutical Education and Research, Ale, Pune. They have always been a constant source of encouragement and support throughout my Ph D programme, which helped me to complete the research work successfully. Their valuable suggestions and timely counsel helped me to design meaningful experiments.

I warmly thank **Dr. Rajesh Kanja**, Assistant Professor (IT- Manager), Pacific Academy of Higher Education and Research University Udaipur, Rajasthan for their continuous encouragement and support for completion of this work. His valuable guidance and motivation contributed to the successful completion of this study.

I gratefully acknowledge **Mr. Ramesh Agarwal,** for his understanding, encouragement and personal devotion which have provided good and smooth basis for my Ph.D. tenure.

It's a pleasure duty to express my sincere grateful thanks to **Dr. D. D. Gaikwad,** and **Dr. Mrunal Shirsath Sir** and **Dr. Rupali Hande** for their help, encouragement and for making available infrastructural facilities and equipment at the institute for the research work. I won't fail in my duty to acknowledge the lessons of courage and wisdom taught by them.

I am grateful to thank **Dr. Pardeshi** madam for educating me aspects of Ayurveda regarding pathophysiology and mode of action various herbs and their references of shlokas in various Ayurvedic Books.

I am thankful to **Dr. Asha Thomas**, D.Y. Patil Institute of Pharmaceutical Science and Research, Pimpri, Pune and **Ms. Sunita Kulkarni** Mam for providing all the necessary facilities and help that were required for the analysis.

Also, thankful my colleague **Reshma Mam, Ashwini Mam, Trupta, Supriya, Pranaya** and all teaching and non-teaching staff of VJSM for their kind support and help.

I owe a deep sense of gratitude towards my Family and especially towards my husband **Er. Sagar H. Tajave,** my family members and my parents for always providing support and inspiration to me throughout my lifetime. They have been an unlimited source of positive mental support.

I would like to thank all whose direct and indirect support helped me in completing my thesis in time.

My distinctive thanks to ***M/s Shorya Thesis Printers & Binders, Udaipur*** for their role in shaping the matter, creative design work and bringing out this document meticulously, neatly and timely

Last but not the least a sincere thanks to all the valuable lives of animals used for the research work. Apologies for any omissions.

Anuradha Subhashchandra Jadhav

PREFACE

This research was undertaken to prove effectiveness of Traditional Herbal medicines available as Indigenous medicine or folk medicine for Kidney stone.

Recurrence of kidney stone after Lithotripsy or Percutaneous Nephrolithotomy is the major problem observed in Diabetic patients. Also Thyroid hormone interferes with formation of kidney stone is the important aspect of research. Amongst various numerous folk medicines herbs were selected on the basis of primary in-vitro analysis.

I express my warmest gratitude to honorable **Dr. Hemant Kothari,** Dean, Pacific University Udaipur, Rajasthan for giving admission to Ph.D. Programme in university. I express my sincere feelings of gratitude to my research guide **Dr. Rajesh Khaturiya,** Professor**,** Pacific University Udaipur, Rajasthan and my Co-guide **Dr. Suresh L. Jadhav** Principal, Vishal Institute of Pharmaceutical Education and Research, Ale, Pune. They have always been a constant source of encouragement and support throughout my Ph.D. programme,

Selected herbal Plants were authenticated from Agharkar Institute Pune. While performing Analytical studies D.Y. Patil Institute, Pimpri, Pune has given their best support and facilities to undertake HPTLC Analysis and Method Development of selected herbal medicines.

It is hoped that the research will be received favorably as an effective remedy for those who are suffering from pain and recurrence of kidney stone.

Author is thankful of Vishal Institute of Pharmaceutical Education and Research, Ale, Pune for making available infrastructural facilities and equipment at the institute for the research work.

INDEX

Sr. No.	Topics	Page No.
1	*Declaration*	ii
2	*Certificate*	iii
3	*Certificate*	iv
4	*Copyright*	v
5	*Acknowledgement*	vi
6	*Preface*	viii
7	*Index*	ix
8	*List of Tables*	xi
9	*List of Figures*	xiv
10	*List of Abbreviations*	xviii
1	**INTRODUCTION**	**1 – 25**
2	**LITERATURE REVIEW**	**26 – 34**
3	**RESEARCH GAP**	**35 – 36**
4	**AIM OBJECTIVES AND PLAN OF WORK**	**37 – 38**
5	**MATERIAL AND METHOD**	**39 - 110**
6	**RESULT AND DISCUSSION**	**111 – 186**
7	**SUMMARY AND CONCLUSION**	**187 – 189**
8	**REFERENCES**	**190 - 208**
	APPENDIX:	
	Research and Review paper publication	**-**
	Conference Certificates	**-**

LIST OF TABLES

Table No.	Title of the Table	Page No.
1.1	Medicinal Plants Used in Ayurveda to Cure Kidney Stones	05
5.1	Structures of chemical Constituents of *Boerhavia diffusa*	42
5.2	Phytochemical Constituent of *Plumeria rubra*	50
5.3	Phytochemical Constituent of *Celosia argentea*	56
5.4	Specifications of Talc	59
5.5	Specifications of Magnesium Separate	60
5.6	Specifications of Lactose	61
5.7	Specifications of Starch	61
5.8	Specifications of Sodium alginate	62
5.9	Specifications of Calcium chloride	63
5.10	List of Materials used	63
5.11	List of Instruments / Equipments used	67
5.12	Specifications of Boerhavia diffusa	71
5.13	Specifications of Plumeria rubra seed pod	72
5.14	Specifications of Celosia argentea seeds	73
5.15	HPTLC Conditions of Leaves of Boerhavia diffusa, Pods of Plumeria rubra and seeds of Celosia argentea extracts	82
5.16	Anti-urolithiasis in vivo experimental design	97
5.17	Combination of Extract	107
5.18	Formulation table of Polyherbal Granules	107
6.1	Morphology and physicochemical specifications of *B. diffusa*	115
6.2	Morphology and physicochemical specifications of *P. rubra* seed pod	115

6.3	Morphology and physicochemical specifications of *C. argentea* seed	116
6.4	Extraction of *Boerhavia diffusa, Plumeria Rubra* and *Celosia argentea*	119
6.5	Physicochemical Parameters of *Boerhavia diffusa* leaves, *Plumeria Rubra* pods and seeds of *Celosia argentea*	130
6.6	Ultra – Violet analysis of *Boerhavia diffusa* leaves	131
6.7	Ultra – Violet analysis of *Plumeria Rubra* pods	131
6.8	Ultra – Violet analysis of *Celosia argentea* seeds	132
6.9	Qualitative Phytochemical Tests of *Boerhavia diffusa* leaves, *Plumeria Rubra pods and Celosia argentea* seeds	133
6.10	pH of Extracts	134
6.11	Phytochemical constituents *B. diffusa* HPTLC	135
6.12	HPTLC chromatogram of aqueous *Boerhavia diffusa* leavesextract at 254 nm	136
6.13	HPTLC chromatogram of *Boerhavia diffusa* leaves extract at 254 nm	137
6.14	Phytochemicals of *P.rubra*	139
6.15	HPTLC chromatogram of *Plumeria Rubra* seed pod extract	141
6.16	HPTLC chromatogram of *Plumeria Rubra* seed pod extract for Lupeol	143
6.17	HPTLC chromatogram of *Plumeria Rubra* Seed pod extract	145
6.18	Phytochemicals present in C. argentea	145
6.19	HPTLC chromatogram of *Celosia argentea* seeds extract	147
6.20	FTIR spectrum interpretation of aqueous extract of *Boerhavia diffusa* leaves	149

6.21	FTIR spectrum interpretation of aqueous extract of *Plumeria Rubra* pods	150
6.22	FTIR spectrum interpretation of aqueous extract of *Celosia argentea* seeds	151
6.23	Linear regression data for calibration plot	153
6.24	Intra-Day and Inter-Day Precision Results	154
6.25	LOD (limit of detection) and LOQ (limit of quantification)	155
6.26	Results of Accuracy	155
6.27	Robustness	156
6.28	In vitro kidney stone dissolution	158
6.29	Optimization of extract combination stone dissolution	158
6.30	The effect of plant extracts on cystine calculi	159
6.31	The effect of plant extracts on carbapatite calculi	160
6.32	The effect of plant extracts on uric acid calculi	160
6.33	Estimation of urine volume and pH	163
6.34	Estimation of calcium, oxalate, phosphorus and magnesium in urine	163
6.35	Estimation of Creatinine and uric acid in serum	164
6.36	Evaluation of Organoleptic Characteristics	174
6.37	Physicochemical evaluation of Polyherbal Granule formulation	177
6.38	Optimized solvent system	178
6.39	Phytochemicals found in Combination Extract	180
6.40	Phytochemicals found in polyherbal granule formulation	182
6.41	Physico-chemical parameters of Polyherbal granule Formulation subjected to stability studies	185

LIST OF FIGURES

Figure No.	Title of the Figure	Page No.
1.1	The overall mechanism of urinary stone formation	10
1.2	Pathophysiology of Kidney stone	15
1.3	Ultrasonography showing presence of Kidney stone. A: Bladder stone, B: left proximal ureter stone, C: Stone located at the pyeloureter junction, D: 5.6 mm large Kidney stone in soft tissue.	20
1.4	Mechanism of Kidney Stone Formation	21
1.5	Role of Parathyroid Hormone in Blood	24
1.6	Role of parathyroid hormone and calcitonin in management of calcium level in blood	25
5.1	A: Boerhavia diffusa Herb, B: Flowering twigs with seed	39
5.2	A: Fresh Plumeria Rubra pod; B: Dry Seed pod; C: Dry Seed pod	47
5.3	Celosia argentea Seeds	53
5.4	Standardization chart of herbal raw materials	69
5.5	Twin Trough chambers for TLC and HPTLC	81
5.6	HPTLC scanner of CAMAG showing TLC plate	81
5.7	Linomat Semiautomatic sampler for HPTLC plates HPTLC Conditions	82
5.8	Kidney stones collected from patient	89
5.9	Composition of Extract	90
5.10	Animal Ethical committee Certificate	92
5.11	Metabolic cages A: showing receiver for urine collection B: Arrangement of metabolic cages on stand	93

5.12	Microscopic analysis of urine was conducted across five groups	96
5.13	Blood removal from retro orbital plexus for serum estimation	100
5.14	Isolated kidneys in formalin solution	103
6.1	A: Authentication cerficate of *Boerhavia diffusa*; B: *Celosia argentea*; C: *Plumeria Rubra*	114
6.2	Soxhlet Extraction of *B. diffusa*	117
6.3	Soxhlet Extraction of *C. argentea*	118
6.4	Soxhlet Extraction of *P.rubra*	118
6.5	Extract of *Boerhavia diffusa* leaves *Celosia*	119
6.6	Extract of *argentea* seeds	119
6.7	Extract of *Plumeria Rubra* pods	119
6.8	*Boerhavia diffusa* leaves	120
6.9	*Plumeria rubra* seed pods	121
6.10	*Celosia argentea* Linn seeds	121
6.11	T. S. of *Boerhavia diffusa* leaf	122
6.12	Powder Characteristics of *Boerhavia diffusa* whole plant	123
6.13	T. S. *Plumeria Rubra* leaf	124
6.14	T. S. *Plumeria Rubra* seed pod shell	124
6.15	T. S. *Plumeria Rubra* seed pod showing medullary rays	125
6.16	Images showing seed of *Plumeria Rubra* showing monocotyledon and embryo	125
6.17	Powder Characteristics *Plumeria Rubra* seed pod powder	126
6.18	T. S. of Celosia argentea Linn seed cotyledon showing reticulate parenchyma with starch grains	127
6.19	T. S. of *Celosia argentea* Linn seed	128

6.20	Powder Characteristics of *Celosia argentea* Linn seed powder	129
6.21	UV Chamber for TLC visualization	131
6.22	Densitogram of *B. diffusa*	135
6.23	Image of *Boerhavia diffusa* leaves extract at 254 nm	136
6.24	Densitogram *B. diffusa* of gallic acid and Quercetin	137
6.25	A: Overlain of Galic acid, B: Overlain of Quercetin, C: Overlain of Galic acid+ Quercetin+ Extract, D:3D spectra of std.+ Extract of *Boerhavia diffusa* leaves extract at 254 nm	138
6.26	A: HPTLC densitogram of *Plumeria Rubra* seed pod extract at 500 nm, B: Densitogram of standard Ursolic acid and Oleic acid, C: Overlain spectra of Ursolic acid and Oleic acid over extract of *Plumeria Rubra*	141
6.27	Image of *Plumeria Rubra* seed pod extract at 254 nm and after derivatization	142
6.28	Image of *Plumeria Rubra* seed pod extract at 366 nm	142
6.29	Densitogram of *Plumeria Rubra* showing presence of Lupeol	143
6.30	3D spectra of Lupeol and *Plumeria Rubra* extract	144
6.31	Overlain spectra for Lupeol and *Plumeria Rubra* Extract extract	144
6.32	Densitogram *C. argentea*	146
6.33	HPTLC densitogram of Aq. Extract of *Celosia argentea* seeds extract at 430 nm	146
6.34	White Remission Derivative Image of *Celosia argentea* seeds	147

6.35	Image of slide of all three extract *Boerhavia diffusa* leaves, *Plumeria Rubra pods and Celosia argentea* seeds extract 254 nm	148
6.36	White Remission Image *Boerhavia diffusa* leaves, *Plumeria Rubra* pods and *Celosia argentea* seeds extract 366nm	148
6.37	I.R. Spectra of aqueous extract of *Boerhavia diffusa* leaves	149
6.38	I.R. Spectra of aqueous extract of *Plumeria Rubra* pods	150
6.39	I.R. Spectra of aqueous extract of *Celosia argentea* seeds	151
6.40	In situ HPTLC spectral of plant extract	152
6.41	Linearity of plant extract	153
6.42	Overlay spectra of Standard and Extract Precision	154
6.43	In vitro kidney stone dissolution method	157
6.44	The effect of plant extracts on cystine calculi	161
6.45	The effect of plant extracts on carbapatite calculi	161
6.46	The effect of plant extracts on uric acid calculi	162
6.47	Effect of PHF 200 and PHF 400 on Serum Calcium (mg/dl) in Ethylene induced Urolithiasis in Rats	165
6.48	Effect of PHF 200 and PHF 400 on Serum Phosphorus (mg/dl) in Ethylene induced Urolithiasis in Rats	166
6.49	Effect of PHF 200 and PHF 400 on Serum BUN (mg/dl) in Ethylene induced Urolithiasis in Rats	167
6.50	Effect of PHF 200 and PHF 400 on Serum Creatinine (mg/dl) in Ethylene induced Urolithiasis in Rats	168
6.51	Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats	169

6.52	Histopathological sections of Kidney showing Damage to renal architecture and it's recovery after the treatment of different extracts	171
6.53	Polyherbal Formulation	174
6.54	Polyherbal Granule	174
6.55	Powder microscopy of Granules	175
6.56	Densitogram and Chromatogram of Galic Acid marker compound	179
6.57	Chromatogram and Densitogram of Rutin marker	179
6.58	Chromatogram and densitogram of Quarcetin and Luteolin marker compound	180
6.59	Densitogram and chromatogram of combination B extract	181
6.60	Chromatogram of Galic acid, Rutin, Quarcetin and Luteolinin Polyherbal Formulation at 254nm	182
6.61	HPTLC finger print profile of Galic acid, Rutin, Quarcetine, Ursolic acid and Luteolin, Aqueous extract & Polyherbal Formulation at 254nm	183
6.62	HPTLC fingerprint profile of Galic acid, Rutin, Quarcetine, Ursolic acid and Luteolin, aqueous extract &Polyherbal Formulation at 366 nm	183
6.63	A: FTIR of extracts of three drugs; B: FTIR of extracts of three drugs with excipients Stability studies	185

LIST OF ABBREVIATIONS

CSIR	:	Council Of Scientific And Industrial Research
CT	:	Computerized Tomography
ESWL	:	Extracorporeal Shock Wave Lithotripsy
URS	:	Ureteroscopy
PCNL	:	Percutaneous Pithotripsy
TCM	:	Traditional Chinese Medicine
ESCOP	:	European Scientific Cooperative On Phytotherapy
GAG	:	Glycosaminoglycans
UTIs	:	Urinary Tract Infections
CaOx	:	Calcium Oxalate
RTA	:	Renal Tubular Acidosis
APRT	:	Adenine Phosphoribosyl Transferase
GSE	:	Grape Seed Extract
EG	:	Ethylene Glycol
AC	:	Ammonium Chloride
ABC	:	Aavarai Bhavanai Chooranam
TKGV	:	Trikantakadikvatha Ghan Vati
PHF-3	:	Polyherbal Formulation-3
BPH	:	Benign Prostatic Hyperplasia
SST	:	Sho-Sai-Ko-To
HPTLC	:	High-Performance Thin Layer Chromatographic
PCNL	:	Percutaneous Nephrolithotomy
ESWL	:	Extracorporeal Shockwave Lithotripsty
FURS	:	Flexible Urinoscopy
MDA	:	Malondialdehyde
E,E	:	Γ-Elemene
GC	:	Gas Chromatography

GCMS	:	Gc-Mass Spectrometry
NMR	:	Nuclear Magnetic Resonance
MS	:	Mass Spectroscopy
TNF-alpha	:	Tumor Necrosis Factoralpha
IL-1 beta	:	Interleukin-1 Beta
NO	:	Nitric Oxide
IFN-gamma	:	Gamma Interferon
TBARS	:	Thiobarbituric acid reactive substances
%RSD	:	Percent Relative Standard Deviation
LQC	:	Low Quality Control
MQC	:	Medium Quality Control
HQC	:	High Quality Control
LOD	:	Limit Of Detection
LOQ	:	Limit Of Quantification
ESWL	:	Extracorporeal Shock Wave Lithotripsy
B	:	Blank
S	:	Standard
T	:	Test
Abs.S	:	Absorbance Of The Standard
ANOVA	:	Analysis Of Variance

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 ROUTE OF DRUG ADMINISTRATION

The route through which a drug is given is typically categorized by the site of administration, such as orally or intravenously. The decision on which route to use is influenced by factors like convenience and patient adherence, as well as the drug's pharmacokinetic and pharmacodynamic properties.

1.1.1 Oral Route of Drug Administration:

This method involves placing the medication in the mouth and swallowing it. Also known as per oral, it is a convenient option for patients who are able to ingest and tolerate oral medications. Drugs with short half-lives can be administered orally in timed-release or sustained-release forms, allowing for gradual absorption over several hours. [1]

1.1.1.1 Advantages of the Oral Route

- **Convenient Administration:** Administering medication orally is straightforward and simple.
- **Patient Acceptance:** This method is widely accepted and preferred by patients.
- **Comprehensive Absorption:** Medication is absorbed throughout the entire gastrointestinal tract.
- **Cost-Effective:** Compared to many other parenteral methods, oral administration is generally more economical.

1.1.1.2 Disadvantages of the Oral Route

- **Inconsistent Absorption Rates:** The absorption of drugs can vary greatly.
- **Drug Degradation:** Some medications are broken down before they can be absorbed into the bloodstream.
- **Limited Permeability:** Many compounds cannot effectively cross the intestinal epithelial membrane to enter the bloodstream.
- **Low Solubility:** Many drugs are not soluble at the low pH levels found in the digestive tract.

- **Liver Inactivation:** Drugs can be inactivated by the liver before reaching systemic circulation.
- **Gastrointestinal Irritation:** Drugs can irritate the mucous lining of the gastrointestinal tract, although this can be mitigated somewhat by coating the drugs.

1.2 RESEARCH APPROACH TO HERBAL PRODUCTS:

Since they form the basis for all life on earth, plants hold a special place in the universe. In every food chain, they are the principal producers. 90% of the calories and 80% of the protein consumed by humans are directly derived from plants. Since the beginning of time, people have exploited plants as possible sources of medicine. The wealth of medicinal herbs originates from India, where people choose to use conventional medicine techniques. As a result, it is crucial that this vast natural resource be enhanced and used in line with the advancement of technology and human requirements.

The current millenium sets the objective of using nutraceuticals to provide treatment without adverse effects. The success of using various plants to treat ailments has increased interest in traditional medicine worldwide. For ages, the ethno-botanical practises of the people of Asia have included the use of medicinal herbs. Only after extensive chemical and pharmaceutical testing did the contemporary medical system diverge from folk medicine and the old medical system. Modern microbiological and chemical techniques can synthesis aromatic and therapeutic molecules, although the process is frequently expensive.

Many people opt for natural remedies from plants to avoid the potential side effects of synthetic drugs. It is estimated that around 20,000 plant species are utilized for medicinal purposes, as noted by Penos et al. [2] Furthermore, a study by Farnsworth et al. [3] in 1985 found that chemical research aimed at identifying the active components xresponsible for the traditional uses of 119 plant-based medications led to the discovery of these active ingredients in 74% of the cases.

The tropical, subtropical, and temperate species found in Asiatic flora serve as systems for herbal-based medicines and are crucial to the region's overall healthcare. According to Farnsworth et al., more than 80% of Asians still rely on traditional and folk treatments for their regular medical requirements. Of India's 1.1 billion people, more than 70% still use traditional herbal medication.[4] According to Scragg et al. [5], phytochemical investigation of plants that have historically been used to treat cancer has produced a variety of chemicals

having anticancer potential. Alkaloids with anti- HIV action have been found to accumulate in plants from many different families. Hussein disclosed a number of medicines and aromatic substances produced from plants [6] These include forskolin from Coleus root, which is used to prevent blood clotting and lower intraocular pressure in glaucoma cases, and anti-malarial drug from Artemisia annua. Forskolin helps the repair of damaged nerves after injury.

Reverse pharmacology, which stems from observational therapeutics, complements other methods in the development of natural drugs.

For a new to the area, the variety of plant usage in medicine can occasionally be overwhelming. However, the possibilities for research strategy offer strong motivation for the discovery of new pharmacophores for multidisciplinary research and a development network. New pharmacophores offer potential contributions to the field of herbal medicinal and preventative agents through the development of novel drug targets and the application of combinatorial chemistry. An example of this is seen in efforts to target curcumin chemically with combinatorial compounds. In India, the Council of Scientific and Industrial Research (CSIR) has initiated a nationwide network program aimed at creating herbal-based formulations for conditions such as diabetes, arthritis, and hepatitis, representing significant efforts in this area.[7]

1.3 DIAGNOSIS AND CONTEMPORARY THERAPEUTIC APPROACHES FOR THE TREATMENT AND MANAGEMENT OF URINARY STONES:

The process of diagnosing urinary stones is multifaceted, incorporating the patient's medical history, dietary patterns, complete blood cell counts, routine urine tests, and serum creatinine levels. Physical examinations are performed with a focus on symptoms commonly linked to urinary stone disease.[8] Furthermore, diagnostic procedures heavily rely on ultrasound and imaging techniques like X-rays and CT scans. Ultrasound is particularly advantageous because it can detect all types of kidney stones, including those that might not be visible on an X-ray. [9]

Urinary stones often pass spontaneously, prompting doctors to manage initial symptoms, primarily through pain relief. However, the rate at which stones pass varies significantly based on their size and location. If a stone cannot pass on its own, medical expulsive therapy is usually recommended. This approach may involve using antibiotics, calcium channel

blockers, pain relievers, and anti-inflammatory drugs. When conservative treatments are ineffective because of the size and complexity of the stones, or if the patient is unable to endure the pain while waiting for the stone to pass naturally, surgical intervention becomes necessary. [10-11]

Common surgical methods for treating kidney stones include extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), percutaneous nephrolithotomy (PCNL), and open surgery. Each of these techniques comes with its own set of advantages and drawbacks. The effectiveness of stone removal depends on various factors such as the patient's age, the size and location of the stones, the number of stones present, the radiological features of the kidneys, and any congenital abnormalities of the renal system. Moreover, both surgical and medical treatments must contend with the problem of stone recurrence. Research indicates that ESWL can lead to acute kidney injury due to the shockwaves and may also increase the risk of post-treatment infections. [12-15].

1.4 HERBAL MEDICINES FOR THE TREATMENT OF URINARY STONES

Numerous treatment plans for urinary stone disorders have been developed in recent decades. However, most of these treatments involve surgery, making them expensive and sometimes difficult to access. Consequently, many individuals opt for or are limited to using traditional herbal remedies. Herbal treatments for urinary stone issues are available in various traditional systems of medicine, including Ayurveda, Traditional Chinese Medicine (TCM), Siddha, and Unani.

Urolithiasis is considered a major health issue. In Ayurveda, urinary stones are known as "mutraashmari" (with "mutra" meaning urine and "ashma" meaning stone). Ayurvedic literature identifies four categories of urinary stones: phosphatic stones (sleshmaashmari), urate stones (pittaashmari), oxalate stones (vataashmari), and seminal concretions (sukraashmari). Ayurvedic treatment approaches for managing urinary stones include herbal remedies, alkaline drinks, and various surgical techniques.

Ayurveda offers various therapies for managing urinary stone diseases, including purification treatments (Shodhana) involving external and internal oleation and sweating induction, as well as alleviation therapies (Shamana). Additionally, Ayurveda recommends panchakarma procedures such as medicated emesis, purgation, and enemas to address these

conditions. This approach primarily involves the oral administration of herbal medications, including mutraladravyas (diuretics), ashmaribhedana (lithotriptics), and teekshnaushna (penetratives).[16].

Table No. 1.1: Medicinal Plants Used in Ayurveda to Cure Kidney Stones

Sr. No.	Botanical name and family	Sanskrit name	Part used	Dose/mode preparation
1.	*Aervalanata* (L.) Juss. (Amaranthaceae)	Gorakshaganja, Astmabayda, Bhadra, Pashanabheda, Pattura	Whole plant	Whole plant
2.	*Anisomelesma labarica (*L.) R. Br. ex-Sims (Lamiaceae)	Sprkka	Whole plant	3-5g, powder
3.	*Anogeissuslatifolia* Wall. Ex Guillem & Perr. (Combretaceae)	Dhava	Stem bark	30-50 ml, decoction
4.	*Apiumgraveolens* L. (Apiaceae)	Karaphsa	Root	5-7g, powder
5.	*Asparagus officinalis* L. (Liliaceae)	Dvipantara	Root	3-6g, powder
6.	*Baliospermm solanifolium* (Burm.) (Euphorbiaceae)	Satavari Hastidanti	Root	1-3g, powder
7.	*Benincasahispida* (Thunb.) Cogn. (C ucurbitaceae)	Kushmand	Fruits	5-10g, powder
8.	*Bergeniacilliata* (Haw.) Sternb. (Saxifragaceae)	Asmabhedaka	Rhizome	3-6g, powder

9.	*Buteamonosperma* (Lam.) Taub. (Lam.)(Leguminosae)	Palasah	Seed	0.5-1g, powder
10.	*Calamusrotang* L. (Arecaceae)	Vetra	Rhizome	50-100 ml, decoction
11.	*Caricapapaya* L. (Caricaceae)	Erandkarkati	Root	5-10g, powder

1.5 ROLE OF WHO IN HERBAL MEDICINE:

The World Health Organization (WHO) acknowledges the crucial contribution of traditional medicine to healthcare. In line with this recognition, the U.S. Congress established the Office of Alternative Medicine within the National Institutes of Health in 1989 to encourage scientific exploration of traditional medicine. That same year, the European Scientific Cooperative on Phytotherapy (ESCOP) was created to enhance the scientific validation and standardization of herbal medicines across Europe. These efforts resulted in increased funding for the study of herbal medicines. For example, in the fiscal year 2005, the National Centre for Complementary and Alternative Medicine at the U.S. National Institutes of Health dedicated about US$33 million to herbal medicine research. Similarly, in 2004, the National Canadian Institute invested close to US$89 million to investigate various traditional therapies. This funding, while modest compared to pharmaceutical industry budgets, highlights significant interest and support from the public, industry, and government in this field.[17]

The significant growth in global interest and utilization of traditional medicines presents two primary challenges. These challenges revolve around international diversity and national policies concerning the regulation, production, and usage of herbs and other complementary medicines. Moreover, they encompass ensuring the quality, safety, and scientific validation of health claims associated with these medicines. [18]

1.5.1 Global Herbal Market And Current Scenario:

The global herbal market is estimated to be worth approximately $62.0 billion. India's current market contribution stands at around $1 billion. Further details regarding India's

position in the global market can be found in Annexure 5. The European Union holds the largest market share in the herbal sector, accounting for 45% of the total global market.

The global market for herbal medicine reached a valuation of USD 151.91 billion in 2021. It is expected to grow from USD 168.86 billion in 2022 to around USD 437.59 billion by 2030, with a projected compound annual growth rate (CAGR) of 11.16% from 2023 to 2030.

Over the past decade, the popularity of herbal medicines and other alternatives to antibiotics has significantly increased. Both patients and healthcare providers are showing heightened interest in herbal products. According to the WHO, 10–50% of individuals in developed countries regularly use herbal products in some form. The primary appeal of herbal products lies in their ability to help the body combat illnesses more effectively than synthetic drugs. In developing countries such as China, Japan, India, Vietnam, South Africa, and Bangladesh, herbal medicines often serve as the only affordable treatment option. These remedies are commonly used to address conditions like coughs, colds, anxiety, gastrointestinal issues, and painful ailments such as joint pain, rheumatism, and stiffness. With the rising popularity of herbal supplements, the market is likely poised for significant growth.

Herbal medicines are increasingly sought after in developing countries for primary health care. This demand arises not only due to their affordability but also because they are culturally accepted, compatible with the human body, and have minimal side effects.

1.6 INTRODUCTION TO LITHOLYTIC ACTIVITY:

Urolithiasis, the formation of urinary stones anywhere in the renal system, is a longstanding chronic health condition documented throughout human history. Traditional medical systems like Ayurveda, Traditional Chinese Medicine (TCM), Siddha, and Unani include extensive literature detailing symptoms, indicators, and treatment approaches for urinary stone diseases. According to Ayurveda, urolithiasis ranks among the eight most challenging disorders. Urinary stone treatment in Ayurveda includes herbal formulae, alkaline drinks, and surgical methods. While, TCM advice using a combination of acupuncture, mexibustion, and multiherbal medications to treat urinary stones. Herbal treatments are still used today to treat and cure urinary stone illnesses among these medicines.

Urinary stones, medically known as calculi, are solid crystalline masses that can develop anywhere within the urinary tract. The condition of stone formation in this tract is termed urolithiasis. The formation of urinary stones typically occurs due to a reduction in natural inhibitors that prevent stone formation in urine or when urine becomes oversaturated with salts. Several environmental and dietary factors, such as low urine volume and diets high in animal protein, contribute significantly to urolithiasis. Metabolic changes like hypercalciuria and hyperuricosuria, as well as deficiencies in nutrients that inhibit stone formation such as citrate, magnesium, and glycosaminoglycans (GAG), also play a crucial role in promoting stone development [19]. Preventing urolithiasis can be challenging since it often manifests without symptoms and may remain unnoticed until it progresses significantly, particularly in cases of recurrence. Small urinary stones can pass spontaneously through urine, with reported spontaneous passage rates of 68% for stones 5 mm or smaller, and 47% for stones larger than 10 mm. [20]

Ancient medical texts such as those from Ayurveda, Chinese traditional medicine, and Greek medicine contain detailed descriptions of urinary stone symptoms, warning signs, and treatments. Common symptoms of urinary stone disease include pain in the back or lower abdomen, presence of blood in the urine, and discomfort during urination. Additionally, individuals may experience nausea, vomiting, and waves of pain that originate in the abdomen, often radiating to the groin, testicles, or vulva, and typically subsiding within 20 to 60 minutes. This type of pain is clinically referred to as renal colic.

Dietary modifications and stone expulsive therapy often prove ineffective for many urinary stone patients due to the size of the stones or their location within the urinary tract. Consequently, these patients often require treatment with advanced interventional techniques. However, evaluating and selecting the most suitable interventional techniques can be challenging, particularly for patients prone to recurrent urinary stones. Alternatively, traditional herbal remedies have been identified as effective, accessible, and cost-effective options. Despite their long history of use and efficacy, widespread adoption of these herbal medications remains hindered, possibly due to inadequate scientific validation and support.[21]

1.6.1 Factors Responsible for formation of Urinary Stone

Urolithiasis has been a significant health concern for many years, yet our understanding of the underlying processes leading to urinary stone formation remains limited. A comprehensive comprehension of these mechanisms is essential for effective patient management, aiming to reduce both the morbidity associated with urolithiasis and related medical costs. Conventionally, it was believed that urinary stones arise from the super saturation of urine and subsequent crystallization of minerals. However, the formation of stones in the urinary tract is not solely attributed to the accumulation of stone salts in urine. The process of urolithiasis is intricate, involving stages such as crystal nucleation, aggregation, retention of crystals by the urothelium, urine saturation, super saturation, and the gradual growth of stones around retained crystals.[22]

It has been found that dietary habits, especially those contributing to metabolic syndrome or inherent abnormalities, are responsible for urine saturation or supersaturation.[20] Many epidemiological and metabolic investigations indicate that dietary habits contribute to the prevalence of urinary stones, influencing the composition of urine. Key dietary factors linked to increased risk include higher protein consumption, elevated intake of salt or oxalate, and reduced calcium intake. [23] Overconsumption of animal proteins in the diet can lead to problems with renal ammonia genesis and/or excretion. This can impair buffering capacity and exacerbate acidic urine due to increased acid excretion. Moreover, independent studies have shown a direct link between fructose consumption and a higher incidence of urinary stones. [24] Individuals with nephrolithiasis and comorbidities such as type II diabetes, obesity, or metabolic syndrome are at increased risk of developing uric acid stones.

1.6.2 Mechanism of Urolithiasis: Crystallization and Retention of Stones:

Urolithiasis becomes medically important when tiny stone fragments remain in the urinary tract and develop into larger stones.[25] The formation of urinary stones includes three main stages: urine oversaturation, crystallization, and the retention of stones within the urinary system.

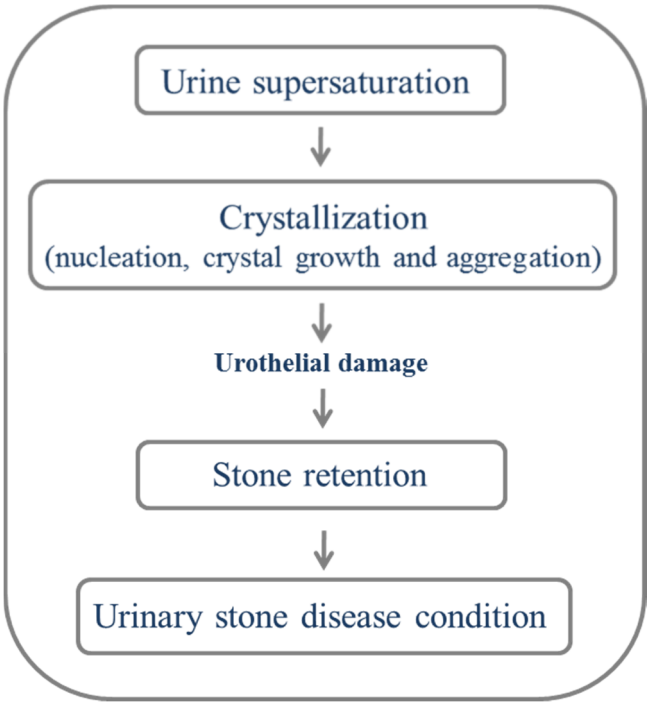


Figure No. 1.1: The overall mechanism of urinary stone formation

1.7 VARIOUS DISORDERS OF KIDNEY:

1.7.1 Chronic kidney diseases:

The most prevalent type of kidney disease is chronic kidney disease (CKD), which is a persistent condition that does not ameliorate with time. It is frequently linked to hypertension, or high blood pressure.

High blood pressure poses a significant risk to kidney health by increasing pressure on the glomeruli, the tiny blood vessels responsible for filtering blood in the kidneys. This sustained pressure can lead to damage over time, causing a gradual decline in kidney function. Eventually, kidney function may deteriorate to the extent that the kidneys are no longer able to adequately perform their functions. At this stage, individuals may require dialysis, a treatment that filters excess fluid and waste products from the blood. While dialysis can assist in managing kidney disease, it is not a cure. Depending on individual circumstances, a kidney transplant may be considered as another treatment option.

Diabetes significantly contributes to chronic kidney disease. It is characterized by elevated blood sugar levels, which gradually impair the blood vessels in the kidneys. Consequently, the kidneys lose their ability to filter blood efficiently, potentially leading to kidney failure due to toxin buildup in the body.[26]

1.7.2 Kidney Stone

Kidney stones represent a frequently encountered kidney issue. These stones form as a result of minerals and other compounds in the bloodstream crystallizing within the kidneys, resulting in the formation of solid masses. Typically, kidney stones exit the body during urination. Although passing kidney stones can be excruciatingly painful, they seldom lead to substantial complications.

Signs and Symptoms

The primary indication of a stone obstructing the ureter or renal pelvis is intense, sporadic pain that radiates from the flank to the groin or inner thigh.[27] This pain occurs due to referred signals from lower thoracic splanchnic nerves to lumbar splanchnic nerves during the passage of the stone from the kidney or proximal ureter to the distal ureter. This sensation known [28] as renal colic, this pain is often described as one of the most severe sensations of pain. Alongside pain, symptoms typically include urinary urgency, restlessness, hematuria, sweating, nausea, and vomiting. The pain recurs in waves lasting 20 to 60 minutes due to peristaltic contractions of the ureter as it attempts to expel the stone.[27]

The anatomical relationship between the urinary tract, reproductive system, and gastrointestinal tract contributes to the referral of pain to the genitals and the occurrence of nausea and vomiting frequently observed in urolithiasis cases.[29] Obstruction of urine flow through one or both ureters can lead to postrenal azotemia and hydronephrosis.[30]

Pain in the lower-left quadrant may resemble diverticulitis due to the overlap of the sigmoid colon and ureter in this region, posing challenges in precise localization of pain.

Risk Factors

Dehydration resulting from insufficient fluid intake significantly contributes to the formation of kidney stones.[27,31] Individuals living in warmer regions are particularly susceptible due to increased fluid loss. Factors that elevate the risk include being overweight, leading a sedentary life, and reduced mobility. [32] Consuming diets high in animal proteins[27], salt, sugars (such as honey, refined sugars, fructose, and high fructose corn syrup) [33] , and large amounts of fruit juice can contribute to kidney stone development by increasing the excretion of uric acid and urinary oxalate. Conversely, beverages like tea, coffee, wine, and beer may lower this risk. Several metabolic disorders, including distal renal tubular acidosis[34], Dent's disease[35] , hyperparathyroidism[36],

primary hyperoxaluria [37], and medullary sponge kidney[38,39], can also cause kidney stones. Medullary sponge kidney affects about 3-20% of individuals prone to developing kidney stones.[38-39] People with Crohn's disease are more susceptible to kidney stones due to hyperoxaluria and magnesium malabsorption associated with the condition.[40-41] Individuals experiencing recurrent kidney stones should undergo screening for these disorders, typically involving a 24-hour urine collection to analyze factors contributing to stone formation.[30]

1.7.3 Glomerulonephritis:

Glomerulonephritis involves the inflammation of glomeruli, small kidney structures crucial for blood filtration. This condition may develop due to factors such as infections, medications, or congenital issues. Often, glomerulonephritis resolves on its own.[42]

1.7.4 Polycystic Kidney Disease:

Polycystic kidney disease (PKD) is an inherited disorder characterized by the formation of multiple fluid-filled cysts in the kidneys. While individual kidney cysts are generally benign and pose little threat, PKD significantly impairs kidney function and can lead to kidney failure. It is crucial to distinguish between solitary kidney cysts, which are usually harmless, and PKD, a more severe condition with serious implications for kidney health.

1.7.5 Urinary tract infections (UTIs):

Urinary tract infections are bacterial infections affecting any portion of the urinary system, with the bladder and urethra being the most frequently affected areas. These infections are typically straightforward to treat and seldom result in further health complications. Nevertheless, untreated cases can escalate, potentially spreading to the kidneys and triggering kidney failure**.**

1.8 TYPE OF KIDNEY STONE:

Kidney stones primarily consist of four major types: calcium stones (comprising 7585% of cases), struvite stones (2-15%), uric acid stones (6-10%), and cystine stones (1-2%). The prevalence and distribution of these stones vary depending on geographical location and the demographics of the population studied. In rare cases, long-term medication use can also contribute to the formation of kidney stones, accounting for approximately 1% of cases. [43].

1.8.1 Calcium Stones:

Calcium stones such as calcium oxalate, calcium urate, and calcium phosphate are linked to hypercalciuria, which can be caused by conditions such as hyperparathyroidismDiseases that enhance calcium absorption from the intestine can result in the kidneys excreting more calcium or phosphate. This condition is often accompanied by elevated levels of uric acid, oxalate, and reduced levels of citrate and magnesium in the urine.[44].

Calcium stones typically consist of calcium oxalate, often mixed with calcium phosphate or calcium urate.[45] Factors contributing to the formation of these stones include elevated calcium levels in the urine (hypercalciuria), reduced urine volume, and low levels of citrate in the urine (hypocitraturia). Hypercalciuria is frequently linked to conditions causing elevated blood calcium levels, such as hyperparathyroidism, certain cancers, sarcoidosis, and high vitamin D intake.[46] When no specific cause is identified, it is termed "idiopathic hypercalciuria," a condition that often runs in families and is influenced by multiple genetic factors, though rare single-gene disorders like Dent's disease can also cause it. This disease is characterized by hypercalciuria, kidney calcification (nephrocalcinosis), and potential kidney failure. Furthermore, alkaline urine can increase the risk of calcium phosphate stone development [47]. Hyperoxaluria, which can result from bowel diseases (enteric hyperoxaluria) or genetic disorders affecting oxalate metabolism (primary hyperoxaluria), also raises the risk of calcium oxalate stone formation.[48]

Dietary oxalate plays a crucial role in kidney stone formation. Foods like spinach, beets, and rhubarb are rich sources of oxalate, which can increase urinary oxalate excretion and raise the risk of developing calcium oxalate stones. Furthermore, excessive intake of vitamin C can lead to increased oxalate production since ascorbic acid is metabolized into oxalate. Calcium can reduce oxalate absorption in the colon by forming insoluble calcium oxalate complexes. [49]

1.8.2 Struvite Stones:

Struvite stones, alternatively referred to as triple phosphate stones or infection stones, arise from upper urinary tract infections triggered by bacteria such as Proteus and Klebsiella that produce urease. Under normal circumstances, urine does not contain high levels of ammonium phosphate. However, struvite stones develop due to heightened ammonia production and an increased urine pH, which lowers phosphate solubility. The presence of

bacterial urease is crucial in struvite stone formation because it raises levels of ammonium and carbonate ions while simultaneously increasing urinary pH.

Struvite stones are composed of magnesium ammonium phosphate and have the potential to grow large enough to occupy the renal collecting system, forming partial or complete staghorn calculi. The formation of these stones is associated with persistent urinary tract infections caused by Gram-negative bacteria that produce urease, including species like Proteus, Pseudomonas, and Klebsiella.[50]

1.8.3 Uric acid stones:

Uric acid stones form due to factors such as high purine intake from diet or medications, and conditions involving rapid cell turnover like malignancy, often seen in patients with gout. These stones typically develop in slightly acidic urine with a pH around 5.5 and are generally not visible on X-ray due to their radiolucent nature. Instead, they can be detected using ultrasound or computed tomography (CT) scans.

Pure uric acid calculi are radiolucent on plain radiographs but visible on ultrasound or CT scans, and they tend to occur in individuals with hyperuricosuria. About 1520% of patients with uric acid stones have a history of gout. A diet high in animal protein, which increases uric acid production during catabolism due to its high purine content, can elevate the risk of uric acid stone formation. Uric acid solubility decreases significantly when urinary pH is below 5.5 but improves above 6.5.[51-53]

1.8.4 Cystine Stones:

Cystine stones are caused by cystinuria, an inherited metabolic disorder affecting the renal tubule's ability to reabsorb cystine.[54] These stones can be challenging to detect on Cystinuria is a rare genetic disorder, estimated to affect approximately 1 in 15,000 adults in the United States, and it represents only about 1% of all cases of nephrolithiasis. It follows an autosomal recessive inheritance pattern and is characterized by excessive excretion of cystine in the urine due to abnormal transport in the renal tubules. Symptoms typically appear as early as the first decade of life and can continue into the fourth decade. These symptoms often include the formation of kidney stones, which tend to be large, numerous, and can affect both kidneys. Diagnosis involves identifying hexagonal crystals in the urine. Patients commonly experience complications such as urinary tract infections,

kidney stone-related obstructions, and recurrent stone formation occurring every 1 to 4 years.[55-56]

1.8.5 Drug-Induced Stones:

Some medications have been linked to the formation of renal stones, which may serve therapeutic purposes in other diseases. Examples of these medications include indinavir, atazanavir, guaifenesin, triamterene, silicate-containing antacids, and sulfa drugs. Although rare, these stones are typically identifiable on X-rays.[56]

1.9 PATHOPHYSIOLOGY:

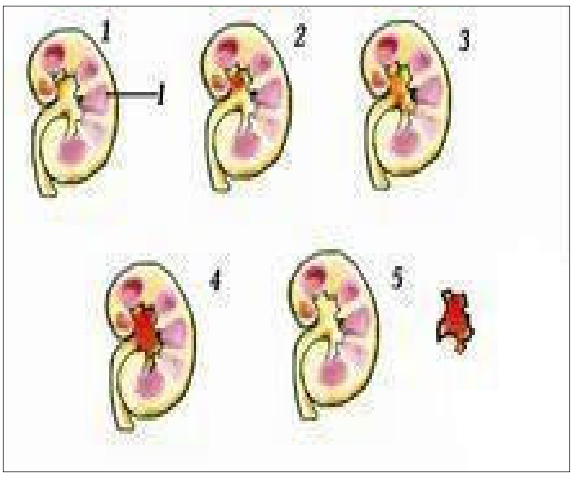


Figure No. 1.2: Pathophysiology of Kidney stone

Kidney stones, typically composed of calcium oxalate crystals measuring around 4–5 mm in size, form within the kidneys. Larger stones, known as staghorn kidney stones, can grow considerably bigger.

- The process begins with calcium and oxalate combining to form the initial crystal nucleus, facilitated by conditions of supersaturation or inhibition.
- These crystals then continue to accumulate at the renal papillae, leading to the gradual growth of kidney stones.
- As kidney stones increase in size, they can accumulate debris. Severe cases involve stones blocking all paths to the renal papillae, causing intense pain and discomfort.
- Complete staghorn stones can form, causing retention. Small fragments breaking off may get lodged in urinary ducts, contributing to further discomfort.

- Stones that dislodge may travel through the ureter. If they cannot be naturally fragmented, surgical intervention becomes necessary for their removal.

1.9.1 Supersaturation of urine:

When urine contains excessive amounts of substances that can form crystals, such as calculogenic substances, tiny crystals known as seed crystals may develop through nucleation. Heterogeneous nucleation, which occurs on solid surfaces, typically progresses more rapidly than homogeneous nucleation, which occurs within a liquid medium without solid surfaces, due to lower energy requirements. Once these seed crystals adhere to cells on the surface of a renal papilla, they can grow and accumulate, eventually forming a structured mass. The rate of stone formation can vary based on the pH of the urine, which may be unusually high or low depending on the chemical composition of the crystals.[57]

The likelihood of uric acid dissolving in urine, and thus the potential for uric-acid stone development, is greatly influenced by the pH level of the urine. For example, when the urine pH is 7.0, uric acid solubility is around 158 mg per 100 mL. However, if the pH drops to 5.0, the solubility significantly decreases to less than 8 mg per 100 mL. This means that the formation of uric-acid stones requires both high levels of uric acid in the urine (hyperuricosuria) and a low urine pH; high uric acid levels alone are not sufficient if the urine pH is not acidic. It's essential to understand that while urine supersaturation is necessary, it is not the only factor needed to cause urinary stones. Although supersaturation is a key factor in the formation of uric acid and cystine stones, calcium-based stones, such as calcium oxalate stones, may have more intricate causes.[58-59]

1.9.2 Randall's plaque:

Randall's plaque, first identified by Alexander Randall in 1937, is composed of calcium phosphate deposits located in the papillary interstitium. These plaques are thought to act as the initial site (nidus) for the development of kidney stones. Although urine supersaturation can lead to crystalluria, it does not inevitably lead to stone formation because the crystals may not attain the necessary size for attachment within the kidneys[60][61]. Furthermore, Randall's plugs can develop within the Duct of Bellini, and this, along with Randall's plaques, can contribute to the formation of stones by producing reactive oxygen species.[62-64]

1.9.3 Pathogenic bacteria:

Some types of bacteria play a role in the development of stones in the urinary tract. For example, Proteus mirabilis, which is urease-positive, generates an enzyme called urease. This enzyme transforms urea into ammonia and carbon dioxide [65], leading to an increase in urinary pH levels. The higher pH levels create a favorable environment for the formation of struvite stones. Moreover, bacteria that do not produce urease can supply components that facilitate the crystallization of calcium oxalate, although the exact mechanism behind this phenomenon remains unclear. [66][67]

1.9.4 Inhibitors of stone formation:

Normal urine contains chelating agents such as citrate, which help prevent the formation and aggregation of calcium-based crystals. Other inhibitors found naturally in urine include calgranulin, a calcium-binding protein from the S-100 family; Tamm-Horsfall protein; glycosaminoglycans; uropontin (a form of osteopontin); nephrocalcin, an acidic glycoprotein; prothrombin F1 peptide; and bikunin, which is rich in uronic acid. Although the precise biochemical pathways through which these substances act are not entirely clear, a deficiency in these inhibitors can lead to crystal aggregation and kidney stone formation.[68] Ensuring adequate intake of magnesium and citrate through the diet can help prevent the development of calcium oxalate and calcium phosphate stones. Additionally, magnesium and citrate work together to inhibit kidney stone formation, though the effectiveness of magnesium in reducing stone formation and growth depends on the administered dosage.[69]

1.9.5 Hypocitraturia:

Hypocitraturia, a condition where urinary citrate excretion is low (commonly defined as under 320 mg/day), is a contributing factor in many kidney stone cases. Citrate is beneficial in preventing stone formation by decreasing the urinary supersaturation of calcium salts through the formation of soluble complexes with calcium ions and by inhibiting the growth and aggregation of crystals. To increase urinary citrate levels and reduce the risk of kidney stones, potassium citrate therapy is often prescribed. Additionally, alkali citrate, which comes in pill, liquid, or powder forms, can be used to boost urinary citrate levels and is available both by prescription and over-the-counter.[70][71]

1.10 DIAGNOSIS:

Diagnosis of kidney stones relies on information gathered from the patient's medical history, physical examination, urinalysis, and radiographic studies. Typically, clinical diagnosis hinges on assessing the pain's characteristics, often described as colicky and intermittent, to determine its location and severity. Pain in the back can indicate obstruction caused by calculi in the kidney. Moreover, during a physical examination, clinicians may observe symptoms such as elevated body temperature and sensitivity around the costovertebral angle on the affected side.[72-74]

1.10.1 Imaging studies:

Calcium-containing stones are relatively radiodense and can often be identified using a traditional radiograph of the abdomen, which includes imaging of the kidneys, ureters, and bladder (KUB film). However, while a KUB radiograph is valuable for monitoring stone size or passage in stone formers, its sensitivity in acute settings may be limited. Approximately 60% of all renal stones are radiopaque. Calcium phosphate stones typically exhibit the highest density, followed by calcium oxalate and magnesium ammonium phosphate stones. In contrast, cystine calculi are minimally radiodense, whereas uric acid stones are generally radiolucent.. [75-79]

People under 50 years old who have a previous history of kidney stones and symptoms indicating their presence, but lacking alarming signs, generally do not require a helical CT scan for imaging.[80].

Moreover, CT scans are typically discouraged for pediatric patients [81]. Nonetheless, in cases where imaging is necessary, a non-contrast helical CT scan with 5-millimeter (0.2-inch) sections is preferred for diagnosing kidney stones and confirming kidney stone disease. Most types of kidney stones are detectable via CT scans, except for those composed of specific drug residues in urine, like indinavir.

When a CT scan is unavailable, an intravenous pyelogram (IVP) can be performed to confirm the diagnosis of urolithiasis. This procedure involves injecting a contrast agent into a vein, followed by obtaining a KUB (kidneys, ureters, bladder) radiograph. The contrast agent improves the visibility of uroliths in the kidneys, ureters, or bladder. Alternatively, stones can be identified using a retrograde pyelogram, where a similar contrast agent is

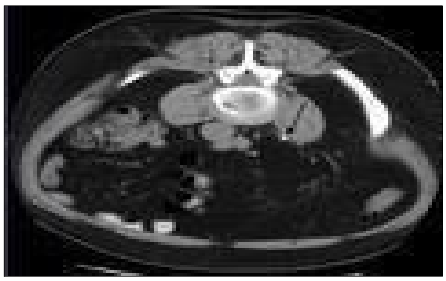
injected directly into the distal ostium of the ureter, the point where the ureter enters the bladder. [82]

Renal ultrasonography can be beneficial in clinical practice due to its ability to provide insights into the presence of hydronephrosis, indicating potential obstruction of urine flow by stones. Unlike KUB, ultrasound imaging can detect radiolucent stones that are otherwise invisible on X-rays. Additionally, renal ultrasonography is advantageous because it is cost-effective and does not expose patients to radiation. It is particularly valuable in populations where radiation exposure is a concern, such as children and pregnant women. In 2009, renal ultrasonography was not considered a replacement for noncontrast helical CT scans in the initial assessment of urolithiasis. This limitation arises because ultrasound may be less effective than CT scans in detecting small stones, especially in the ureters, and may fail to identify other significant underlying conditions that could be causing the symptoms. [83-85]

In contrast, according to a 2014 study, ultrasonography was recommended as the primary diagnostic imaging test. Additional imaging studies should be conducted based on clinical judgment by the physician. Opting for ultrasonography over CT scans initially leads to reduced radiation exposure with comparable clinical outcomes.[86]

(A)

This KUB radiograph reveals kidney stones on both sides. Additionally, there are phleboliths located in the pelvis, which might be mistaken for bladder stones.

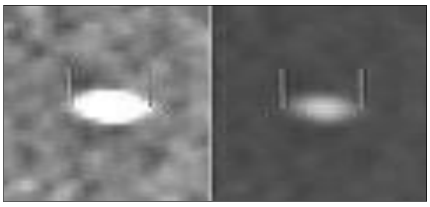


(B)

An axial CT scan of the abdomen without contrast reveals a 3-mm stone located in the left proximal ureter, clearly indicated by an arrow.

(C)

Renal ultrasound revealed a stone positioned at the junction between the renal pelvis and the ureter, alongside signs of hydronephrosis.



(D)

Detection of a kidney stone measuring 5.6 mm using soft tissue rather than skeletal CT window.

Figure No. 1.3: Ultrasonography showing presence of Kidney stone. A: Bladder stone, B: left proximal ureter stone, C: Stone located at the pyeloureter junction, D: 5.6 mm large Kidney stone in soft tissue.

1.11 KIDNEY STONE COMPOSITION AND RECURRENCE:

Calcium-containing stones are the predominant type of kidney stone worldwide [84]. In Olmsted County, Minnesota, a study found that calcium stones constituted 94% of all kidney stone cases, with 76% primarily composed of calcium oxalate, 18% of calcium phosphate, 5% uric acid stones, 1% struvite magnesium ammonium phosphate, and 0.1% cystine stones [85]. Similar trends were noted in Germany, where an analysis of 45,783 urinary stones from 2007 to 2020 identified calcium oxalate (CaOx) as the most prevalent type at 71.4%, followed by calcium phosphate at 10.2%, and uric acid at 8.3%.[86]

Uric acid nephrolithiasis constitutes approximately 8–10% of kidney stone cases and its prevalence is increasing globally. It is frequently observed in individuals who are obese or have metabolic syndrome. This rise in incidence correlates with the increasing rates of metabolic syndrome, obesity, and diabetes worldwide. Uric acid stones are more prevalent

among older adults. Hyperuricosuria is not the primary cause; instead, it is the acidity of the urine that plays a crucial role. [87].

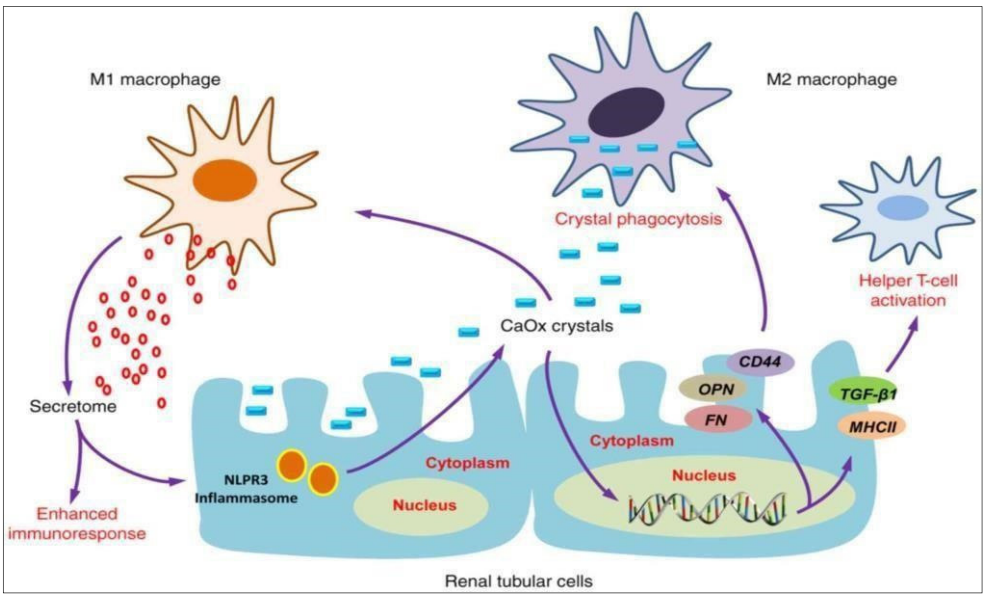


Figure No. 1.4: Mechanism of Kidney Stone Formation

In a retrospective study conducted at a major stone center in the United States, researchers observed a significant rise in the prevalence of uric acid stones among 1516 patients between 1980 and 2015. Initially accounting for 7% of kidney stones, uric acid stones increased to 14% during this period. The study found that individuals with uric acid stones tended to be older, had higher BMI, and lower urinary pH compared to those with calcium stones [88].

Another study across seven states in the US, involving 4339 kidney stone cases, reported a 12% incidence of uric acid stones. It noted that stone composition varied minimally between regions except for Florida [47].

Norwegian surgical data over four decades revealed an increase in uric acid stones from 2.0% to 9.1% during the 2014–2017 period [89,90]. Struvite stones, which are associated with infection due to urea-splitting bacteria, constitute 7–8% of kidney stones worldwide [91].

Certain rare inherited metabolic disorders, such as cystinuria, primary hyperoxaluria, distal renal tubular acidosis (RTA), xanthinuria, Lesch–Nyhan syndrome, Dent disease, and adenine phosphoribosyltransferase (APRT) deficiency, frequently manifest as kidney stones in early childhood and pose a significant risk of recurrence.

Cystinuria, the most prevalent among rare monogenic disorders, causes approximately 6% to 8% of kidney stones in children and 1% to 2% in adults [92]. Primary hyperoxalurias, estimated to affect about 1 to 3 individuals per million population, are crucial to identify [93]. Left untreated, these conditions can lead to recurrent kidney stones, especially in primary hyperoxaluria type 1, which may progress to kidney failure. Currently, epidemiological data highlight uric acid and other rare stones as posing the highest risk for symptomatic recurrence, underscoring the importance of understanding stone composition for effective disease prevention.

Stone recurrence is broadly defined to encompass both symptomatic stones that are clinically evident and asymptomatic changes detected through radiographic imaging of kidney stone burden [94] There has been inconsistency in terminology and classification in this field, alongside varying reported rates of recurrence. Earlier studies have suggested recurrence rates for nephrolithiasis as high as 50%.[95, 96] A community-based study conducted in Minnesota and Florida with a 5-year follow-up found that 19% of patients experienced symptomatic recurrence, while 25% reported recurrence, albeit self-reported. Radiographic evidence revealed asymptomatic recurrence, including new stone formation in 35% of patients, stone growth in 24%, and spontaneous stone passage in 27%.[97] The study proposed a novel "rule of halves" based on its findings: "half of initial symptomatic stone formers have an underlying asymptomatic kidney stone at baseline, half of whom will pass the stone within the next 5 years, and half of these will experience symptoms (pain or gross hematuria) during stone passage.[98].

In a study of symptomatic kidney stone recurrence in Olmsted County, Minnesota, the rates per 100 person-years following initial episodes were as follows: 3.4 (95% CI, 3.2–3.7) after the first episode, 7.1 (95% CI, 6.4–7.9) after the second episode, 12.1 (95% CI, 10.3–13.9) after the third episode, and 17.6 (95% CI, 15.1–20.0) after the fourth or subsequent episodes ($p < 0.001$ for trend). Several independent risk factors for increased recurrence were identified, including younger age, male sex, higher body mass index, family history of stones, pregnancy, and a history of brushite, struvite, or uric acid stones [99].

A systematic review encompassing twenty-one studies from 1976 to 2011 reported a median five-year recurrence rate of 26% among first-time kidney stone formers [100]. Furthermore, a comprehensive meta-analysis that included 40 retrospective and 13 prospective studies involving 488,130 patients identified twelve risk factors associated with kidney stone

disease recurrence: younger age, higher BMI, family history of kidney stones, personal history of kidney stones, hypertension, uric acid stones, white race, suspected prior kidney stone episode, previous surgery, presence of concurrent asymptomatic stones, presence of pelvic or lower pole kidney stones, and completion of a 24-hour urine test [101-102]

1.12 ROLE OF HERBAL MEDICINES IN MANAGEMENT OF KIDNEY STONE:

Urolithiasis is recognized as one of the eight major health issues, and in Ayurveda, urinary stones are commonly referred to as mutraashamari (mutra for urine, ashma for stone, and ari for enemy). Ayurvedic texts describe four main types of urinary calculi: phosphatic stones (sleshmaashmari), urate stones (pittaashmari), oxalate stones (vataashmari), and spermoliths or seminal concretions (sukraashmari). Ayurvedic medicine employs herbal remedies, alkaline beverages, and surgical techniques to effectively treat and manage urinary stones.

To address urinary stone illnesses, Ayurvedic treatments typically include Shodhana therapies (involving external and internal oleation and induction of sweating), Shamana therapy, and Panchakarma procedures like medicated emesis, purgation, and enemas. These treatments involve the oral administration of herbal medicines such as mutraladravyas (diuretics), ashmaribhedana (lithotriptics), and teekshnaushna (penetrative).[103]

1.13 ROLE OF PARATHYROID HORMONE AND CALCITONIN IN MANAGEMENT OF CALCIUM LEVEL IN BLOOD AND URINE:

Calcitonin plays a crucial role in maintaining calcium and phosphate levels in the bloodstream. Its mechanism involves counteracting the effects of parathyroid hormone, thereby lowering calcium levels in the blood. Additionally, calcitonin decreases the excretion of calcium and magnesium in urine by enhancing reabsorption within the loop of Henle.

Parathyroid hormone (PTH) plays several critical roles in regulating calcium and phosphate levels in the body through its actions on the kidneys. In the nephron, the majority of calcium reabsorption occurs in the proximal convoluted tubule and the ascending loop of Henle. PTH primarily acts on the distal convoluted tubule and the collecting ducts of the nephron,

where it enhances calcium reabsorption directly. By doing so, PTH reduces urinary calcium loss. Additionally, PTH stimulates the activation of vitamin D in the kidneys, which further aids in calcium absorption from the intestines. Importantly, PTH inhibits phosphate reabsorption in the proximal convoluted tubule. This action is crucial because phosphate ions in the blood tend to form insoluble salts with calcium, thereby reducing the level of ionized calcium in the bloodstream. Therefore, by reducing phosphate reabsorption, PTH helps to maintain adequate levels of ionized calcium in the blood.[104]

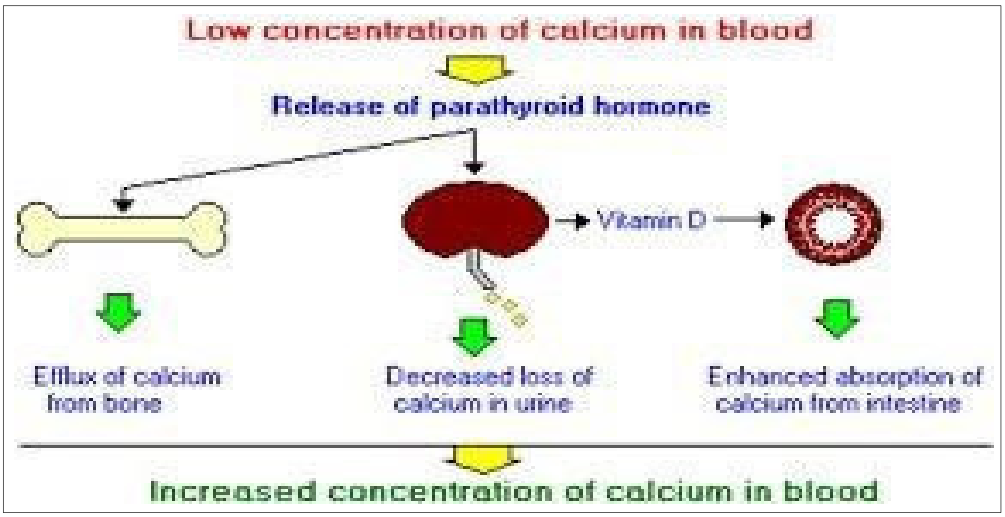


Figure No. 1.5: Role of Parathyroid Hormone in Blood

Calcitonin is involved in regulating calcium and other mineral levels within the kidneys. It inhibits phosphate reabsorption by the kidneys and enhances the reabsorption of calcium and magnesium, thereby increasing calcium excretion through urine.

Parathyroid hormone (PTH) initiates its action at the kidneys by promoting the synthesis of 1 alpha-hydroxylase in the proximal convoluted tubule. This enzyme is crucial for converting inactive vitamin D (25-hydroxycholecalciferol) into its active form, 1,25-dihydroxycholecalciferol. Active vitamin D facilitates calcium reabsorption in the distal convoluted tubule through calbindin-D, a vitamin D-dependent calcium-binding protein. In the small intestine, vitamin D promotes calcium absorption via both an active transcellular pathway requiring energy and a passive paracellular pathway that utilizes tight junctions.[105]

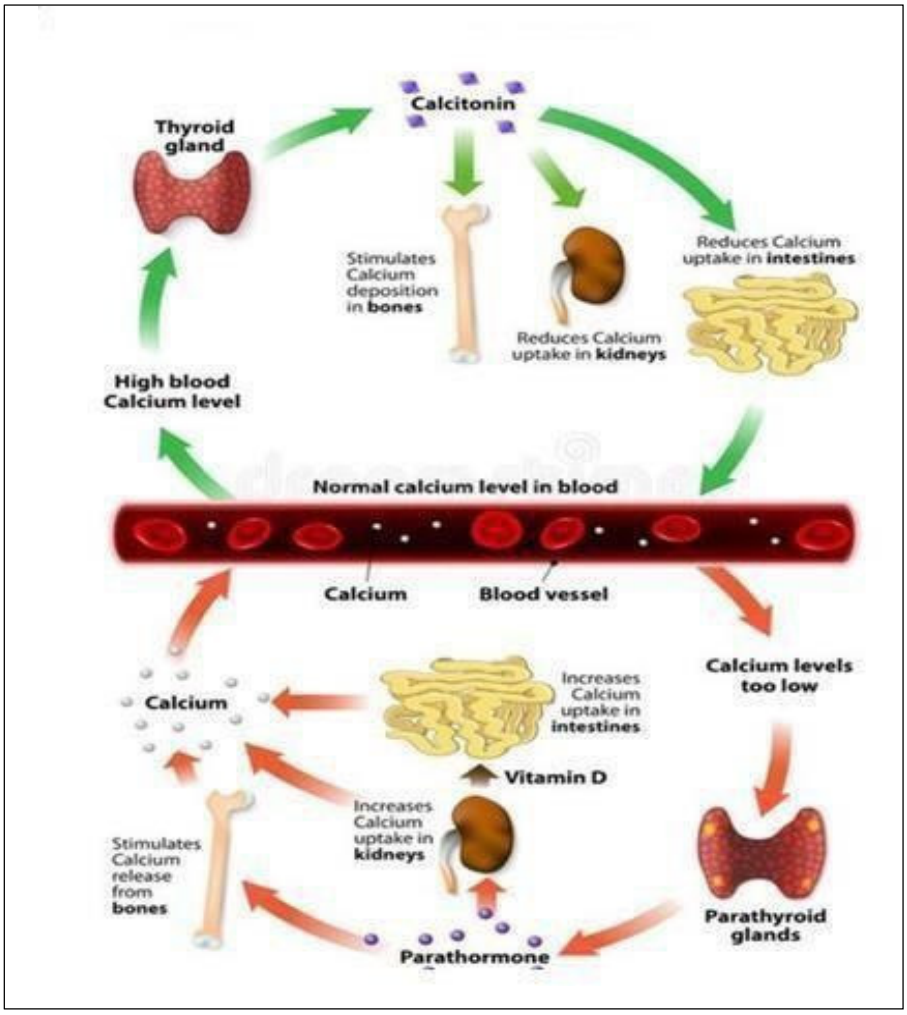


Figure No. 1.6: Role of parathyroid hormone and calcitonin in management of calcium level in blood

CHAPTER 2

LITERATURE REVIEW

REVIEW OF LITERATURE

Jagtap PN et al (2023) The study selected potent antiurolithiatic herbs to assess the efficacy of a polyherbal tablet. This tablet consisted of Tribulus terrestris, Musa balbisiana colla, Bryophyllum pinnata, and Commiphora wightii. The antiurolithiatic activity of the tablets was tested using Wistar rats to combat renal calculi induced by ethylene glycol.[106]

Alomair MK et al (2023) investigated the potential anti-urolithic properties of grape seed extract (GSE) using a rat model of experimental urolithiasis induced by ethylene glycol (EG) and ammonium chloride (AC). Their findings indicate that GSE demonstrated protective effects against EG-induced renal stones, as shown by improvements in kidney function, reduction in histological damage, and decreased formation of oxalate crystals. These effects are likely attributed to the antioxidant and anti-inflammatory properties exhibited by the extracts.[107]

Golla S et al (2023) The current study assessed the potential anti-urolithic effects of Cyperus rotundus tubers extract using in silico, in vitro, and in vivo approaches. In silico analysis focused on the constituents of Cyperus rotundus and the pathological protein oxalate oxidase (PDB Id: 2ETE). In vivo experiments examined the effects of the ethanolic extract (administered at doses of 100, 200, and 400 mg/kg body weight) on rats induced with sodium oxalate urolithiasis for seven days. The results demonstrated that the ethanolic extract of Cyperus rotundus tuber prevented sodium oxalate-induced stone formation, corroborated by molecular docking studies.[108]

Kishor P et al (2022) carried out preparation and standardization of polyherbal extract of *T. terrestris* L., *Avera lanata* L., *Crataeva religiosa* hook. And *Emblica officinalis* L. I suspension base showed good stability and redispersibility. Male mice chosen, existing research show that having the property like stone discharge through female mice had been substantially lower.[109]

Sharma YK et al (2022) *Citrus limon L., Citrus aurantium L., and Citrus medica L.* are known for their medicinal properties, including antiurolithic activity, treatment of stomach ache, vomiting, as well as their antifungal and antibacterial effects. In a study, urolithiasis was induced in rats using ethylene glycol. The combined extract of all three plants at a dose of 300 mg/kg showed significant efficacy compared to when each plant was administered

individually. The analysis included measurement of calcium, phosphate, magnesium, creatinine, uric acid, protein, urea nitrogen, and oxalate levels.[110]

Kamaraj MC et al (2022) The DPPH free radical scavenging assay was employed to assess free radical scavenging activity. Rutin served as a positive control. An acute toxicity study was carried out using Wister albino rats, which indicated no adverse effects on liver and renal functions fifteen days post-administration. These findings provide scientific support for considering the polyherbal formulation as a promising treatment for chronic kidney disease.[111]

Wanjari MM et al (2022) In both acute and sub-acute toxicity studies, administration of gokshuradiguggulu showed no significant changes in parameters or adverse effects compared to the control group treated with the vehicle. No instances of mortality or moribund states were observed across any groups in either study. The administration of gokshuradiguggulu at both acute and repeated doses over 28 days did not demonstrate any toxicity or adverse effects at the administered doses, with a determined No Observed Adverse Effect Level (NOAEL) of 2700 mg/kg.[112]

Venkatesan H et al (2022) In this study, we evaluated the antiurolithiatic effects of Aavarai Bhavanai Chooranam (ABC) in Wister albino rats with ethylene glycol-induced urolithiasis. We analyzed urine levels of calcium, magnesium, oxalate, inorganic phosphate, protein, and creatinine. ABC treatment was found to increase urine output and maintain alkalinity in the urinary tract, which likely contributes to its antilithiatic properties. Furthermore, ABC demonstrated protective effects on kidney structure and function, enhancing physiological mechanisms that deter stone formation. These findings support the clinical efficacy of ABC in kidney health maintenance.[113]

Ogoun T R et al (2022) The study aimed to examine the impact of Yoyo cleanser bitters, a popular Nigerian polyherbal preparation, on the biochemical functions of the liver and kidney in albino rats. The findings suggest that the polyherbal formulation, administered at the specified doses and duration, did not induce cholestasis or impair the liver's ability to excrete bilirubin, as indicated by levels of AST, ALP, albumin, total bilirubin, and protein. Furthermore, the formulation did not appear to have nephrotoxic effects on the kidneys, as evidenced by the decreased levels of creatinine observed in the study.[114]

Mishra A et al (2022) WHO has identified Urolithiasis, also known as urinary calculi or stones, as a prevalent urinary tract condition globally. It involves the formation of calcifications within the urinary system. Urolithiasis poses a significant worldwide health concern. Several therapeutic agents, such as NSAIDs, aminoglycoside antibiotics, and chemotherapeutic agents, have been incorporated into medical treatments in recent years. However, these medications carry risks of adverse effects on the kidneys, potentially leading to acute renal failure, chronic interstitial nephritis, and nephrotic syndrome. [115]

Jagtap PN et al, (2021) The polyherbal formulation, Lithout tablets, was prepared using the Wet Granulation method. The study findings indicate that animals administered with the standard drug (Cystone) and Lithout tablets at a dosage of 200 mg/kg showed considerable anti-urolithiatic activity. Furthermore, they exhibited protection against tubular interstitial damage in both kidneys and liver in ethylene glycol-induced urolithiatic rats. [116]

Khan A et al, (2021) Conducted a comprehensive literature review on the efficacy of different herbal treatments in rat models of calcium oxalate (CaOx) urolithiasis. Favorable outcomes such as decreased stone size, reduced number of stones, and facilitated passage were evaluated.[117]

Swati Sharma et al, (2021) The prepared Trikantakadikvatha churn tablets demonstrate crystal inhibition over time, with the extent of inhibition varying with drug concentration. At a concentration of 1000 µg/ml, the tablets achieved a maximum crystal inhibition of 76.25%. Furthermore, the developed dosage forms of Trikantakadikvatha ghan vati (TKGV) are more stable than the decoction (kvatha) and show promising results in the treatment of Urolithiasis (Mutrakrichra).[118]

Sachin M. Mahajan et al, (2021) The polyherbal suspension was formulated using alcoholic extracts of Curcuma caesia, Citrullus lanatus, Evolvulus alsinoides, Gymnema sylvestre, Tinospora cordifolia, Caesalpinia bonduc, and Withania coagulans. The resulting Polyherbal Formulation-C exhibited a pleasant appearance and texture. No changes were observed in sedimentation, flow rate, pH, viscosity, or other physicochemical parameters. This suspension, composed of ethanolic extracts, appears to be effective and safe for use in combinational therapy.[119]

Supriya S. Chimagave et al, (2020) The antiarthritic activity of the polyherbal formulation against Freund's complete adjuvant-induced paw edema demonstrates significant effects at

all three doses (250, 500, and 750 mg/kg p.o), markedly reducing paw swelling. This formulation provides a substantial protective effect against paw edema induced by Freund's complete adjuvant in rats.[120]

Harikesh Maurya et al, (2019) A polyherbal dispersible tablet formulated with A. officinal, *B. diffusa*, C. papaya, C. fistula, C. intybus, F. hispida, F. indica, C. nurvala, S. virgaurea, and V. negundo was developed for managing kidney disorders. The PHF-3 formulation demonstrated satisfactory disintegration and in vitro dispersion time, attributed to the inclusion of cross povidone, and was identified as the best formulation. Stability studies and IR compatibility tests confirmed that PHF-3 could represent a new, easily swallowable dispersible tablet, potentially enhancing drug permeability and improving bioavailability for nephrotic patients.[121]

Patel N et al, (2019) To evaluate the acute oral toxicity of the Uricare Tablet, an anti- BPH herbo-mineral formulation, on Swiss albino mice and its efficacy against Benign Prostatic Hyperplasia (BPH), a study was conducted. No mortality was observed in any group. The Uricare Tablet demonstrated significant effects on body weight, urine volume, and various prostatic and biochemical parameters. The results indicate that the Uricare Tablet does not produce any toxic effects at a dose of 2000 mg/kg, and the normal values of kidney markers and physical prostate parameters suggest its effectiveness against BPH.[122]

Baheti D et al, (2013) A studied increase in biochemical parameters such as ACP, ALP, AST, and ALT levels, along with a decrease in LDH levels in the kidney homogenate, indicated the induction of urolithiasis. Daily oral treatment with a polyherbal formulation indicated the induction of urolithiasis. Daily oral treatment with a polyherbal formulation (PHF) consisting of Plectranthus mollis Spreng, Didymocarpus pedicellata, Teraxacum officinale, Dendrophthoe elastic Desr, and Citrus medica at doses of 300 and 400 mg/kg not only significantly decreased the quantity of calcium oxalate deposited in the kidneys but also reversed all the biochemical changes induced by calcium oxalate urolithiasis, thereby supporting its traditional use.[123]

Gilhotra UK et al, (2013) Hydroalcoholic extracts of Kalanchoe pinnata and Rotula aquatica, as well as their combination, were formulated into herbal tablets and evaluated for their antilithiatic properties using an in vitro method. The homogeneous precipitation

method was employed, and the study was conducted in glass tubes using TRIS buffer at pH 7.4. The herbal tablet formulation containing Kalanchoe pinnata and Rotula aquatica demonstrated an inhibitory effect on calcium oxalate crystallization, indicating potential benefits in the treatment of renal lithiasis.[124]

Bodakhe KS et al, (2013) The study investigated the protective effects of Cystone on oxidative stress and calcium oxalate crystal formation in hyperoxaluria-induced urolithiasis. In untreated animals, EG treatment resulted in a higher urine volume, lower urinary pH, and increased urinary excretion of oxalate, calcium, and phosphate. Cystone treatment demonstrated a protective role by enhancing renal antioxidant status and promoting diuresis, thereby mitigating oxidative stress and calcium oxalate crystal deposition. [125]

Deng G et al (2011) Xiao-Chai-Hu-Tang, also known as Sho-sai-ko-to (SST), is a traditional East Asian herbal formulation comprised of seven botanical extracts commonly used to treat liver diseases. Studies have indicated that SST can lower transaminase levels and decrease the incidence of hepatocellular carcinoma in patients with hepatitis B. To explore its potential benefits for hepatitis C patients ineligible for interferon-based treatments, we performed a phase II clinical trial to assess whether SST merits additional investigation.[126]

M. G. Shekar Kumaran, et al (2011) The Ayurvedic formulation studied led to a notable reduction in the size of renal calculi, unlike the placebo group where no size reduction was observed. Patients receiving the Ayurvedic treatment showed improvements in clinical symptoms such as hematuria, frequent urination, and tenderness in the kidney, ureter, and bladder (KUB) area. Additionally, there was a significant decrease in the number of urinary red and white blood cells and serum uric acid levels in these patients. This formulation appears to be safe and effective for individuals with smaller kidney stones, enhancing the rate of stone expulsion. [127]

Jain PK et al, (2010) A novel, straightforward, sensitive, precise, and reliable highperformance thin layer chromatographic (HPTLC) method has been developed for estimating andrographolide in herbal extracts and pharmaceutical formulations. The method resulted in compact spots for andrographolide, with an Rf value of 0.49. Calibration plot data indicated a strong linear relationship, with an r^2 value of 0.9986 within the

concentration range of 200 ng to 1000 ng based on peak area. The detection and quantification limits were established at 3.5 ng and 11.7 ng, respectively.[128]

Mohamme D. et al (2010) This study involves formulating a gel using Pothos scandens Linn (P. scandens) leaf extract and evaluating its effectiveness in healing burn wounds. The dried leaves of P. scandens were extracted with ethanol and underwent preliminary phytochemical evaluation as well as wound healing activity studies. Various gel formulations containing 4% (w/v) ethanolic extract of P. scandens were prepared with polymers carbopol 934 and carbopol 940 at different concentrations.[129]

Das et al (2010) The formulation containing 2.5% Stevia extract demonstrated superior stability compared to other formulations and the control sample. Evaluation of various parameters indicated no toxicity or skin irritation, suggesting this Stevia extract formulation is both safe and beneficial for use.[130]

Ajazuddin et al (2010) This review provides an overview of the present state of novel herbal formulation development, detailing their preparation methods, active ingredient types, particle size, entrapment efficiency, administration routes, biological activities, and diverse applications.[131]

Shirfule AL et al (2009) A polyherbal formulation comprising *Tribulus Terrestris* L., *Ricinus communis* L. roots, *Solanum indicum* L., *Hygrophila spinosa* L. seeds, and *Solanum surattense* L. aerial parts is utilized for the management of kidney stones. The active components, identified as saponins, were analyzed using high-performance thinlayer chromatography. Patients with naturally induced urolithiasis were administered 1 g of the powder mixed with an equal volume of honey and fresh curd (1 teaspoonful) orally for 28 days. Ultrasonography performed before and after treatment indicated complete elimination of calculi or reduced stone growth in the majority of patients.[132]

Sutar NG et al, (2009) The antipyretic activity of an ethanolic extract from *Moringa oleifera* Lam. seeds was investigated in albino rats under normal body temperature and yeast-induced pyrexia conditions. The ethanol extract was administered orally at doses of 100, 200, and 300 mg/kg body weight. It exhibited a significant, dose-dependent reduction in both normal body temperature and yeast-induced fever. The antipyretic effect of the

ethanolic seed extract was found to be comparable to that of Paracetamol (150 mg/kg body weight, oral route), a standard antipyretic agent. [133]

Lakshmi V et al (2009) The antidiuretic activity of *Moringa oleifera* pods has been established. In the present study, the aqueous extract of Moringa oleifera pods was tested for diuretic activity in albino rats at two oral doses (400 mg/kg and 800 mg/kg). The diuretic effect of the extract was compared with that of the standard drug hydrochlorothiazide. The findings concluded that Moringa oleifera exhibits diuretic action.[134]

Jaiswal D et al (2009) The effects of *Moringa oleifera* Lam. leaves aqueous extract on hyperglycemic rats were investigated. In animals with sub and mild diabetes, the extract caused a significant decrease in glucose levels, with reductions of 31.1% and 32.8% respectively, during OGTT (Oral Glucose Tolerance Test). Moreover, there was a notable decrease in urine sugar levels from +4 to nil, and urine protein levels from +2 to trace. These findings provide scientific validation for the traditional use of M. oleifera in treating diabetes mellitus.[135]

Hamman JH et al (2008) The latest research has explored additional effects and uses of Aloe vera leaf gel. These include its potential to improve the absorption and availability of co-administered compounds when used in liquid preparations of whole leaf or inner fillet gel. Furthermore, Aloe vera has shown promise in enhancing skin permeation. Additionally, there are significant pharmaceutical applications, such as using dried Aloe vera gel powder as an excipient in sustained-release pharmaceutical formulations.[136]

P.M. Dandgi et al, (2008) Polyherbal suspensions were developed using extracts that exhibited notable activity and were assessed for their physicochemical properties and hepatoprotective effects, alongside LIV-52 as a standard reference. It was observed that the F3 formulation, comprising chloroform, petroleum ether, and aqueous extracts of Ferula asafoetida, petroleum ether and ethanol extracts of *Momordica charantia* Linn., and petroleum ether and ethanol extracts of *Nardostachys jatamansi*, displayed considerable hepatoprotective activity possibly attributed to the synergistic effects of these extracts.[137]

Kaur A D et al (2008) A novel, straightforward, sensitive, accurate, and sturdy high performance thin-layer chromatography (HPTLC) method was developed to quantify conessine in herbal extracts and pharmaceutical formulations. The chromatographic

separation employed a twin trough glass chamber saturated with a mobile phase comprising toluene, ethyl acetate, and diethyl amine (6.5:2.5:1, v/v/v) at room temperature (25 ± 2 °C) via linear ascending development. This system reliably produced well-defined spots for conessine with an Rf value of 0.82. Calibration plots exhibited excellent linearity with an r2 value of 0.9998 over a concentration range of 1–10 µg, as determined by peak area analysis. [138]

Ram Kumar Roy et al, (2007) The onset of hair growth was significantly shortened to one-third compared to control animals when treated with the formulated product. Moreover, the duration for complete hair regrowth was reduced by 32%. Analysis of the hair growth cycle following treatment with both the formulation and minoxidil (2%) revealed a higher count of hair follicles in the anagen phase compared to the control group. These findings support the well-known hair growth-promoting properties attributed to the plants traditionally. Additionally, the formulated product demonstrates promising potential for treating alopecia.[139]

Ahemad B. et al (2006) The methanol extract of Moringa fruit was investigated for its potential anti-inflammatory properties using a rat paw edema model induced by carrageenan. The results showed dose-dependent inhibition of edema, indicating significant potency of the extract. It contains anti-inflammatory compounds that could be beneficial for treating acute and chronic inflammatory conditions. Additionally, Moringa oleifera Lam. (Moringaceae) was studied for its analgesic effects against thermal stimuli using Eddy's hot plate test and Analgesiometer test, as well as for its antipyretic effects.[140]

Siddiq A et al, (2005) The antioxidant potential of *Moringa oleifera* leaves could be linked to their rich content of flavonoids, polyphenols, and tocopherols. The findings suggest that M. oleifera leaves have the potential to be utilized as a promising source of natural antioxidants and nutraceuticals.[141]

Siddhuraju P et al (2003) The antioxidant activities and free radical scavenging capacities of water, aqueous methanol, and aqueous ethanol extracts from freeze-dried *Moringa oleifera* Lam. leaves sourced from various agro-climatic regions were assessed. Results indicate that all leaf extracts effectively scavenged peroxyl and superoxyl radicals. This

study underscores the potential of Moringa leaves as a rich natural source of antioxidants, owing to their significant antioxidant activity.[142]

Lalas S et al, (2002) According to the study, aqueous, methanol (80%), and ethanol (70%) extracts of freeze-dried *Moringa oleifera* leaves demonstrated significant radical scavenging and antioxidant properties. These extracts exhibited the ability to scavenge peroxyl and superoxyl radicals. The primary bioactive compounds identified were quercetin and kaempferol. Additionally, the oil extracted from dried Moringa oleifera seeds exhibited superior antioxidant activity compared to butylated hydroxytoluene and α-tocopherol.[143]

CHAPTER 3

RESEARCH GAP

RESEARCH GAP

Urolithiasis is a prevalent health issue, with kidney diseases affecting a substantial portion of the global population, estimated at about 20-22%. Among various types of stones, Calcium oxalate and uric acid calculi are the most frequently encountered in nephrolithiasis. The recurrence of kidney stones is a significant concern, attributed to the nucleation and supersaturation of substances like calcium and uric acid in the kidneys. This phenomenon is predominantly observed in males.

Stones development:

1. Reduce in urine volume and an increase in the concentration of stone-forming substances in the kidney.
2. Increase of excretion of calcium oxalate, urate, cystine, xanthine, & phosphate.
3. Increased level of Parathyroid hormone and decreased level of calcitonin.

Treatment of many operative procedures for curing urinary stone disorders, have been established in recent decades. There are currently numerous techniquesavailable likepercutaneous nephrolithotomy (PCNL), extracorporeal shockwave lithotripsty (ESWL) and flexible urinoscopy (FURS) with laser lithotripsy. These procedures are very painful and also there no assurance of non-reoccurrence of the stones after Lithotripsy.

A wide variety of allopathic treatments are readily available on the market. However, since these drugs are excreted through the kidneys, they can potentially burden the kidneys. Therefore, traditional herbal remedies have been employed to address kidney diseases caused by various factors. Some marketed preparations are available but contains many herbal drugs together. Many markets Polyherbal formulation doesn't have combination of all three plants. Therefore, there was need to formulate, develop and evaluate Polyherbal formulation in order to understand better its chemical composition, safety and in vitro and in vivo efficacy related of this polyherbal formulation in curing kidney stone. Hence current research was taken on Isolation, Identification, Estimation of phytochemicals responsible for antilithiatic activity of Polyherbal granule formulation. ***Boerhavia diffusa, Celosia argentea*** **and** ***Plumeria Rubra*** was evaluated for antiurolithiatic and nephroprotective

activity in Wistar rats. From the literatures present project is seems more promising than existing Litholytic agents. The project will provide effective and economic polyherbal oral formulation for the treatment Urolithiasis. The proposed study will provide evidence-based research for Herbal Drugs.

CHAPTER 4

AIM, OBJECTIVES, PLAN OF WORK

AIM, OBJECTIVES AND PLAN OF WORK

4.1 AIM:

"Development and Evaluation of Oral Polyherbal Formulation for Litholytic Activity in Ethylene Glycol-Induced Urolithiasis in Wistar Rats."

The present study is carried out to evaluate the Litholytic activities of following herbal preparation against Polyherbal formulations in Wistar rats.

Polyherbal plant:

1. *Boerhavia diffusa*
2. *Plumeria Rubra*
3. *Celosia argentea*

4.2 THE PRESENT STUDY IS PLANNED WITH THE FOLLOWING OBJECTIVES:

1. Selection of the appropriate plants which possess litholytic, diuretic Potential.
2. Authentication of selected plants.
3. Evaluation of preliminary standardization parameter of selected crude drugs
4. Phytochemical estimation by TLC, HPTLC.
5. In vitro study of herbal formulation
6. In vivo study of herbal formulation
7. To design and formulate safe and efficacious herbal formulation.
8. Evaluation of herbal formulation.

4.3 PLAN OF WORK

4.1 Plan of work

1. Conducting a thorough review of existing literature
2. Choosing a suitable plant species
3. Creating a comprehensive plant profile
4. Acquiring, gathering, and authenticating the chosen plant species
5. Extraction of crude drugs by soxhlation
6. Pharmacological and Phytochemical investigation of crude drug
7. In vitro study of Extract
8. In vivo study of Extract
9. Formulation development of polyherbal medicaments
10. Stability study of polyherbal formulation

CHAPTER 5

MATERIAL AND METHOD

MATERIALS AND METHODS

5.1 PLANT PROFILE

5.1.1 *Boerhavia diffusa:*

- **Botanical Name:** *Boerhavia diffusa*
- **Family:** Nyctaginaceae
- **Nature:** A creeping herbaceous plant, perennial and extensively branched, characterized by robust spindle-shaped roots. Its branches spread widely, with a purplish stem that thickens at the nodes. [144]

(A)

(B)

Figure No. 5.1: A: ***Boerhavia diffusa*** **Herb, B: Flowering twigs with seed**

- **Veracular Names:**[145]

English	:	Spreading hogweed
Chinese	:	Huang Xi Xin
French Guiana	:	Ipecacuanha de Cayenne
Spanish	:	Hierba de cabra
Assamese	:	RangaPunarnabha;
Bengali	:	Rakta Punarnava;
Gujarati	:	Dholisaturdi, Motosatodo;
Hindi	:	Gadapurna, Lalpunarnava;
Malayalam	:	ChuvannaTazhutawa;
Marathi	:	Ghetuli, Vasuchimuli, Satodimula, Punarnava, and Khaparkhuti.
Part used	:	Root, Leaves and seeds

5.1.1.1 Description:

Boerhaavia diffusa is a widely distributed plant found across various regions including India, the Pacific islands, and southern United States. The flowers are small, approximately 5 mm in diameter. Pollen grains are spherical, about 65 microns in diameter. Its small fruits are sticky and typically grow a few inches above the ground, facilitating dispersal by adhering to small migratory birds passing nearby [146].

Habit: It is a creeping perennial herb with numerous branches and robust fusiform roots.

Stem: The branches spread outwards (divaricate). The stem is purplish in color and thickened at the nodes.

Leaves: Opposite in arrangement, oblique, ovate or nearly circular, with rounded tips. The edges are smooth with a slightly pinkish hue, and they have wavy margins. The lower surface of the leaves bears small, white scales and the base is rounded.

Inflorescence: The flowers are arranged in small umbels that form a corymbose structure, occurring in axillary and terminal panicles.[147]

Flowers:

- Bracteoles are acute. The perianth tube is narrowed above the ovary, with a funnel-shaped limb that is dark pink and bears five vertical bands externally.
- Stamens are 2 or 3, slightly protruding and of unequal length.
- The ovary is superior and oblique, containing a single erect ovule and stigma.[148]

Fruit: The fruit is an achene that is rounded and has six ribs.

Seed: Seeds are minute, albuminous with endosperm, and have a curved embryo.

5.1.1.2 Phytochemical Constituents:

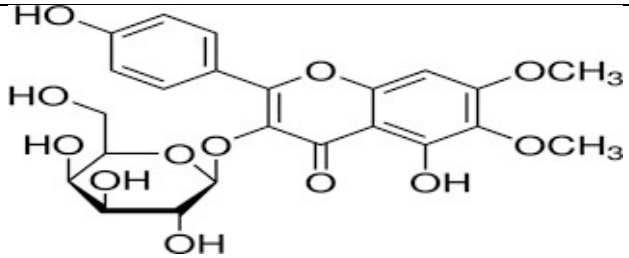
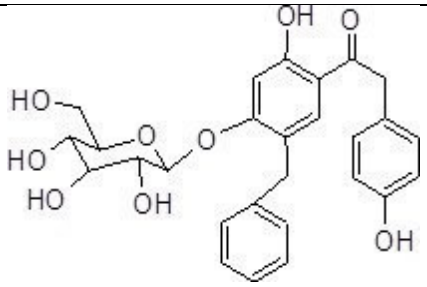
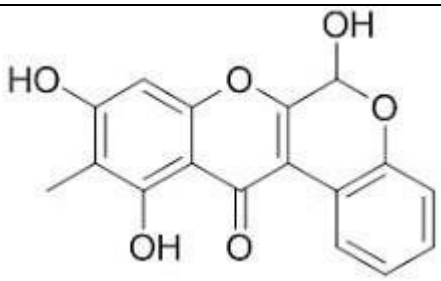
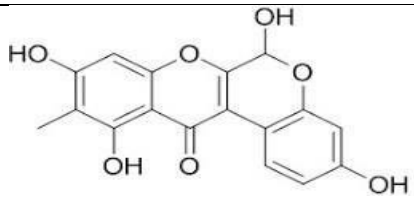
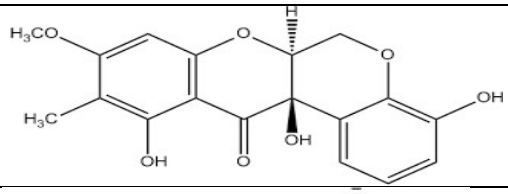
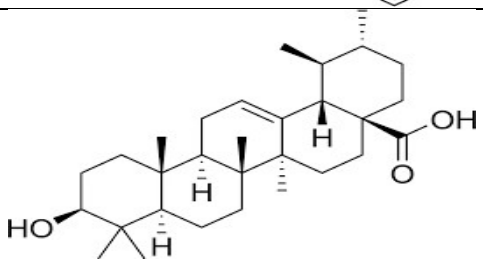
Boerhaavia diffusa contains numerous compounds including flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins. Specific compounds isolated and studied for their biological activities include punarnavine (C17H22N2O, melting point 236–237°C), boeravinone A-F, hypoxanthine 9-L-arabinofuranoside, ursolic acid, punarnavoside, liirodendrin, and a glycoprotein with a molecular weight of kDa.[149]

Punarnava (*Boerhavia diffusa*) is known for its rich phytochemical composition. Analysis has revealed the presence of β-Sitosterol, α-2-sitosterol, palmitic acid, ester of β-sitosterol, tetracosanoic acid, hexacosanoic acid, stearic acid, arachidic acid, urosilic acid, Hentriacontane, β-Ecdysone, and triacontanol [150].

Phytochemical screening of the roots from garden-grown *B. diffusa* plants of various ages indicated that the highest alkaloid content (2%) is found in the roots of 3-yearold mature plants. The herb and roots are also rich in proteins and fats. Specifically, the herb contains 15 amino acids, including 6 essential amino acids, while the root contains 14 amino acids, including 7 essential amino acids. Potassium nitrate is abundant in the plant, alongside punarnavine.

Previous research has identified flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins in *B. diffusa.* These findings corroborate earlier studies, suggesting that these phytochemicals, particularly those in leaf extracts, may contribute to the herb's antibacterial properties. Variations in antimicrobial effects among different solvent extracts are likely due to differences in phytochemical composition and species variations.[151]

Table No. 5.1: Structures of chemical Constituents of *Boerhavia diffusa*

Sr. No.	Phytochemical Constituent Name	Structure
1.	**Eupalitin-3-*O*-*β*-D-galactopyranoside**	
2.	**Punarnavoside**	
3.	**Boeravinone B**	
4.	**Boeravinone E**	
5.	Boeravinone C	
6.	Ursolic Acid	

7.	Liriodendrin	
8.	Hypoxanthine 9-Larabinofuranoside	

5.1.1.3 Pharmacological activities:

5.1.1.3.1 Anti-bacterial activity:

Boerhavia diffusa (*B. diffusa*) leaves exhibit strong antibacterial effects against a variety of Gram-negative and Gram-positive bacteria, possibly attributable to their phytochemical composition. The ethanol extract demonstrated inhibitory effects on Gram-positive bacteria such as Staphylococcus aureus, Bacillus subtilis, Staphylococcus faecalis, and Micrococcus luteus, as well as on all Gram-negative bacteria included in this study. Meanwhile, the methanol extract showed inhibitory effects against all Gram-positive bacteria studied, except Micrococcus luteus, and against Gram-negative bacteria including Klebsiella pneumoniae, Pseudomonas vulgaris, Serratia marcescens, and Shigella flexneri. The antibacterial activity of various extracts of the stem bark of Prosopis cineraria (Linn.) Druce was evaluated using the agar well diffusion method.[152]

5.1.1.3.2 Hypoglycemic activity:

The hypoglycemic activity of an alcoholic extract from the entire plant of *B. diffusa* was observed to possess hepatoprotective properties against carbon tetrachloride-induced hepatotoxicity in rats and mice. Another study investigated the impact of orally administered aqueous leaf extract of *B. diffusa* on normal and alloxan-induced diabetic rats,

demonstrating a significant decrease in blood glucose levels and a notable increase in plasma insulin levels in both normal and diabetic rats. These effects were more pronounced compared to glibenclamide. Additionally, the chloroform extract of *B. diffusa* leaves exhibited a dose-dependent reduction in blood glucose levels in streptozotocin-induced NIDDM rats, comparable to the effects of glibenclamide, thereby validating its traditional use in Ayurvedic medicine for managing diabetes. Furthermore, studies on the leaf extract of *B. diffusa* indicated a dose-dependent reduction in blood glucose levels, possibly through rejuvenation of pancreatic β-cells or through extrapancreatic mechanisms. [153]

5.1.1.3.3 The anti-nociceptive activity:

B. diffusa was evaluated using the acetic acidinduced abdominal writhing test in mice. Pre-treatment of the animals with naloxone (5 mg/kg, i.p.) reversed the analgesic effect of morphine and the juice, but not the decoction. According to reports, the active antinociceptive component of *B. diffusa* is predominantly found in the juice of fresh leaves. This component exhibits significant antinociceptive effects in various pain models.

5.1.1.3.4 Hepatoprotective activity:

The aqueous root extract of *B. diffusa* (2ml/kg) demonstrated significant hepatoprotective effects against thioacetamide-induced liver damage. It showed considerable protection against several serum parameters including GOT, GPT, ACP, and ALP, although it did not affect GLDH and bilirubin levels. Furthermore, the study indicated that administration of the aqueous extract (2ml/kg) was more effective in hepatoprotection compared to the powdered form of the drug. [154]

5.1.1.3.5 Antiproliferative and antiestrogenic activity:

Antiproliferative and antiestrogenic properties of methanol extract of *B. diffusa* (BME) in MCF-7 breast cancer cell lines.

5.1.1.3.6 Anti-inflammatory activity:

The ethanol extract from leaves demonstrated significant anti-inflammatory effects at a dosage of 400 mg/kg, showing reductions of 30.4%, 32.2%, 33.9%, and 32% in paw edema induced by carrageenin, serotonin, histamine, and dextran, respectively, in rat models. Additionally, the ethanol extract from stem bark exhibited an IC50 value of 100 ng/ml against COX-1, indicating its potential for treating inflammatory conditions. Furthermore,

anti-inflammatory activity was evaluated using latex extract from the plant, specifically in a carrageenan-induced inflammation model.

5.1.1.3.7 Anticonvulsant activity:

Research demonstrated that the crude methanolic extract of *B. diffusa* along with its liriodendrin-rich fraction exhibited a dose-dependent protective effect against PTZ-induced convulsions. Regarding its antistress, adaptogenic, and immunomodulatory properties, ethanol extracts derived from the roots of *B. diffusa* demonstrated enhanced stress tolerance in both swim endurance and cold restrain stress tests. Immunomodulatory effects were evidenced by an increase in carbon clearance, indicative of stimulation of the reticuloendothelial system. Furthermore, there was observed augmentation in the DTH (Delayed Type Hypersensitivity) response to SRBC (Sheep Red Blood Cells) in mice, suggesting enhanced cell-mediated immunity and stimulation of lymphocytes and accessory cell types.[155]

5.1.1.3.8 Nephroprotective activity:

Boerhavia diffusa exhibits protective effects on the kidneys and their diseases. Several studies, primarily conducted on laboratory animals with limited human trials, indicate its potential in kidney protection and management of toxicity. The plant extract demonstrates the ability to normalize elevated levels of urea, creatinine, uric acid, and BUN in serum, as well as lipid peroxidation (LPO) in the kidneys. It also affects ion concentration normalization and improves cellular damage when administered in various extracts. *Boerhavia diffusa* treatment shields the kidneys from crystal-induced renal cell injury caused by calcium oxalate (CaOx) deposition in rat kidneys. Additionally, it sustains urine volume by enhancing Na+ and K+ excretion. Various studies confirm *Boerhavia diffusa*'s diuretic effect, lowering serum levels of creatinine, uric acid, potassium, chloride, phosphorus, calcium, and oxalate, while increasing urine volume. *Boerhavia diffusa* extract decreases malondialdehyde (MDA) levels, boosts antioxidant enzymes (SOD, CAT, GST, GPx), and mitigates crystal formation in hyperoxaluria-induced nephrotoxicity in rats.

5.1.2 *Plumeria Rubra*

Plant Profile

- **Botanical name:** Plumeria Rubra
- **Family:** Apocynaceae
- **Nature:** Spirally clustered at the ends of the stems, the elliptic leaves are dark green, measuring 8 to 16 inches in length, with gently wavy edges.[156]
- **Vernacular Name:**

Hindi	:	Chameli
Tamil	:	Perungalli
Marathi	:	Khairchampa
English	:	Frangipani
Telgu	:	Veyyivarahal
Benghal	:	Dalanaphula [157]
Part used	:	Flower, Leaves.

Figure No. 5.2: A: Fresh *Plumeria Rubra* pod; B: Dry Seed pod; C: Dry Seed pod

5.1.2.1 Description:

Plumeria rubra typically grows as a spreading shrub or small tree, reaching heights of 2–8 m (20–25 ft) and similar widths. Its thick succulent trunk and blunt, sausage-like branches are covered in a thin grey bark. The branches, which can be brittle, exude a white latex sap when broken, known to cause skin and mucous membrane irritation. The large green leaves, arranged alternately and clustered at branch ends, measure 30 to 50 cm (12–20 in) long. These deciduous leaves fall during cooler months. The terminal flowers appear in summer,

often abundant and strongly fragrant, with five petals in colors ranging from pink to white, sometimes with yellow at the center.

Initially tubular, the flowers open to diameters of 5–7.5 cm (2–3 in), occasionally producing 20-60 winged seeds.

Leaves: They are simple, alternate, oblong to elliptic, thick and leathery, reaching up to 14 inches long and 1½ inches wide. Their margins are strongly recurved, hairless on the upper surface with a whitish underside, and numerous lateral veins almost perpendicular from the midribs.

Regarding the flowers: They are salver form, with five waxy white petals and yellow centers, arranged in cymes at branch tips.

Fruits: As for the fruits, they are not depicted, typically follicles that are brown and long-pointed, often borne in pairs.[158]

5.1.2.2 Phytochemical Constituent:

Flowers of the Plumeria plant contain resin, quercetin, and traces of kaempferol and cyanidin glycosides. Fresh leaves and bark are rich in plumeride and resinic acid. The bark additionally contains fulvoplumerin. A mixture of terpenoids and sterols includes plumieride. Latex coagulum obtained from branches yields caoutchouc and resinous matter. The chemical composition of the essential oil, obtained by hydrodistillation of the leaves, includes (Z)-β-farnesene, α-patchoulene, limonene, (E)-β-farnesene, α-copaene, and phytol. Conversely, the flower oil is characterized by significant quantities of (E)-non-2-en-1-ol, limonene, phenylacetaldehyde, ntetradecanal, γ-elemene, and (E,E)-α-farnesene. Compounds isolated from the ethanol-soluble fraction of methanol extract of *Plumeria Rubra* include Rubranonoside glucopyranosyl naringenin, a new flavanone glycoside named rubranin, a sphingolipid named rubradoid, a new iridoid galactoside, rubrajaleelol, and rubrajaleelic acid. [159]

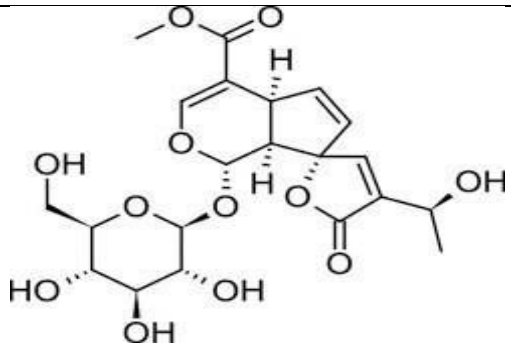
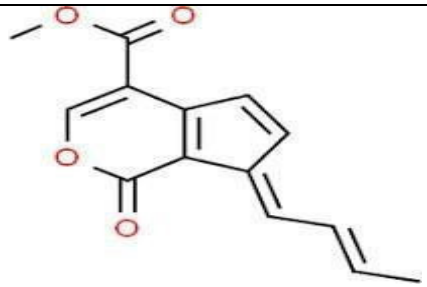
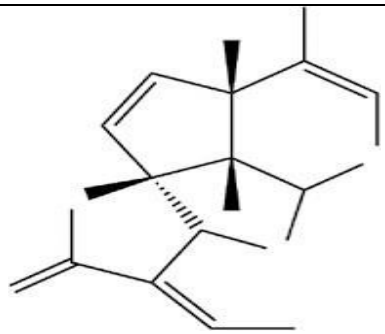
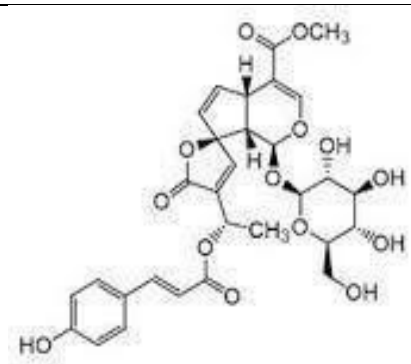
The essential oil of *Plumeria Rubra* was found to contain a diverse array of compounds, including 13 hydrocarbons, 17 alcohols, 13 esters, 9 aldehydes, and 15 miscellaneous compounds. Further analysis of the alcoholic extract from the leaves isolated five specific compounds: lupeol nanoate, lupeol heptanoate, rubrinol glucoside, β-sitosterol-β-D-glucoside, and plumeiride coumarate. These compounds were identified based on their physical and chemical properties as well as spectral analyses. A total of 41 compounds have

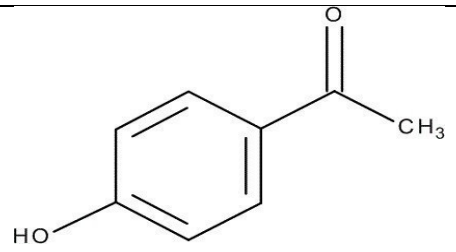
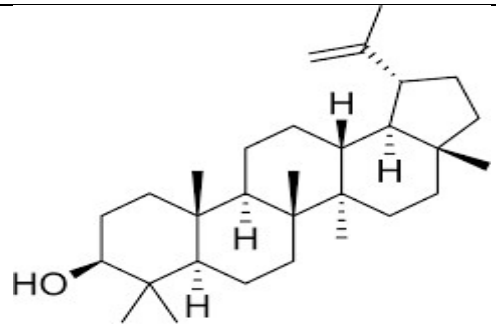
been identified from *Plumeria Rubra*, with the oil composition consisting of 10 esters (43.3%), 11 hydrocarbons (8.1%), 10 alcohols (29.8%), 4 carboxylic acids (8.5%), 2 ethers (1.0%), 3 aldehydes (2.5%), and benzyl salicylate (20.9%). *Plumeria Rubra* is notably rich in ursolic acid, a natural pentacyclic triterpenoid carboxylic acid.

Gas chromatography analysis of *Plumeria Rubra* flower oil identified 31 volatile components, predominantly alkanoic acids such as lauric, myristic, and palmitic acids. Other constituents included hydrocarbons, alcohols, ethers, and aldehydes. Compounds isolated from *Plumeria Rubra* heartwood, namely plumericin and isoplumericin, exhibited molluscicidal, cytotoxic, and antibacterial properties, while hydroxyacetophenone showed mild cytotoxicity. Bioactivity-guided fractionation studies of *P. rubra* have identified cytotoxic compounds in the bark collected from Indonesia. Active constituents such as fulvoplumierin, allamcin, allamandin, and 2,5- dimethoxy-p-benzoquinone are among the reported iridoids and quinone derivatives. [160]

The flower volatile constituents of *Plumeria Rubra* L. cultivated in the foothills of northern India were examined using gas chromatography (GC) and GC-mass spectrometry (GC-MS). A total of 31 components were identified, which constituted 94.0% of the essential oil and 89.2% of the steam volatile extract. The major classes of constituents identified included benzyl esters (49.0% in essential oil, 41.4% in steam volatile extract), aliphatic alkanes (25.8%, 7.2%), oxygenated monoterpenes (0.1%, 27.1%), oxygenated sesquiterpenes (9.5%, 8.8%), and diterpenes (9.4%, 0.2%). Key compounds identified were benzyl salicylate (26.7% in essential oil, 33.5% in steam volatile extract), benzyl benzoate (22.3%, 7.9%), geraniol (trace, 17.2%), (E, E)-geranyl linalool (9.4%, 0.2%), tricosane (8.3%, 1.1%), linalool (0.1%, 8.0%), nonadecane (7.0%, 3.8%), (E)-nerolidol (7.0%, 5.5%), and pentacosane (4.4%, 0.3%). A comparative analysis with existing data on floral compositions of *P. rubra* highlighted significant qualitative and quantitative differences. Notably absent in the Indian specimens were alkanoic acids, neryl phenyl acetate, phenylacetaldehyde, and β-phenylethyl alcohol, previously reported in other *P. rubra* samples. Additionally, two novel iridoid diastereomers were isolated and structurally characterized from the flowers of *P. rubra* L. cv. Acutifolia using spectroscopic methods. From the heartwood of *Plumeria Rubra*, compounds such as plumericin, isoplumericin, 4- hydroxyacetophenone, plumeride, 13-coumaroylplumieride, and protoplumericine were isolated. The study also detected significant levels of immune reactive cardiac glycosides in *Plumeria rubra*.[161]

Table No. 5.2: Phytochemical Constituent of *Plumeria rubra*

Sr. No.	Name of Phytochemical Constituent	Structure
1.	Plumeride	
2.	Fulvoplumierin	
3.	Plumericin	
4.	13-coumaroylplumieride	

5.	4-hydroxyacetophenone	
6.	Lupeol	

5.1.2.3 Pharmacological Activity:

5.1.2.3.1 Anxiolytic effect:

The anxiolytic potential of the ethanolic extract from *Plumeria Rubra* flowers and its fraction was assessed using the elevated plus maze model for anxiety. The study suggests that both the flower extract and its insoluble butanolic fraction show promising anxiolytic effects.[162]

5.1.2.3.2 Antioxidant, cytotoxic and hypolipidemic activity:

The study on the methanolic extract of *Plumeria Rubra* flowers revealed its antioxidant capacity, cytotoxic effects, and ability to lower lipid levels. The DPPH assay showed a notable antioxidant activity with 72% free radical inhibition, and the total phenolic content was found to be 167.3 µg/ml. At a concentration of 1.67 mg/ml, significant free radical scavenging was noted. Moreover, the extract exhibited strong hypolipidemic properties, resulting in a 60% reduction in cholesterol levels. However, the MTT assay on HCT 116 cell lines indicated that the extract did not affect the proliferation of colon cancer cells.

5.1.2.3.3 Hepatoprotective activity:

The hepatoprotective effect of an alcoholic extract from *Plumeria rubra* pods was investigated in male albino rats with CCL4-induced hepatic injury. The results indicated significant hepatoprotective effects at doses of 200mg/kg and 100mg/kg of the extract.[21]

5.1.2.3.4 Antifertility Activity:

The ethanolic extract of *Plumeria rubra* was evaluated for its abortifacient properties. It was observed that doses of 50, 100, and 200 mg/kg body weight produced a dose-dependent adverse effect on the fertility index and the number of implantations in the uterine horn of female rats. This was evidenced by an increase in the percentage of post-implantation embryonic loss.

5.1.2.3.5 Antimicrobial Activity:

The antimicrobial potential of *Plumeria rubra* extracts was evaluated using the cup plate method and by measuring the minimum inhibitory concentration (MIC) against various microorganisms, including Escherichia coli, Bacillus subtilis, Staphylococcus aureus, and Aspergillus niger. The methanol extract showed notable effectiveness against the bacterial strains, with its performance comparable to that of Ciprofloxacin, a standard antibiotic. In contrast, the aqueous extract displayed antifungal activity, with its results benchmarked against Fluconazole, a standard antifungal drug.[163]

5.1.2.3.6 Antiviral activity:

Plumeria rubra, which contains fulvoplumerin, acts as an inhibitor of the reverse transcriptase enzyme of human immunodeficiency virus type 1 (HIV-1).

5.1.2.3.7 Anti-inflammatory and Antioxidant Activity:

The methanolic extract of *Plumeria rubra* flowers demonstrated significant antioxidant and anti-inflammatory properties. Phytochemical analysis revealed that *Plumeria rubra* is rich in flavonoids and phenols.[164]

5.1.2.3.8 Anti-oxidative and proteolytic activities: were investigated using *Plumeria rubra* lattices. The findings demonstrated significant enzymatic and proteolytic capabilities inherent to *Plumeria rubra*.

5.1.2.3.9 Anti-microbial activity:

Phytochemical analysis of the crude extract indicated the presence of tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides, and reducing sugars. However, phlobatannins were notably absent in the methanol extract of *Plumeria rubra* flowers. The crude extracts exhibited significant inhibitory effects at a concentration of 20 mg/ml, except against Corynebacterium pyogenes and Bacillus anthracis from *Plumeria rubra* leaves. Tannins, alkaloids, flavonoids, saponins, gums, and terpenoids were

identified in *Plumeria rubra* flowers. Research suggests that tannins, particularly abundant in the extract, possess potent anti-inflammatory properties. Additionally, flavonoids have demonstrated analgesic and anti-inflammatory effects in various studies.[165]

5.1.2.3.10 Nephro protective activity:

Increased production of reactive oxygen species (ROS) in renal tissue leads to organ damage, as indicated by alterations in Malondialdehyde (MDA) and Superoxide Dismutase (SOD) levels. In rats treated with Cisplatin, MDA levels were notably higher compared to the control group. *Plumeria rubra* effectively reduced MDA levels in renal tissue, potentially through its ability to scavenge oxygen free radicals in the kidneys. Additionally, it significantly elevated SOD levels, which improved kidney function and histopathology. Treatment with *Plumeria rubra* facilitated renal function recovery, evidenced by the regenerative capacity observed in renal tubules.

5.1.3 *Celosia argentea*:

Plant Profile:

- **Botanical name:** *Celosia argentea*
- **Family:** Amaranthaceae

Nature: The plant stands upright and bears simple leaves in alternate arrangement. Its flowers form dense terminal spikes, where papery pinkishwhite to silvery-white flowers interlace, and occasionally displaying bright pink tips at the ends of the flower spikes.

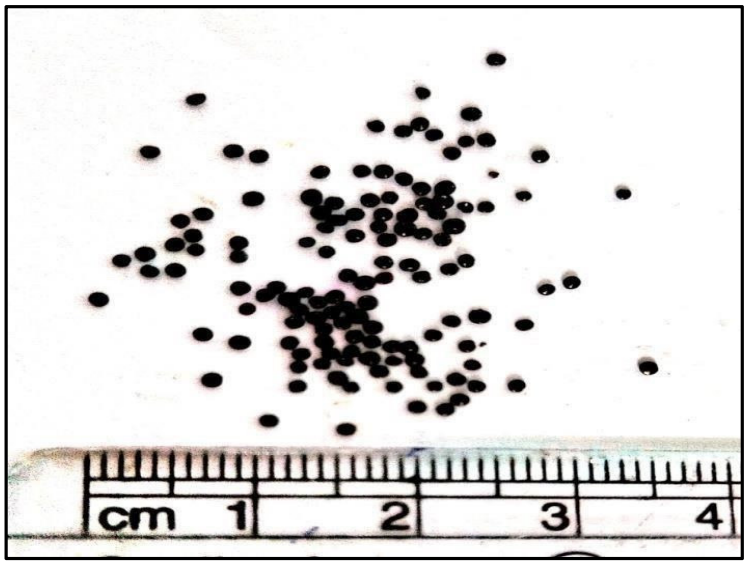


Figure No. 5.3: *Celosia argentea* Seeds

- **Vernacular Names:** English: Common coxcomb, Crested coxcomb, Feather coxcomb Telugu: Gurugu, Panchechettu Hindi: Sufaidmurga, Indivara, Survali, Laal Murga, Sanskrit: Vitunnaka, Sitivara, Sunishannaka Panjabi: Srwali Marathi: Kombda Kannada: Kanne Hoo Chinese: Bairihong, Ye ji guan huo, Guoweicao, Ji guan huo French: Amarantecrete de coq, Celosieargentee, Celociecrete de coq [166]
- **Parts Used:** Seed, Flower, Plant
- **Propagation of the Herb:** Seeds are sown in early to mid-spring in a warm greenhouse. Germination typically occurs within 2 weeks. Once the seedlings are large enough to handle, transplant them into individual pots and plant them outdoors after the last expected frosts.[167]

5.1.3.1 Description:

Celosia argentea L. is a rapidly growing annual herb, upright and coarse-textured, reaching heights of 0.4 to 2 meters. It features either a simple or branched structure with smooth stems and branches. The plant is characterized by numerous ascending branches and vibrant-colored bedding plants. Stems and branches are prominently ridged and often furrowed, and generally lack hairiness.

The leaves are arranged alternately, lanceolate-oblong to narrowly linear, entire, measuring 4 to 14 cm in length. They taper to acute or obtuse tips with a short mucro on the midrib extension, and are smooth with a bitter taste and scent. Leaves are light green, with laminae ranging from 2 to 15 cm long and 0.1 to 3.2 cm wide, narrowing towards a slender, indistinctly demarcated petiole originating from the central stem. Upper and branch leaves are smaller and notably reduced in size, often accompanied by small, leaf-like sterile shoots in leaf axils.

Seed Description: The seeds, known as Semen Celosiae, are the prominent medicinal part of *Celosia argentea*. They are shiny, oblate, and measure 1.25–1.5 mm in diameter, with a lenticular shape and a glossy black or reddish-black surface. The seeds feature a prominent middle prominence and a laterally scooped hilum. Their seed coat is very finely reticulated and thin, with a crumbly texture. [168]

Despite similarities with Semen Cristata in profile, color, and texture, the seeds of *Celosia argentea* are distinguished as the most crucial component for medicinal use.

Flower: The flowers are densely packed in cylindrical spikes, initially pink and gradually turning white.

Fruit: The fruit is a capsule with a globose shape, containing approximately 12 reticulate seeds.

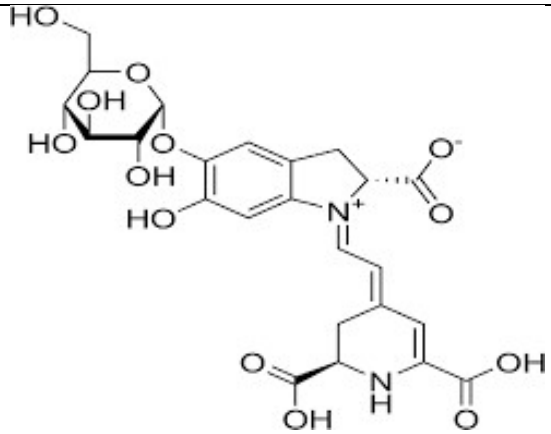
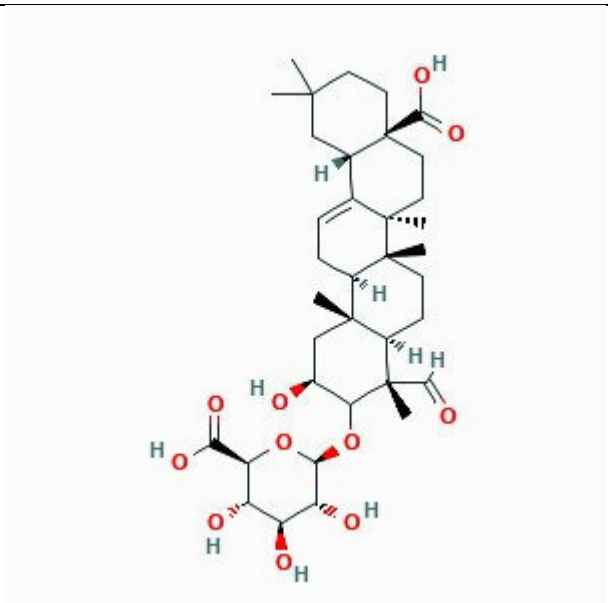
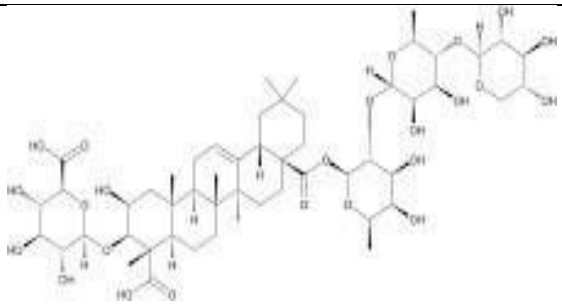
Leaf Characteristics: Apices: Acute Arrangement: Alternate in a spiral pattern Bases: Cuneate Margins: Entire Shapes: Elliptic Types: Simple

Habit: *Celosia argentea* is an erect, glabrous annual herb extensively branched with a profuse growth pattern.[169]

5.1.3.2 Phytochemical Constituent:

A variety of compounds with diverse properties have been identified in *Celosia argentea* plants, including phenolics, steroids, diterpenes, and flavonoids. Recent studies have isolated and characterized three new triterpenoid saponins—celosin E, celosin F, and celosin G—alongside the known compound cristatain, using advanced techniques such as nuclear magnetic resonance (NMR) and mass spectrometry (MS). These compounds, along with cristatain, have demonstrated potential antitumor and antiinflammatory properties in preliminary in vitro screenings. Phytochemical analysis of *Celosia argentea* has also revealed the presence of betalains, nicotinic acid, celogenamide A, celogentin A–D, Celogentin-H, celogentin-J, celogentin-K, and moroidin. Additionally, the inflorescence of *Celosia argentea* has been studied for its potential use as an economical and eco-friendly biosorbent for removing methylene blue from waste water. The combination of *Celosia argentea* inflorescence with stem of Cicer arietinum and cob of Zea mays was investigated in batch adsorption studies, varying the amount of adsorbent, concentration, temperature, and pH. These findings underscore the growing interest in *Celosia argentea* due to its active phytochemical constituents and promising pharmacological activities.[170]

Table No. 5.3: Phytochemical Constituent of *Celosia argentea*

Sr. No.	Name of Phytochemical Constituent	Structure
1.	Betalain	
2.	Cristatain	
3.	Celosin F	

4.	Moroidin	
5.	Celogentin C	
6.	Nicotinic Acid	
7.	Oleanolic acid	

5.1.3.3 Pharmacological Activity:

5.1.3.3.1 Immunological activity:

Celosian, identified as a chemical component of *C. argentea,* exhibits immunostimulatory properties. This acidic polysaccharide, derived from the seeds of the plant, has been found to possess significant antihepatotoxic effects in animal models of chemical and immunological liver injuries. Studies indicate that Celosian acts as an immunostimulant by inducing the production of tumor necrosis factor-alpha (TNF-alpha), interleukin-1 beta (IL-1 beta), nitric oxide (NO), and gamma interferon (IFN-gamma) through various in-vitro experimental approaches.[171]

5.1.3.3.2 The anti-inflammatory activity: potential of *C. argentea* was explored through various studies. In vivo experiments focused on the flavonoid fraction obtained from the alcoholic leaf extract, evaluating its effectiveness in animal models such as carrageenan-induced rat paw edema for acute inflammation and cotton pellet-induced chronic inflammation. Additionally, triterpenoid saponins known as celosin E, celosin F, celosin G, and cristatain were identified from the seeds of *C. argentea*. These compounds were subsequently screened for anti-inflammatory properties using in vitro methods.

5.1.3.3.3 Anti-cancer activity: The anti-cancer potential of triterpenoid saponins derived from the seeds of *C. argentea*, specifically celosin E, celosin F, celosin G, and cristatain, has been evaluated through in vitro methods.[172]

5.1.3.3.4 Hepatoprotective activity: The hepatoprotective potential of a 70% ethanolic extract derived from *C. argentea* seeds was evaluated against carbon tetrachloride (CCl4)-induced hepatic damage in rats. The study observed a significant decrease in lipid peroxidation levels (TBARS) and an increase in antioxidant defense parameters compared to the rats treated only with CCl4, suggesting a protective effect on the liver.

5.1.3.3.5 Potential antioxidant activity: *Celosia argentea* was investigated due to its high phenolic content. Three extracts from *C. argentea*—aerial parts, seeds, and roots— were evaluated for their ability to scavenge radicals using in-vitro methods [173]. Among these extracts, the seed extract exhibited the highest total phenolic content compared to the aerial parts and root extracts. The study found that the seed extract showed the strongest ability to scavenge harmful radicals, followed by the aerial parts extract. In contrast, the root extract demonstrated negligible antioxidant potential. These findings suggest that the seed extract of *C. argentea*, rich in polyphenols, could potentially protect against oxidative damage by scavenging and suppressing harmful free radicals.[174]

5.1.3.3.6 Antimitotic activity: The compound moroidin, derived from the seeds of *C. argentea*, exhibits potent inhibition of tubulin polymerization, indicating significant antimitotic effects.

5.1.3.3.7 Antibacterial activity: Antibacterial efficacy was examined in a study involving crude alcoholic extracts of Datura alba and *C. argentea* leaves against pathogens from burn patients. Using the disc-diffusion method, both extracts demonstrated substantial zones of inhibition against all pathogens tested, comparable to Silver Sulphadiazine cream. Datura

alba extract exhibited over 50% greater antibacterial activity compared to *C. argentea*, highlighting its superior effectiveness.[175]

5.1.3.3.8 Wound healing activity: The research investigated the therapeutic potential of a 10% w/w alcohol extract of *C. argentea* incorporated into an ointment, using a rat burn wound model. The results substantiated the positive effects of the *C. argentea* extract on wound healing, indicating its capability to enhance the proliferation and migration of dermal fibroblasts. *C. argentea* is well-regarded in India for its medicinal properties in wound care, akin to other plants like Aloe vera, Azadirachta indica, Carica papaya, Cinnamomum zeylanicum, Curcuma longa, Ocimum sanctum, Nelumbo nucifera, among others. [176,177]

5.1.3.3.9 The antiurolithiatic activity: this drug is supported by modern research. Increased urine output serves two primary purposes. Firstly, it promotes mechanical diuresis, which helps prevent urinary stagnation and the formation of calculi (stones). Secondly, by producing more dilute urine, it reduces the supersaturation of stoneforming components. Thus, this drug acts not only as an antiurolithiatic agent but also as a preventive measure against further stone formation.[178]

5.1.4 EXIPIENT PROFILE

5.1.4.1 Talc:

Table No.5.4: Specifications of Talc

Sr. No.	Test	Test / Limits	Method
1.	Description	A White or almost white powder, Free from greetiness; readily adheres to skin, odourless	[179]
2.	Identification	Test A, B, C.	[179]
3.	Acidity & Alkalinity	Meets the requirement of IP' 2014	[179]
4.	Acid soluble substances	NMT 2.0 %	[179]
5.	Water soluble	NMT 10 mg	[179]
6.	Carbonates	Meets requirement of IP' 2014	[179]

7.	Chlorides	NMT 250ppm	[179]
8.	Organic compounds	NMT slightly yellow or gray	[179]
9.	Loss on drying	NMT 1.0 %	[179]
10.	Microbial contamination	1.0 g is free from E coli and 10.0 g free from Salmonellae & Shigella	[179]

5.1.4.2 Magnesium Stearate:

Table No. 5.5: Specifications of Magnesium Stearate

Sr. No.	Test	Test / Limits	Method
1.	Description	A very fine, light White powder; Odourless or with faint odour of stearic acid	[179]
2.	Identification	Test A,B,C,D.	[179]
3.	Appearance of solution	Solution meets the requirement of IP' 2014	[179]
4.	Acidity & Alkalinity	Meets the requirement of IP' 2014	[179]
5.	Acid value	195 to 210	[179]
6.	Free stearic acid	NMT 3%	[179]
7.	Zinc stearate	Meets requirement of IP' 2014	[179]
8.	Heavy metal	NMT 10 ppm	[179]
9.	Chlorides	NMT 250 ppm	[179]
10.	Sulphates	NMT 0.6%	[179]
11.	Loss on drying	NMT 6.0 %	[179]
12.	Assay	NLT 3.8% and NMT 5.0 % of Mg NLT 40% of stearic acid and NMT 90.0 % of stearic & palmitic acid	[179]

5.1.4.3 Lactose:

Table No. 5.6: Specifications of Lactose

Sr. No.	Test	Test / Limits	Method
1.	Description	A fine or almost white powder, crystalline powder; odorless.	[179]
2.	Identification	Test A,B,C	[179]
3.	Acidity or Alkalinity	NMT 0.4 ml of 0.1 M NaOH to get pink colour	[179]
4.	Specific rotation	+54.4° to +55.9°	[179]
5.	Light absorption	0.04 at 400nm	[179]
6.	Arsenic	NMT 1 ppm	[179]
7.	Heavy metal	NMT 5ppm	[179]
8.	Loss on drying	NMT 4.5 to 5.5	[179]
9.	Sulphated ash	NMT 0.1 %	[179]
10.	Microbial limit	NMT 100 CFU per 1 gm. Free from E.coli, S. Typhi and Shigella Species in 10.0 g.	[179]

5.1.4.4 Starch

Table No. 5.7: Specifications of Starch

Sr. No.	Test	Test / Limits	Method
1.	Description	A fine or a white powder, odourless and tastelesspowder.	[180]
2.	Chemical name CAS	Starch [9005-25-8]	[180]
3.	Acidity or Alkalinity	pH4.0-8.0.	[180]
4.	Bulk density	0.45-0.58 g/cm 3	[180]

5.	Moisture content	71 degree celcius	[180]
6.	Solubility	Practically insoluble in cold ehanol, Starch swell 5-10% In water at 37 degree celcius	[180] [180]
7.	Functional Category	Tablet and capsule diluent, disintergrator, tablet binder	[180]
8.	Safety Regulatory status	Edible food substance, widely used GRAS listed, FDA	[180] [180]

5.1.4.5 Sodium alginate

Table No. 5.8: Specifications of Sodium alginate

Sr. No.	Test	Test / Limits	Method
1.	Description	An odourless and tasteless White to pale yellowish – brown coloured powder.	[180]
2.	Chemical name CAS	Soldium alginate [9005-38-3]	[180]
3.	Acidity or Alkalinity	pH7.2 (1% aqueous solution).	[180]
4.	Heavy Metals	< 20 ppm	[180]
5.	Microbial limit	NMT 1000CFU per 1 gm. Free from E.coli, S. Typhi and Shigella Species in 10.0 g.	[180]
6.	Solubility	Practically insoluble in ethanol, slowly soluble in water forming viscous colloidal solution	[180]
7.	Functional Category Safety Regulatory status	Stabilizing agent, suspending agent, viscosity increasing agent LD50 (rat oral) 5gm/Kg GRAS listed, FDA, accepted Food additive	[180]

5.1.4.6 Calcium chloride

Table No. 5.9: Specifications of Calcium chloride

Sr. No.	Test	Test / Limits	Method
1.	Description	A fine or almost white powder, crystalline powder; granules.	[180]
2.	Chemical name CAS	Calcium Chloride anhydrous [10043-52-4]	[180]
3.	Acidity or Alkalinity	pH 4.5 – 9.	[180]
4.	Bulk density	0.835g/cm2	[180]
5.	Solubility	Freely soluble in water and ethanol	[180]
6.	Functional Category	Antimicrobial preservative, water absorbing agent	[180]
7.	Safety	LD50 (rat oral) 1gm/Kg	[180]
8.	Regulatory status	GRAS listed, FDA, FCC (food Chemicals codex)	[180]

5.1.5.1 Experimental Details

The drugs, excipients, chemicals/ reagents and equipment used for various experiments are enlisted as follows:

5.1.5.1.1 Materials

Table No. 5.10: List of Materials used

Name of materials	Supplied / Gifted by
Leaves of Boerhaviadiffusa	Collected from Local areas
Pods of Plumeriarubra	Collected from Local areas
Seeds of *Celosia argentea*	Collected from Local areas
Methanol	Research-Lab Fine Chem., Mumbai

Phloroglucinol	Research-Lab Fine Chem., Mumbai
Hydrochloric acid	Research-Lab Fine Chem., Mumbai
Potassium Mercuric Iodide	Research-Lab Fine Chem., Mumbai
Iodine	Hilab Chemicals, Shrirampur
Potassium Iodide	Hilab Chemicals, Shrirampur
Picric acid	Research-Lab Fine Chem., Mumbai
Potassium bismuth iodide	Hilab Chemicals, Shrirampur
α- napthol	Reliance Scientific, Pune
Sulphuric acid	Research-Lab Fine Chem., Mumbai
Fehling solution A	Sahyadri Scientific, Islampur
Fehling solution B	Sahyadri Scientific, Islampur
Copper acetate	Sahyadri Scientific, Islampur
Glacial acetic acid	Research-Lab Fine Chem , Mumbai
Benedict's Solution	Research-Lab Fine Chem , Mumbai
Chloroform	Molychem, Mumbai
Ammonia	Research-Lab Fine Chem , Mumbai
Pyridine	Research-Lab Fine Chem., Mumbai
Sodium nitroprusside	Sahydri Scientific, Islampur
Sodium hydroxide	Research-Lab Fine Chem., Mumbai
Millon's reagent	Research-Lab Fine Chem., Mumbai
Copper sulphate	Ecolab, Pune
Potassium hydroxide	Research-Lab Fine Chem., Mumbai
Ninhydrin reagent	Research-Lab Fine Chem., Mumbai
Acetone	Research-Lab Fine Chem., Mumbai
Potassium hydroxide	Research-Lab Fine Chem , Mumbai

Phenolphthalein	Research-Lab Fine Chem., Mumbai
Ferric chloride	Pure Chem., Pune
Gelatin	Research-Lab Fine Chem., Mumbai
Lead acetate	Research-Lab Fine Chem., Mumbai
Bromine	Research-Lab Fine Chem., Mumbai
Potassium bromide	Research-Lab Fine Chem., Mumbai
Silica gel	Molychem, Mumbai
Gallic Acid	Ajinkya supplier, Pune
Rutin	Ajinkya supplier, Pune
Luteolin	Ajinkya supplier, Pune
Quercetin	Ajinkya supplier, Pune
Ether	Molychem, Mumbai
Phosphate Buffer	Research-Lab Fine Chem , Mumbai
Toluene	Research-Lab Fine Chem , Mumbai
Formic acid	Research Lab, Mumbai
Ethyl acetate	Research-Lab Fine Chem , Mumbai
Carbon tetrachloride.	Research-Lab Fine Chem , Mumbai
Potassium Iodide	Hilab Chemicals, Shrirampur
Sodium thiosulphate	ThermosilScientific, Pune
Carbopol 934	Research-Lab Fine Chem , Mumbai
HPMC K 100	Research-Lab Fine Chem , Mumbai
Propylene Glycol	Research-Lab Fine Chem , Mumbai
Triethanolamine	Hilab chemicals, Shrirampur
Propyl paraben	Chem Pure, Ltd.
Ethylene Glycol	Research-Lab Fine Chem , Mumbai
Ursolic acid	Ajinkya Enterprizes, Pune
Starch	Sahyadri Scientific, Islampur
Lactose	Sahyadri Scientific, Islampur

Magnesium stearate	Sahyadri Scientific, Islampur
Talc	Sahyadri Scientific, Islampur
Sodium alginate	Sahyadri Scientific, Islampur
Calcium Chloride	Sahyadri Scientific, Islampur
Cyclohexane	Sahyadri Scientific, Islampur

5.1.5.1.2 Instrument / Equipment

Table No. 5.11: List of Instruments / Equipments used

Name of the Equipment / Instrument	Make	Model
Electronic Balance	Shimadzu	AY220
Soxhlet	Sahydri Scientific	1000
Rotary evaporator	Medica instruments	Evator
Chromatography visualization (UVCabinet)	Camag- winCATS	-
Digital melting point apparatus	Veego	Vpmds, Nashik
Sonicator	Citizen	CD-4820
pH meter	Elico, India	LI 613
UV-visible double beam spectrophotometer	Shimadzu	UV-1800
FTIR spectrophotometer	Perkin Elmer	Spectrum65
Chromatogram development chambers	Camag	
Microscope	Imicron, India	Radical RM-3
Centrifuge	Remi	R8C
High speed centrifuge	Beckman Coulter	Allegra 64 R Centrifuge USA

Precoated silica gel 60 F254 TLC plates (10 × 10 cm, layer thickness 0.2 mm)	E. Merk K GaA, Darmstadt, Germany	-
The HPTLC system consisted of Linamat V Autosprayer connected to a nitrogen cylinder, a twin trough chamber (10×10cm), a derivation chamber and a plate heater	Camag, Muttenz, Switzerland	-
Refractometer	Abbe	-
Binoculardissecting microscope	Labomed	Vision 2000
Magnetic Stirrer	RemiMotar	1MLH
Rheometer	Stress-Tech	Reologica, Sweden
Viscometer	Brookfield Engineering Lab	MLVT115
Stabilitychamber	Biotechno lab	BTL
Immersion device for derivatisation	Camag	-
TLC/HPTLC plate heater	Camag	-
Digital Homogeniser	Remi	RQT-127 A/D

Software

1. Graph pad Instat v 3.1.
2. WinCATS Windows
3. Xpversion1.4.6

5.2 PHARMACOGNOSTICAL AND PHYTOPHYSICOCHEMICAL INVESTIGATIONS OF CRUDE DRUGS

5.2.1 Aim:

To prepare aqueous extract of crude drugs and evaluate its Pharmacognostic and Phytophysico chemical properties.

5.2.2 Objective:

The prime objectives of carrying out the dissertation work were as follows:

1. To prepare Aqueous extract of *Boerhavia diffusa, Plumeria Rubra* and *Celosia argentea*
2. To carry out morphological, microscopic, physicochemical evaluation like ash values, extractive values, moisture content, foreign organic matter and fluorescence analysis of *Boerhavia diffusa*, *Plumeria Rubra* and *Celosia argentea.*
3. To evaluate the prepared Aqueous and Methanolic extract of *Boerhavia diffusa*, *Plumeria Rubra* and *Celosia argentea* for various parameters like physical examinations, phytochemical investigations, pH, TLC and FTIR.

5.2.3 Introduction:

In recent years, there has been a global increase in the use of medicinal plant products within healthcare systems. The growing demand for alternative medicine worldwide has fueled expansion in the natural product market and renewed interest in traditional medicine systems. Herbal drug technology plays a crucial role in transforming botanical materials into medicines, emphasizing the importance of standardization, quality control, and the effective integration of modern scientific techniques with traditional knowledge. Standardization, according to the American Herbal Products Association, involves ensuring consistent quality by controlling and minimizing variations in the composition of materials through rigorous quality assurance practices applied to both agricultural and manufacturing processes.

To ensure the quality of herbal drugs, standardization methods must consider various factors such as the correct identification of the sample, organoleptic evaluation, pharmacognostic evaluation, volatile matter content, and quantitative assessments like ash and extractive values. Additionally, phytochemical evaluation, tests for xenobiotics, microbial load,

toxicity, and biological activity are crucial. The phytochemical profile is particularly important because it directly influences the drug's activity. Fingerprint profiles provide guidelines for understanding the drug's phytochemical makeup, which helps ensure quality. Quantifying marker compounds also serves as an additional measure for quality assessment. Therefore, phytochemical evaluation for standardization includes these essential elements. Preliminary testing for the presence of different chemical groups.

1. Measurement of key chemical groups such as alkaloids, phenolics, triterpenic acids, and tannins. Development of fingerprint profiles.
2. Creation of fingerprint profiles using multiple markers.
3. Determination of significant chemical constituents.

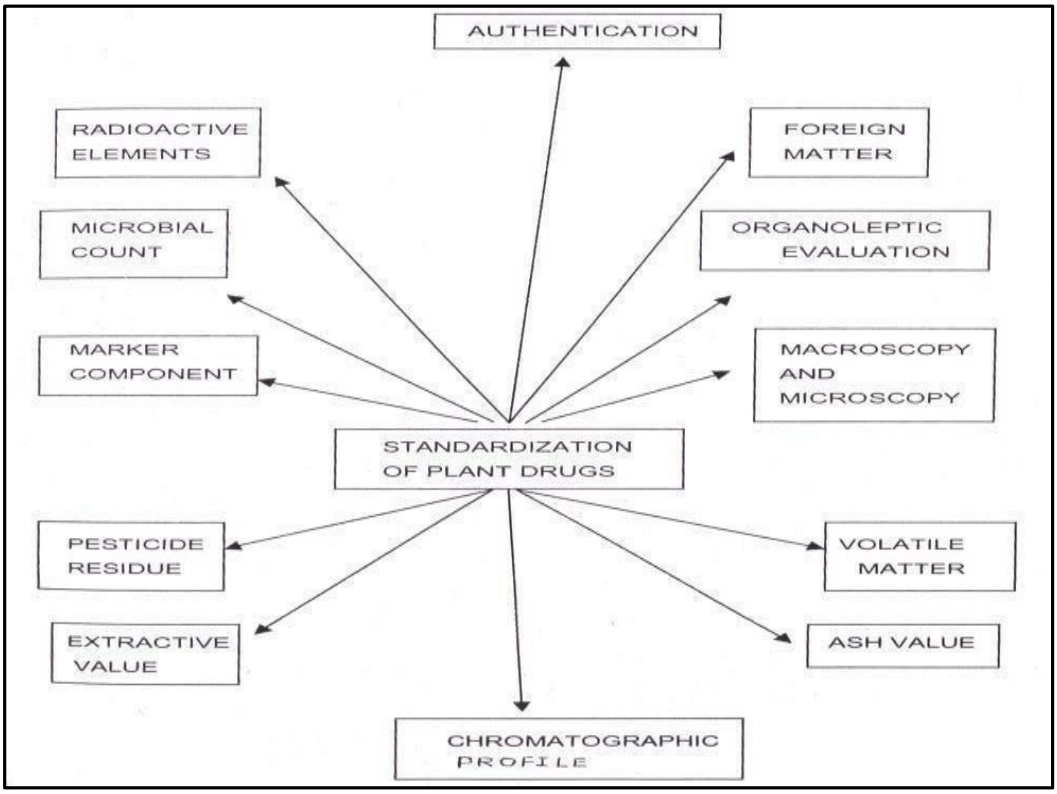


Figure No. 5.4: Standardization chart of herbal raw materials

To achieve these objectives, the raw materials used in creating the herbal hair formulation were assessed based on their pharmacognostical, physicochemical, and phytochemical properties.

5.2.4 Experimental:

5.2.4.1 Procurement and Authentication of drugs:

The plants used in this study include the whole plant of *Boerhavia diffusa* Linn, collected from Khandala, Maharashtra, and mature pods of *Plumeria Rubra* Linn, gathered from Rajuri, Junnar, Maharashtra. Seeds of *Celosia argentea* Linn were acquired from the Mankarnika Ayurvedic store in Chinchwad, Pune. The plants and seeds were identified and authenticated by Dr. R. K. Chaudhary, Scientist at the Agharkar Research Institute, an autonomous body under DST, GOI, Pune. Herbarium specimens have been preserved in the laboratory. The plant material was thoroughly washed with running tap water, rinsed with distilled water, and shade-dried for seven days. After drying, the plant material was ground using a laboratory herbal grinding mill. The coarse powder (60#) of the dried plants was stored in air-tight containers for further pharmacognostical, physicochemical, and phytochemical evaluation.

5.2.4.2 Preparation of Extracts [181]

The extraction process involves separating the desired constituents from a crude drug, commonly using the Soxhlet extraction method. Traditionally employed for solid samples, Soxhlet extraction involves placing the sample in a porous thimble within the main chamber of the Soxhlet apparatus. The solvent continuously refluxes through the thimble with the aid of a condenser and siphon side arm, repeating the extraction cycle multiple times. This well-established and robust technique allows for unattended extraction, though it necessitates a lengthy extraction time and substantial solvent use.

Steps:

1. Continuously extract a component from a solid mixture. Boil the solvent, allowing its vapors to rise through the larger side arm.
2. Condensed solvent droplets fall into the porous cup, dissolving the desired component from the solid mixture.
3. When the smaller side arm overflows, it triggers a siphoning action.
4. The residual solvent drains from the porous cup as fresh solvent droplets continue to fall. The solvent, now containing the dissolved component, is siphoned back into the boiler below.

A. Boerhavia diffusa

50 grams of dried, coarse *Boerhavia diffusa* powder were subjected to continuous extraction with water as the solvent using a Soxhlet apparatus for 48 hours. Once extraction was complete, the aqueous extract was concentrated under reduced pressure at 40ºC using a vacuum dryer. The resulting dried extract was then stored in desiccators.

B. Plumeria Rubra

50 grams of dried, coarse *Plumeria Rubra* powder were continuously extracted with water using a Soxhlet apparatus over a 48-hour period. Once the extraction was complete, the aqueous extract was concentrated under reduced pressure at 40ºC using a vacuum dryer. The resulting dried extract was then stored in desiccators.

C. Celosia argentea

50 grams of dried coarse powder of *Celosia argentea* were subjected to continuous extraction with water using a Soxhlet apparatus for 48 hours. Upon complete extraction, the aqueous extract was concentrated under reduced pressure at 40°C using a vacuum dryer to obtain a dried extract, which was then stored in a desiccator.

5.2.4.3 Specification of *Boerhavia diffusa*, *Plumeria Rubra* and *Celosia argentea*

Table No. 5.12: Specifications of *Boerhavia diffusa*[182]

Sr. No.	TEST	SPECIFICATION
1.	Description	Powder Green in colour
2.	Identification	a) Microscopic under100X magnification it shows one layer of palisade, spongy parenchyma 2-4 layered with small air spacesanomocyticstomata, covering trichomes, calcium oxalate crystals, orange red resinous matter present in mesophyll. b) By Thin layer chromatography it shows major spot i.e. *Eupalitin 3-O-β-D Galctopyranoside* (Rf: 0.51) as phytoconstituent.

3.	Foreign Organic Matters	Nil
4.	Ethanol Soluble extractive	Not less Than 6.0 %
5.	Water Soluble extractive	Not less Than 11 %
6.	Loss on Drying (Moisture content)	Not More Than 3.0%
7.	Total Ash	Not More Than 10%
8.	Acid insoluble ash	Not More Than 1.0%

Table No. 5.13: Specifications of *Plumeria Rubra* seed pod

Sr. No.	TEST	SPECIFICATION
1.	Description	Powder Dark brown in colour
2.	Identification	a) Microscopic under 100 X magnification transverse section of seed pod showed palisade cell, midrib shows spongy parenchyma and 5-6 pairs of vascular bundle calcium oxalate crystal, b) By Thin layer chromatography it shows major spot i.e. Lupeol (Rf: 0.46) as phytoconstituent.
3.	Foreign Organic Matters	Nil
4.	Ethanol Soluble extractive	Not less Than 3.93 %
5.	Water Soluble extractive	Not less Than 6.03%

6.	Loss on Drying (Moisture content)	Not More Than 2.8%
7.	Total Ash	Not More Than 14.80%
8.	Acid insoluble ash	Not More Than 0.7%

Table No. 5.14: Specifications of *Celosia argentea* seeds[183]

Sr. No.	TEST	SPECIFICATION
1.	Description	Powder faint black in colour
2.	Identification	a) Microscopic under100X magnification shows outer dark brown covering enclosing testa, oil globules, starch grain, crystals of calcium oxalate. b) By Thin layer chromatography it shows major spot i.e. Oleanolic acid (Rf: 0.43) as phytoconstituent.
3.	Foreign Organic Matters	Not More Than 0.6%
4.	Ethanol Soluble extractive	Not less Than 1.2 %
5.	Water Soluble extractive	Not less Than 0.6%
6.	Loss on Drying (Moisture content)	Not More Than 0.05%
7.	Total Ash	Not More Than 10%
8.	Acid insoluble ash	Not More Than 1.8%

5.2.4.4 Morphological Evaluation[184]

Morphological evaluation involves assessing drugs based on characteristics such as color, odor, taste, size, shape, and special attributes like texture. The leaves of *Boerhavia diffusa*, pods of *Plumeria Rubra*, and seeds of *Celosia argentea* were examined and compared against established references to describe their morphological features.

5.2.4.5 Microscopic evaluation

Microscopic examination was conducted on freehand sections of the leaves of *Boerhavia diffusa*, pods of *Plumeria Rubra*, and seeds of *Celosia argentea*. The sections were stained with phloroglucinol and concentrated hydrochloric acid to verify the presence of lignin.

The powders of *Boerhavia diffusa*, *Plumeria Rubra*, and *Celosia argentea* were examined under a microscope following treatment with phloroglucinol and concentrated hydrochloric acid.

5.2.4.6 Determination of ash values[185]

Identifying inorganic components such as phosphates, carbonates, and silicates of sodium, potassium, magnesium, and calcium within the substance. In some cases, the presence of inorganic substances like calcium oxalate, silica, and carbonates in the raw material can influence the 'Total ash value'. These substances can be eliminated by treating the material with acid, which dissolves them (since they are soluble in hydrochloric acid), allowing for the determination of the acid-insoluble ash value.

5.2.4.6.1 Determination of Total ash values

To determine the total ash content, 2 grams of powdered materials (leaves of *Boerhavia diffusa*, pods of *Plumeria Rubra*, and seeds of *Celosia argentea*) were individually placed in pre-weighed silica crucibles. The materials were spread evenly and accurately weighed. The crucibles were then heated gradually up to 450°C to ensure complete incineration without carbon residues. After cooling in a desiccator, the crucibles with ash were weighed, and the percentage of ash was calculated based on the difference between the weight of the crucible before and after incineration.

5.2.4.6.2 Determination of acid-insoluble ash

To determine acid-insoluble ash, 1 gram of ash obtained previously was mixed with 25 ml of dilute hydrochloric acid and gently boiled at 70-80ºC for 5 minutes. The mixture was then filtered through filter paper, which was subsequently washed with hot water and ignited until a constant weight was achieved. The percentage of acid-insoluble ash relative to the air-dried drug was then computed.

5.2.4.6.3 Determination of water-soluble ash

To determine the water-soluble ash content, one gram of ash obtained previously was boiled with 25 ml of water for 5 minutes. The insoluble matter was collected on an ashless filter paper, washed with hot water, and ignited for 15 minutes at a temperature below 450ºC. The weight of the insoluble matter was subtracted from the total ash weight to obtain the water-soluble ash content. The percentage of water-soluble ash relative to the air-dried drug was then calculated.

5.2.4.6.4 Determination of extractive values [184]:

Determination of alcohol-soluble extractives

To determine the alcohol-soluble extractives, 5 grams of the powdered, air-dried material was macerated with 100 mL of alcohol in a sealed flask for 24 hours, with frequent shaking in the initial 6 hours followed by 18 hours of standing. The mixture was then filtered rapidly, ensuring no solvent loss. Subsequently, 25 mL of the filtrate was evaporated to dryness in a tared flat-bottomed dish, dried at 105°C until a constant weight was achieved, and then weighed. The percentage of alcohol-soluble extractive was calculated based on the weight of the air-dried material and is expressed as a percentage.

Determination of water-soluble extractives

To determine the water-soluble extractives, 5 grams of the powdered air-dried substance was macerated with 100 mL of a chloroform-water mixture in a closed flask for 24 hours. The mixture was shaken frequently during the first 6 hours and left to stand for an additional 18 hours. After maceration, the solution was filtered rapidly, ensuring no solvent loss. A portion (25 mL) of the filtrate was evaporated to dryness in a tared flatbottomed shallow dish, dried at 105°C until a constant weight was achieved, and then weighed. The

percentage of water-soluble extractives was calculated based on the weight of the air-dried substance and is expressed as a percentage.

5.2.4.6.5 Determination of loss on drying (Moisture Content)[186]

The determination of loss on drying (moisture content) involved accurately weighing 1.5 grams of powdered material in a dried and tared flat weighing bottle. The sample was then dried in an oven at 105°C until a constant weight was achieved. The moisture content was calculated as a percentage relative to the initial weight of the sample. The weight loss due to drying was measured and recorded as the moisture content.

5.2.4.6.6 Determination of foreign organic matter[186]

100 grams of *Boerhavia diffusa* leaves, *Plumeria Rubra* pods, and *Celosia argentea* seeds were weighed individually and spread out on a white tile without overlapping. The leaves, pods, and seeds were visually inspected for any foreign organic matter, which was manually removed. After ensuring complete separation, the remaining matter was reweighed, and the percentage weight present in each sample was determined, yielding results between 270-272% w/w.

5.2.4.6.7 Fluorescence Analysis

The fluorescence properties of the leaves, pods, and seed powders under visible and ultraviolet light were investigated. This was achieved by immersing the powders in various reagent solutions and observing them under the appropriate wavelengths in a UV chamber (273-275 nm).

5.2.5 Phytochemical Evaluation[187-188]

The drugs extract underwent multiple chemical analyses to ascertain the existence of alkaloids, carbohydrates, glycosides, saponins, proteins, amino acids, phytosterols, fixed oils, fats, phenolic compounds, and tannins.

Detection of Alkaloids

1. Mayer's test

The samples underwent treatment with potassium mercuric iodide (known as Mayer's reagent). The appearance of a cream-colored precipitate confirmed the presence of alkaloids.

2. Wagner's test

The samples underwent treatment with iodine solution in potassium iodide (known as Wagner's reagent), and the appearance of a brown precipitate indicated the existence of alkaloids.

3. Hager's test

The samples underwent treatment with a concentrated picric acid solution (known as Hager's reagent), where the appearance of a yellow precipitate signified the detection of alkaloids.

4. Dragendorff's test

The samples underwent treatment with potassium bismuth iodide, known as Dragendorff's reagent. The observation of a reddish-brown precipitate following this treatment confirmed the presence of alkaloids.

Detection of Carbohydrates

1. Molisch's test

The samples were mixed with 2 drops of an alcoholic solution of α-naphthol, shaken thoroughly, and then 1 ml of concentrated sulfuric acid was carefully added along the sides of the test tube. The mixture was allowed to stand, and the presence of carbohydrates was indicated by the appearance of a violet ring.

2. Fehling's test

The samples underwent boiling on a water bath followed by the addition of 1ml each of Fehling's solutions A and B. The formation of a red precipitate was taken as an indication of the presence of carbohydrates.

3. Barfoed's test

The extracts were treated with copper acetate in glacial acetic acid (Barfoed's reagent) and heated in a boiling water bath for 2 minutes. The formation of a red precipitate indicates the presence of carbohydrates.

4. Benedict's test

The samples underwent treatment with Benedict's reagent followed by heating on a boiling water bath for 2 minutes. The formation of a distinctive-colored precipitate serves as an indicator for the presence of carbohydrates.

Detection of Glycosides

1. Borntrager's test

The samples underwent treatment with chloroform and a 10% ammonia solution. The development of a pink coloration served as an indicator for the presence of glycosides.

2. Legal's test

The extracts underwent treatment with pyridine, sodium nitroprusside, and 10% sodium hydroxide. The development of a pink coloration indicates the presence of glycosides.

Detection of Saponins

The samples were mixed with distilled water and agitated for 15 minutes. The presence of saponins was indicated by the development of foam.

Detection of Proteins and amino acids

1. Millon's test

The samples underwent treatment with Millon's reagent, and the emergence of a white precipitate confirmed the presence of proteins.

2. Biuret test

The extracts were treated with 2% copper sulfate, ethanol, and an excess of potassium hydroxide pellets. The formation of a pink color indicates the presence of proteins.

3. Ninhydrin test

The samples underwent treatment with ninhydrin and acetone, where the development of a purple hue confirmed the existence of amino acids.

Detection of Phytosterols

1. Libermann-Burchard's test

The samples were dissolved in acetic anhydride. Subsequently, a few drops of concentrated sulfuric acid were added slowly down the sides of the test tube. Any color changes observed indicate the presence of phytosterols.

Detection of Fixed Oils and Fats

1. Spot test

A small amount of extracts was placed between two filter papers. The appearance of oil stains on the paper serves as an indicator of the presence of fixed oils.

2. Saponification test

The samples underwent treatment with 0.5 N alcoholic potassium hydroxide solution and phenolphthalein. This mixture was heated on a water bath for 2 hours. The formation of soap during this process indicates the presence of fixed oils and fats.

Detection of Tannins

Small amounts of alcohol and aqueous extracts were individually diluted in water and analyzed for the presence of phenolic compounds and tannins.

1. Ferric Chloride test

To the test solutions, a few drops of 5% ferric chloride solution were added. The appearance of a blue-black or green-black color indicates the presence of phenolic compounds and tannins.

2. Gelatin test

A few drops of a 1% gelatin solution in 10% sodium hydroxide were added to the test solutions. The formation of a white precipitate indicates the presence of tannins.

Detection of Phenolic compounds

1. Lead acetate test

A few drops of a 10% lead acetate solution were added to the test solutions. The formation of a bulky white precipitate indicates the presence of phenolic compounds.

2. Alkaline reagent test

Test solutions were treated with a 10% ammonium hydroxide solution. The presence of flavonoids is indicated by yellow fluorescence.

3. Aqueous bromine test

A few drops of aqueous bromine solution were added to the test samples. The formation of a yellow precipitate indicates the presence of tannins.

Detection of Gums and Mucilage's

The samples were dissolved in distilled water, followed by the addition of absolute alcohol while stirring continuously. The appearance of a white precipitate suggests the presence of gums and mucilages.

Detection of Volatile Oils

In a volatile oil estimation apparatus, 50 grams of powdered crude drugs were subjected to hydro-distillation. The distillate was collected in a graduated tube, where the aqueous portion automatically separated from the volatile oils.

pH of Extracts

The pH of the sample was measured by agitating 1g of powdered extract in 10 ml of freshly prepared cooled distilled water in a volumetric flask for 5 minutes. After filtration, the pH was determined using a digital pH meter.

5.2.6 Optimization of TLC Solvent System[189]

Various solvent systems were tested to establish a TLC system for *Boerhavia diffusa* leaves, *Plumeria Rubra* pods, and *Celosia argentea* seeds. The selection of the mobile phase for further study was based on maximum separation of constituents in the extracts, informed by a review of relevant literature.

5.2.6.1 Thin Layer Chromatography

A stock solution containing aqueous extracts of *Boerhavia diffusa* leaves, *Plumeria Rubra* pods, and *Celosia argentea* seeds was prepared. Fluorescent precoated K5 silica plates were used as the stationary phase. A 5 µl solution was applied as a band measuring 5 mm by 2 mm on each plate and allowed to dry before development. The solvent level in the developing tank was adjusted to 2-3 mm below the origin line on the plate. Development

was considered complete when the solvent front had traveled a distance not less than ¾ of the plate length and no more than 5 mm below the top. After drying, the plates were examined under daylight. The retention factor (Rf value) for each spot was calculated by dividing the distance traveled by the spot by the distance traveled by the solvent front.

Figure No. 5.5: Twin Trough chambers for TLC and HPTLC

5.2.6.2 HPTLC fingerprinting of extracts[189,195]

HPTLC fingerprinting of extracts involved initial filtration through a 0.45µm filter. The HPTLC analysis was conducted using conditions specifically optimized for the detection of marker compounds such as Gallic acid, Rutin, Quercetin, Luteolin, Eupalitin, Ursolic acid, and Oleic acid in extracts derived from the leaves of *Boerhavia diffusa*, pods of *Plumeria Rubra*, and seeds of *Celosia argentea*.

Figure No. 5.6: HPTLC scanner of CAMAG showing TLC plate

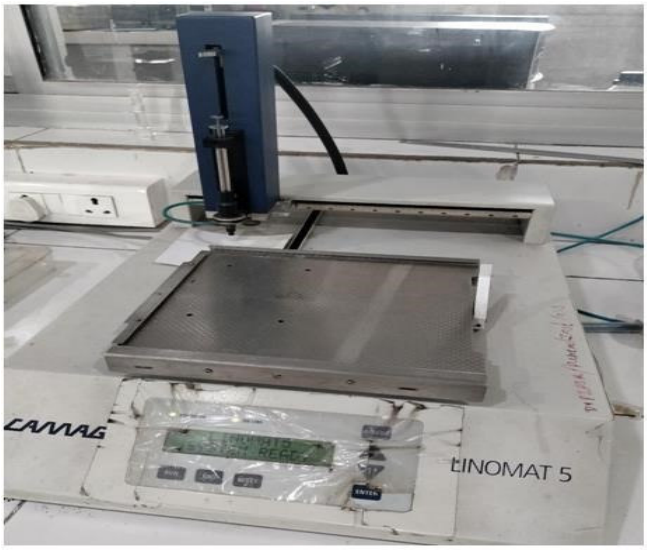


Figure No. 5.7: Linomat Semiautomatic sampler for HPTLC plates HPTLC Conditions

Table No. 5.15: HPTLC Conditions of Leaves of *Boerhavia diffusa*, Pods of *Plumeria Rubra* and seeds of *Celosia argentea* extracts

Standard	B. D. extract	C. A. extract	P. R. extract
Solvent system	Ethyl acetate: Methanol : Formic acid (5:0.5:0.5 v/v)	Toluene: Ethyl acetate : Formic acid (8:2:0.1 v/v)	Toluene : Ethyl acetate : Formic acid (8:2:0.1 v/v)
Layer	Silica Gel GF 254	Silica Gel GF 254	Silica Gel GF 254
Application	Camag100µLsample syringe	Camag100µLsample Syringe	Camag100µLsample Syringe
Chamber Condition	Twin trough glass	Twin trough glass	Twin trough glass
Saturation time and Temperature	20 mins (25±2ºC)	20 mins (25±2ºC)	20 mins (25±2ºC)
Development distance	70 mm	70 mm	70 mm
Migration time	20 mins	20 mins	20 mins
Detection	254 nm	650 nm	500 nm

Development Mode	Ascending dimensional and one	Ascending dimensional And one	Ascending dimensional and one
Slit dimensions	6 mm×0.45 Micro	6mm×0.45 Micro	6 mm×0.45 Micro
Scanning Speed	20 mm/s	20 mm/s	20 mm/s

5.2.6.3 HPTLC fingerprinting of Pods of *Plumeria Rubra* and seeds of *Celosia argentea* extract by conditioning method.

After scanning at 366 nm, the plates underwent a 5-second immersion in Anisaldehyde sulphuric acid reagent, followed by a 20-minute conditioning at 110ºC. Subsequently, the plates were cooled and their fingerprints were recorded by scanning at 540 nm.

5.2.6.4 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of extracts from Leaves of *Boerhavia diffusa*, Pods of *Plumeria Rubra*, and seeds of *Celosia argentea* were acquired using a Perkin Elmer FTIR spectrophotometer employing the KBr disc technique. Drug powder samples were mixed with KBr to form an infrared-transparent matrix. KBr discs were prepared by compressing the powder, and scans were conducted across the mid-infrared spectrum ranging from 4000 to 400 cm^-1 at a resolution of 1 cm^-1.

5.2.7 HPTLC Method Development and Validation [189]

Aim:

To develop and validate method of active compounds.

Objective

The prime objectives of carrying out the dissertation work were as follows:

1. HPTLC method development for active compounds.
2. Validate developed HPTLC method of active compounds.

Introduction

It is essential to validate analytical methods developed for the subsequent use to estimate the controls accurately. However, the developed and selects analytical HPTLC method was validated.

Typical Validation Characteristic which should be considered are listed below.

1. Linearity
2. Range
3. Accuracy
4. Precision
5. Limit of Detection
6. Limit of Quantitation
7. Specificity
8. Robustness

Experimental

The plant extracts were stored in airtight containers at room temperature. Thin-layer chromatography (TLC) plates precoated with silica gel 60 F254 (20 × 20 cm, 0.2 mm thickness) obtained from E. Merck Ltd. were employed for the chromatographic analysis.

The supplier of the TLC plates was located in Mumbai, India.

Reagents and standards

The laboratory utilized high-quality organic solvents and chemicals for scientific analysis. Specifically, in Nashik, India, a Camag Linomat 5 semiautomatic sample applicator (Camag, Switzerland) equipped with a 100 µl Hamilton syringe and WinCATS software (CAMAG Ver. 1.4.1), along with a Camag TLC Scanner 3 and Twin Trough Chamber, were employed.

Method of preparing a standard solution

Plant extract stock solutions (1000 µg/mL) were prepared individually by dissolving 10 mg of accurately weighed standard in 10 mL of methanol. From each stock solution, a 100 µg/mL solution was prepared by transferring 1 mL of the stock solution to a 10 mL volumetric flask and adjusting the volume with methanol.

Chromatographic conditions

Chromatographic separation utilized HPTLC plates (10 × 10 cm), precoated with 0.2 mm thick silica gel 60 F254 on aluminum support. Sample application was performed using a Camag Linomat 5 sample applicator from Muttenz, Switzerland, equipped with a 100 µl Hamilton syringe. Standard solutions of markers and extracts were applied as 6.0 mm wide bands, positioned 10.0 mm from the bottom edge of the chromatographic plate. The development was carried out in an ascending manner up to 80 mm using a mobile phase at room temperature (24 ± 2°C) in a Camag glass twin-trough chamber, pre-saturated with mobile phase vapour for 30 minutes. After development, the plates were dried and scanned at 270 nm using a Camag TLC Scanner 3, equipped with a deuterium lamp and win CATS software.

Optimization of Mobile phase

The standard stock solution containing 100g/ml of (plant extract) was applied onto a TLC plate and subjected to development using various solvent systems. Numerous preliminary trials were undertaken to ascertain the optimal mobile phase composition. This optimization aimed to ensure the delivery of accurate, precise, and reproducible results for the quantification of (Active constituent).

Assay

For the assay, both standard and sample (extract) solutions were applied three times on a TLC plate. A concentration of 100g/ml of the active components was used for the standard solutions. Calibration curves were generated from the peak areas of these standard solutions. The sample (extract) solution was used to quantify the markers. By comparing the measured areas of the sample with the calibration curves, the amount of active components per gram of formulation was determined.

Validation of methods

The optimized HPTLC method was validated with respect to the following parameters in compliance with ICH recommendations Q2 (R1).

5.2.7.1 Linearity

Linearity refers to the ability of an analytical method to yield test results that are directly proportional to the concentration of the analyte within a specified range. To determine

linearity, a graph plotting peak area against standard concentration is created, from which the correlation coefficient (r^2) and the line equation are derived.

5.2.7.2 Specificity

The capacity to assess the analyte in the presence of components that are predicted to be present in the sample matrix is referred to as specificity. The method's specificity was determined by comparing the Rf value, and the peak purity was determined by comparing the spectrum of standard Active components with the sample.

5.2.7.3 Precision

Precision refers to the consistency of an analytical method's performance under standard conditions and is typically reported as the percent relative standard deviation (%RSD) for a significant number of samples. According to ICH guidelines, precision should be assessed at three levels: low quality control (LQC), medium quality control (MQC), and high-quality control (HQC). Repeatability, also known as intra-assay precision, measures precision over a short time period under the same conditions. It is evaluated using at least nine determinations within the specified range of the method. Intra-day precision is tested multiple times within a single day, while inter-assay precision is assessed across different days.

5.2.7.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The Limit of Detection (LOD) refers to the smallest quantity of an analyte in a sample that can be detected, but not necessarily quantified, under specific experimental conditions. In contrast, the Limit of Quantification (LOQ) is the lowest amount of analyte in a sample that can be quantified with acceptable precision and accuracy under the same conditions. To determine LOD and LOQ, the formula used is × SD s k× s SD, where k k is a constant (3.3 for LOD and 10 for LOQ), SD represents the standard deviation of the analytical signal, and s s denotes the slope of the calibration curve.

5.2.7.5 Accuracy

Accuracy should be expressed as a percentage of the recovery by analyzing a known added amount of analyte in the sample or as the deviation between the mean and the true value, including confidence intervals. To assess accuracy, at least nine determinations should be performed across at least three concentration levels within the specified range (for example,

three concentrations with three repetitions of the full analytical method). The percentage recovery was determined by conducting recovery tests in triplicate at three concentration levels: 80%, 100%, and 120%. A known amount of a standard mixture of active components was added to these samples, which were then analyzed, and the results were compared to the expected values.

5.2.7.6 Robustness

The robustness of an analytical procedure evaluates its capacity to withstand small, intentional changes in method parameters without affecting performance. This characteristic indicates the procedure's reliability under typical conditions. Robustness was assessed three times at concentrations of 300 ng/spot and 400 ng/spot, incorporating slight adjustments in mobile phase composition and saturation time. The resulting data were analyzed by calculating the % RSD of concentration.

5.3 IN VITRO LITHOLYTIC ACTIVITY

Aim: To study invitro litholytic activity

Objective:

The prime objectives of carrying out the dissertation work were as follows:

1. Preliminary screening of *Boerhavia diffusa*, *Celosia argentea* and *Plumeria Rubra* individually for their Litholytic activity.
2. To carry out Dissolution method of plant's extract of all three plants.
3. Investigate the impact of plant extracts on different types of kidney stones, specifically cystine stones, carbapatite stones, and uric acid stones.

Introduction:

Litholytic activity refers to the ability of a substance or agent to dissolve or break down urinary stones, also known as kidney stones or renal calculi. This property is essential in the context of urology and nephrology as kidney stones can cause significant pain, obstruction, and other complications if not effectively treated. Litholytic agents can be natural or synthetic and may act through various mechanisms to facilitate the dissolution or fragmentation of kidney stones. [197, 198]

5.3.1 Types of Litholytic Agents:

Chemical Litholytic: These are substances that chemically dissolve kidney stones. They may work by altering the composition of the stone or by increasing the solubility of its components. Common chemical litholytic agents include certain acids or salts that target specific stone types.

Mechanical Litholytics: These methods physically disintegrate kidney stones, frequently using shock waves or mechanical fragmentation. A prominent example of a mechanical litholytic approach is Extracorporeal Shock Wave Lithotripsy (ESWL).

Herbal and Natural Litholytics: Some plants and herbal remedies have been traditionally used for their litholytic properties. These natural agents may contain compounds that aid in stone dissolution or facilitate the passage of smaller stone fragments.

5.3.2 Mechanisms of Litholytic Activity:

Dissolution: Chemical agents may directly interact with the stone's components to dissolve it gradually, reducing the stone's size over time.

Fragmentation: Mechanical litholytic agents, like ESWL, generate shock waves or vibrations that break the stone into smaller, more manageable pieces.

Inhibition of Stone Formation: Some litholytic agents may act preventively by inhibiting the formation of new stones or limiting stone growth.

5.3.3 Evaluation of Litholytic Activity:

In vitro Studies: Researchers can conduct experiments using simulated urine or solutions containing the stone's constituents to assess the efficacy of potential litholytic agents.

In vivo Studies: Animal models with induced kidney stones can be used to evaluate the effect of litholytic agents on actual stones within a living system.

Clinical Trials: Human clinical trials are crucial for determining the safety and effectiveness of litholytic agents in real-world scenarios.

5.3.4 Factors Influencing Litholytic Activity:

Composition of Stones: Various kidney stones, such as calcium oxalate, struvite, uric acid, and cystine, may react differently to different litholytic treatments.

Size and Position of Stones: The size and position of the kidney stones in the urinary tract are critical in determining the appropriate litholytic approach.

Patient Considerations: The patient's overall health, existing medical conditions, and potential drug interactions must be considered when selecting litholytic agents.

Clinical Applications:

Litholytic agents can be used as primary treatments for certain types of kidney stones, especially in cases where surgery may be risky or not feasible. They can be used as adjunctive therapies along with other stone management procedures to enhance stone clearance.

5.3.5 Experimental

5.3.5.1 Preliminary screening of Individual plant for their litholytic activity

Individual Aqueous decoction of *Boerhavia difussa, Plumeria Rubra and Celosia argentea* was prepared and divided into two parts. Real kidney stones of humans were collected from 'Shree Hospital'Alephata, weighed and also measured accordingly. Preweighed kidney stone were transfered to one part of aqueous decoction. Second part of decoction was served as control. Both the flasks were kept in incubator at 37°C with intermittent shaking in-between the experiment. After 24hrs stone was collected and it has dried after 18 hours in an oven set at 40 °C. Again, the collected stones were observed morphologically as well as weighed again to find out dissolution of stone due to aqueous decoction of *Boerhavia difussa, Plumeria Rubra and Celosia argentea*.

Figure No. 5.8: Kidney stones collected from patient

5.3.5.2 Optimization of combination:

The present study investigates the litholytic potential of three plant extracts: *Boerhavia diffusa*, *Plumeria Rubra* seed pods, and *Celosia argentea* seeds. Initial screenings revealed that all three plants exhibit litholytic properties. Based on existing literature, four different compositions of these extracts were evaluated for their effectiveness in dissolving kidney stones. The combinations are as follows: Combination A: 2 parts *B. diffusa*, 1 part *P. rubra* seed pods, and 1 part *C. argentea*. Combination B: 3 parts *B. diffusa*, 1 part *P. rubra* seed pods, and 1 part *C. argentea*. Combination C: 4 parts *B. diffusa*, 1 part *P. rubra* seed pods, and 1 part *C. argentea*. Combination D: 1 part *B. diffusa*, 1 part *P. rubra* seed pods, and 1 part *C. argentea*. These combinations were tested for their ability to dissolve kidney stones.

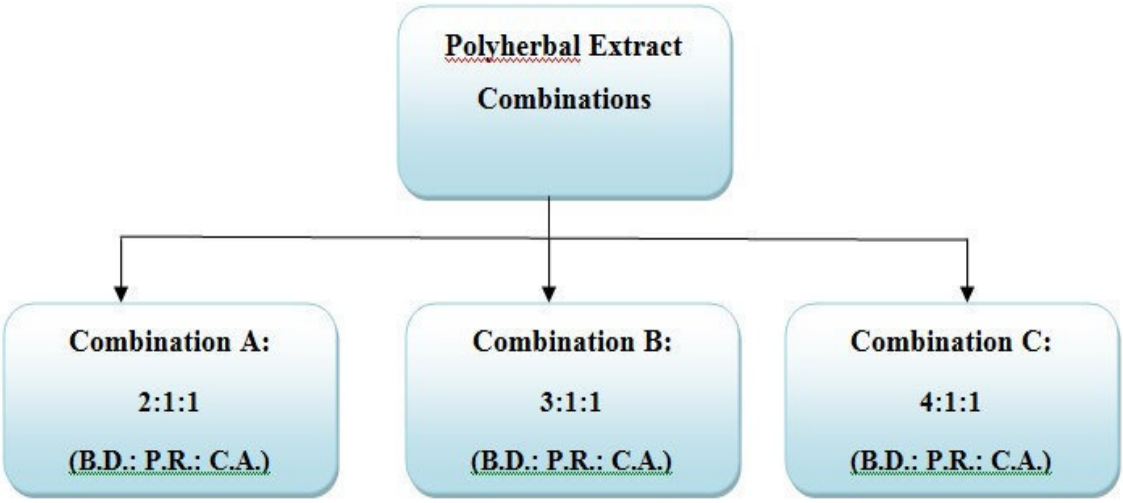


Figure No. 5.9: Composition of Extract

Although all the treatments (aqueous extracts of individual drugs and polyherbal extracts) demonstrated significant reduction in stone size, the polyherbal extract treatment using combination C (4 parts *B. diffusa* + 1 part *C. argentea* + 1 part *P. rubra* seed pod) exhibited superior results in stone dissolution compared to combination A (2 parts *B. diffusa* + 1 part *C. argentea* + 1 part *P. rubra* seed pod) and combination B (3 parts *B. diffusa* + 1 part *C. argentea* + 1 part *P. rubra* seed pod). The combination with 1 part *B. diffusa* + 1 part *C. argentea* + 1 part *P. rubra* seed pod showed negligible effects and was therefore excluded from further study.

5.3.5.3 Dissolution method

Aqueous extracts of all three plants were prepared by combining them in a ratio of 4:1:1 and boiling the mixture for 30 minutes in 100 ml of physiological solution (9 g NaCl per liter), which also served as a control environment to observe differences in calculus weight. After filtration, 60 ml of the extract was dispensed into glass Erlenmeyer flasks. Kidney calculi were added to each flask and suspended at room temperature using a permeable bag. The weight loss of the calculi was measured weekly for six weeks by weighing them after drying for 18 hours in an oven set at 40°C. Each experiment was conducted in triplicate. The activity of the extract was assessed by calculating the rate of calculus dissolution over time and comparing the final weight with the initial weight before incubation with the extract. The percentage dissolution was determined using the formula: (W_initial - W_final) / W_initial * 100, where W_initial and W_final are the weights of the calculus before and after incubation with plant extracts, respectively.

5.4 IN VIVO STUDIES

Aim: To study In vivo Litholytic Activity

Objective:

1. To carry out acute oral toxicity study.
2. To carry out study on adult male wistar rat by Ethylene glycol urolithiasis model.
3. To carry out urine analysis
4. To carry out serum analysis
5. To carry out Histopathological study.

5.4.1 Introduction:

Protocol

This study was carried out in accordance with the approval of CPCSEA/IAEC under reference number 1401/PO/RE/S/11/IAEC/2020-21/07/02.

Certificate

This is to certify that the project proposal no **1409/PO/RE/s/11/IAEC/2020-21/07/02** entitled **Development and evaluation of Litholytic action of Polyherbal Formulation in Wistar Rats.** submitted by Ms. **Jadhav Anuradha Subhashchandra** has been approved/recommended by the IAEC of VJSM's Vishal Institute of Pharmaceutical education and research. Ale, in its meeting held on **24.7.2021** and **48** Wistar Albino Rats have been sanctioned under this proposal for a duration of next **three** months.

Authorized by	Name	Signature	Date
Chairman:	Dr. D. D. Gaikwad		24.7.2021
Member Secretary:	Prof. V. R. Kumbhar		24.7.2021
Main Nominee of CPCSEA:	Dr. M. M. Ghaisas	24.7.2021	24.7.2021

Figure No. 5.10: Animal Ethical committee Certificate

5.4.2 Material Selection:

Wistar albino male rats weighing between 150 and 250 g were chosen for the study on antiurolithiatic activity. The rats were acclimatized to standard laboratory conditions in metabolic cages, provided with standard rat diet, and had access to water ad libitum. All animal care and experimental procedures were conducted following CPCSEA/IAEC approval no: 1401/PO/RE/S/11/IAEC/2020-21/07/02 guidelines. The rats were procured from Lachmi Biofarms Pvt. Ltd., Pune, Maharashtra, India, and housed in groups of six in clean cages with bedding changed twice weekly. Analytical grade ethylene glycol sourced from GS Lab, Pune, Maharashtra, India, was used for inducing urinary stones. CYSTONE® tablets, manufactured and marketed by The Himalaya Drug Company, Makali, Bangalore, India, served as the standard antiurolithiatic drug for the experiments.

Acclimatization and Quarantine:

Quarantine involves isolating recently arrived animals from those already present in the facility until the health and potentially the microbiological status of the newcomers have been assessed.

The newly acquired Wistar albino rats were isolated for one week to prevent the introduction of diseases to the existing animals and to allow them to achieve psychological, physiological, and dietary stability before use.

Housing

The animals were housed in a well-ventilated animal facility maintained at a constant temperature of 55 to 60% relative humidity. They were housed in spacious enclosures with metabolic cages and paddy husk serving as bedding material.

(A)

(B)

Figure No. 5.11: Metabolic cages A: showing receiver for urine collection B: Arrangement of metabolic cages on stand

Water and dietary regimen

The animals were provided with a standard pellet diet and had access to clean water. They were given unlimited food and water except during fasting periods. The bedding was refreshed twice weekly.

Animal tagging

Each animal cage used in the study was correctly labeled for identification purposes. To ensure proper identification, animals within each cage were marked with picric acid on either the head, torso, or tail.

5.4.3 Study of acute toxicity[199]

The acute toxicity study has been conducted according to OECD guidelines 423. The aqueous extract of *B. diffusa* demonstrated non-toxic characteristics in oral toxicity tests at a dosage of 2000 mg/kg, with experimental animals showing no signs of toxicity.

Similarly, the aqueous extracts of *C. argentea* and *P. rubra* were well tolerated at doses up to 2000 mg/kg body weight, prompting the selection of lower doses (200 mg/kg and 400 mg/kg, equivalent to 1/10th and 1/20th of the maximum dose) for further studies. Further investigations are warranted to assess potential long-term toxic effects.

5.4.4 In vivo evaluation of anti-urolithiasis[200, 201]

The efficacy of aqueous plant extracts in combating urolithiasis was studied using urolithiatic Wistar rats. Urolithiasis was induced by administering ethylene glycol (0.75% v/v) orally in drinking water. The anti-urolithiatic effects of the plant extracts were compared with those of a standard medication.

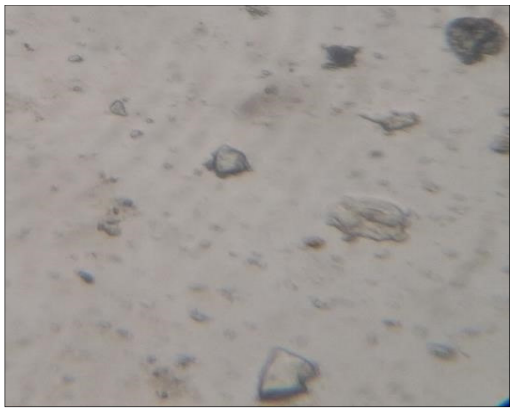
5.4.4.1 Analysis of Urine

The anti-lithiatic and prophylactic activities of aqueous extracts from polyherbal combinations of *B. diffusa*, *C. argentea*, and *P. rubra* were assessed by measuring the levels of stone-forming constituents (calcium, oxalate, phosphorus) as well as stone inhibitors (magnesium) in urine and serum.

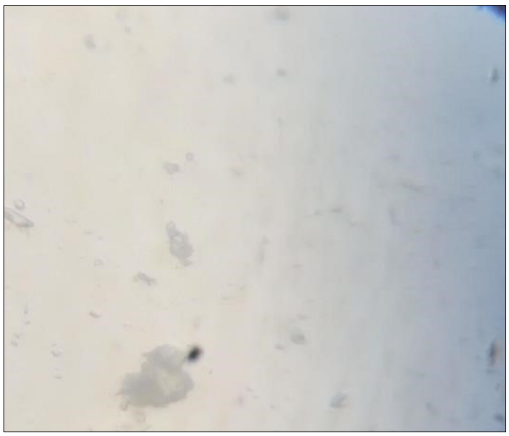
5.4.4.2 Microscopic observation

Urinary supersaturation with minerals is a primary cause of stone formation. These constituents that lead to stone formation tend to aggregate and become visible in urine as crystals. Microscopic examination of urine samples is crucial for diagnosing urolithiasis. In the urine sediment (at 10X magnification) of the normal group animals, no crystals were observed (Figure 5.12). Conversely, urine samples from animals in the positive control group showed numerous calcium oxalate crystals that were widely scattered. These crystals exhibited a characteristic double-bordered rectangular shape under plain white light. The crystals' shape and size varied across different groups. Although all treatments (aqueous extracts of individual drugs and polyherbal extracts) significantly reduced stone size, treatment with polyherbal extract combination B (4 parts *B. diffusa*, 1 part *C. argentea*, and 1 part *P. rubra* seed pod) demonstrated superior results with minimal stone formation compared to combination A (2 parts *B. diffusa*, 1 part *C. argentea*, and 1 part *P. rubra* seed pod). However, small crystal fragments were observed in all treatment groups.

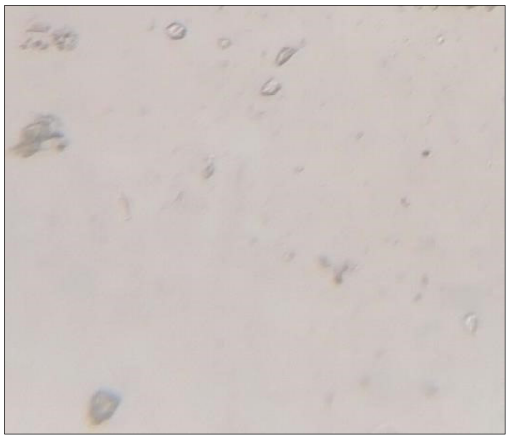
A: Normal Group



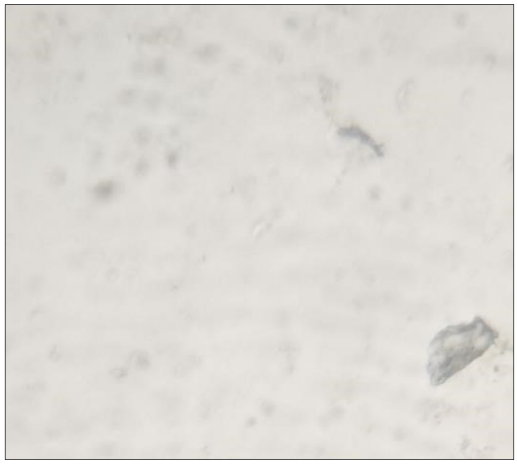
B: Diseased Control



C: Standard Drug



D: Combination A



E: Combination B

Figure No. 5.12: Microscopic analysis of urine was conducted across five groups:

A) Normal group, B) Diseased control group with ethylene glycol-induced stones, C) Standard Drug (Cystone) group, D) Combination A group showing a few stones, and E) Combination B group showing minimal stones in urine microscopy.

5.4.5 Urolithiasis Model Induced by Ethylene Glycol[201]

Following a week of acclimatisation, the rats were separated into five groups of six animals each.

Table No. 5.16: Anti-urolithiasis in vivo experimental design

Group	Treatment	No. of Animals
I	Regular diet and water	6
II	Ethylene Glycol (0.75% v/v) for 28 days	6
III	Ethylene Glycol (0.75% v/v) for 28 days + standard drug 750mg/kg (15-28th day)	6
IV	Ethylene Glycol (0.75% v/v) for 28 days + extract 200mg/kg (1-28th day)	6
V	Ethylene Glycol (0.75% v/v) for 28 days + Effective extract 400mg/kg (1-28th day)	6

5.4.5.1 Evaluation of Anti-Urolithiatic Activity Analysis of urine:

On the 28th day of the experiment, all animals housed in metabolic cages were taken out, and urine samples were gathered. Throughout the urine collection period, the animals had unrestricted access to drinking water. To prevent bacterial growth and metal hydrolysis, a small amount of concentrated hydrochloric acid was added to each urine sample before storing them at 4°C. The urine samples were subsequently analyzed for volume, pH, calcium, phosphate, oxalate, and magnesium.

i) Calcium in Urine Estimation:

Calcium Test:

Calcium testing is crucial for monitoring bone diseases and abnormalities in calcium regulation. Elevated serum calcium levels are linked to conditions such as hyperparathyroidism, metabolic bone disorders, and hypervitaminosis. Conversely, decreased serum calcium levels are associated with hyperparathyroidism, rickets, Steatorrhea, nephritis, and calcium-wasting diseases. Assessment of urinary calcium levels

aids clinicians in evaluating calcium handling by the kidneys during parathyroid gland disorders. Furthermore, urinary calcium levels play a significant role in diagnosing kidney stones.

Principle:

In the research and drug discovery, there is a growing interest in simple, automation-compatible methods for measuring calcium levels in biological samples. Biochain's calcium assay kit facilitates direct testing of calcium in these samples without requiring any prior preparation. The kit employs a phenosulphonephthalein dye, which forms a highly stable blue compound upon binding with free calcium. The intensity of the blue color at 612 nm directly correlates with the calcium concentration in the sample.

Kit contents include materials for 500 tests using 96-well plates. It comprises Reagent A (50 mL), Reagent B (50 mL), and a Calcium standard solution (1 mL, 20 mg/dL Ca2+).

The kit is shipped at room temperature and should be stored at 4°C. Shelf life is 12 months from receipt.

For reagent preparation,

Mix equal volumes of Reagent A and B and allow them to equilibrate to room temperature before use.

1. To perform the procedure using a 96-well plate:

Dilute 5 mL of both standards and samples into the wells of a clear bottom 96-well plate. Diluted standards can be stored at 40°C for future use.

PREMIX (ml)	H2O	Ca (mg/dl)
100+0	100	20
80+20	100	16
60+40	100	12
40+60	100	8
20+80	100	4
10+90	100	2
0+100	100	0

2. To begin the assay, 200 mL of the working reagent was added and gently mixed.
3. Following a 3-minute incubation at room temperature, the optical density was measured at 570-650 nm with peak absorbance observed at 612 nm. For the cuvette procedure,
4. The diluted standards and samples were prepared by transferring 15 mL into appropriately labeled containers.
5. Subsequently, 1000 mL of working reagent was added and thoroughly vortexed.
6. After a 3-minute incubation period, the mixture was transferred to a cuvette and the optical density was measured at 612 nm.

Calculation:

Replace the blank optical density (OD) values with the standard OD values and create a plot of OD versus standard Ca2+ concentrations. Utilize linear regression analysis to determine the slope. The calcium concentration in a sample can be calculated using the formula: Calcium concentration in sample = (OD sample - OD blank) / Slope. The OD values at 612nm for both the sample and the blank (which could be water or ethanol) are recorded.

ii) Estimation of Magnesium in Urine

Magnesium

Magnesium, similar to potassium, plays a crucial role as an intracellular cation. It serves as a catalyst for various enzymes and is essential for the activation of amino acids and protein synthesis. Elevated levels can be associated with conditions such as dehydration, Addison's disease, and uremia. Conversely, lower magnesium levels may occur due to malabsorption, treatment of diabetic coma, chronic renal disease, chronic alcoholism, pancreatitis, and hyperthyroidism.

Principle

In an alkaline medium, magnesium reacts with Calmagite to form a red-colored complex. The addition of specific chelating agents removes interference from calcium, proteins, and detergents. The intensity of the color produced is directly proportional to the magnesium content in the sample.

Preparation of Reagents

All reagents are supplied ready for immediate use. Store away from direct light. **Preparation of Working Reagent** For extensive assay series, prepare a working solution by mixing equal parts of L1 (buffer reagent) and L2 (color reagent). Store the working reagent tightly sealed at 2-8°C for up to one month.

Sample Material Urine: To analyze, acidify a 24-hour urine sample to a pH of 2-3 by adding approximately 10-15 ml of HCl. Dilute with distilled water at a 1:3 ratio before testing. Multiply the obtained result by four.

Procedure Measure absorbance at 510nm wavelength using appropriate filters. **Temperature** Conduct tests at standard room temperature with a 1 cm light path.

5.4.5.2 Serum parameters: Following treatment with ethylene glycol for 28 days, a 24-hour urine sample was collected and assessed for stone-forming and stone-inhibiting parameters as described above. Animals were anesthetized using either chloroform or ether, and blood was collected from the retroorbital plexus (see Fig. 5.13). Subsequently, the animals were sacrificed, and blood samples were obtained for further estimation of serum parameters.

Figure No. 5.13: Blood removal from retro orbital plexus for serum estimation

i) Estimation of Uric Acid in Serum

Uric acid is the final product of purine metabolism. It is primarily excreted by the kidneys and, to a lesser extent, broken down by microbes in the intestine. Elevated uric acid levels can result from conditions such as gout, arthritis, reduced kidney function, and fasting.

Conversely, conditions like Wilson's disease, Fanconi syndrome, and yellow liver atrophy can lead to low uric acid levels**.**

Principle: The conversion of uric acid to allantoin and hydrogen peroxide is catalyzed by uricase, a protein enzyme. The reaction between hydrogen peroxide and a phenolic molecule with four aminoantipyrine molecules generates a red quinoneimine dye complex. The intensity of this color change correlates with the concentration of uric acid in the sample. Uric acid + H2O → Allantoin + H2O2 (catalyzed by Uricase) H2O2 + 4 Aminoantipyrine + Phenolic → Red Quinoneimine dye + H2O (catalyzed by Peroxidase)

Contents: L1: Buffer reagent (20ml, 60ml, 2X60ml, 2X120ml) L2: Enzyme reagent (5ml, 15ml, 2X15ml, 2X30ml) S: Uric acid Standard (5ml, 5ml, 5ml, 5ml) (8mg/dl) Storage

All reagents remain stable at 2-8°C until the expiry date printed on the label.

Preparation of Working Reagent:

Combine the contents of one bottle of L2 (Enzyme Reagent) with one bottle of L1 (Buffer reagent) to prepare the working reagent. This working reagent remains stable for at least four weeks when stored at 2-8°C.

When stored**,** the working reagent may develop a faint pink color, which does not affect its performance. Alternatively, the required amount of working reagent can be prepared by mixing 4 parts of L1 (buffer reagent) with 1 part of L2 (enzyme reagent). Another option is to use 0.8 ml of L1 and 0.2 ml of L2 instead of 1 ml of the working reagent immediately before conducting the test. Regarding sample stability, serum uric acid remains stable in samples stored at 2-8°C for 3-5 days.

For the procedure:

Wavelength/filter: 520 nm / Yellow Green

Temperature: 37°C / Room temperature

Light path: 1 cm

Pipette the appropriate volumes into clean, dry test tubes labeled as Blank (B), Standard (S), and Test (T). Mix thoroughly and incubate the test tubes in boiling water (100°C) for 10 minutes. After cooling under running tap water, measure the absorbance of the Standard (Abs. S) and Test sample (Abs. T) against the Blank.

System parameters: Reaction endpoint: Sample volume used was 0.02 ml. The wavelength employed was 520 nm with a reagent volume of 1.00 ml. Zero setting was based on the reagent blank, and the reaction slope was observed to be increasing. Incubation temperature was maintained at 37°C (room temperature). Linearity was established at 20 mg/dl. Incubation times were 5 minutes and 15 minutes, respectively, and the unit of measurement used was mg/dl. The standard concentration was set at 8 mg/dl.

ii. Estimation of Creatinine in Serum

Creatinine is a metabolite of creatine phosphate found in skeletal muscle. Its daily production correlates with muscle mass and it is excreted entirely by the kidneys. Elevated levels are indicative of renal dysfunction, reduced renal blood flow (due to conditions like shock, dehydration, or congestive heart failure), diabetes, acromegaly, and other disorders. Conversely, levels are decreased in muscular dystrophy.

Principle: In an alkaline environment, picric acid reacts with creatinine to form a vibrant orange-red complex with alkaline picrate. The intensity of this coloration is proportional to the creatinine concentration in the sample.

Contents: 15 tests: L1 - 60ml Picric acid reagent, L2 - 75ml Buffer reagent, S - 5ml Creatinine standard (40mg/dl) 35 tests: L1 - 140ml Picric acid reagent, L2 - 12ml Buffer reagent, S - 5ml Creatinine standard (40mg/dl) 70 tests: L1 - 2 x 140ml Picric acid reagent, L2 - 25ml Buffer reagent, S - 10ml Creatinine standard (40mg/dl)

Storage/Stability: All reagents are stable at room temperature until the expiration date indicated on the label.

Reagent Preparation: Reagents are ready-to-use. Avoid mouth pipetting.

Sample Material: Serum creatinine remains stable in serum for up to one day at 2-8°C.

Procedure: The wavelength/filter used was 520nm (green), and the temperature during the experiment was maintained at room temperature. The light path was standardized to 1cm. Test tubes were mixed thoroughly and kept at room temperature for exactly 20 minutes. Absorbance readings of the standard (Abs. S) and test samples (Abs. T) were measured against the blank.

System Parameters: The reaction type was endpoint. Sample volume used was 0.1ml, and the wavelength employed was 520nm. Reagent volume utilized was 1.1ml. Zero setting

was done with the reagent blank. Reaction slope was noted as increasing. Incubation temperature was maintained at room temperature. Linearity was determined at 8mg/dl. Incubation time was fixed at 20 minutes. Units were measured in mg/dl with the standard set at 8mg/dl.

Calculation: Creatinine concentration in mg% was calculated using the formula: Abs. T / Abs. S x 2.

5.4.6 Histopathology[202]: Upon completion of the experimental period, rats were anesthetized using ether or chloroform and then sacrificed. The abdomen was incised to remove and clean the kidneys, which were preserved in 10% neutral formalin. One kidney was fixed in formalin and embedded in paraffin. Sections of 5 µm thickness were prepared and stained with hematoxylin-eosin solution. These sections were examined under plain and polarized microscopes to document histopathological changes.

Figure No. 5.14: Isolated kidneys in formalin solution

5.4.7 Statistical Analysis: The results of the assays were evaluated using one-way analysis of variance (ANOVA), followed by Student's unpaired t-test with Dunnett's correction using Graph Pad software Version 6.0. The data are presented as mean ± SEM. The comparison was made between the positive control group and the Cystone treated group (standard), with results considered as 100%. Differences between the groups treated with various extracts and combinations of extracts were compared to the positive control group. The significance level was set at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

5.5 FORMULATION AND EVALUATION OF POLYHERBAL GRANULES[203,204]

Aim: To formulate and evaluate Polyherbal Granules by using wet granulation method.

Objective:

The prime objectives of my present investigation include:

- To prepare Decoction of leaves *Boerhavia diffusa* using by taking powderd crude drug, evaporate the decoction.
- Make fine power of Seeds of *Celosia argentea*, Pod of *Plumeria Rubra* pass them through sieve no 22, weigh them as per specified dose in official books or references, mix them separately in above decoction after cooling which is in semisolid consistency along with other excipients.
- To prepare polyherbal granules by wet granulation method.
- To evaluate the prepared polyherbal oil for various parameters like colour, odour, pH, Angle of repose Flowability, to perform FTIR of granule for compatibility study of active ingredient and tablet blend of polyherbal formulation.
- To evaluate Antilithiatic activity of the formulations by using rat animal model.
- To carryout stability studies of optimized formulation.

5.5.1 Introduction

Ayurveda is an ancient Sanskrit Indian system of using herbs, spices and other natural items to benefit good health, both inside and out or “The science of life”. Ayurveda which has its origin in Atharvaveda, is an Ancient Indian Medical Science. The basic aim of Ayurveda is to enhance the immunity of an individual and its efficacy lies in curing the chronic ailments of mankind.

पुनर्नवा

पुनर्नवा पुनर्भूः स्यात् सदामण्डलपत्रकः ।
श्वेतमूलश्च वृश्चीवो वर्षाभूः दद्रपत्रकः||७५२||
शोफघ्नी जटिला सदयोविशाखा दीर्घपत्रकः
वर्षाभूर्मधुरा तिक्ता कषाया कटुका सरा ७५३ ।
क्षारोष्णा दीपनी रूक्षा शोफानिलकफापहा
हृदया रुच्या जयेदर्शाव्रणपाण्डुगरोदरम् ७५४ | कै.नि.

देवकुकुटकः शीतो वृश्यो मूत्ररुजापहः ।
अश्मयां नाशकः प्रोक्तः पूर्ववैधैर्मनीषिभिः ॥ नि.र.
देवकुरडू-शीत, वृष्य व मुत्ररोग, आणि अश्मरी नाशक आहे.

श्वेतस्तु चम्पकः प्रोक्तः सरस्तितः कटुः स्मृतः ।
तुवरोष्णः कुष्ठकण्ट्वणशुलकफापहः ॥
यातं चोदररोगं च आध्मानं चैव नाशयेत् ।भा. नि.॥

रक्ता पुनर्नवा तिक्ता सारका शीतला ।
वातला ग्राहका चैव पाके कट्टी रसायनी ॥
कफपित्ते रक्तदोष प्रदरं शोफपाण्डुनुत्।भा. नि.॥

श्वेता पुनर्नवा तिक्ता चोष्णा कट्वी च तूवरा ।
रुच्याग्निदीपनी रूक्षा मधुरा पटु सारका ॥
हृद्या शोफं कफं वातं कासमर्शोव्रणं जयेत् ।
पाण्डून् विषोदरं शूलं हृद्रोगोरक्षतापहा ॥
पुनर्नवा पर्णशाका चातिरूक्षा कफापहा । वाताग्निमान्धगुल्मघ्नी प्लीहशूलविनाशिका | (नि.र.)

कुरडू

श्रीहस्तिनी सिंहकेशी चुबुकः क्षेत्रनाशिनी |

कृष्णसूक्ष्मफला ज्ञेया सितावारस्तु कुर्कुटा ||६६१||

कुरण्टिका हिमा स्वाद्वी स्वादुपाका कटुः पटुः ।

तिक्ता रूक्षा सरा वृष्या गुर्वी मारुतपित्तला ||६६२|| वस्तिमारुतकृच्छ्राश्मकफरक्तं नियच्छति |६६३| कै.नि.

चंपा

चंपकं कटुकं तिक्तं कषायं मधुरं हिमम्

निहंन्ती कफपित्तास्र मूत्रकृच्छ विषक्रिमीन | कै.नि.॥

Borhavia diffusa (Punarnava) dravya has properties such as madhur, tikta, kashaykaturasa, ushnavirya, rukshakhar. among these maximum properties work as kaphashamak action.and formation of Ashmari (renal urinary stone) predominantly responsible as kaphadosha and punarnavadravya mostly action of kaphaghna action it works as both way preventive process formation of Ashmari, as well as dissolve the ashmari which is formed due to ushnaguna (hot potency) by bhedan action land due to sara properties it work as diuretic (mootral action) that relieves from symptoms inflammation.

Plumeria Rubra (champak) dravya has properties katutiktakashay, mostly Kaphashamak, allivatedkapha dosha which is major cause of the formation of ashmari (Stone). Madhur rasa, sheetaguna which diminished pitta dosha and related their symptoms burning sensation during urination ((mootrakruccha), fever and also reduced the tendencyto the formation of stones.

Celosia argentea (Kuradudravya) has the properties of katutikta rasa and rukshaguna, which allivated the kapha dosha and role as kaphashamak property it also work as preventive mechanism of stone and formed stone isvremoved through urinary tract by Sara guna properties promote the motion of urine flow and work as diuretic (mootral).

The Vatashamak properties of kuradu show the action of pain in renal stones. [205-207]

5.5.2 Pathophysiology of Kidney stone as per Ayurvedic references:

Etiological factor (unwholesome diet) Vitiation of Tridosha, specifically predominantly Kapha Dosha Aggrevates Kapha Dosha ncreased specifically properties of Kapha dosha

like snigdha, dravapicchil and kledatwa property aggrevated vitiated kapha dosha (sthansanshray) carried into mootravahasrotasa (urinary system) by urinary tract (Adhomootravadhadhaminis) due to slightly vitiated vatadosha ,pitta dosha property ruksha and ushnaguna respectively they (shushka /shoshan) absorb thekapha Dosha due to absorption of kapha dosha specifically in urinary tract formation of Adhmari (Renal stone, Urinary stone) due to shushkakapha dosha formation of Ashmari ,blockage of urinarytract (mootravahasrotasa) that shows the following sign and symptoms such as pain around umbilical region (nabhipradeshishool), fever (jwar), Dysuria, burning sensation (mutrakruccha) Swelling due to blockage of urine flow (shoth).

5.5.3 Polyherbal formulation[203,204,208]:

Polyherbal formulation was made initially in two combinations depending on literature available and correlation of doses given for humans. Combination A contains

Combination A:.2 parts of *B. diffusa*: 1 part of each *C. argentea* and *P. rubra* (2:1:1) and Combination B: 4 parts of *B. diffusa*: 1 part of each *C. argentea* and *P. rubra* (4:1:1),

Optimization of given combination was done by microscopic urine analysis, Combination B was found effective as compared to Combination A.

Table No. 5.17: Combination of Extract

Sr. No.	Ingredient	Combination A	Combination B
1.	*Boerhavia diffusa* powder	2 Parts	4 Parts
2.	*Plumeria Rubra* powder	1 Parts	1 Parts
3.	*Celosia argentea* powder	1 Parts	1 Parts

Table No.5.18: Formulation table of Granules

Sr. No.	Name of Ingredient	Quantity Taken
1.	*B. diffusa* whole plant powder	16gm
2.	*C. argentea* seed powder	4gm
3.	*P. rubra* powder of seed pod	4gm

Method of preparation of granules formulation:

- Prepare Decoction of leaves *Boerhavia diffusa* using by taking 16 gm and 20gm for PHF 1 and PHF 2 of powdered crude drug respectively, boil in 100ml water, reduced the original volume to get one third part of it, pass through sieve no 22, evaporate the decoction.
- Make fine power of Seeds of *Celosia argentea*, Pod of *Plumeria Rubra* pass them through sieve no 22, weigh 2gm and 2.5 gm each mix them separately in above decoction after cooling which is in semisolid consistency along with other excipients.
- Prepare polyherbal granules by wet granulation method.
- Evaluate the prepared polyherbal oil for various parameters like colour, odour, pH, Angle of repose flowability, to perform FTIR of granule for compatability study of active ingredient and tablet blend of polyherbal formulation.
- Evaluate Antilithiatic activityof the formulations by using rat animal model.
- Carryout stability studies of optimized formulation.

5.5.4 Evaluation of granules formulation:[184,185,209]

The granule formulations were evaluated for different pharmaceutical parameters.

5.5.4.1 Macroscopic evaluation: In macroscopic evaluation the appearance, colour, odor and taste were determined.

5.5.4.2 Microscopic evaluation: Samples of the sieved powder (5mg each) were individually treated with iodine, chloral hydrate, Phloroglucinol, or potassium iodide. A drop of glycerin was subsequently added and the samples were mounted. The characteristics of the powdered samples were observed using a binocular microscope equipped with a camera.

5.5.4.3 Determination of pH: The pH of the formulated granules was measured using a digital pH meter. This involved dissolving 1 gram of granules in 100 milliliters of water.

5.5.4.4 Loss on drying at 105°C: To determine the moisture content (loss on drying), first dry the evaporating dish for 30 minutes under the specified conditions. Next, accurately weigh approximately 5 to 10 grams of granular powder into the pre-weighed evaporating

dish. Dry the powder sample at 105°C for 3 hours, then weigh it. Continue drying and weighing at 30-minute intervals until the difference between two successive weighings is no more than 0.25%.

5.5.4.5 Determination of Ash Values[184,185,209]

A) **Total Ash Value:** Two grams of granules were precisely measured into a silica crucible that had been heated and cleaned beforehand. The granules were heated gradually to a temperature between 500 and 600°C until they turned white, indicating the absence of carbon. Afterward, the crucible was cooled in a desiccator, and the weight of the total ash and dried material was determined.

B) **Acid Insoluble Ash Value:** The total ash in the crucible was treated with 25 mL of hydrochloric acid (HCl) and gently boiled for 5 minutes. The insoluble residue was gathered using an ashless filter paper, washed thoroughly with hot water until the filtrate reached neutrality. The filter paper containing the insoluble material was then transferred back into the crucible and heated until a constant weight was achieved. After cooling, the residue was weighed.

5.5.4.6 Determination of Extractive Value[184,185,209]

A) **Water Soluble Extractive Value:** An amount of 5 grams of granules was precisely measured and placed into a conical flask with a glass stopper. The granules were macerated with 100 mL of chloroform water for a duration of 18 hours. After maceration, the mixture was filtered, and approximately 25 mL of the filtrate was transferred into a porcelain dish. The solution was evaporated to dryness on a water bath. Subsequently, it was dried at 105°C for 6 hours, allowed to cool, and then weighed.

B) **Alcohol Soluble Extractive Value:** Ethanol replaced chloroform-water as the solvent, while the remaining procedure remained identical to that used for determining the water-soluble extractive value.

5.5.4.7 Particle size (80-100 mesh for Granules):[184,185]

The granule separation process utilized the sieve method, where a series of sieves were arranged in increasing mesh size order. Granules were carefully weighed and placed onto

the top sieve, followed by shaking the assembly for 15 minutes. After shaking, the sieves were individually removed, and the weight of granules retained on each sieve was recorded.

5.5.4.7.1 Bulk Density:

Initially, 10 grams of granules were placed into a graduated measuring cylinder and gently tapped on a wooden surface to settle. The bulk density was then determined using a specified formula. For accurate measurement, the granules were transferred to a volumetric flask designed for tap density analysis. The bulk density (in g/ml) was calculated by dividing the weight of the sample by the volume it occupied.

5.5.4.7.2 Tap Density: The tap density of granules was assessed by subjecting them to 50 taps using a tap density apparatus. To determine the tap density, the tapped volume of the granules was measured, and the ratio of the sample weight to the tapped volume was calculated.

The tap density (g/ml) can be calculated using the following formula. = weight of sample in gm/ volume occupied by the sample.

5.5.4.7.3 Angle of Repose:

The angle of repose was measured using the funnel method. This involved allowing the powder to flow through a funnel positioned on a stand until it formed a heap. The angle of repose was determined from the measurements of the height of the heap and the radius of the base of the heap formed by the flowing powder.

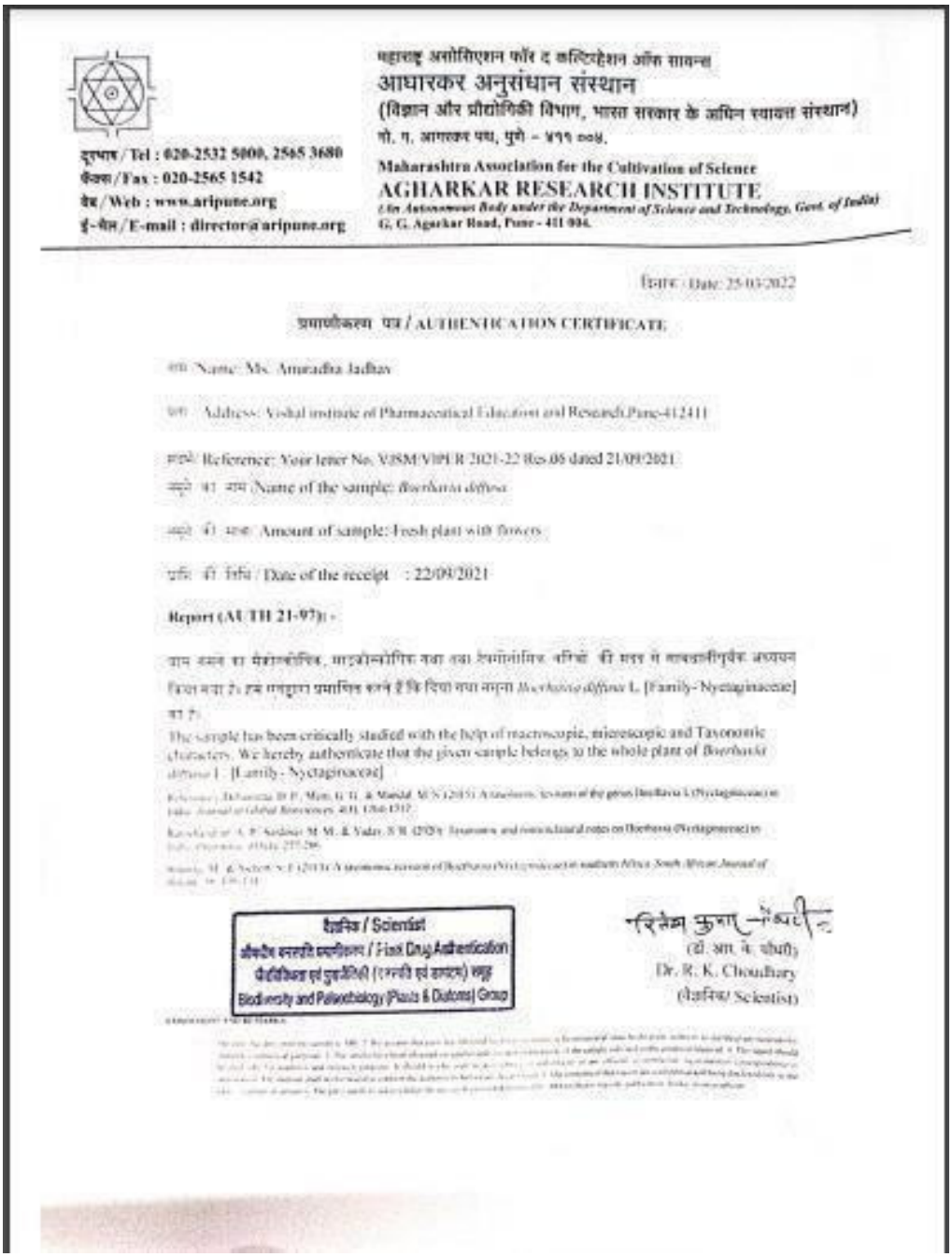
CHAPTER 6

RESULT AND DISCUSSION

RESULT AND DISCUSSION

6.1 PHARMACOGNOSTICAL AND PHYTOPHYSICOCHEMICAL INVESTIGATIONS OF CRUDE DRUGS RESULTS

6.1.1 Authentication of plant material: The plants used in the study included the whole plant of *Boerhavia diffusa* Linn, sourced from Khandala, Maharashtra. Matured pods of *Plumeria Rubra* Linn were collected from Rajuri, Junnar Maharashtra. Seeds of *Celosia argentea* Linn were purchased from Mankarnika Ayurvedic medical store, Chinchwad Pune. Authentication of the plants and seeds was conducted by Dr. R.K. Chaudhary, a Scientist at Agharkar Research Institute, Pune, an autonomous body under DST, Government of India. The collected plant material underwent thorough washing with running tap water, followed by rinsing with distilled water. Subsequently, it was shade-dried for seven days, after which the dried plants were ground using a laboratory herbal grinding mill. The coarse powder (60#) obtained from the dried plants was stored in an air-tight container for preparing aqueous extracts intended for further in vivo study.



महाराष्ट्र असोसिएशन फॉर द कल्टिव्हेशन ऑफ सायन्स
आधारकर अनुसंधान संस्थान
(विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार के अधीन स्वायत्त संस्थान)
जी. ग. आगरकर पथ, पुणे - ४११ ००४.

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G. G. Agarkar Road, Pune - 411 004.

दिनांक / Date: 25-03-2022

प्रमाणीकरण पत्र / AUTHENTICATION CERTIFICATE

नाम / Name: Ms. Amrudha Jadhav

पता / Address: Vishal institute of Pharmaceutical Education and Research,Pune-412411

संदर्भ / Reference: Your letter No. VISM/VIPER/2021-22/Res.08 dated 21/09/2021

नमूने का नाम / Name of the sample: *Boerhavia diffusa*

नमूने की मात्रा / Amount of sample: Fresh plant with flowers

प्राप्ति की तिथि / Date of the receipt : 22/09/2021

Report (AUTH 21-97):-

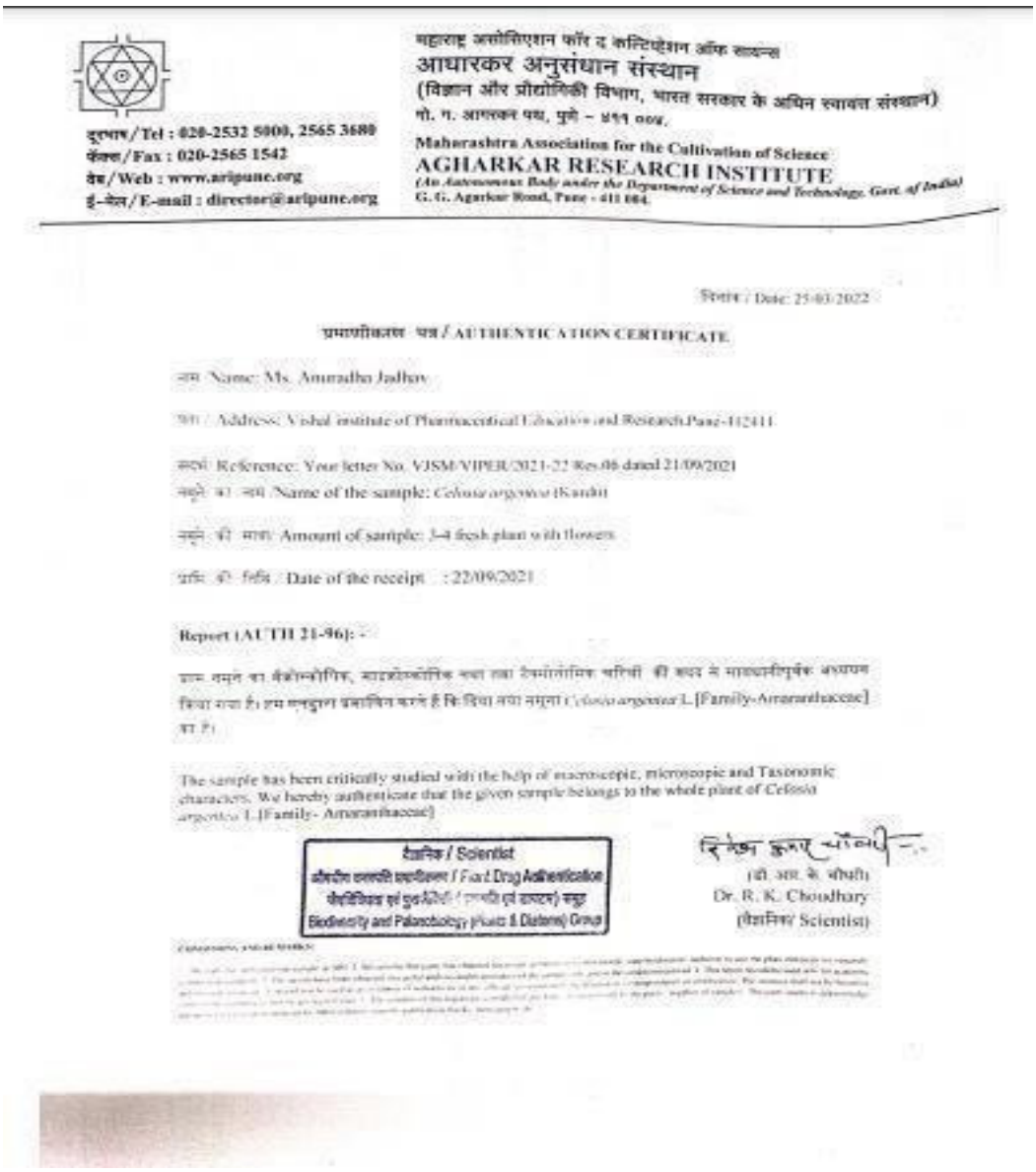
नाम नमूने का मैक्रोस्कोपिक, माइक्रोस्कोपिक तथा टैक्सोनॉमिक वर्णनों की मदद से सावधानीपूर्वक अध्ययन किया गया है। हम एतद्द्वारा प्रमाणित करते हैं कि दिया गया नमूना *Boerhavia diffusa* L. [Family- Nyctaginaceae] का है।

The sample has been critically studied with the help of macroscopic, microscopic and Taxonomic characters. We hereby authenticate that the given sample belongs to the whole plant of *Boerhavia diffusa* L. [Family - Nyctaginaceae]

वैज्ञानिक / Scientist
औषधीय वनस्पति प्रमाणीकरण / Plant Drug Authentication
पैलियोबिओलॉजी एवं डायटम (शैवाल एवं डायटम) समूह
Biodiversity and Paleobiology (Plants & Diatoms) Group

(डॉ. आर. के. चौधरी)
Dr. R. K. Choudhary
(वैज्ञानिक/ Scientist)

A



महाराष्ट्र असोसिएशन फॉर द कल्टिव्हेशन ऑफ सायन्स
आधारकर अनुसंधान संस्थान
(विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार के अधिन स्वायत्त संस्थान)
गो. ग. आगरकर पथ, पुणे – ४११ ००४.
Maharashtra Association for the Cultivation of Science
AGHARKAR RESEARCH INSTITUTE
(An Autonomous Body under the Department of Science and Technology, Govt. of India)
G. G. Agarkar Road, Pune - 411 004.

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ई-मेल/E-mail : director@aripune.org

दिनांक / Date: 25/03/2022

प्रमाणीकरण पत्र / AUTHENTICATION CERTIFICATE

नाम / Name: Ms. Anuradha Jadhav

पता / Address: Vishal institute of Pharmaceutical Education and Research,Pune-412411

संदर्भ / Reference: Your letter No. VJSM/VIPER/2021-22 Res.06 dated 21/09/2021

नमूने का नाम / Name of the sample: *Celosia argentea* (Kandi)

नमूने की मात्रा / Amount of sample: 3-4 fresh plant with flowers

प्राप्ति की तिथि / Date of the receipt : 22/09/2021

Report (AUTH 21-96): -

प्राप्त नमूने का वैक्रोस्कोपिक, माइक्रोस्कोपिक तथा तथा टैक्सोनोमिक चरित्रों की मदद से सावधानीपूर्वक अध्ययन किया गया है। हम एतद्द्वारा प्रमाणित करते हैं कि दिया गया नमूना *Celosia argentea* L.[Family-Amaranthaceae] का है।

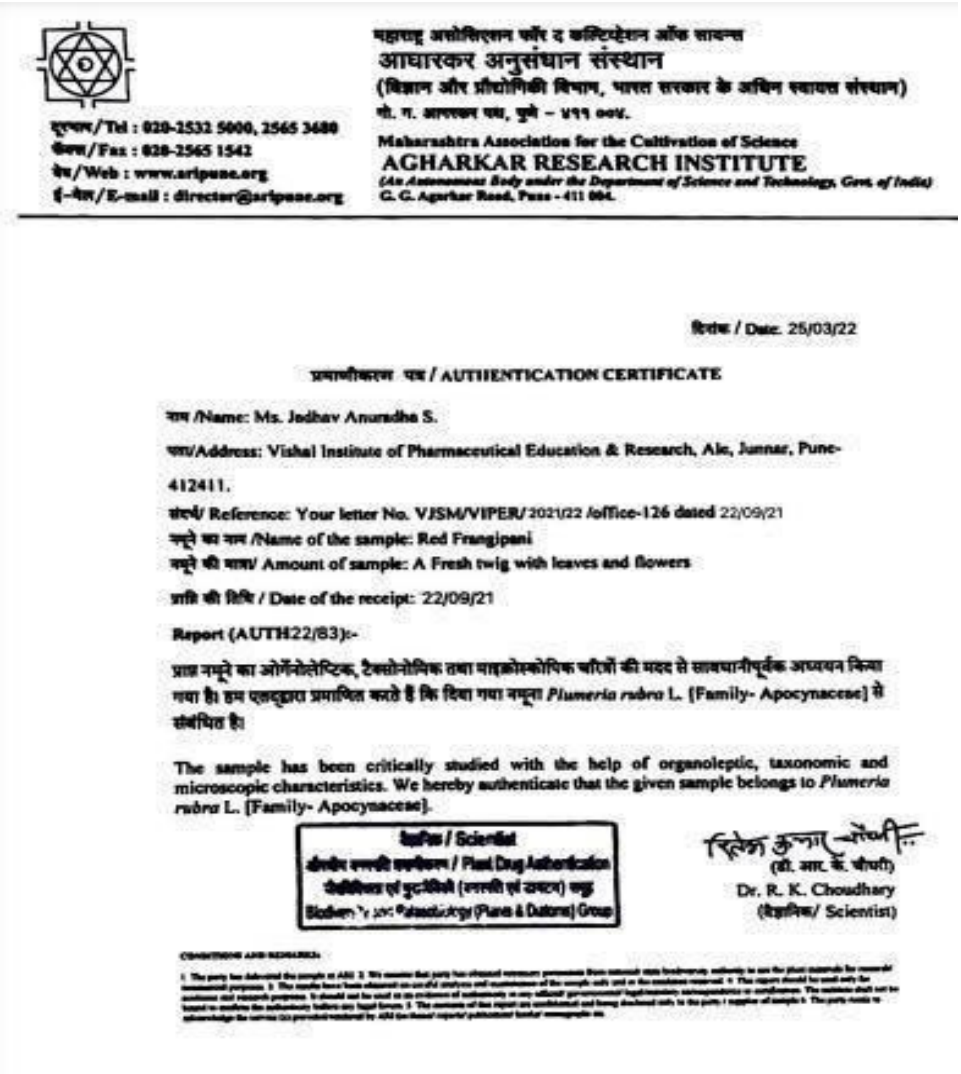
The sample has been critically studied with the help of macroscopic, microscopic and Taxonomic characters. We hereby authenticate that the given sample belongs to the whole plant of *Celosia argentea* L.[Family- Amaranthaceae]

वैज्ञानिक / Scientist
औषधीय वनस्पति प्रमाणीकरण / Plant Drug Authentication
जैवविविधता एवं पुरा-वैज्ञानिकी (पत्तनी एवं डायटम) समूह
Biodiversity and Palaeobiology (Plants & Diatoms) Group

(डॉ. आर. के. चौधरी)
Dr. R. K. Choudhary
(वैज्ञानिक/ Scientist)

Enclosures, and Remarks:

B



महाराष्ट्र असोसिएशन फॉर द कल्टिव्हेशन ऑफ सायन्स
आघारकर अनुसंधान संस्थान
(विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार के अधिन स्वायत्त संस्थान)
गो. ग. आगरकर मार्ग, पुणे – ४११ ००४.

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Maharashtra Association for the Cultivation of Science
AGHARKAR RESEARCH INSTITUTE
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G. G. Agarkar Road, Pune - 411 004.

दिनांक / Date: 25/03/22

प्रमाणीकरण पत्र / AUTHENTICATION CERTIFICATE

नाम /Name: Ms. Jadhav Anuradha S.

पता/Address: Vishal Institute of Pharmaceutical Education & Research, Ale, Junnar, Pune-412411.

संदर्भ/ Reference: Your letter No. VJSM/VIPER/2021/22 /office-126 dated 22/09/21

नमूने का नाम /Name of the sample: Red Frangipani

नमूने की मात्रा/ Amount of sample: A Fresh twig with leaves and flowers

प्राप्ति की तिथि / Date of the receipt: 22/09/21

Report (AUTH22/83):-

प्राप्त नमूने का ओर्गेनोलेप्टिक, टैक्सोनोमिक तथा माइक्रोस्कोपिक चीजों की मदद से सावधानीपूर्वक अध्ययन किया गया है। हम एतद्द्वारा प्रमाणित करते हैं कि दिया गया नमूना *Plumeria rubra* L. [Family- Apocynaceae] से संबंधित है।

The sample has been critically studied with the help of organoleptic, taxonomic and microscopic characteristics. We hereby authenticate that the given sample belongs to *Plumeria rubra* L. [Family- Apocynaceae].

वैज्ञानिक / Scientist
औषधीय वनस्पती प्रमाणीकरण / Plant Drug Authentication
जैवविविधता एवं पुराजीवविज्ञान (वनस्पती एवं डायटम) समूह
Biodiversity and Palaeobiology (Plants & Diatoms) Group

(डॉ. आर. के. चौधरी)
Dr. R. K. Choudhary
(वैज्ञानिक/ Scientist)

CONDITIONS AND REMARKS:

C

Figure No. 6.1: A: Authentication cerficate of *Boerhavia diffusa*; B: *Celosia argentea*; C: *Plumeria Rubra*

6.1.2 Morphology and Specifications of *B. diffusa, P.rubra, C.argentea*

Table No. 6.1: Morphology and physicochemical specifications of *B. diffusa*

Sr. No.	TEST	SPECIFICATION
1.	Colour	Powder Green in colour
2.	Taste	Bitter and Sweet
3.	Foreign Organic Matters	Nil
4.	Ethanol Soluble extractive	10.24%
5.	Water Soluble extractive	22.50 %
6.	Loss on Drying (Moisture content)	2.1%
7.	Total Ash	6.0%
8.	Acid insoluble ash	0.25%

Table No. 6.2: Morphology and physicochemical specifications of *P. rubra* seed pod

Sr. No.	TEST	SPECIFICATION
1.	Colour of fresh seed pod	Dark reddish brown
2.	Colour of dry powder	Powder Dark brown in colour
3.	Taste	Pungent in taste
4.	Shape	Elongated, tube like
5.	Size	10-15 cm available in pair
6.	Foreign Organic Matters	Nil
7.	Ethanol Soluble extractive	14.66%
8.	Water Soluble extractive	5.25%
9.	Loss on Drying (Moisture content)	1.8%
10.	Total Ash	12.26%
11.	Acid insoluble ash	0.5%

Table No. 6.3: Morphology and physicochemical specifications of *C. argentea* seed

Sr. No.	TEST	SPECIFICATION
1.	Colour	Powder faint blackish in colour
2.	Taste	Mild in taste
3.	Size	1-2 mm
4.	Appearance of seeds	Black and lustrous
5.	Foreign Organic Matters	0.6%
6.	Ethanol Soluble extractive	4.96%
7.	Water Soluble extractive	1.27%
8.	Loss on Drying (Moisture content)	0.05%
9.	Total Ash	10.26%
10.	Acid insoluble ash	1.8%

6.1.3 Extraction

The extraction of three plants was conducted both individually and in combinations of 2:1:1 and 4:1:1 ratios using the Soxhlet extraction method continuously for 48 hours. The resulting extracts underwent physical examination, including evaluation of color and consistency, as detailed in Table 6.1.

Figure No. 6.2: Soxhlet Extraction of *B. diffusa*

Figure No. 6.3: Soxhlet Extraction of *C. argentea*

Figure No. 6.4: Soxhlet Extraction of *P.rubra*

Table No. 6.4: Extraction of *Boerhavia diffusa, Plumeria Rubra* and *Celosia argentea*

Sr. No.	Extracts	Colour	Consistency	% Yield
1.	*Boerhavi adiffusa* leaves	Green	Semisolid	3.3
2.	*Plumeria Rubra pods*	Dark brown	Semisolid- Jelly like	24.66
3.	*Celosia argentea* seeds	Faint black	Sticky	4.96

Figure No. 6.5: Extract of *Boerhavia diffusa* leaves *Celosia*

Figure No. 6.6 : Extract of *argentea* seeds

Figure No. 6.7: Extract of *Plumeria Rubra* pods

6.1.4 Morphological Evaluation

A. *Boerhavia diffusa*

Morphological analysis indicated that *Boerhavia diffusa* leaves are simple, opposite, and petiolate. They are thick with an undulate margin and vary in shape from ovate to orbicular, measuring between 2 to 4 cm in length and 1.5 to 3 cm in width. The apex of the leaves is rounded, and the base ranges from rounded to subcordate. The upper surface is green while the lower surface is silvery white, and the petiole is as long as the lamina (Figure 6.8). The organoleptic evaluation of the powdered leaves of *Boerhavia diffusa* showed that it has a dark green color, a slight odor, and a distinctive taste.

Figure No. 6.8: *Boerhavia diffusa* leaves

The morphological studies revealed the information about *Plumeria Rubra* pods are dark reddish brown in colour, having strong odour, slight in taste, elongated tube-like structure, available in pair or doble pod about 10-15 cm in length, coarse in texture and having rough fracture. (Figure 6.9). The organoleptic assessment of the powder derived from *Plumeria Rubra* pods indicated it had a dark brown color, was odorless, and had a mildly bitter taste.

Figure No. 6.9: *Plumeria rubra* seed pods

C. Celosia argenteaseeds

The morphological analysis indicated that the seeds of *Celosia argentea* are spherical to lenticular or subreniform in shape, measuring between 1.5 to 3 mm in diameter and about 1 mm in thickness. They are margined, with a shallow depression near a small elevated region at the margin where the micropyle and hilum are located. The seed coat typically shows cracking at this point, and they are characterized by a black and glossy color (Figure 6.10). In terms of organoleptic evaluation, the powdered *Celosia argentea* seeds were noted to have no distinct odor, and they appeared faintly black in color. They exhibited a slightly bitter and unpleasant taste.

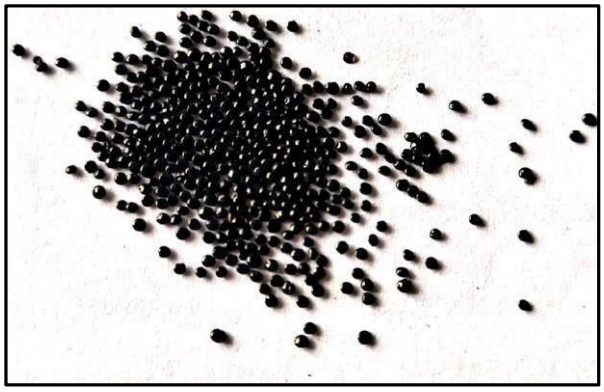


Figure No. 6.10: *Celosia argentea* Linn seeds

6.1.5 Microscopic Evaluation

Microscopic evaluation enables a detailed examination of a drug, allowing for its identification based on known histological characteristics.

A. Boerhavia diffusa:

In Figure 6.11, the cross-section of a leaf through the midrib region shows distinct layers including upper and lower single-layered epidermis, mesophyll, and the midrib itself. Both epidermal layers consist of single-layered cells with rectangular straight walls. The mesophyll is comprised of palisade parenchyma and spongy parenchyma. Within the midrib, collenchyma and a vascular bundle are observed. The palisade parenchyma, which contains chlorophyll, is arranged in a single layer, while the spongy parenchyma exhibits irregular arrangement with notable intercellular spaces also containing chlorophyll. The vascular bundle is situated centrally within the midrib.

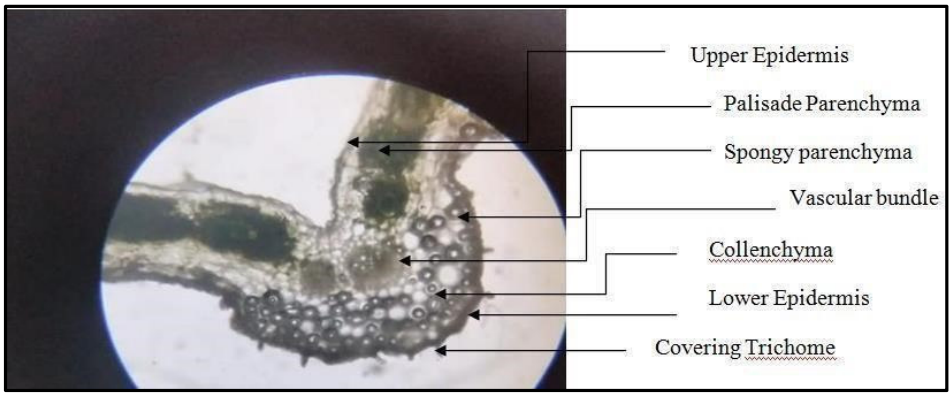
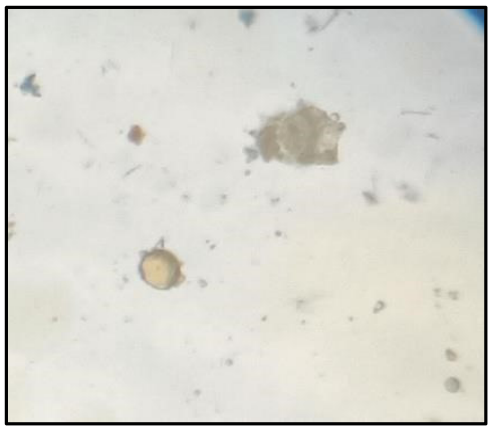


Figure No. 6.11: T. S. of *Boerhavia diffusa* leaf

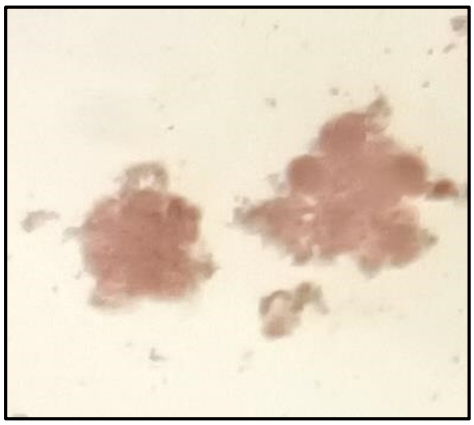
Microscopic study of *Boerhavia diffusa* leaf powder showed different characters such as Vessel, Prismatic crystal, pollen grain, covering trichomes, lignified parenchyma, epidermal cells and Fibers.

Vessel

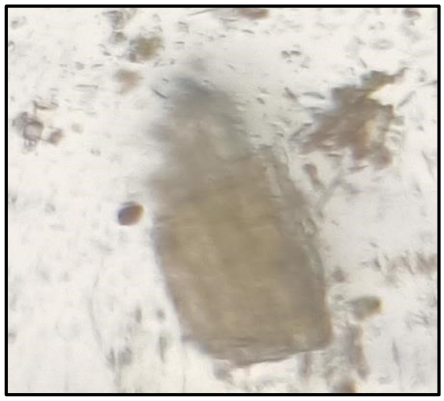


Prismatic crystal

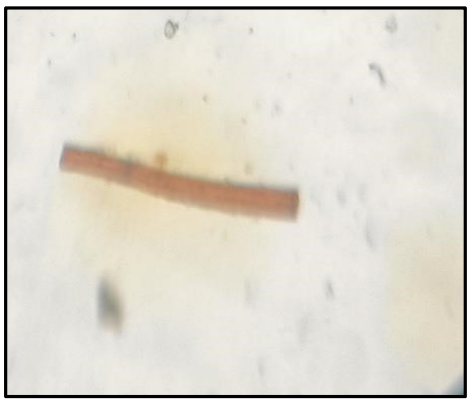
Covering Trichomes



Lignified parenchyma



Epidermal cells



Fibers

Figure No. 6.12: Powder Characteristics of *Boerhavia diffusa* whole plant

B. Plumeria Rubra

Figures 6.13 to 6.16 illustrate a transverse section of the pedicel, which is rounded in outline. The section shows an outer, hairy epidermis, followed by a cortex composed of 1-2 rows of hypodermal chlorenchymatous cells, 1-2 rows of collenchymatous cells, and 3-4 rows of parenchymatous cells. The pericycle consists of intermittent groups of lignified fibers. The vascular tissue forms a continuous ring of collateral vascular bundles, enclosing a wide parenchymatous pith with small groups of premedullary phloem.

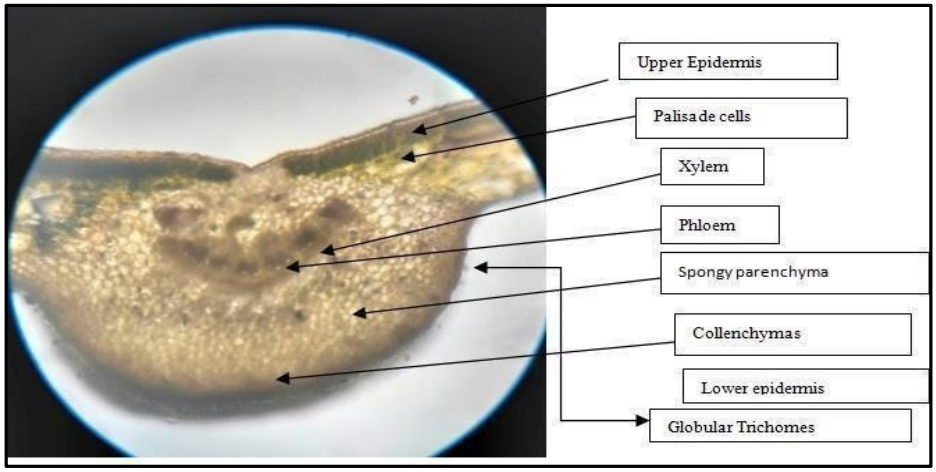


Figure No. 6.13: T. S. *Plumeria Rubra* leaf

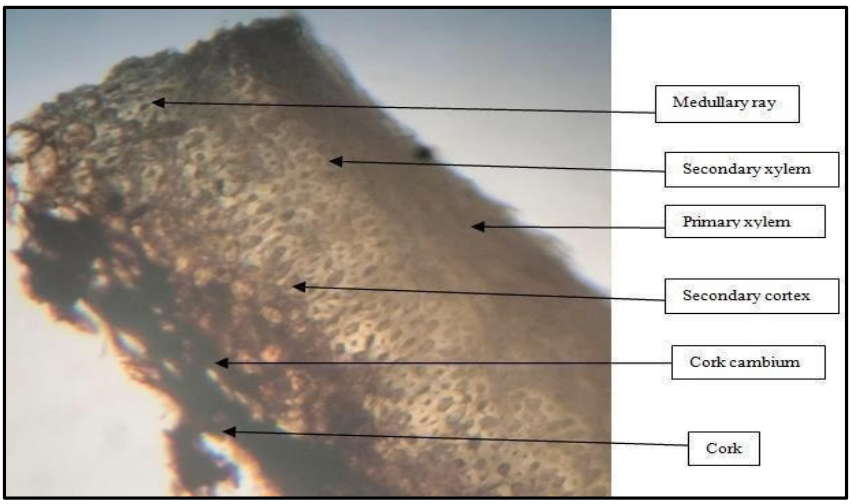


Figure No. 6.14: T. S. *Plumeria Rubra* seed pod shell

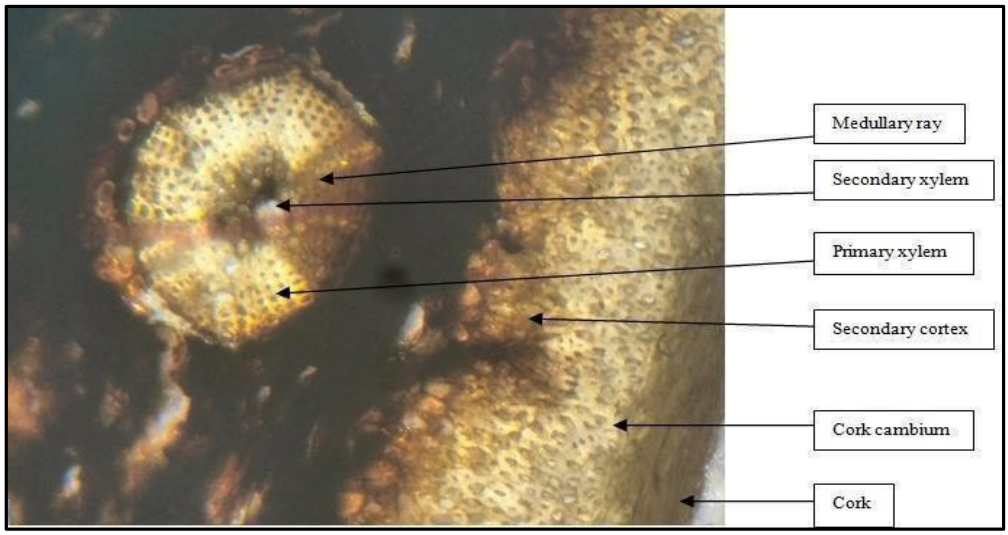


Figure No. 6.15: T. S. *Plumeria Rubra* seed pod showing medullary rays

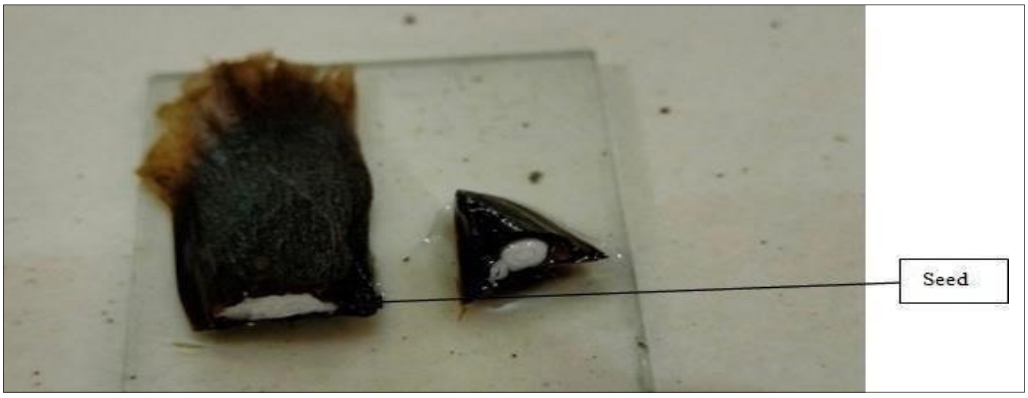
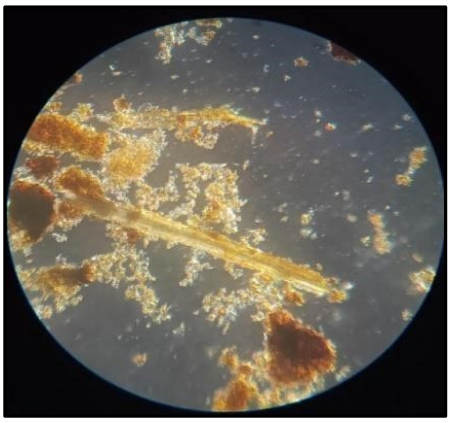
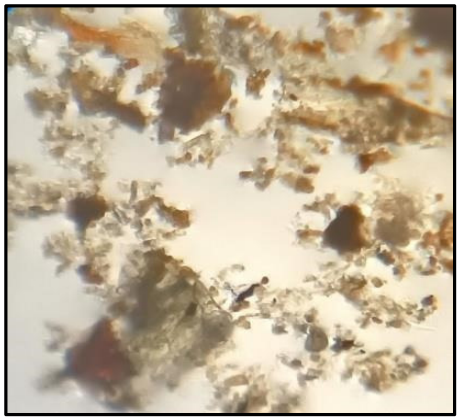


Figure No. 6.16: Images showing seed of *Plumeria Rubra* showing monocotyledon and embryo

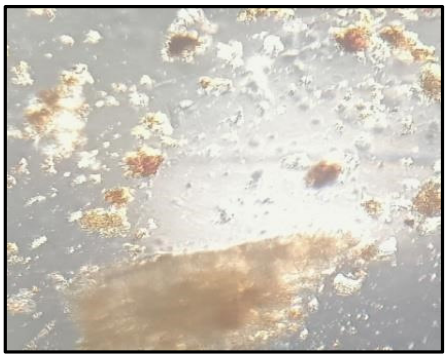
Microscopic study of *Plumeria Rubra* seed pod powder showed different characters such as unicellular trichmome, lignified cells, vascular bundle and starch grains



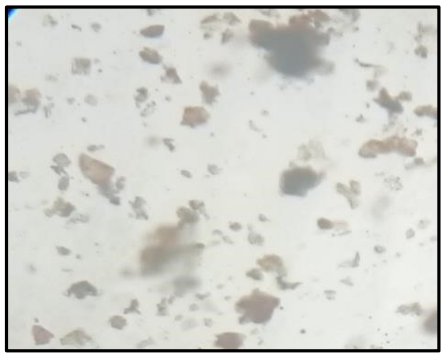
Unicellular trichomes



Lignified cells



Vascular bundle



Starch grains

Figure No. 6.17: Powder Characteristics *Plumeria Rubra* seed pod powder

C. Celosia argentea

In Figure 6.18, the cross-section of *Celosia argentea* seeds displays a monocotyledonous seed structure. The thickened parenchyma cells form a reticulate pattern and are connected to perisperm layers containing starch grains. The embryo cells, which are of varying shapes and sizes, are prominently filled with mucilage.

Figure No. 6.18: T. S. of Celosia argentea Linn seed cotyledon showing reticulate parenchyma with starch grains

Figure 6.19 reveals the transverse section of *Celosia argentea* Linn seed outer thick dark brown colour edtesta enclosing the wide central horizontally placed starchy perisperm and oil globules.

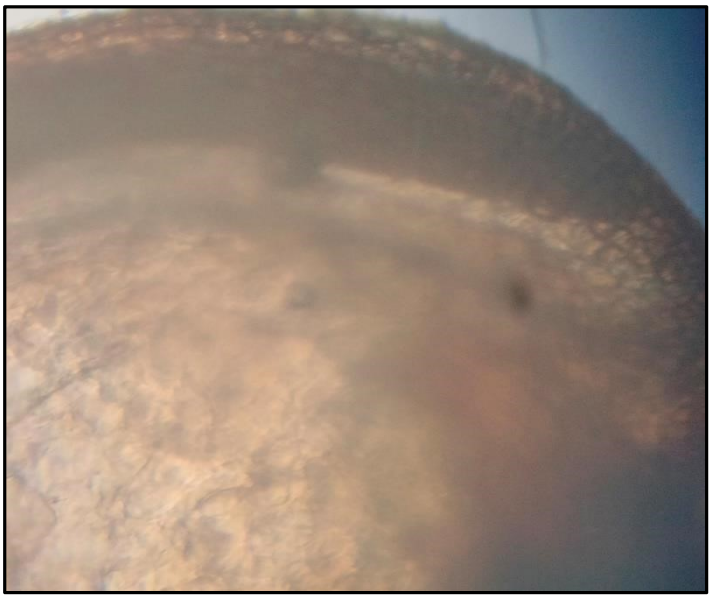
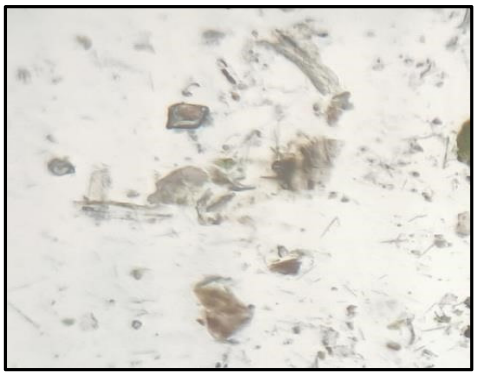


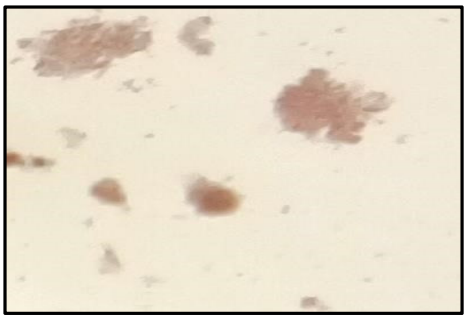
Figure No. 6.19: T. S. of *Celosia argentea* Linn seed

The microscopic examination of *Celosia argentea* Linn seed powder revealed various distinct features including starch grains, aleurone grains, cotyledon cells of testa, parenchymatous cells of cotyledons, and oil glands.



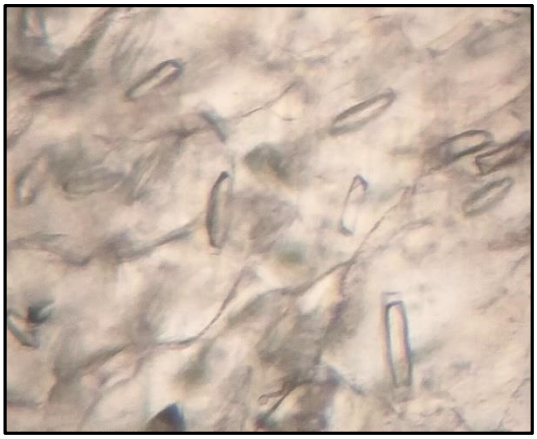
Starch grains

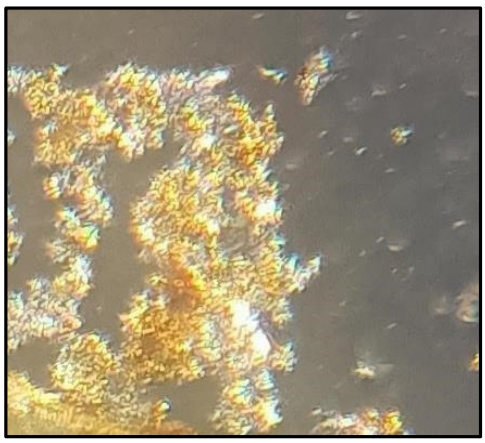
Aleurone grains



Lignified cells

Epidermal cells of testa





Parenchymatous cells of cotyledons **Oil glands**

Figure No. 6.20: Powder Characteristics of *Celosia argentea* Linn seed powder

6.1.6 Physicochemical Evaluation

Physicochemical evaluation is crucial for determining the identity, quality, and purity of crude drugs. Developing pharmacopoeial specifications for plant materials enables quality control chemists to effectively verify and approve these materials. Physical constants play a significant role in detecting adulteration or mishandling of drugs.

Ash values are particularly important in assessing the quality and purity of crude drugs. They reveal the presence of various impurities such as carbonates, oxalates, and silicates. Watersoluble ash estimation helps quantify the amount of inorganic compounds present in drugs, while acid-insoluble ash, primarily composed of silica, indicates contamination with earthy materials. Acid-insoluble ash specifically measures silica content, including sand. Lower values for these parameters suggest reduced inorganic matter and silica in the crude drug.

The extractive values are important for assessing the chemical composition of crude drugs and for determining the concentration of specific constituents soluble in particular solvents. Methanol and aqueous extracts showed the highest extractive values for leaves of *Boerhavia diffusa*, pods of *Plumeria Rubra*, and seeds of *Celosia argentea*. The percentage of active chemical constituents in the crude drug is reported based on air-dried weight. Minimizing moisture content is crucial to prevent the decomposition of crude drugs, such as chemical changes or microbial contamination (e.g., bacterial, yeast, or fungal growth) during storage.

Foreign organic matter refers to any parts of an organ or organs not specified in the drug's definition and description. Monographs of crude drugs specify the maximum acceptable limit for foreign organic matter. Exceeding this limit leads to a deterioration in the drug's quality.

Table 6.5 presents the physicochemical characterization of powders derived from *Boerhavia diffusa* leaves, *Plumeria Rubra* pods, and *Celosia argentea* seeds.

Table No. 6.5: Physicochemical Parameters of *Boerhavia diffusa* leaves, *Plumeria Rubra* pods and seeds of *Celosia argentea*

Sr. No.	Physicochemical Parameters	*B.D.*	*PR*	*CA*
1.	Ash Values			
	• Total ash	12.3%w/w	15.5%w/w	7.6%w/w
	• Acid insoluble ash	0.3%w/w	0.5%w/w	0.5%w/w
	• Acid soluble ash	12.5%w/w	4.5%w/w	2.2%w/w
2.	Extractive Values			
	• Alcohol soluble extractive	9.2 %w/w	5.0%w/w	11.50%w/w
	• Water soluble extractive	22.8%w/w	31.6%w/w	15.40%w/w
3.	Moisture Content	4.5%w/w	2.5%w/w	0.5%w/w
4.	Foreign organic matter	Nil	Nil	0.5 %w/w

6.1.7 Fluorescence Analysis

When exposed to UV and visible light and treated with different reagents, powders emit diverse color emissions, aiding in the identification of drugs in powdered form.

A. ***Boerhavia diffusa*:** UV analysis of *Boerhavia diffusa* leaves is conducted in a UV chamber as illustrated in Figure 6.21.

Figure No. 6.21: UV Chamber for TLC visualization

Table No. 6.6: Ultra – Violet analysis of *Boerhavia diffusa* leaves

Sr. No.	Treatment	Visible light	UV (254nm)	UV (366nm)
1.	Drug Powder	Green	Fluorescent Green	Green
2.	Drug + NaoH	Dull Green	Dark green	Green
3.	Drug + HNO_3	Yellowish green	Faint Green	Greenish Yellow
4.	Drug + H_2SO_4	Green	Green	Violet
5.	Drug + Methanol	Dark green	Green	Pinkish white

A. Plumeria Rubra

Table No. 6.7: Ultra – Violet analysis of *Plumeria Rubra* pods

Sr. No.	Treatment	Visible light	UV (254nm)	UV (366nm)
1.	Drug Powder	Faint yellow	Faint green	Light pink
2.	Drug + NaoH	Brown	Green	violet
3.	Drug + HNO_3	Green	Green	pink
4.	Drug + H_2SO_4	Gray	Brown	brown
5.	Drug + Methanol	brown	Green	Faint creamy

B. Celosia argentea

Table No. 6.8: Ultra – Violet analysis of *Celosia argentea* seeds

Sr. No.	Treatment	Visible light	UV (254nm)	UV (366nm)
1.	Drug Powder	Brown	Dark Green	Pink
2.	Drug + NaoH	Yellowish	Greenish Yellow	Faint green
3.	Drug + HNO_3	Brown	Cream Yellow	violet
4.	Drug +H_2SO_4	Dark Violet	Greenish yellow	Pink
5.	Drug +Methanol	Gray	Yellowish Green	Blue

6.1.8 Phytochemical Evaluation

Phytochemical evaluation involves the detection of various phytoconstituents present in crude drug extracts and the establishment of their chemical composition profile.

A. ***Boerhavia diffusa*** Preliminary phytochemical screening of the aqueous extract of *Boerhavia diffusa* leaves revealed the presence of alkaloids, glycosides, saponins, phytosterols, tannins, and flavonoids. Carbohydrates, proteins & amino acids, fixed oils & fats, gums & mucilage, and volatile oil were absent.

B. ***Plumeria Rubra*** Preliminary phytochemical screening of the aqueous extract of *Plumeria Rubra* pods indicated the presence of alkaloids, carbohydrates, glycosides, phytosterols, fixed oils & fats, flavonoids, and gums & mucilage. Saponins, proteins & amino acids, tannins, and volatile oil were absent.

C. ***Celosia argentea*** Preliminary phytochemical screening of the aqueous extract of *Celosia argentea* seeds demonstrated the presence of alkaloids, carbohydrates, glycosides, saponins, proteins & amino acids, phytosterols, tannins, flavonoids, and gums & mucilage. Fixed oils & fats and volatile oil were absent.

D. Phytochemical constituents detected in crude extracts of *Boerhavia diffusa* leaves, *Plumeria Rubra pods and Celosia argentea* seeds are shown in table 6.9.

Table No. 6.9: Qualitative Phytochemical Tests of *Boerhavia diffusa* leaves, *Plumeria Rubra pods and Celosia argentea* seeds

Sr. No.	Tests	*B.D.*	*PR*	*CA*
1.	**Tests for Alkaloids**			
	• Mayer's test	+	+	+
	• Wagner's test	+	+	+
	• Hager's tests	+	+	+
	• Dragendorff's test	+	+	+
2.	**Tests for Carbohydrates**			
	• Molish's test	_	_	+
	• Fehling test	_	_	+
	• Barfoed's test	_	_	+
	• Benedict's test	_	_	+
3.	**Tests for Glycosides**			
	• Borntrager's test	+	+	+
	• Legal's test	+	+	+
4.	**Test for Saponins**			
	Test solution+20ml distilled H_20	+	+	+
5.	**Test for Phytosterol**			
	• Libermann-Burchard's test	+	+	+
6.	**Tests for Fixed oils & fats**			
	• Spot test	_	+	+
	• Saponification test	_	+	+

7.	**Tests for Tannins**			
	• Ferric chloride test	-	_	-
	• Gelatin test	-	_	-
	• Aqueous bromine test	-	_	-

+ve signifies a positive outcome, while -ve signifies a negative outcome.

6.1.9 pH of Extracts:

pH of *Boerhavia diffusa* leaves*, Plumeria Rubra pods and Celosia argentea* seeds extract mentioned in the table 6.10.

Table No. 6.10: pH of Extracts

Sr. No.	Extract	pH
1.	*Boerhavia diffusa* leaves	7.0
2.	*Plumeria Rubra pods*	7.5
3.	*Celosia argentea* seeds	7.5

6.1.10 Optimization of TLC Solvent System

6.1.10.1. *Boerhavia diffusa*

Best solvent system for *Boerhavia diffusa*

For aqueous extract – Ethyl acetate: Methanol: Formic acid (5: 0.5: 0.5v/v).

The TLC studies of aqueous extract show best separation using Toluene: Acetone: Formic Acid (11: 6: 1 v/v) as a mobile phase.

High Performance Thin Layer Chromatography for Eupalitin

The densitogram revealed five spots under UV light (254 nm) at Rf values of 0.20, 0.27, 0.37, 0.64, and 0.72. Specifically, the spot at Rf 0.64 exhibited a dark green coloration when exposed to UV radiation (254 nm). Eupalitin is recognized as the primary active constituent found in *Boerhavia diffusa* leaves. Its presence is indicated by a blue fluorescence under UV light at Rf 0.64 during Thin Layer Chromatography, confirming its status as the main component of *Boerhavia diffusa* leaves.

Table No. 6.11: Phytochemical constituents *B. diffusa* HPTLC

Sr. No.	Name Phytocontituent	Rf	Amount of phytoconstituent (%)
1.	Eupalitin	0.64	53.63
2.	Gallic acid	0.27	3.14
3.	Quercetin	0.53	14.54

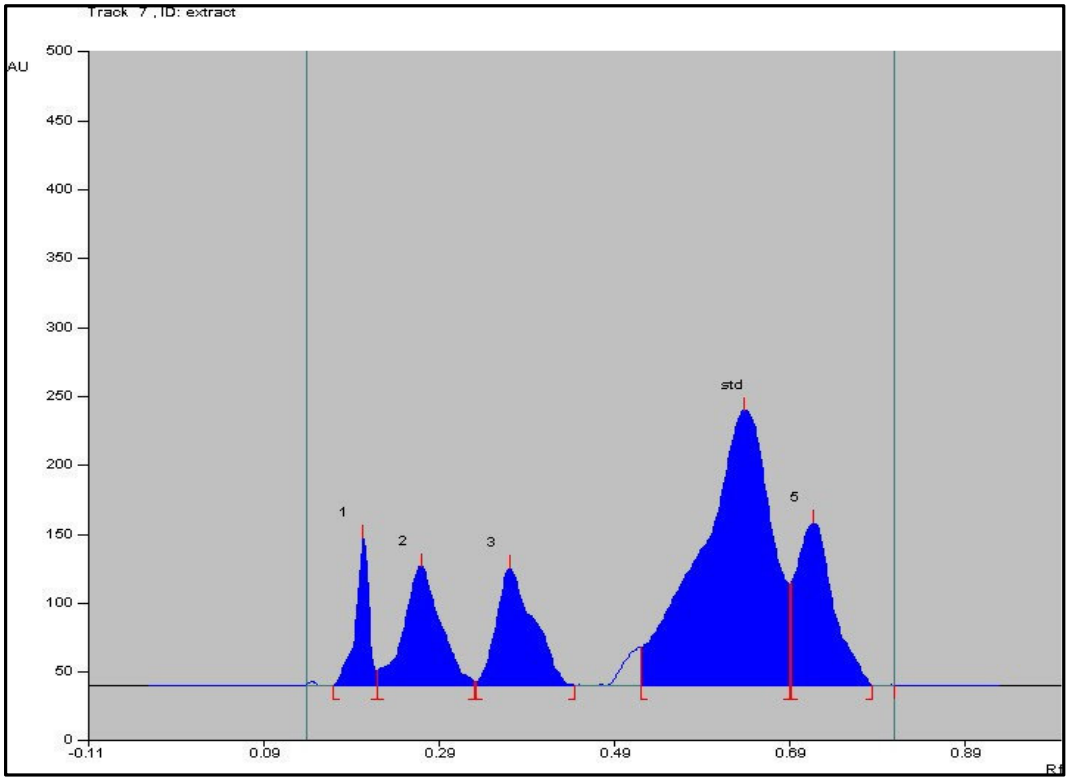


Figure No. 6.22: Densitogram of *B. diffusa*

Table No. 6.12: HPTLC chromatogram of aqueous *Boerhavia diffusa* leavesextract at 254 nm

Track 7, ID: extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.17 Rf	0.2 AU	0.20 Rf	107.3 AU	17.94 %	0.22 Rf	11.3 AU	1315.3 AU	5.31 %	unknown *
2	0.22 Rf	11.4 AU	0.27 Rf	86.9 AU	14.54 %	0.33 Rf	2.6 AU	2996.7 AU	12.10 %	unknown *
3	0.33 Rf	2.8 AU	0.37 Rf	85.7 AU	14.34 %	0.44 Rf	0.2 AU	2996.1 AU	12.10 %	unknown *
4	0.52 Rf	27.8 AU	0.64 Rf	200.0 AU	33.46 %	0.69 Rf	74.0 AU	13278.6 AU	53.63 %	std
5	0.69 Rf	74.4 AU	0.72 Rf	117.9 AU	19.71 %	0.78 Rf	0.0 AU	4173.0 AU	16.85 %	unknown *

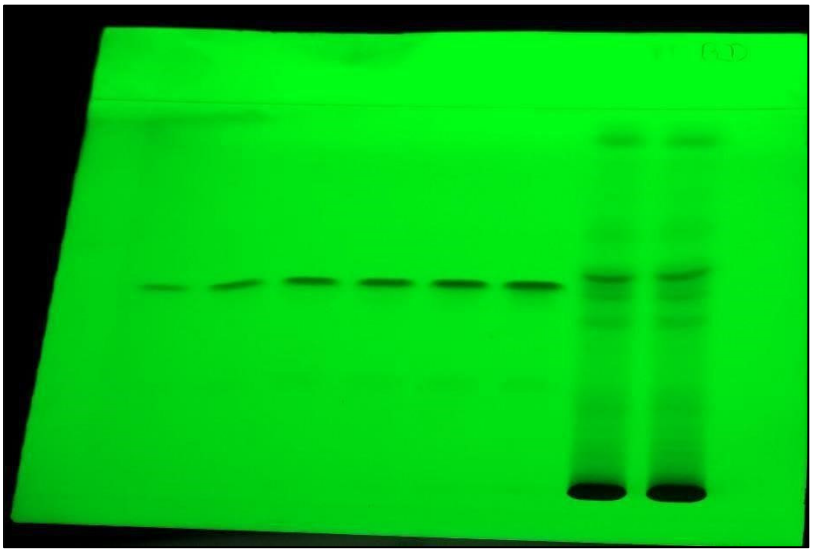


Figure No. 6.23: Image of *Boerhavia diffusa* leaves extract at 254 nm

High Performance Thin Layer Chromatography of Boerhavia diffusa leaves extract at 254 nm for Galic acid and quercetin

The obtained densitogram showed 12 spots at 0.04, 0.12, 0.14, 0.18, 0.23, 0.27, 0.39, 0.46, 0.53, 0.58, 0.71 and 0.81 UV light (254 nm). The spot at Rf 0.27 was light green and 0.53 was dark green under UV radiation (254 nm). Gallic acid and Quercetin have been reported the main active ingredient of *Boerhavia diffusa* leaves. On Thin Layer Chromatography it gives blue colour fluorescence under UV light at Rf 0.27 and 0.53 indicating the presence of Gallic acid and quercetin respectively, which the main.

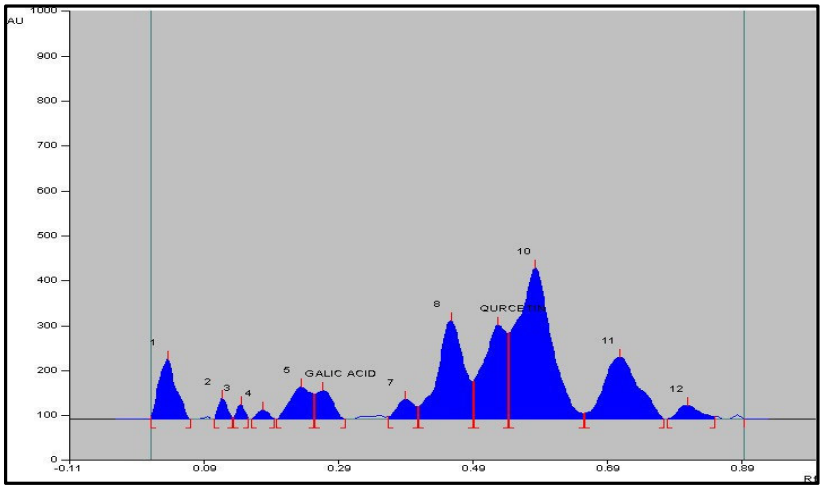
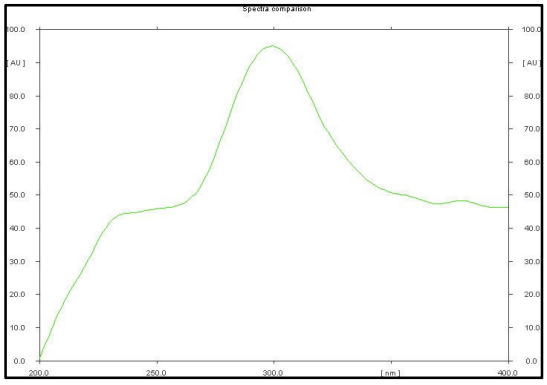
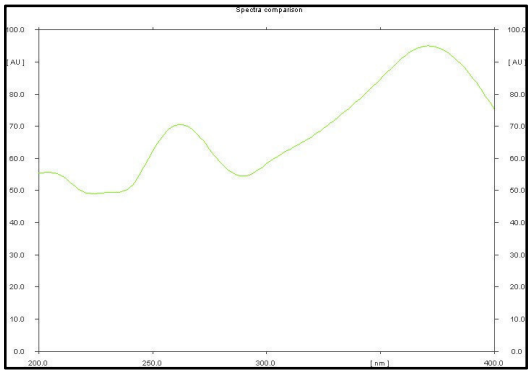


Figure No. 6.24: Densitogram *B. diffusa* of gallic acid and Quercetin

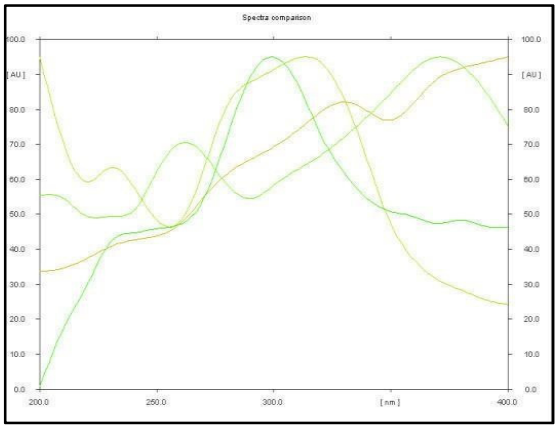
Table No. 6.13: HPTLC chromatogram of *Boerhavia diffusa* leaves extract at 254 nm

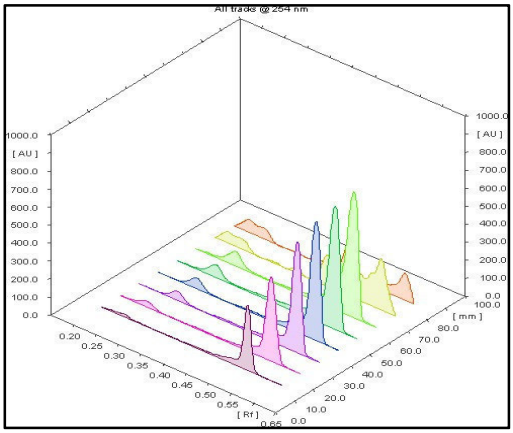
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.01 Rf	6.3 AU	0.04 Rf	133.1 AU	9.92 %	0.07 Rf	0.1 AU	2749.9 AU	6.61 %	unknown *
2	0.10 Rf	0.3 AU	0.12 Rf	45.6 AU	3.40 %	0.13 Rf	3.4 AU	487.5 AU	1.17 %	unknown *
3	0.13 Rf	3.7 AU	0.14 Rf	32.7 AU	2.44 %	0.16 Rf	1.3 AU	301.7 AU	0.73 %	unknown *
4	0.16 Rf	0.3 AU	0.18 Rf	19.8 AU	1.48 %	0.19 Rf	0.5 AU	277.0 AU	0.67 %	unknown *
5	0.20 Rf	0.0 AU	0.23 Rf	71.5 AU	5.33 %	0.25 Rf	55.1 AU	1843.4 AU	4.43 %	unknown *
6	0.25 Rf	55.5 AU	0.27 Rf	63.2 AU	4.71 %	0.30 Rf	0.1 AU	1306.1 AU	3.14 %	GALIC ACID
7	0.36 Rf	5.8 AU	0.39 Rf	44.2 AU	3.29 %	0.41 Rf	27.9 AU	934.2 AU	2.25 %	unknown *
8	0.41 Rf	28.0 AU	0.46 Rf	219.2 AU	16.34 %	0.49 Rf	33.2 AU	6940.8 AU	16.69 %	unknown *
9	0.49 Rf	84.2 AU	0.53 Rf	208.7 AU	15.56 %	0.54 Rf	31.5 AU	6046.6 AU	14.54 %	QURCETIN
10	0.54 Rf	192.3 AU	0.58 Rf	336.0 AU	25.04 %	0.65 Rf	12.9 AU	14022.5 AU	33.71 %	unknown *
11	0.66 Rf	12.9 AU	0.71 Rf	137.5 AU	10.25 %	0.77 Rf	0.4 AU	5863.7 AU	14.10 %	unknown *
12	0.78 Rf	0.4 AU	0.81 Rf	30.3 AU	2.26 %	0.85 Rf	6.4 AU	820.4 AU	1.97 %	unknown *





A: Galic Acid **B: Quercetin**





C: Gallic acid and Quercetin with Extract **D: 3D spectra of Standard and extract**

Figure No. 6.25: A: Overlain of Galic acid, B: Overlain of Quercetin, C: Overlain of Galic acid+ Quercetin+ Extract, D:3D spectra of std.+ Extract of *Boerhavia diffusa* leaves extract at 254 nm

6.1.10.2 *Plumeria Rubra*

Table No. 6.14: Phytochemicals of *P.rubra*

Sr. No.	Name Phyto constituent	Rf	Amount of phytoconstituents (%)
1.	Ursolic acid	0.61	4.60
2.	Oleic acid	0.57	21.71
3.	Lupeol	0.68	14

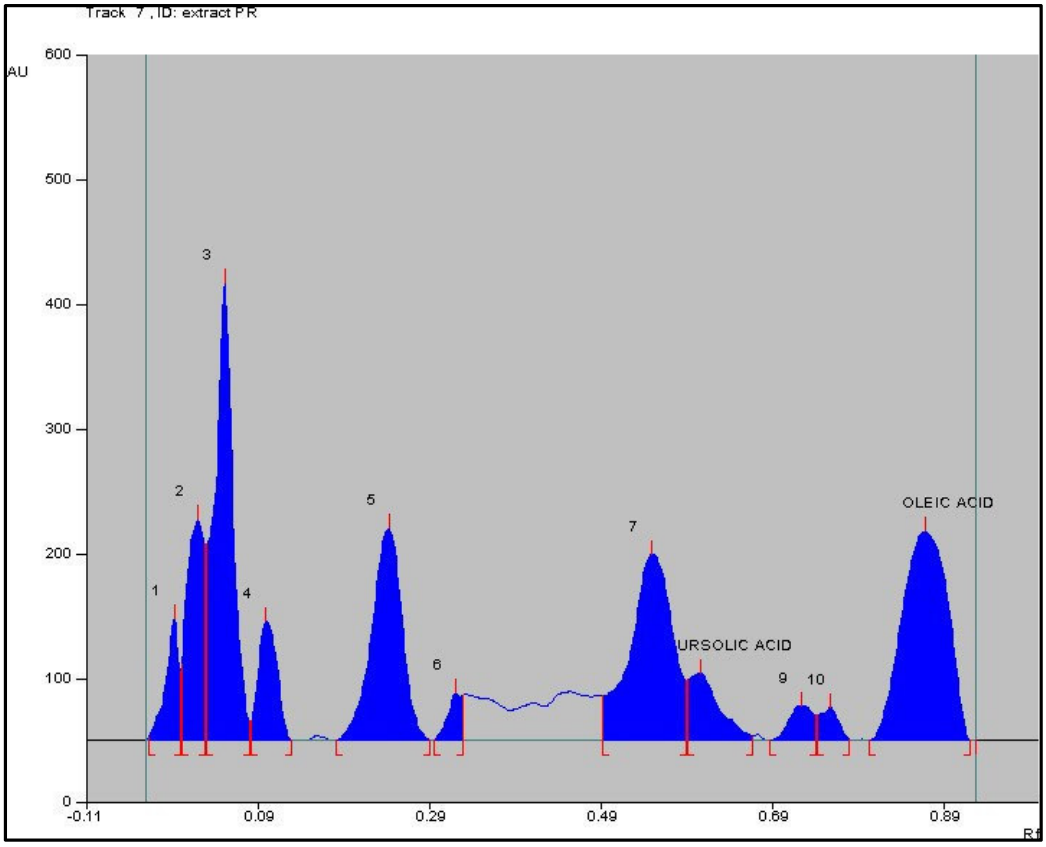
Plumeria Rubra

Best solvent system for *Plumeria Rubra*

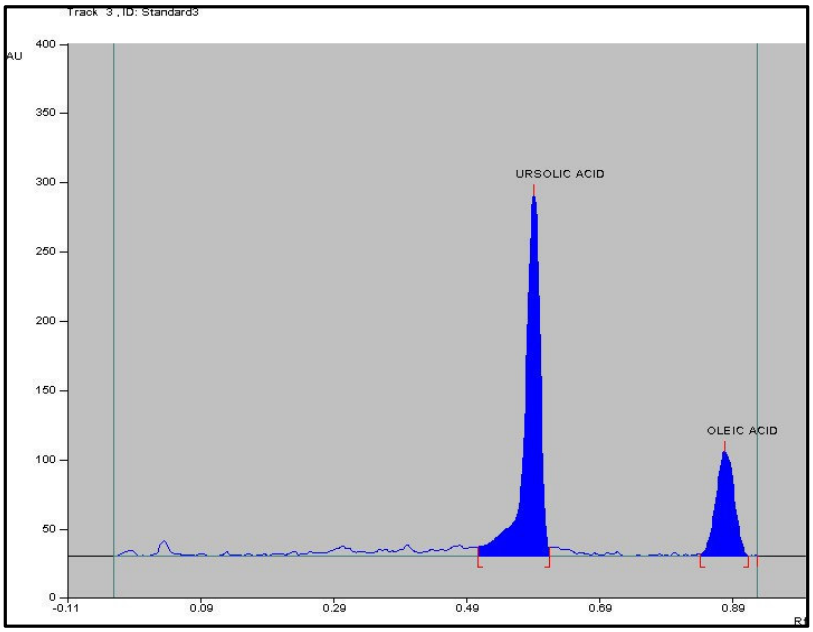
The TLC studies of aqueous extract show best separation using Toluene: Ethyl acetate: Formic acid (8:2:0.1 v/v) as a mobile phase.

High Performance Thin Layer Chromatography

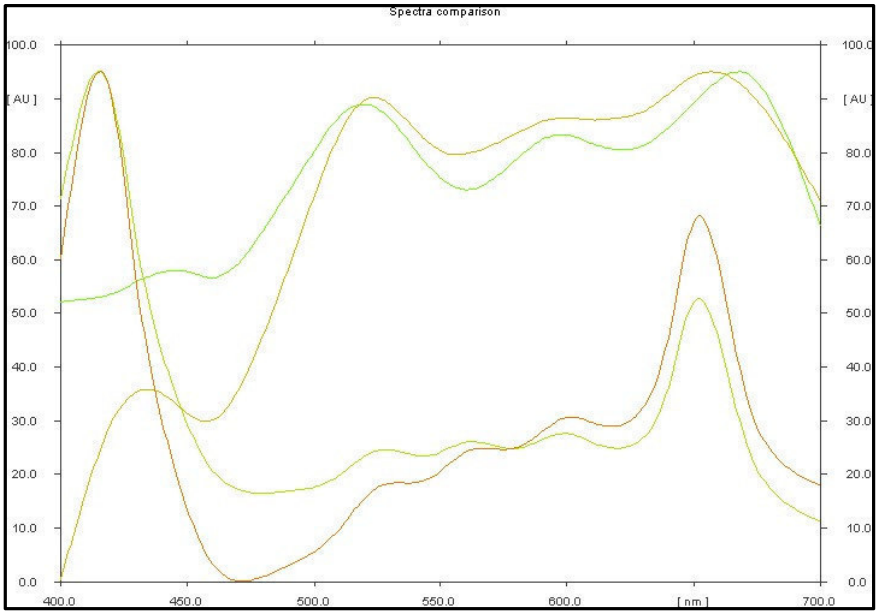
The obtained densitogram showed 10spots at Rf 0.02, 0.05, 0.10, 0.24, 0.32, 0.55, 0.61, 0.72, 0.76 and 0.87in UV light at 500 nm. The spot at Rf 0.61 was dark brown and Rf 0.87 was yellowish cream fluorescence under UV radiation (366 nm). Ursolic acid and oleic acid has been reported the main active ingredient of *Plumeria Rubra* seed pod.



A: HPTLC densitogram of *Plumeria Rubra* seed pod extract at 500 nm



B: Densitogram of standard Ursolic acid and Oleic acid



C: Overlain spectra of Ursolic acid, Oleic acid over aq extract of *Plumeria Rubra*

Figure No. 6.26: A: HPTLC densitogram of *Plumeria Rubra* seed pod extract at 500 nm, B: Densitogram of standard Ursolic acid and Oleic acid, C: Overlain spectra of Ursolic acid and Oleic acid over extract of *Plumeria Rubra*

Table 6.15: HPTLC chromatogram of *Plumeria Rubra* seed pod extract

Track 7, ID: extract PR

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.04 Rf	2.4 AU	-0.01 Rf	98.0 AU	7.15 %	-0.00 Rf	55.2 AU	1227.2 AU	3.56 %	unknown *
2	0.00 Rf	61.3 AU	0.02 Rf	177.3 AU	12.93 %	0.03 Rf	56.9 AU	3049.0 AU	8.85 %	unknown *
3	0.03 Rf	157.5 AU	0.05 Rf	366.8 AU	26.75 %	0.08 Rf	15.0 AU	6550.6 AU	19.02 %	unknown *
4	0.08 Rf	15.3 AU	0.10 Rf	95.8 AU	6.99 %	0.13 Rf	0.2 AU	1749.6 AU	5.08 %	unknown *
5	0.18 Rf	0.0 AU	0.24 Rf	170.1 AU	12.41 %	0.29 Rf	0.1 AU	4980.8 AU	14.47 %	unknown *
6	0.29 Rf	0.4 AU	0.32 Rf	38.0 AU	2.77 %	0.33 Rf	36.1 AU	520.6 AU	1.51 %	unknown *
7	0.49 Rf	35.3 AU	0.55 Rf	149.0 AU	10.87 %	0.59 Rf	48.0 AU	6183.4 AU	17.96 %	unknown *
8	0.59 Rf	48.4 AU	0.61 Rf	53.9 AU	3.93 %	0.67 Rf	2.9 AU	1583.2 AU	4.60 %	URSOLIC ACID
9	0.69 Rf	0.0 AU	0.72 Rf	27.9 AU	2.03 %	0.74 Rf	19.8 AU	645.2 AU	1.87 %	unknown *
10	0.74 Rf	19.9 AU	0.76 Rf	26.6 AU	1.94 %	0.78 Rf	0.4 AU	468.3 AU	1.36 %	unknown *
11	0.80 Rf	0.1 AU	0.87 Rf	167.7 AU	12.23 %	0.92 Rf	0.4 AU	7475.5 AU	21.71 %	OLEIC ACID

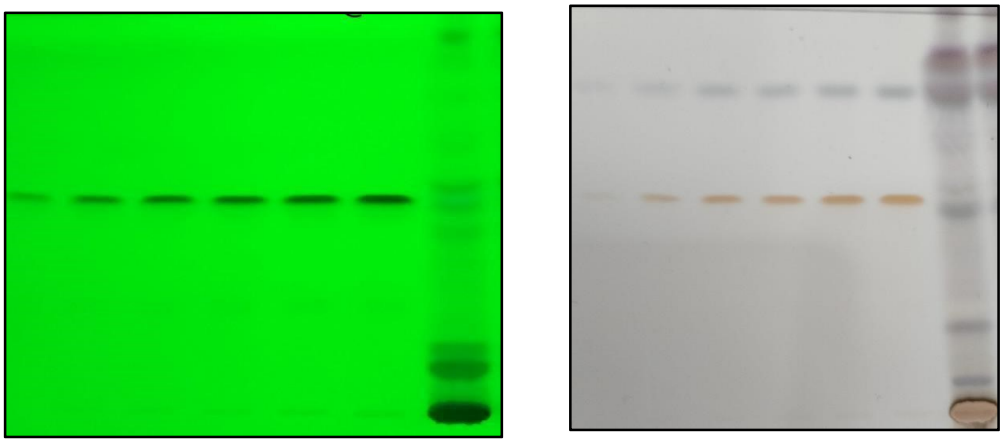


Figure No. 6.27: Image of *Plumeria Rubra* seed pod extract at 254 nm and after derivatization

Figure No. 6.28: Image of *Plumeria Rubra* seed pod extract at 366 nm

This is also found that Plumaria rubra seed pods showed presence of **Lupeol** The obtained densitogram showed 13spots at Rf 0.04, 0.12, 0.17, 0.24, 0.27, 0.30, 0.38,0.46, 0.50,0.59, 0.71, 0.80 and 0.89 in UV light at 500 nm. The spot at Rf 0.71 was Lupeol. Thus, Ursolic acid, oleic acid and Lupeol has been reported the main active ingredient of *Plumeria Rubra* seed pod.

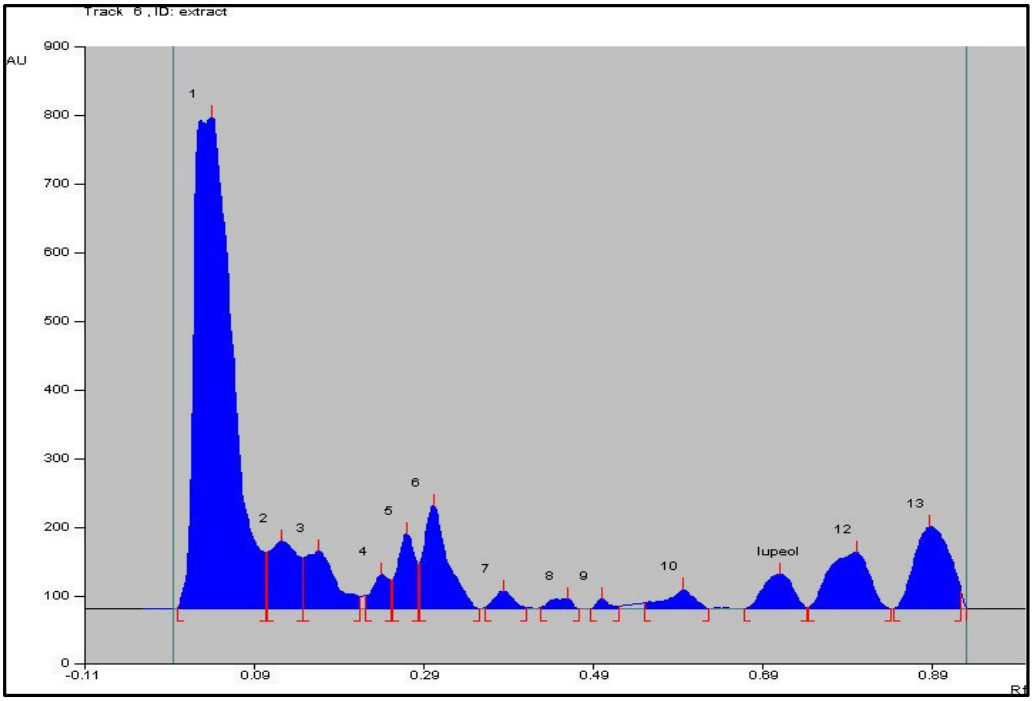


Figure 6.29: Densitogram of *Plumeria Rubra* showing presence of Lupeol

Table 6.16: HPTLC chromatogram of *Plumeria Rubra* seed pod extract for Lupeol

Track 6, ID: extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.00 Rf	1.5 AU	0.04 Rf	716.7 AU	46.38 %	0.10 Rf	33.1 AU	26370.1 AU	54.58 %	unknown *
2	0.10 Rf	82.9 AU	0.12 Rf	98.8 AU	6.39 %	0.15 Rf	73.9 AU	2727.3 AU	5.65 %	unknown *
3	0.15 Rf	74.1 AU	0.17 Rf	84.7 AU	5.48 %	0.21 Rf	18.3 AU	2520.8 AU	5.22 %	unknown *
4	0.22 Rf	18.8 AU	0.24 Rf	50.2 AU	3.25 %	0.25 Rf	40.9 AU	858.7 AU	1.78 %	unknown *
5	0.25 Rf	42.2 AU	0.27 Rf	109.0 AU	7.05 %	0.28 Rf	34.0 AU	1846.4 AU	3.82 %	unknown *
6	0.28 Rf	66.1 AU	0.30 Rf	151.0 AU	9.77 %	0.35 Rf	0.2 AU	3468.7 AU	7.18 %	unknown *
7	0.36 Rf	2.2 AU	0.38 Rf	25.2 AU	1.63 %	0.41 Rf	1.7 AU	470.6 AU	0.97 %	unknown *
8	0.43 Rf	2.0 AU	0.46 Rf	14.4 AU	0.93 %	0.47 Rf	0.5 AU	312.5 AU	0.65 %	unknown *
9	0.49 Rf	0.2 AU	0.50 Rf	15.1 AU	0.98 %	0.52 Rf	2.8 AU	170.1 AU	0.35 %	unknown *
10	0.55 Rf	9.0 AU	0.59 Rf	28.3 AU	1.83 %	0.63 Rf	0.1 AU	768.4 AU	1.59 %	unknown *
11	0.67 Rf	0.1 AU	0.71 Rf	50.2 AU	3.25 %	0.74 Rf	2.0 AU	1442.8 AU	2.99 %	lupeol
12	0.74 Rf	2.2 AU	0.80 Rf	82.0 AU	5.31 %	0.84 Rf	0.1 AU	3219.4 AU	6.66 %	unknown *
13	0.84 Rf	0.3 AU	0.89 Rf	119.6 AU	7.74 %	0.92 Rf	25.9 AU	4134.4 AU	8.56 %	unknown *

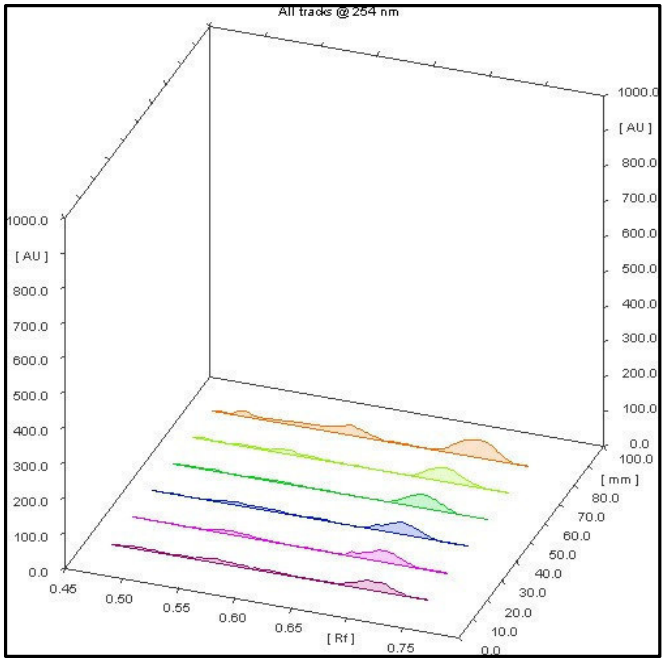


Figure 6.30: 3D spectra of Lupeol and *Plumeria Rubra* extract

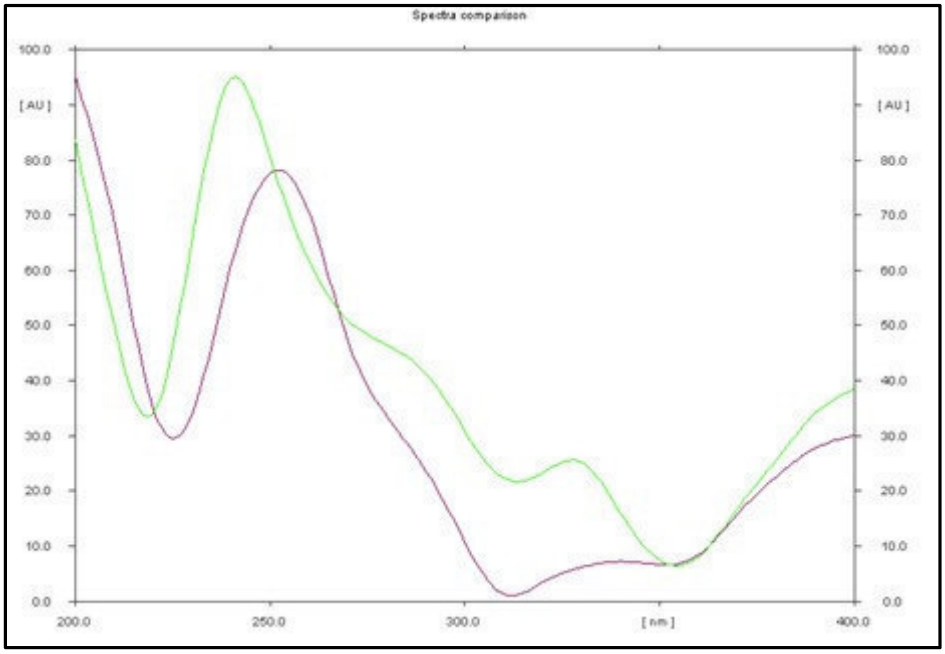


Figure 6.31: Overlain spectra for Lupeol and *Plumeria Rubra* Extract extract

Table 6.17: HPTLC chromatogram of *Plumeria Rubra* Seed pod extract

Show all of selected substance | Clear all

Track	Rf	Assigned Substance	Max. Signal	Display	r(s,m)	r(m,e)
1	0.73	Lupeol	106 AU @ 200 nm	✔	0.999928	1.000000
2	0.68	AutoGenerated9	66 AU @ 200 nm		0.999828	0.999990
2	0.71	Lupeol	84 AU @ 200 nm		0.999830	0.999921
3	0.68	AutoGenerated9	113 AU @ 200 nm		0.998130	0.998920
4	0.68	STD	139 AU @ 241 nm	✔	0.999012	0.999935
5	0.67	STD	299 AU @ 400 nm		0.999874	0.999968
6	0.73	Lupeol	145 AU @ 200 nm		0.999946	0.999984

6.1.10.3. *Celosia argentea*

Best solvent system for *Celosia argentea*

The TLC experiments with the aqueous extract indicate optimal separation with a mobile phase consisting of toluene, ethyl acetate, and formic acid (8:2:0.1 v/v). The derivatizing agent used was Anisaldehyde sulfuric acid.

Table No. 6.18: Phytochemicals present in C. argentea

Sr. No.	Name Phyto constituent	Rf	Amount of phytoconstituents (%)
1.	Gallic acid	0.20	3.11
2.	Quercetin	0.50	11.80

High Performance Thin Layer Chromatography

High Performance Thin Layer Chromatography (HPTLC) analysis revealed multiple spots under UV light (500 nm) with Rf values of 0.02, 0.12, 0.15, 0.20, 0.32, 0.40, 0.46, 0.50, 0.63, 0.68, and 0.73. Among these, the spot at Rf 0.54 appeared yellowish-brown. Previous studies have identified gallic acid and quercetin as the primary active compounds in *Celosia argentea* seeds. Gallic acid was detected at Rf 0.20, while quercetin was identified at Rf 0.50, confirming their presence in *Celosia argentea* seeds.

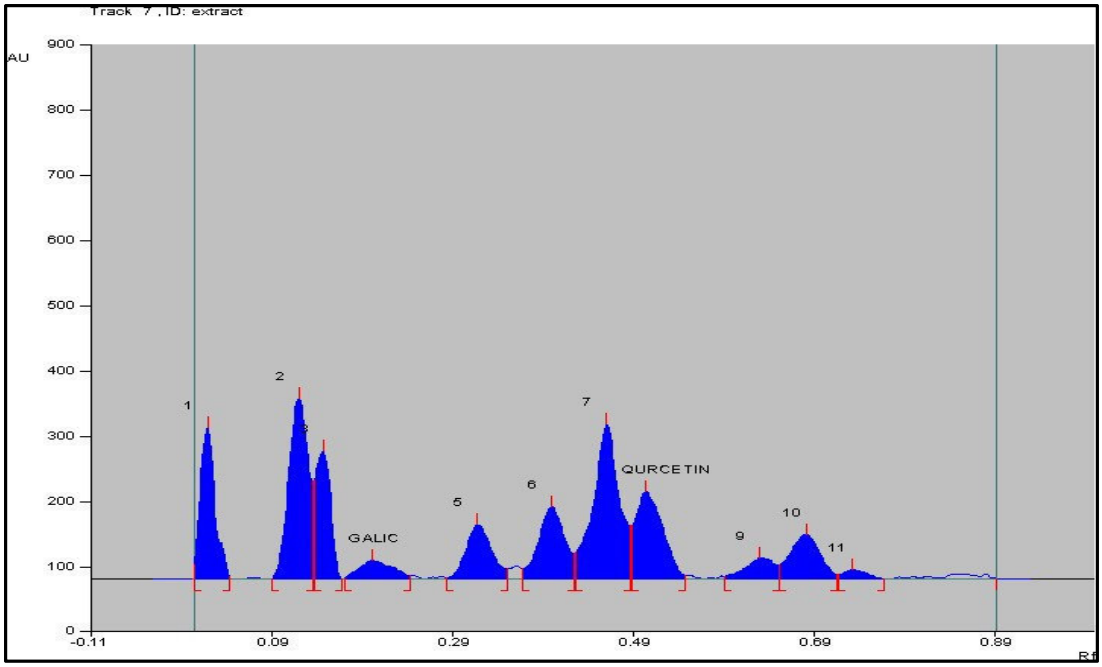


Figure No. 6.32: Densitogram *C. argentea*

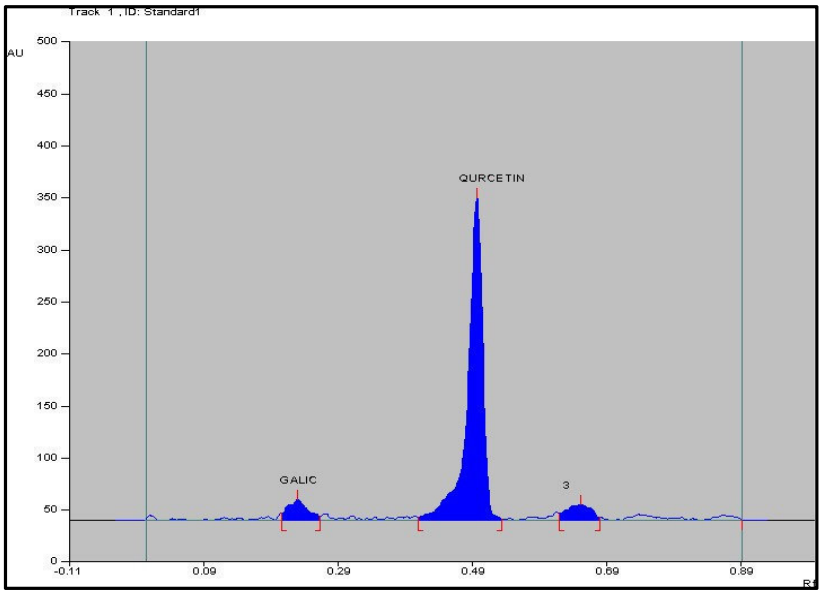


Figure No. 6.33: HPTLC densitogram of Aq. Extract of *Celosia argentea* seeds extract at 430 nm

Table No. 6.19: HPTLC chromatogram of *Celosia argentea* seeds extract

Track 7, ID: extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.00 Rf	21.6 AU	0.02 Rf	233.3 AU	16.48 %	0.04 Rf	3.4 AU	3139.7 AU	11.04 %	unknown *
2	0.09 Rf	0.7 AU	0.12 Rf	276.6 AU	19.54 %	0.13 Rf	50.5 AU	5003.7 AU	17.59 %	unknown *
3	0.14 Rf	152.9 AU	0.15 Rf	196.4 AU	13.88 %	0.17 Rf	0.6 AU	2705.0 AU	9.51 %	unknown *
4	0.17 Rf	0.3 AU	0.20 Rf	29.0 AU	2.05 %	0.24 Rf	4.6 AU	885.7 AU	3.11 %	GALIC
5	0.28 Rf	2.1 AU	0.32 Rf	83.4 AU	5.89 %	0.35 Rf	15.6 AU	1966.6 AU	6.91 %	unknown *
6	0.37 Rf	16.7 AU	0.40 Rf	111.0 AU	7.84 %	0.42 Rf	39.8 AU	2650.9 AU	9.32 %	unknown *
7	0.42 Rf	40.4 AU	0.46 Rf	237.2 AU	16.76 %	0.49 Rf	32.3 AU	5672.9 AU	19.94 %	unknown *
8	0.49 Rf	82.9 AU	0.50 Rf	133.7 AU	9.45 %	0.55 Rf	4.9 AU	3356.7 AU	11.80 %	QURCETIN
9	0.59 Rf	4.3 AU	0.63 Rf	31.7 AU	2.24 %	0.65 Rf	21.8 AU	877.0 AU	3.08 %	unknown *
10	0.65 Rf	22.0 AU	0.68 Rf	68.5 AU	4.84 %	0.72 Rf	6.7 AU	1879.8 AU	6.61 %	unknown *
11	0.72 Rf	6.8 AU	0.73 Rf	14.6 AU	1.03 %	0.77 Rf	0.2 AU	306.5 AU	1.08 %	unknown *

At Visible (Spraying reagent: Anisaldehyde sulphuric acid reagent)

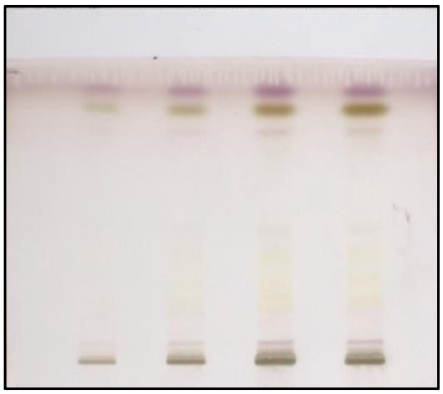


Figure No. 6.34: White Remission Derivative Image of *Celosia argentea* seeds

6.1.10.4 High Performance Thin Layer Chromatography of aqueous extract of Boerhavia diffusa, Plumeria Rubra seed pod and Celosia argentea seeds

The HPTLC analysis of *Boerhavia diffusa* leaves, *Plumeria Rubra* pods, and *Celosia argentea* seeds methanolic extracts demonstrated effective separation using a mobile phase consisting of Toluene acetate: Methanol acid (5:4:0.5:0.5 v/v). Anisaldehyde sulphuric acid was employed as the derivatizing agent.

At 366 nm (After Spraying reagent)

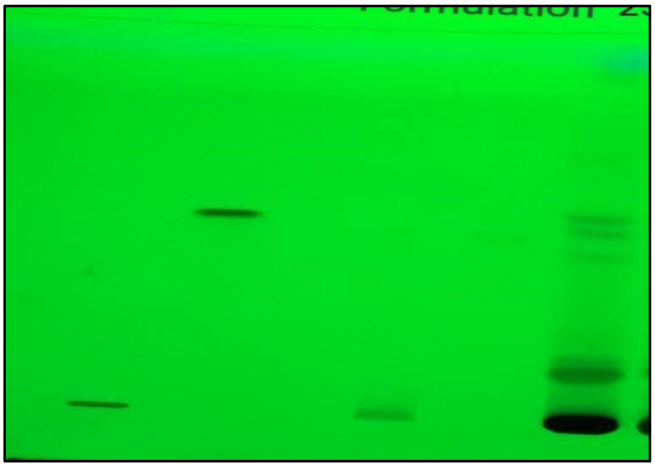


Figure No. 6.35: Image of slide of all three extract *Boerhavia diffusa* leaves, *Plumeria Rubra pods and Celosia argentea* seeds extract 254 nm

At Visible (Spraying reagent: Anisaldehyde sulphuric acid reagent)

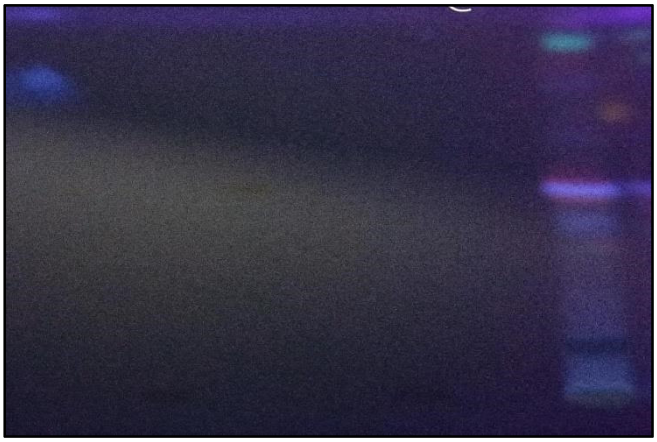


Figure No. 6.36: White Remission Image *Boerhavia diffusa* leaves, *Plumeria Rubra* pods and *Celosia argentea* seeds extract 366nm

Common solvent system for *Boerhavia diffusa* leaves, *Plumeria Rubra* pods and *Celosia argentea* seeds Methanolic extracts: Toluene: Ethyl acetate: Methanol: Formic acid (5:4:0.5:0.5 v/v)

6.1.11 Fourier-transform infrared spectroscopy (FTIR)

A. Boerhavia diffusa

The FTIR spectrum of aqueous extract of *Boerhavia diffusa* leaves is shown in Figure 6.37. The interpretation of functional IR bands is shown in Table 6.20.

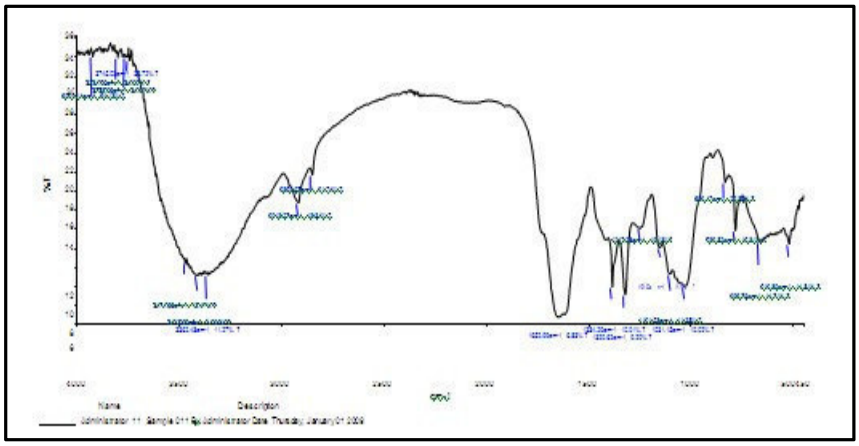


Figure 6.37: I.R. Spectra of aqueous extract of *Boerhavia diffusa* leaves

Table No. 6.20: FTIR spectrum interpretation of aqueous extract of *Boerhavia diffusa* leaves

Wave number(cm^{-1})	Functional Group
3433 cm^{-1}	-O-H Stretch
3030 cm^{-1}	Ar-C-H Stretch
$^{-1}$2917cm	-C-H (CH_3) Stretch
$^{-1}$1635 cm	C=O Stretch
1284 cm^{-1}	C-OStretch

B. Plumeria Rubra

The FTIR spectrum of aqueous extract of *Plumeria Rubra* podsis shown in Figure 6.38.The interpretation of functional IR bands is shown in Table 6.21.

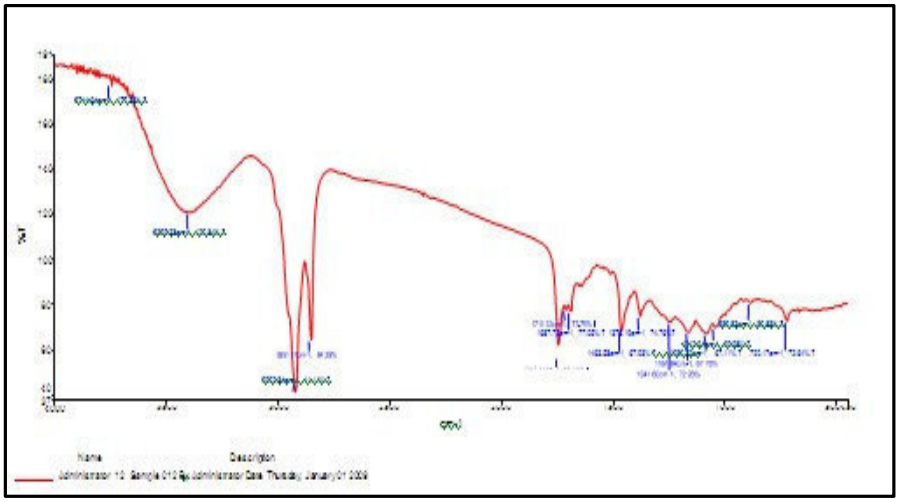


Figure No. 6.38: I.R. Spectra of aqueous extract of *Plumeria Rubra* pods

Table No. 6.21: FTIR spectrum interpretation of aqueous extract of *Plumeria Rubra* pods

Wave number(cm^{-1})	Functional Group
3359 cm^{-1}	-O-H Stretch
3030 cm^{-1}	Ar-C-H Stretch
$^{-1}$2945cm	-C-H Stretch
$^{-1}$1722 cm	C=O Stretch
519 cm^{-1}	C=C Stretch
1444 cm^{-1}	-C-H def
1284 cm^{-1}	-C-O Stretch

C. Celosia argentea

The FTIR spectrum of aqueous extract of *Celosia argentea* seeds is shown in Figure 6.39. The interpretation of functional IR bands is shown in Table 6.22.

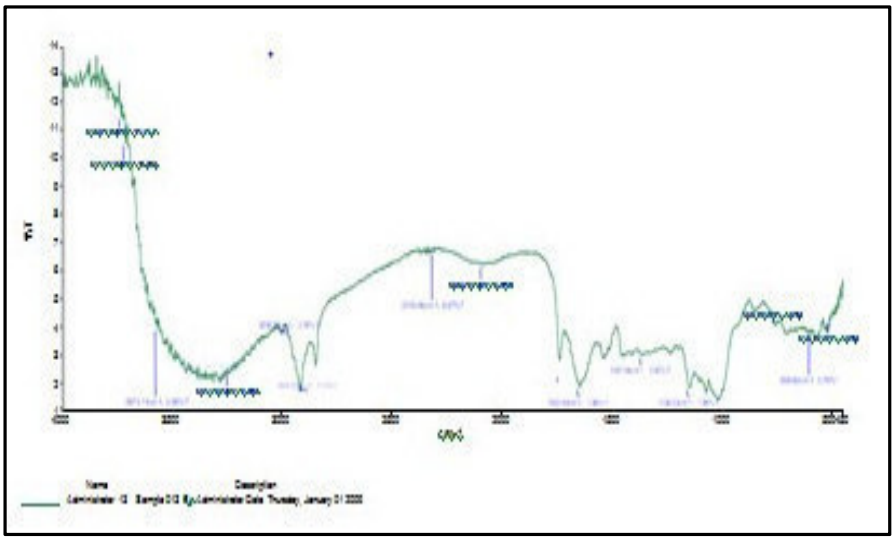


Figure No. 6.39: I.R. Spectra of aqueous extract of *Celosia argentea* seeds

Table No. 6.22: FTIR spectrum interpretation of aqueous extract of *Celosia argentea* seeds

Wave number(cm^{-1})	Functional Group
3408 cm^{-1}	-O-H Stretch
3008 cm^{-1}	Ar-C-H Stretch
$^{-1}$2853cm	CH_3-C-H Stretch
$^{-1}$1740 cm	C=O Stretch
1464 cm^{-1}	-C-H def
1234 cm^{-1}	C-O
1064 cm^{-1}	C-C Stretch

Conclusion

This study presents distinctive diagnostic characteristics of *Boerhavia diffusa* leaves, *Plumeria Rubra* pods, and *Celosia argentea* seeds to aid in the comprehensive identification of these botanicals. Morphological and microscopic examinations are essential preliminary

procedures for precisely establishing the botanical origins. Furthermore, physiochemical and qualitative chemical analyses of the leaves, pods, and seeds confirm the authenticity and quality of the botanicals for identification purposes. These results offer important insights for future pharmacological and therapeutic investigations, as well as for the standardization of plant materials.

6.1.12 B. HPTLC METHOD DEVELOPMENT AND VALIDATION

Results

In situ HPTLC spectral of plant extract taken was found at 270 nm and was selected as scanning wavelength (Fig. no.6.40)

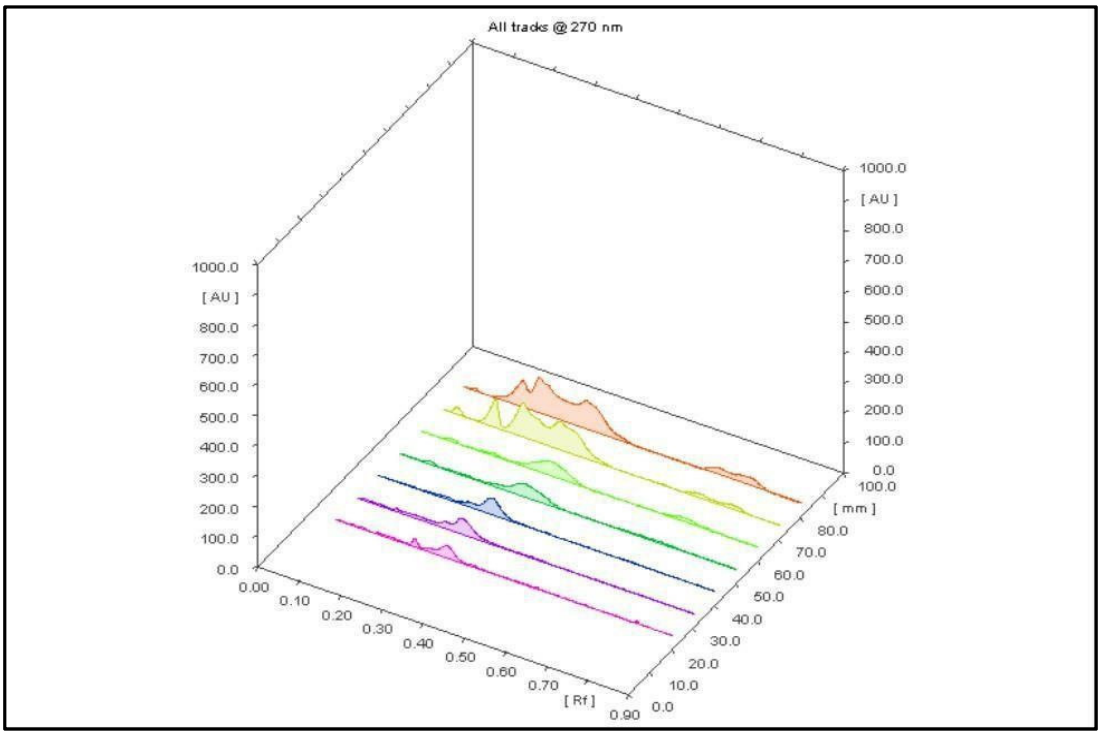


Figure No. 6.40: In situ HPTLC spectral of plant extract

With a mobile phase of Methanol: Ethyl acetate: Formic acid (5:0.5:0.5), good resolution and crisp peaks with minimal tailing were obtained.

HPTLC Method Validation

6.1.12.1 Linearity

Plotting medication concentration vs peak area for each component revealed a linear relationship. Plant extract demonstrated a linear response in the 200-800 ng/spot

concentration range (Fig.no.6.41). The high correlation coefficient values verified the linearity. The results are shown in (Table no. 6.23).

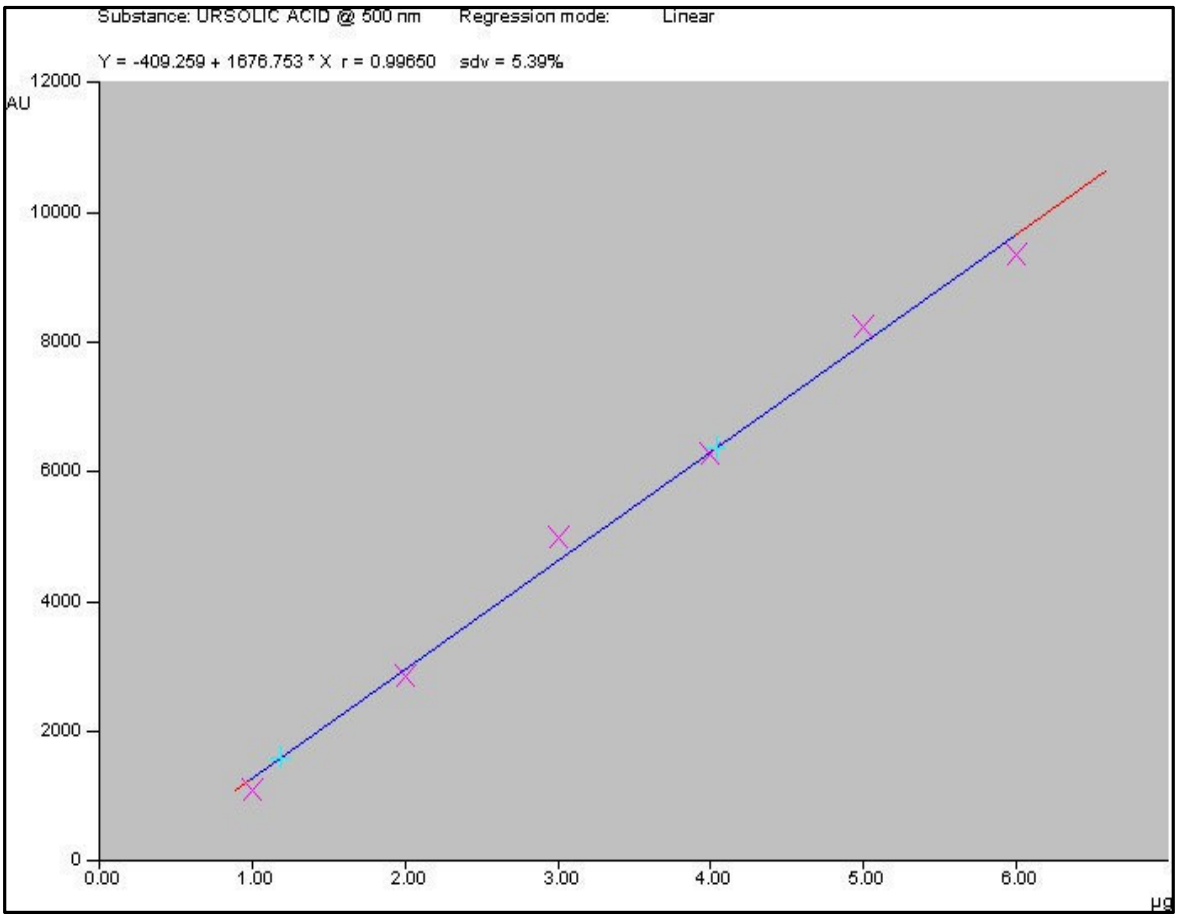


Figure No. 6.41: Linearity of plant extract

Table No. 6.23: Linear regression data for calibration plot

Sr. No.	Parameter	Value
1.	Linearity	200-600
2.	Regression Coefficient	0.99650
3.	Slope	1676.753
4.	Intercept	-409.250

6.1.12.2 Specificity

When the spectra of standard and plant extract were superimposed (Fig.no.6.42) with plant extract, it was discovered that the ingredients in the extract did not interfere with the peaks of standard. As a result, the proposed technique was proven to be Specific.

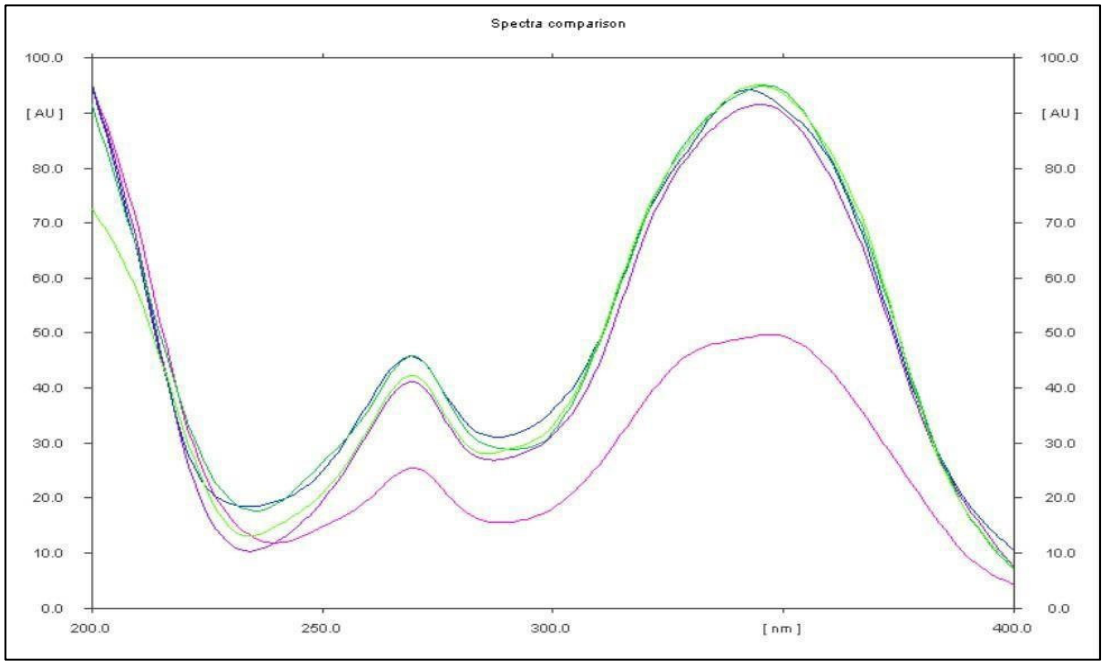


Figure No. 6.42: Overlay spectra of Standard and Extract Precision

Intraday precision refers to variance of the procedure at three different concentration levels during the same day, whereas interday precision refers to change between days. Table no. 6.24 shows that the % RSD values for both intraday and interday precision were found to be within acceptable limits.

Table No. 6.24: Intra-Day and Inter-Day Precision Results

Concentration (ng/spot)	Interday			Intraday		
	Mean	SD	%RSD	Mean	SD	%RSD
300	8743.2	60.98	0.42	8720.9	87.93	0.67
500	12097.45	245.66	1.14	13067.98	115.8	0.87
700	150786.85	30.25	1.20	15044.66	54.67	1.07

6.1.12.3 LOD (limit of detection) and LOQ (limit of quantification)

Plant extract LOD and LOQ findings are reported in Table No. 6.25.

Table No. 6.25: LOD (limit of detection) and LOQ (limit of quantification)

Sr. No.	LOD (ng/spot)	LOQ (ng/spot)
1.	17.37	53.90

6.1.12.4 Accuracy

The method's accuracy is expressed as a percentage of the known increased amount of analyte in the sample. The percent recovery was calculated by running recovery trials in triplicate at three concentration levels, namely 80%, 100%, and 120%, using a known amount of standard plant extract mixture. Table no. 6.26 shows the results collected.

Table No. 6.26: Results of Accuracy

Sr. No.	Level	% Recovery	% RSD	Mean
1.	50	99.14	0.97	99.19
2.	100	101.65	1.34	
3.	150	98.96	1.05	

6.1.12.5 Robustness

For 300 and 400 ng/spot, the % RSD of the peak area was computed in triplicate for variations in mobile phase composition and saturation time length. As shown in Table 6.27, the values of % RSD were less than 2%, indicating that the devised approach is robust.

Table No. 6.27: Robustness

Parameters	300 ng/spot %RSD	400 ng/spot % RSD
Mobile Phase Composition		
Methanol : EAA:FA (5:0.5:0.5) v/v/v	0.67	0.55
Methanol : EAA:FA (5:0.5:1.0)v/v/v	0.84	0.73
Saturation time		
+ 5 min	0.45	0.76
-5 min	0.57	0.69

Conclusion

The application of a simple, rapid and accurate HPTLC method may be recommended for quality assurance and fingerprinting to establish the authenticity of marker compound in poly formulation. The method was validated to track the active principles in complex mixture of herbal ingredients used in traditional medicines like “Ayurveda”. The method could be extended for the marker-based standardization of other herbal products.

6.2 IN VITRO LITHOLYTIC ACTIVITY

Results:

6.2.1 Preliminary screening of Individual plant for their litholytic activity:

Each Plant aqueous extract was prepared and checked primarily for their dissolution potential of kidney stone.

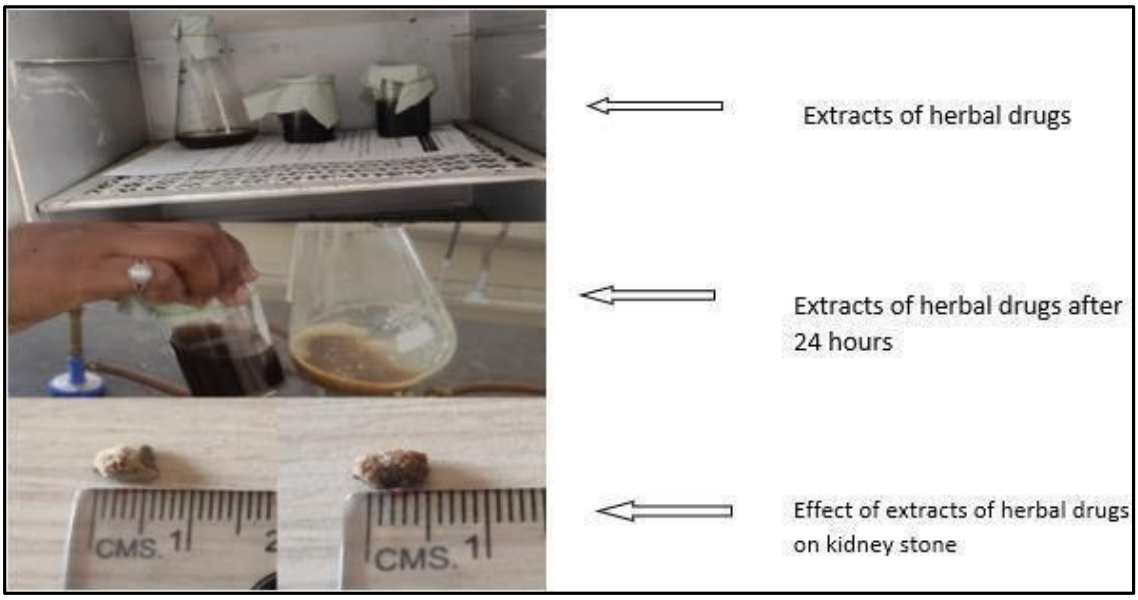


Figure No. 6.43: In vitro kidney stone dissolution method

After preliminary in-vitro study it is found that Aqueous extract of *Boerhavia difussa* showed decrease in weight by 10 mg as well as decrease in diameter of stone by 1mm after the treatment of 24 hours. Colour of PHF containing kidney stone was becoming buff as compared to control after 24 hrs and was found effective. Aqueous extract of *Celosia argentea* showed decrease in weight by 08 mg and diameter of stone was reduced by 1.1mm. Aqueous extract of *Plumeria Rubra* showed dissolution by 12 mg while diameter was reduced by 0.9 mm. Therefore, it is observed from preliminary screening that, selected herbs showed effectiveness in in vitro dissolution of stone and can be proceeded for further systematic in vitro and in vivo study using ethylene glycol method.

Table No. 6.28: In vitro kidney stone dissolution

Selected Aqueous Herbal Extract	Weight of Stone		Diameter of Stone	
	Before treatment	After treatment	Before treatment	After treatment
Boerhavia diffusa	95 mg	83 mg	8 mm	7.1 mm
Celosia argentea	70 mg	62 mg	5 mm	3.9 mm
Plumeria Rubra	80 mg	70 mg	6 mm	5 mm

6.2.3 Optimization of combination ratio of polyherbal extracts for in vitro analysis on cystine calculi, Carbapatite calculi and Uric acid calculi:

Although all the treatments (aqueous extracts of individual drugs and polyherbal extracts) showed considerable reduction in size of the stones, since results shown that Aqueous extract of *B. diffusa* shown more significant effects amonst indidual extracts of three plants. Antilithiatic treatment with polyherbal extract of combination C (4 parts of *B. diffusa*+1 part each of *C. argentea* and 1 part of *P. rubra* seed pod) showed better results with better dissolution of stones in 24 hrs. as compared to that of combination A (2 parts of *B. diffusa*+1 part each of *C. argentea* and 1 part of *P. rubra* seed pod.), Combination B (3 parts of *B. diffusa*+1 part each of *C. argentea* and 1 part of *P. rubra* seed pod.) and Combination of 1 parts of *B. diffusa*+1 part each of *C. argentea* and 1 part of *P. rubra* seed pod shown very negligible effect so this is not taken for further study.

Therefore, Combination C is used for further in vivo studies.

Table No. 6.29: Optimization of extract combination stone dissolution

Polyherbal combination	Weight of Stone		Diameter of Stone		% Dissolution after 24 Hrs.
	Before treatment	After treatment	Before treatment	After treatment	
Combination A	78 mg	70 mg	6 mm	5.5 mm	10.25
Combination B	82 mg	71 mg	6.2 mm	5.1 mm	13.41
Combination C	80 mg	65 mg	6 mm	4.9 mm	18.75

Systematic in vitro analysis of plant extracts on cystine calculi, Carbapatite calculi and Uric acid calculi:

6.2.3.1 The effect of plant extracts on cystine calculi was investigated.

The three medicinal plant extracts had effects on the dissolution of cystine calculi starting from the second week of the experiment and caused a weight loss of more than 9% for the aqueous extract of all the three plant extracts. As compared to control solution of NaCl this shows weight reduction of 4.8 %.

6.2.3.2 The effect of plant extracts on carbapatite calculi was investigated.

The three medicinal plant extracts had effects on the dissolution of carbapatite calculi starting from the second week of the experiment and caused a weight loss of more than 9.5% for the aqueous extract of all the three plant extracts. As compared to control solution of NaCl this shows weight reduction of 4.8 %.

6.2.3.3 The effect of plant extracts on uric acid calculi was investigated.

The three medicinal plant extracts had effects on the dissolution uric acid calculi starting from the second week of the experiment and caused a weight loss of more than 8.9 % for the aqueous extract of all the three plant extracts. As compared to control solution of NaCl this shows weight reduction of 4.8 %.

Table No. 6.30: The effect of plant extracts on cystine calculi

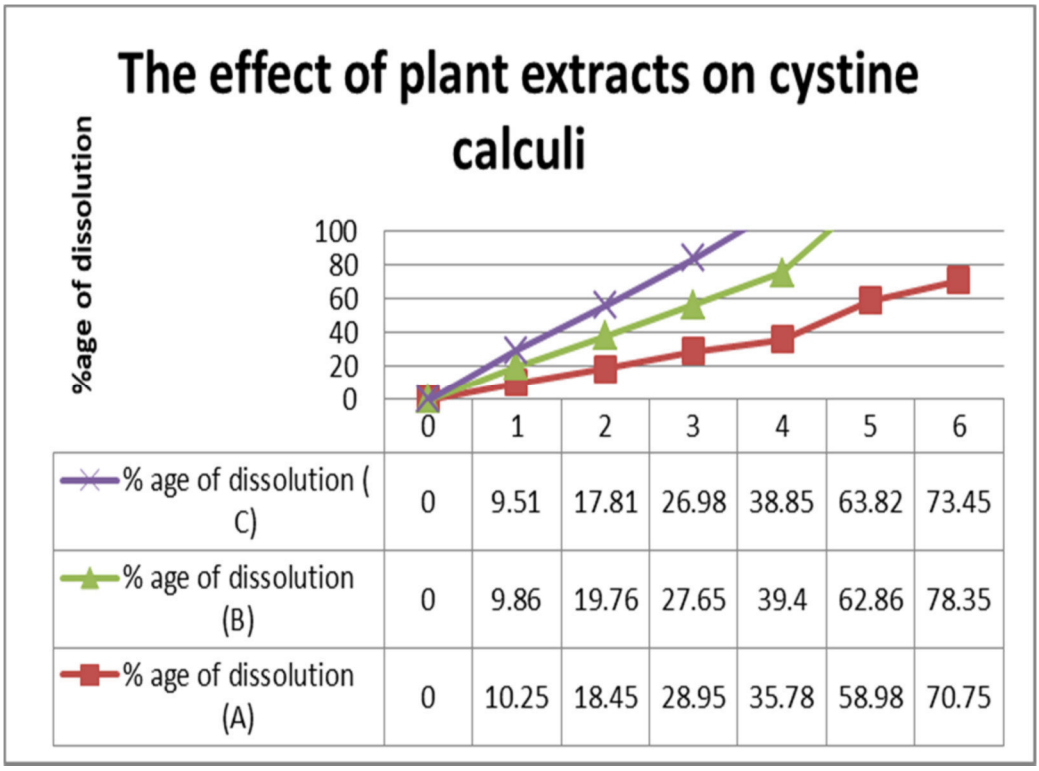
Sr. No.	Time (week)	% age of dissolution (A)	% age of dissolution (B)	% age of dissolution (C)
1.	0	0	0	0
2.	1	10.25	9.86	9.51
3.	2	18.45	19.76	17.81
4.	3	28.95	27.65	26.98
5.	4	35.78	39.40	38.85
6.	5	58.98	62.86	63.82
7.	6	70.75	78.35	73.45

Table No. 6.31: The effect of plant extracts on carbapatite calculi

Sr. No.	Time (week)	% age of dissolution (A)	% age of dissolution (B)	% age of dissolution (C)
1.	0	0	0	0
2.	1	9.25	8.89	9.81
3.	2	17.83	18.63	16.65
4.	3	26.77	24.98	25.08
5.	4	32.65	36.77	35.61
6.	5	45.65	43.75	48.35
7.	6	60.45	59.81	63.48

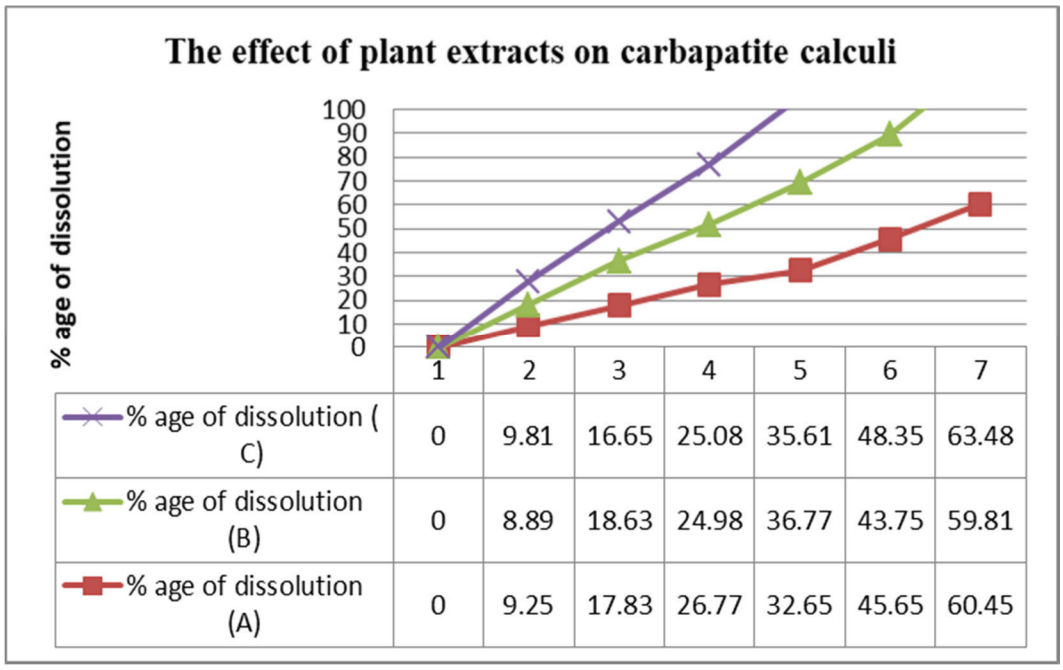
Table No. 6.32: The effect of plant extracts on uric acid calculi

Sr. No.	Time (week)	% age of dissolution (A)	% age of dissolution (B)	% age of dissolution (C)
1.	0	0	0	0
2.	1	10.08	9.66	8.99
3.	2	16.68	18.88	17.35
4.	3	24.56	26.95	25.05
5.	4	30.76	34.82	35.67
6.	5	40.68	42.96	46.77
7.	6	68.56	64.55	60.95



	0	1	2	3	4	5	6
% age of dissolution (C)	0	9.51	17.81	26.98	38.85	63.82	73.45
% age of dissolution (B)	0	9.86	19.76	27.65	39.4	62.86	78.35
% age of dissolution (A)	0	10.25	18.45	28.95	35.78	58.98	70.75

Figure No. 6.44: The effect of plant extracts on cystine calculi



	1	2	3	4	5	6	7
% age of dissolution (C)	0	9.81	16.65	25.08	35.61	48.35	63.48
% age of dissolution (B)	0	8.89	18.63	24.98	36.77	43.75	59.81
% age of dissolution (A)	0	9.25	17.83	26.77	32.65	45.65	60.45

Figure No. 6.45: The effect of plant extracts on carbapatite calculi

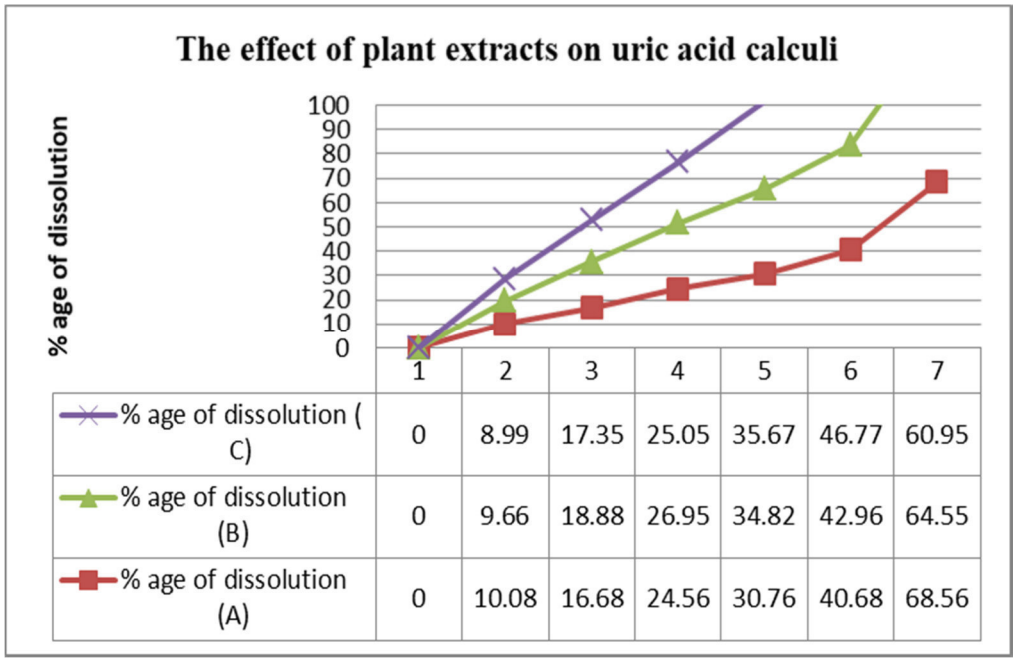


Figure No. 6.46: The effect of plant extracts on uric acid calculi

Conclusion

From Preliminary in vitro analysis it is found that each drug has antilithiatic potential. In vitro antilithiatic treatment with polyherbal extract of combination C (4 parts of *B. diffusa*+1 part each of *C. argentea* and 1 part of P.rubra seed pod) showed better results with better dissolution of stones in 24 hrs. as compared to that of combination A (2 parts of *B. diffusa*+1 part each of *C. argentea* and 1 part of P.rubra seed pod and combination B (3 parts of *B. diffusa*+1 part each of *C. argentea* and 1 part of P.rubra seed pod). Therefore Combination C is used for further in vivo studies. Also On the basis of effect of plant extract on three types of kidney stones viz. cystine calculi, Carbapatite calculi and Uric acid calculi , polyherbal formulation was found effective than and can be studied by in vivo Ethylene glycol model using wistar rats.

6.3 *IN-VIVO* STUDIES Results

***In-Vivo* Anti-Urolithiaitic Activity**

6.3.1 Urine Analysis

i) Estimation of urine volume and pH

Table No. 6.33: Estimation of urine volume and pH

PARAMETERS	GROUP-I	GROUP-II	GROUP-III	GROUP-IV	GROUP-V
Urine Volume	2.57±0.11	1.60±0.23###	5.40±0.14***	3.35±0.54***	5.19±0.32***
pH	7.25±0.04	5.7±0.13**	7.29±0.45***	6.30±0.24***	7.24±0.04***

Values are expressed as Mean ± SEM, n=6.

*P ≤ 0.05 ,

P ≤ 0.01 and*P ≤ 0.001 compared with disease control

From Table no. 6.33, It can be seen that Urolithiasis induced animals shows decreased in urine volume (Group II), whereas the treated groups (II, III, IV) shows increased in urine volume when compared with Group II rats.

In ethylene glycol induced rats pH was reduced when compared with normal control group. Treatment with standard drug 750mg/kg and ethanolic extracts of plants were found to increase the urine pH in a dose dependent manner.

6.3.2 Estimation of Calcium, Oxalate, phosphorus and magnesium in Urine

Table No. 6.34: Estimation of calcium, oxalate, phosphorus and magnesium in urine

PARAMETERS	GROUP-I	GROUP-II	GROUP-III	GROUP-IV	GROUP-V
Calcium	9.54±0.10	13.56±0.08## #	10.24±0.11***	12.81±0.15**	10.36±0.169***
Oxalate	1.86±0.07	4.39±0.17###	2.11±0.07***	3.8±0.12**	2.55±0.10***
Phosphorous	5.15±0.20	7.13±0.17###	5.18±0.14***	6.33±0.14**	5.28±0.11***
Magnesium	4.16±0.08	1.73±0.12###	3.33±0.11***	2.11±0.09*	3.18±0.07***

Values are expressed as Mean ± SEM, n=6.

*P ≤ 0.05,

P ≤ 0.01 and*P ≤ 0.001 compared with disease control

From table no. 6.34, In disease control animals calcium, phosphorus and oxalate excretion were significantly increased, While the magnesium level decreased when compared with group I animals. When supplement with plant extract significantly lowered the elevated levels of calcium, phosphorus and oxalate when compared with group II animals, and restore the magnesium level when compared with normal animals.

6.4 SERUM ANALYSIS:

6.4.1 Estimation of Creatinine and uric acid in serum

Table No. 6.35: Estimation of Creatinine and uric acid in serum

PARAMETERS	I	II	III	IV	V
Creatinine	2.65±0.12	3.33±0.08###	2.43±0.11***	2.91±0.07***	2.63±0.11***
Uric Acid	2.7±0.05	4.43±0.09###	2.95±0.07***	3.98±0.09**	3.21±0.08***

Values are expressed as Mean ± SEM, n=6.

*P ≤ 0.05,

P ≤ 0.01 and*P ≤ 0.001 compared with disease control

In calculi-induced rats (Group II), the elevated serum levels of Creatinine, uric acid However, treatment with plant extract restored the serum levels of Creatinine, uric acid. The ethanolic plant extract (Group IV and V) and (Group III) significantly ($p<0.05$) reduced the elevated serum uric acid level as compared to group II.

Serum Parameters

6.4.2 Effect of PHF 200 and PHF 400 on Serum Calcium (mg/dl) in Ethylene induced Urolithiasis in Rats

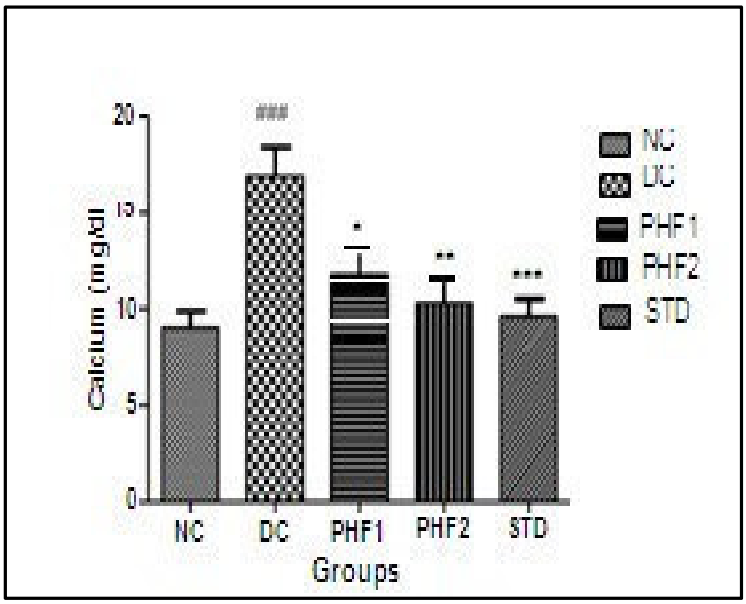


Figure No. 6.47: Effect of PHF 200 and PHF 400 on Serum Calcium (mg/dl) in Ethylene induced Urolithiasis in Rats

Figure 6.47: Effect of PHF 200 and PHF 400 on Serum Calcium (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and PHF 2 on Serum Calcium in EG induced Urolithiasis in Rats in Figure

1. The treatment of rats with EG (0.75 %, w/v) induced significant (p<0.001) elevation in Serum Calcium when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.05; p<0.01; p<0.001) increment in Serum Calcium when compared with DC rats.

6.4.3 Effect of PHF 200 and PHF 400 on Serum Phosphorus (mg/dl) in Ethylene induced Urolithiasis in Rats

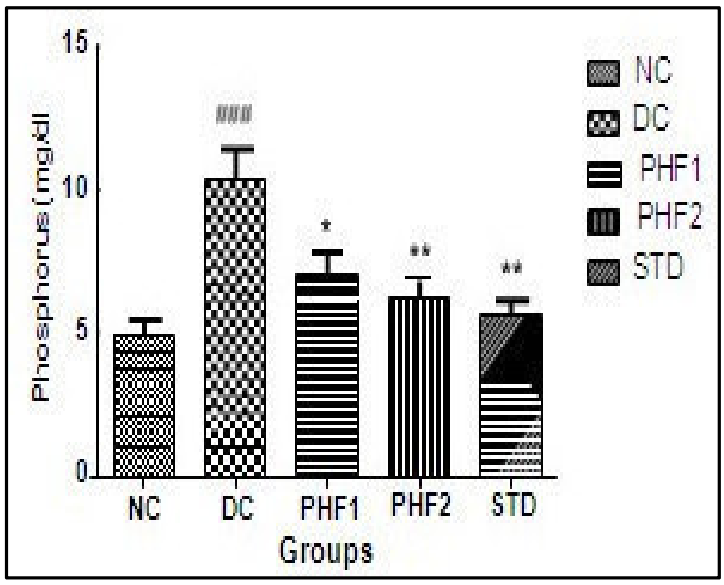


Figure No. 6.48: Effect of PHF 200 and PHF 400 on Serum Phosphorus (mg/dl) in Ethylene induced Urolithiasis in Rats

Figure 6.48: Effect of PHF 200 and PHF 400 on Serum Phosphorus (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and PHF 2 on Serum Phosphorus in EG induced Urolithiasis in Rats in Figure 6.45. The treatment of rats with EG (0.75 %, w/v) induced significant ($p<0.001$) elevation in Serum Phosphorus when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$; $p<0.01$) increment in Serum Phosphorus when compared with DC rats.

6.4.4 Effect of PHF 200 and PHF 400 on Serum BUN (mg/dl) in Ethylene induced Urolithiasis in Rat

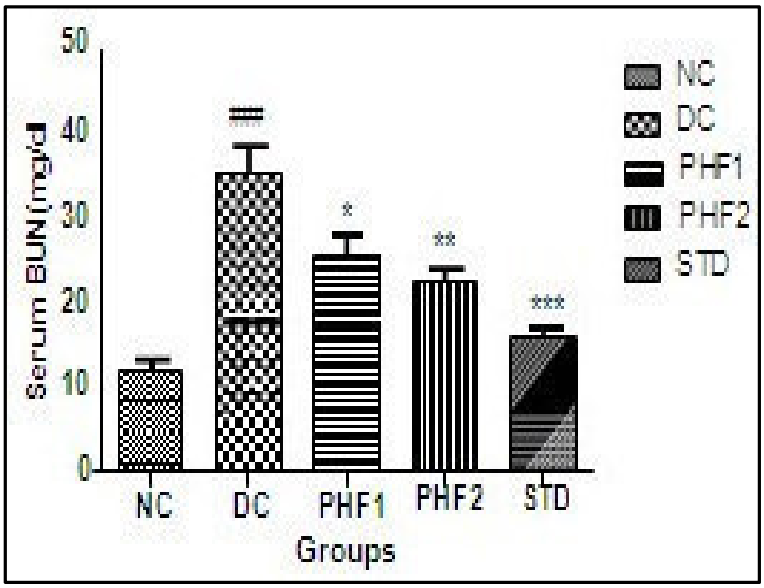


Figure No. 6.49: Effect of PHF 200 and PHF 400 on Serum BUN (mg/dl) in Ethylene induced Urolithiasis in Rats

Figure 6.49: Effect of PHF 200 and PHF 400 on Serum BUN (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One- way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and 2 400 on Serum BUN (mg/dl) in EG induced Urolithiasis in Rats in Figure 6.49. The treatment of rats with EG (0.75 %, w/v) induced significant ($p<0.001$) elevation in Serum BUN when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$; $p<0.001$) increment in Serum BUN when compared with DC rats.

6.4.5 Effect of PHF 200 and PHF 400 on Serum Creatinine (mg/dl) in Ethylene induced Urolithiasis in Rats.

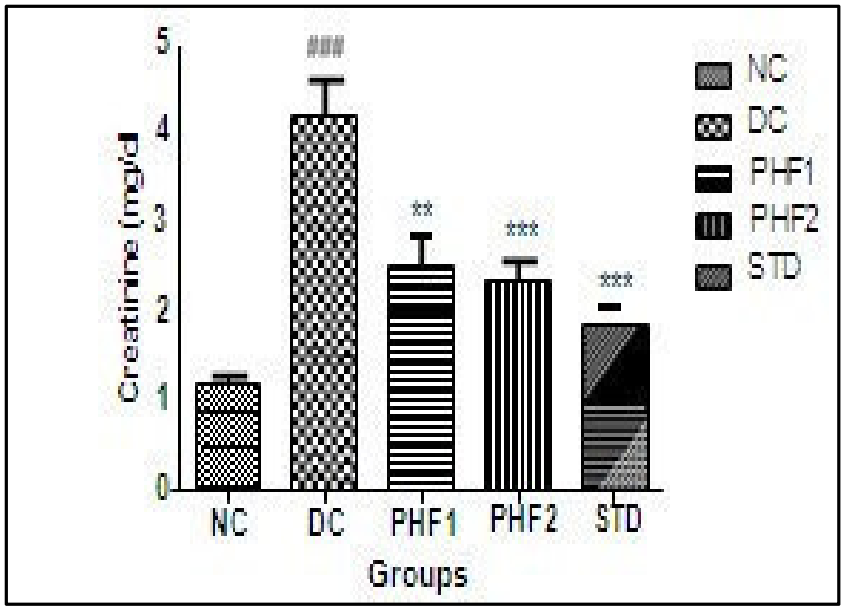


Figure No. 6.50: Effect of PHF 200 and PHF 400 on Serum Creatinine (mg/dl) in Ethylene induced Urolithiasis in Rats

Figure 6.50: Effect of PHF 1 and PHF 2 on Serum Creatinine (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One- way ANOVA followed by Tukey's Kramer test. ###p<0.001 versus NC rats and B***p<0.001 versus DC rats.

The effects of PHF 1 and 2 on Serum Creatinine in EG induced Urolithiasis in Rats in Figure 6.47. The treatment of rats with EG (0.75 %, w/v) induced significant (p<0.001) elevation in Serum Creatinine when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.01; p<0.001) increment in Serum Creatinine when compared with DC rats.

6.4.6 Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats

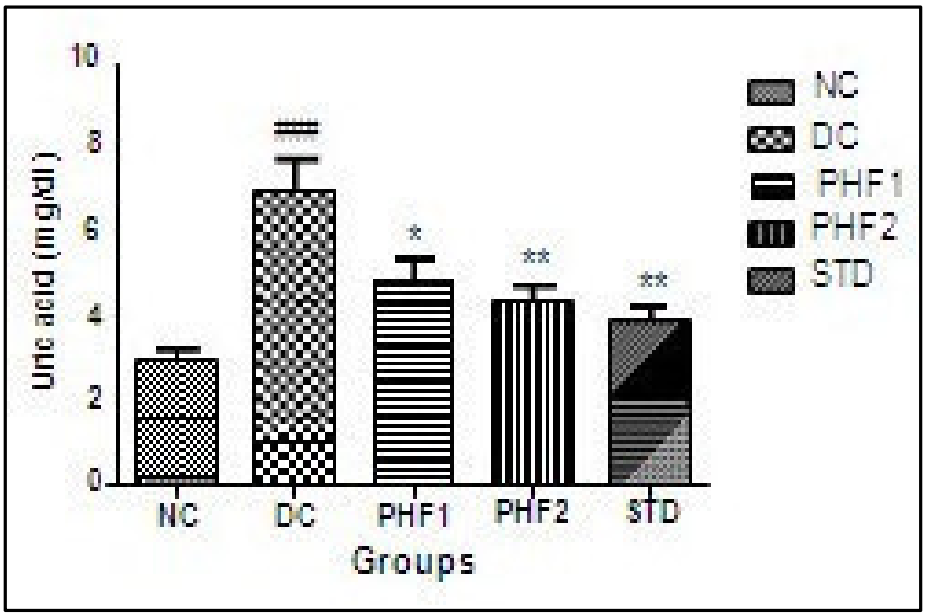


Figure No. 6.51: Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats

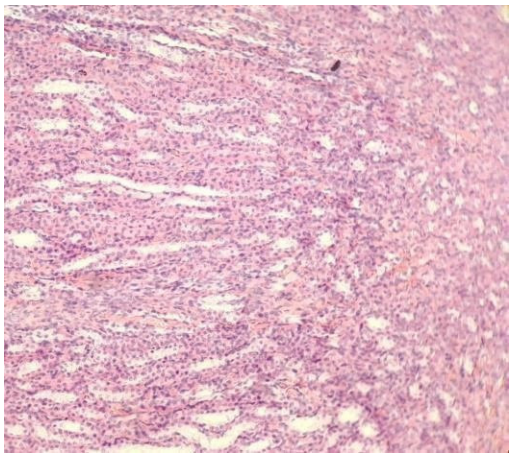
Figure 6.51: Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed byOne-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and 2 on Serum Uric acid in EG induced Urolithiasis in Rats in Figure 6.48.

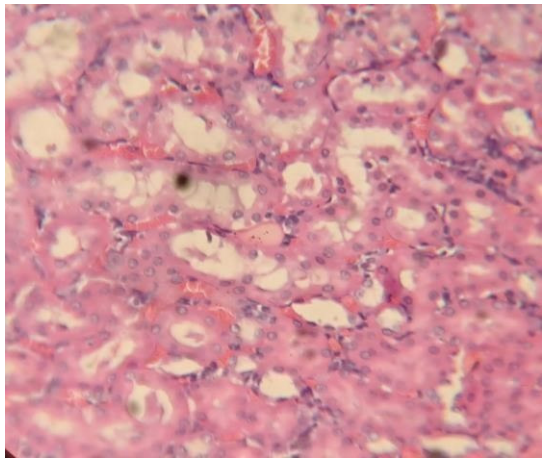
The treatment of rats with EG (0.75 %, w/v) induced significant ($p<0.001$) elevation in Serum Uric acid when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$; $p<0.001$) increment in Serum Uric acid when compared with DC rats

6.5 HISTOPATHOLOGY STUDY INTERPRETATION:

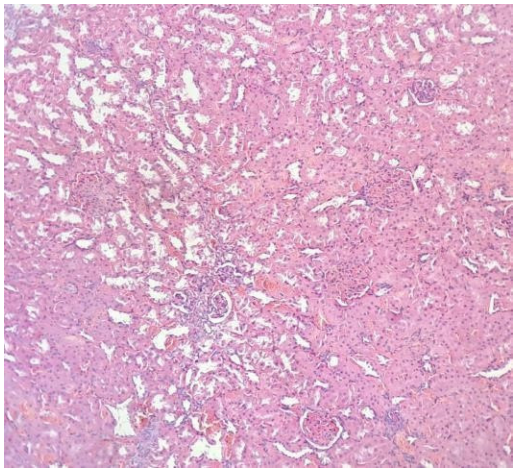
The histopathological reports further confirmed the urine analysis results. When urolithiasis is induced by Ethylene glycol it shows damage to glomerulus, interstitial tissues of rat but no such changes were seen with normal rat group. Sections of positive control animals showed many deposit scores of stone, distorted structure with severe necrosis of renal tubule. It also consists of chronic inflammatory cells composed of small lymphocytes, shows diffuse eosinophilic sclerosis, severe tubular necrosis. Internal structure evident with inflammatory damage. Polyherbal extract treatment reversed the damage caused by urolithiasis. Those group treated with 200mg/ Kg dose showed edematous stroma with separation of tubules. Many glomeruli showed diffuse eosinophilic sclerosis and reports of histopathology suggest tubular necrosis, thoughtubular necrosis is evident, the severity of the damage is less as compared to positive control group but more as compared to group treated with cystone. In the animals those treated with 400mg/ kg the structure of kidney, showed outer cortex and inner medulla in good condition. The cortex showed numerous convoluted tubules. Glomeruli were found good in numbers. Stroma showed moderate degree of edema and minimal necrosis. The Histology showed the extent of damage has been improved or is less as compared to positive control.



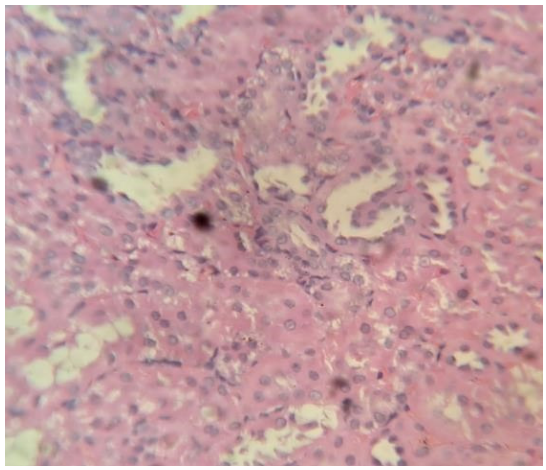
Group 1 : Normal



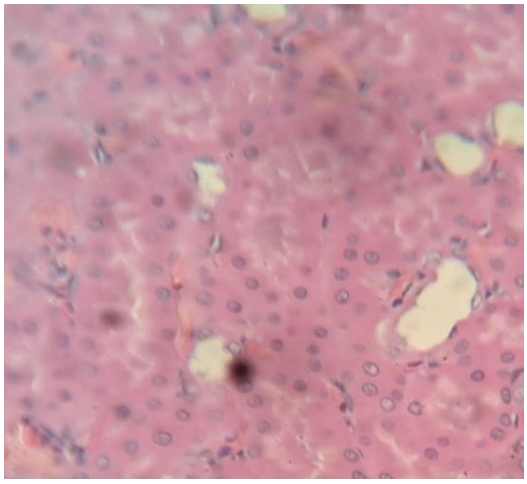
Group 2: Positive control



Group 3: Cystone



Group 4: Extract 200 mg/Kg



Group 5: Extract 400mg/Kg

Figure No. 6.52: Histopathological sections of Kidney showing Damage to renal architecture and it's recovery after the treatment of different extracts

Group - 1 - Normal control.

Gross Examination: Received two kidneys in one container. The larger kidney measures 2 x 1.6 x 0.6 cm and the smaller kidney measures 2 x 1.4 x 1 cm. Cut surfaces of both the kidneys are grayish brown and firm.

Microscopic Examination: Section studied show renal tissue showing outer cortex and inner medulla and surrounded by fibro collagenous capsule and adipose tissue. Few of the glomeruli show congestion. The tubules are lined by cuboidal epithelium. The stroma shows few congested blood vessels. There is no evidence of tuberculosis. There is no evidence of malignancy.

Group - 2 - Disease control - EG model.

Gross Examination: Received two kidneys in one container. The larger kidney measures 2 x 1.5 x 1 cm and the smaller kidney measures 2 x 1.4 x 1 cm. Cut surfaces of both the kidneys are grayish brown and firm.

Microscopic Examination: Sections from the renal tissue show renal cortex and medulla and is covered by thin fibro collagenous capsule and adipose tissue. A fair number of glomeruli show congestion and edema. Many tubules are dilated and their lumia show presence of polyhedral and rhomboid shaped eosinophilic material (suggestive of calcium oxalate crystals). The interstitium shows chronic inflammatory infiltrate of lymphocytes, plasma cells, and histiocytes. Occasional Foreign body type of giant cells are seen. Stoma shows few areas of hemorrhage. There is no definite evidence of malignancy.

Group - 3 - Standard drug administration.

Gross Examination: Received two kidneys in one container. The larger kidney measures 2.5 x 1.2 x 1 cm and the smaller kidney measures 2.5 x 1.5 x 0.8 cm. Cut surfaces of both the kidneys are grayish brown and firm.

Microscopic Examination: Sections from the renal tissue show renal cortex and medulla and is covered by thin fibro collagenous capsule and adipose tissue. A few glomeruli show congestion and edema. A few tubules are dilated and their lumia show presence of polyhedral and rhomboid shaped eosinophilic material (suggestive of calcium oxalate crystals). The interstitium shows few foci of chronic inflammatory infiltrate of lymphocytes, plasma cells, and histiocytes. Stoma shows few areas of haemorrhage. There is no definite evidence of Tuberculosis or malignancy.

Group 4 - Drug administration - PHF 200mg / kg.

Gross Examination: Received two kidneys in one container. The larger kidney measures 2.5 x 1.6 x 0.8 cm and the smaller kidney measures 2 x 1.4 x 0.8 cm. Cut surfaces of both the kidneys are grayish brown and firm.

Microscopic Examination: Sections from the renal tissue show renal cortex and medulla and is covered by thin fibro collagenous capsule and adipose tissue. A few glomeruli show congestion. A few tubules are dilated and their lumia show presence of polyhedral and rhomboid shaped eosinophilic material (suggestive of calcium oxalate crystals). The

interstitium shows a few foci of chromic inflammatory infiltrate of lymphocytes, plasma cells, and histiocytes. Stoma shows a few areas of haemorrhage. There is no definite evidence of Tuberculosis or malignancy.

Group 5 - Drug administration - PHF 400mg / kg.

Gross Examination: Received two kidneys in one container. The larger kidney measures 2.5 x 1.6 x 1.0 cm and the smaller kidney measures 2 x 1.4 x 0.8 cm. Cut surfaces of both the kidneys are grayish brown and firm.

Microscopic Examination: Sections from the renal tissue show renal cortex and medulla and is covered by thin fibro collagenous capsule and adipose tissue. Very less number of glomeruli show congestion. A few tubules are dilated and their lumia show minute presence of polyhedral and rhomboid shaped eosinophilic material (suggestive of calcium oxalate crystals). The interstitium shows a few foci of chronic inflammatory infiltrate of lymphocytes, plasma cells, and histiocytes. Stoma shows a few areas of haemorrhage. There is no definite evidence of Tuberculosis or malignancy.

Conclusion: From microscopic Urine analysis of two drug combination i.e. Combination A:.2 parts of *B. diffusa*: 1 part of each C.argentea and P.rubra (2:1:1) and Combination B: 4 parts of *B. diffusa*: 1 part of each C.argentea and P.rubra (4:1:1), Combination B was found effective as compared to Combination A. For further in vivo study Combination B is used. For in vivo study Ethylene glycol urolithiasis model was selected. After 28 days urine analysis as well as serum analysis was done, it was found from urine analysis that, in disease control animals' calcium, phosphorus and oxalate excretion were significantly increased, While the magnesium level decreased when compared with group I animals. When supplemented with plant extract significantly lowered the elevated levels of calcium, phosphorus and oxalate when compared with group II animals, and restore the magnesium level when compared with normal animals. Also from serum analysis it was found that the the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$; $p<0.001$) increment in Serum Calcium, when compared with DC rats.Treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$; $p<0.01$) increment in Serum Phosphorus when compared with DC rats. Treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$;

p<0.001) incrementin Serum BUN when compared with DC rats. Treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.01; p<0.001) increment in Serum Creatinine when compared with DC rats. Treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.05; p<0.01; p<0.001) increment in Serum Uric acid when compared with DC rats.

6.5 FORMULATION AND EVALUATION OF POLYHERBAL GRANULES[206]

Results

6.5.1 Macroscopic evaluation:

The results of Organoleptic characteristics, are summarized in Table 6.36.

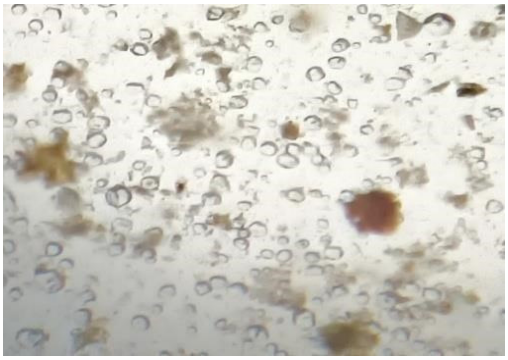
Table No. 6.36: Evaluation of Organoleptic Characteristics

Sr. No.	Parameters	PHF1	PHF2
1.	Colour	Green	Greenish black
2.	Odour	Characteristic	Characteristic
3.	Taste	Particular	Particular

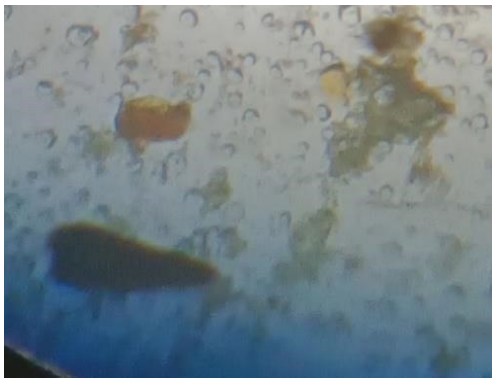
Figure No. 6.53: Polyherbal Formulation

Figure No. 6.54: Polyherbal Granule

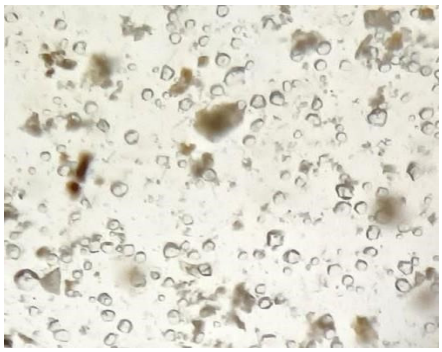
6.5.2 Microscopic evaluation: Powder characteristic of granules showed presence of lignified cells, starch grains, cuticle, contain xylem vessel, lignified sclerides, endosperm, starch grain and epidermis.



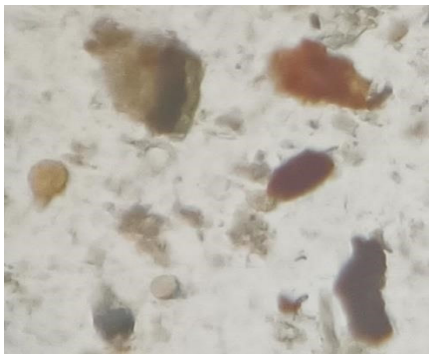
Lignified cells



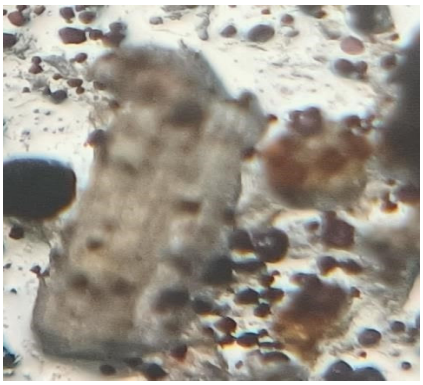
Oil glands



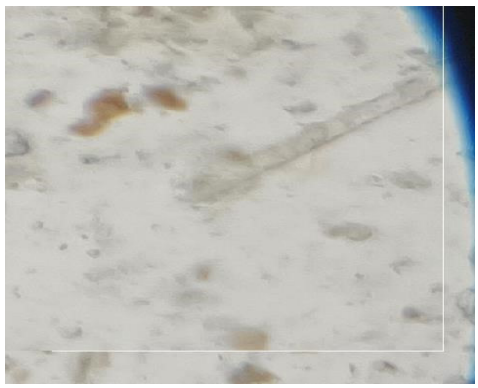
Starch grains



Cuticle cells



Xylem fiber



Fiber of plants

Figure No. 6.55: Powder microscopy of Granules

6.5.3 Determination of pH:

Polyherbal granule formulation was evaluated for pH and result found to be 6.37 As shown in Table.

6.5.4 Loss on drying at 105°C:

Polyherbal granule formulation was evaluated for Moisture Content and the result found to be 3.2% w/w. As shown in table 6.35.

6.5.5 Determination of Ash Values

A) The Polyherbal formulation were evaluated and the obtain total ash valueis 6.254% w/w, water soluble ash value is 3.56% w/w and acid insoluble ashvalueis0.94%w/w. As shown in Table 6.37.

6.5.6 Determination of Extractive Values:

The Polyherbal granule formulation were evaluated for extractive value determination and the obtain result for Water soluble extractive Value 12.26%w/w and Alcohol soluble extractive Value3.36%w/w. As shown in Table 6.35.

6.5.7 Particle size (80-100 mesh for Granules):

This was done by sieve method. Sieves were arranged in an ascending order. Granules was weighed and added to the top sieve and the assembly was shaken for 15mins. The amount of granules retained on sieve No.80 was found 16.12 gm and those on Seive no #100 was found to be 0.24.

6.5.7.1 Bulk Density:

15g of granules was taken in a graduated measuring cylinder and tapped on a wooden surface. Bulk density is calculated by using the formula. Bulk density found was 0.41gm/ml.

6.5.7.2 Tap Density:

Tap density of granules was determined after 50 tapping with the help of tap density apparatus. For the determination of tap density we check the tap volume of granule and determine the ratio of weight taken and tap volume of granule sample. The Tap density of granules was found to be 0.44gm/ml.

6.5.7.3 Angle of Repose:

Angle of repose was determined by using funnel method. The granules were allowed to flow through a funnel fixed on a stand to form a heap. The height and the radius give the angle of repose. The angle of repose was found to be 33.37 indicating good flow property.

Table No. 6.37: Physicochemical evaluation of Polyherbal Granule formulation

Parameters	Observations	Standard values
Appearance	Granular	--
Colour	Dark Greenish Brown	--
Odour	Slightly bitter	--
Taste	Mild bitter and characteristic	--
pH of PHF	7.8	7.4
Loss on drying	3.2 %	
Bulk Density	0.41gm/ml	Good (0.30-1.6)
Tap density	0.44 gm/ml	Good (0.40-0.45)
Hausner ratio	1.07	Good (1.12-1.18)
Total ash value	6.254	--
Water soluble extractive	12.26	--
Alcohol soluble extractive	3.36	--
Angle of repose	33.37	Good (31-35)

6.6 HPTLC FINGERPRINTING OF POLYHERBAL GRANULE FORMULATION:

HPTLC study of polyherbal granules was carried out using the different biomarker compounds corresponding to the therapeutically active ingredients to ensure the presence of active ingredients in the formulation. HPTLC of formulation needed the optimization of solvent system as solvent system for three plants were different as per literature as wellas practical performed. HPTLC fingerprints represents presence of Gallic acid, Rutin, Quercetin and Luteolin in the same solvent system.

6.6.1 Optimized Solvent system:

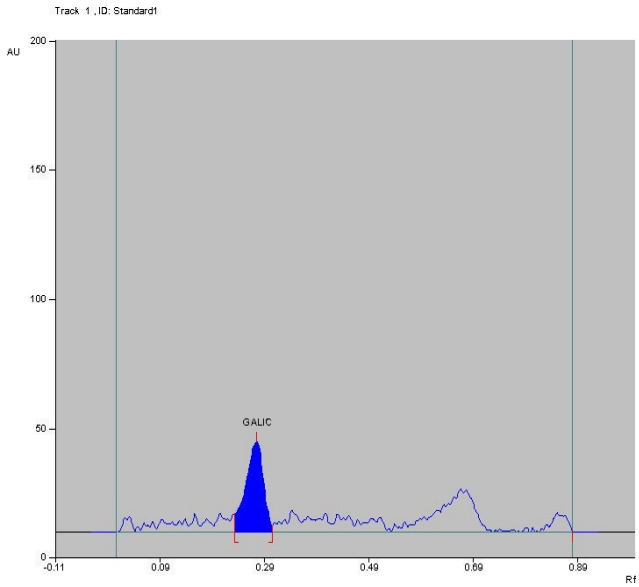
Table 6.38: Optimized solvent system

Toluene	Ethyl acetate	Methanol	Formic acid
6	4	0.5	0.5

6.6.2 Application of standards, Aqueous extract and formulation:

Marker compounds as shown in following table Galic acid, Rutin, Quarcetine, Ursolic acid and Luteolin, are applied on same slide in the 1.0µl and 2.0µl along with aqueous extract of polyherbal formulation and Aqueous Polyherbal formulation and run in above optimized solvent system.

1. Galic Acid:

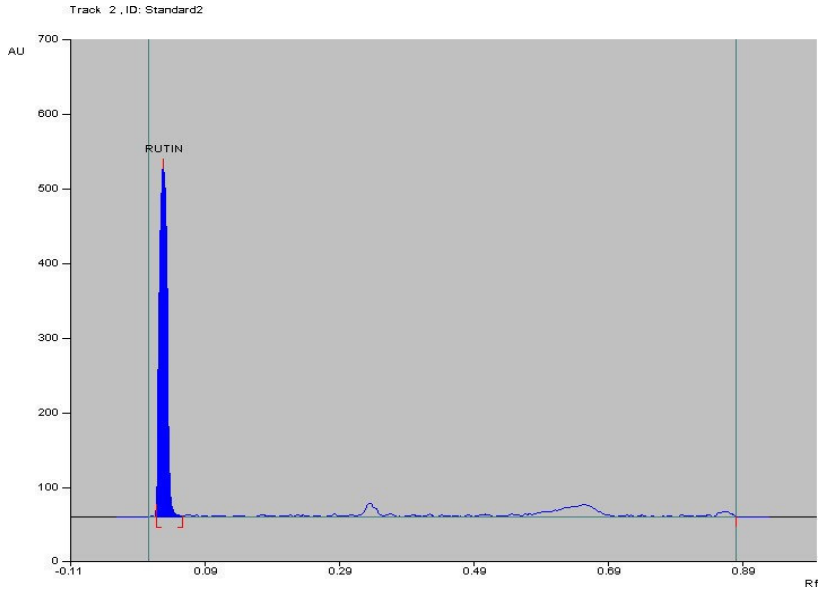


Track 1, ID: Standard1

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.23 Rf	7.0 AU	0.28 Rf	35.1 AU	100.00 %	0.30 Rf	2.4 AU	989.2 AU	100.00 %	GALIC

Figure No. 6.56: Densitogram and Chromatogram of Galic Acid marker compound

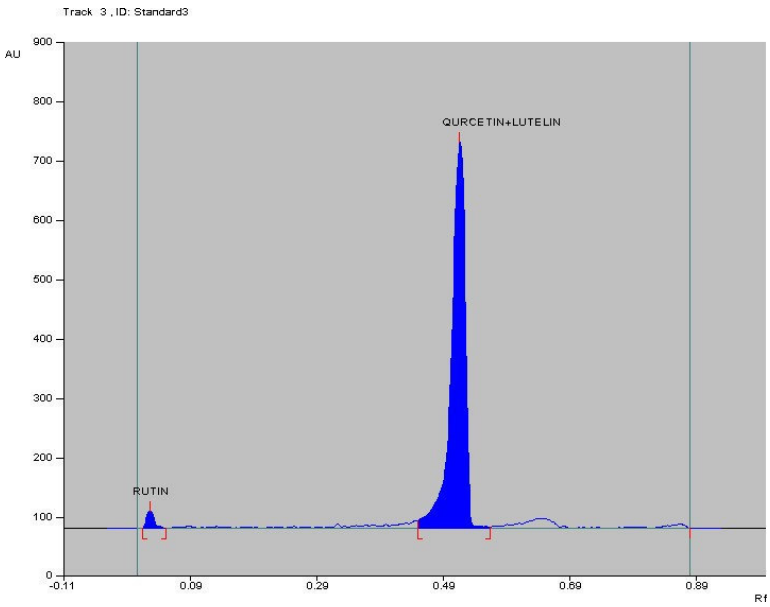
2. Rutin:



Track 2, ID: Standard2

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.02 Rf	17.4 AU	0.03 Rf	468.2 AU	100.00 %	0.06 Rf	0.9 AU	4483.9 AU	100.00 %	RUTIN

Figure No. 6.57: Chromatogram and Densitogram of Rutin marker



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.01 Rf	0.2 AU	0.03 Rf	29.5 AU	4.34 %	0.05 Rf	0.5 AU	305.5 AU	2.49 %	RUTIN
2	0.45 Rf	12.2 AU	0.52 Rf	651.1 AU	95.66 %	0.56 Rf	1.7 AU	11949.0 AU	97.51 %	QURCETIN+LUTELIN

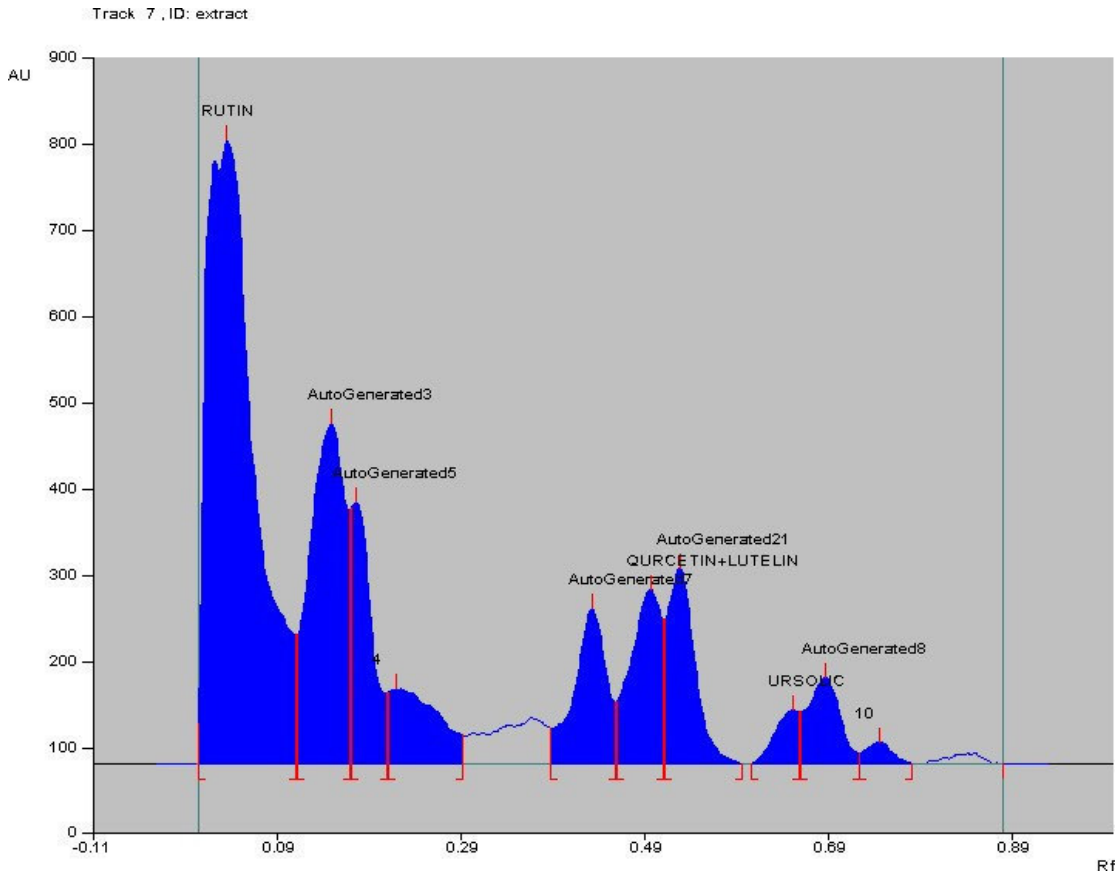
Figure No. 6.58: Chromatogram and densitogram of Quarcetin and Luteolin marker compound

6.6.3 Presence of Marker compounds in extract:

Extract of *B. diffusa, P. rubra* and *C. argentea* shows presence of Rutin, Quercetin, Luteolin and ursolic acid as shown in table 6.39.

Table 6.39: Phytochemicals found in Combination Extract

Sr. No.	Bioactive molecules found in extract	Rf value	Amount present
1.	Rutin	0.03	42.19
2.	Quercetin	0.50	7.47
3.	Luteolin	0.50	7.47
4.	Ursolic acid	0.65	1.75



Track 7, ID: extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.00 Rf	46.1 AU	0.03 Rf	724.1 AU	31.37 %	0.11 Rf	49.8 AU	31910.8 AU	42.19 %	RUTIN
2	0.11 Rf	150.4 AU	0.15 Rf	395.0 AU	17.12 %	0.17 Rf	35.1 AU	12743.6 AU	16.85 %	AutoGenerated3
3	0.17 Rf	296.3 AU	0.17 Rf	303.4 AU	13.14 %	0.21 Rf	32.6 AU	5691.5 AU	7.52 %	AutoGenerated5
4	0.21 Rf	83.1 AU	0.22 Rf	87.0 AU	3.77 %	0.29 Rf	33.4 AU	3950.4 AU	5.22 %	unknown *
5	0.39 Rf	41.1 AU	0.43 Rf	180.6 AU	7.82 %	0.46 Rf	71.3 AU	5202.9 AU	6.88 %	AutoGenerated7
6	0.46 Rf	71.4 AU	0.50 Rf	202.5 AU	8.77 %	0.51 Rf	37.7 AU	5652.1 AU	7.47 %	QURCETIN+LUTELIN
7	0.51 Rf	167.7 AU	0.53 Rf	227.2 AU	9.84 %	0.59 Rf	0.2 AU	5621.1 AU	7.43 %	AutoGenerated21
8	0.61 Rf	0.8 AU	0.65 Rf	62.9 AU	2.72 %	0.66 Rf	30.6 AU	1320.7 AU	1.75 %	URSOLIC
9	0.66 Rf	60.7 AU	0.69 Rf	99.8 AU	4.32 %	0.72 Rf	12.3 AU	2942.6 AU	3.89 %	AutoGenerated8
10	0.72 Rf	12.4 AU	0.74 Rf	25.5 AU	1.10 %	0.78 Rf	0.2 AU	599.3 AU	0.79 %	unknown *

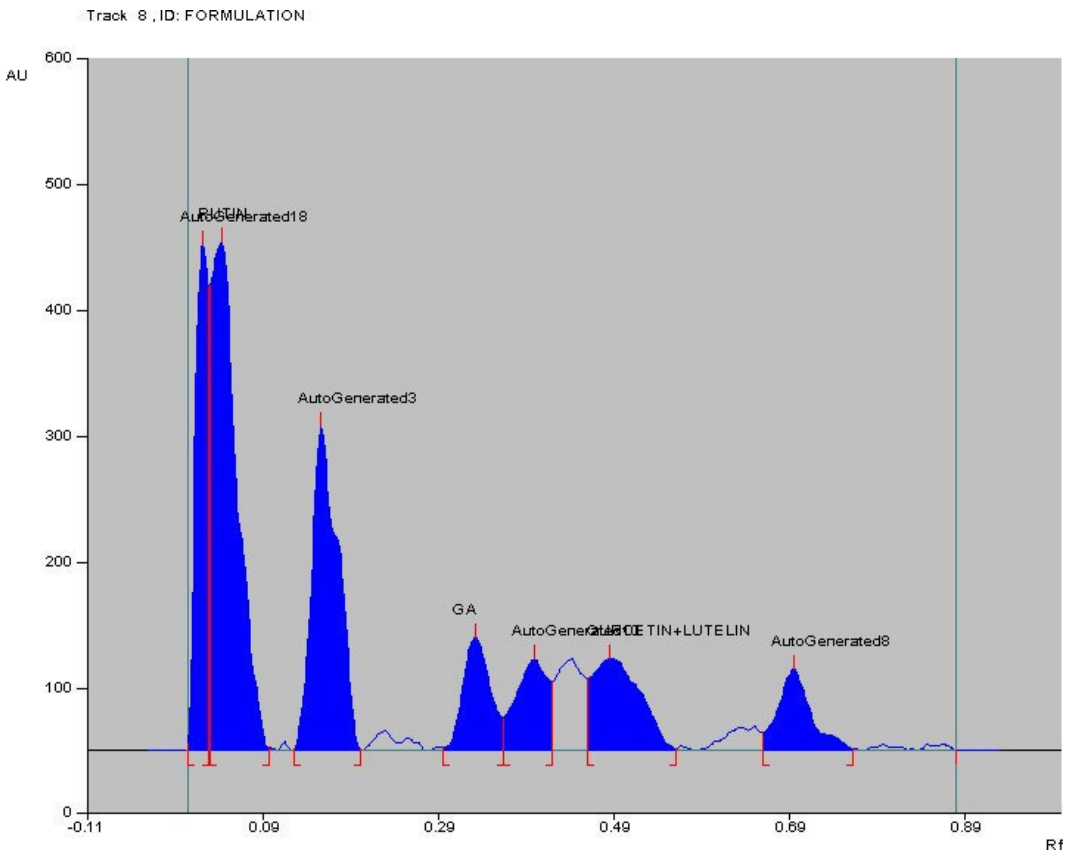
Figure No. 6.59: Densitogram and chromatogram of combination B extract

6.6.4 Presence of marker compounds in Polyherbal Formulation:

Polyherbal granules were studied by using HPTLC for checking presence of pharmacologically active biomarkers responsible for their antilithiatic activity. As Rutin is responsible for its Nephroprotective activity HPTLC study reveals that give formulation may give promising results against kidney stone and will work as Nephroprotective.

Table No. 6.40: Phytochemicals found in polyherbal granule formulation

Sr. No.	Name of Bioactive Compound	Rf value	Amount Present
1.	Rutin	0.04	31.92
2.	Gallic acid	0.33	7.41
3.	Quercetin	0.48	10.70
4.	Luteolin	0.48	10.70



Track 8, ID: FORMULATION

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.00 Rf	5.3 AU	0.02 Rf	402.3 AU	29.51 %	0.03 Rf	38.6 AU	4969.5 AU	15.91 %	AutoGenerated18
2	0.03 Rf	369.7 AU	0.04 Rf	403.9 AU	29.63 %	0.10 Rf	1.8 AU	9973.3 AU	31.92 %	RUTIN
3	0.13 Rf	0.8 AU	0.16 Rf	257.4 AU	18.88 %	0.20 Rf	0.7 AU	6435.7 AU	20.60 %	AutoGenerated3
4	0.29 Rf	2.0 AU	0.33 Rf	89.8 AU	6.58 %	0.36 Rf	25.3 AU	2316.4 AU	7.41 %	GA
5	0.36 Rf	25.6 AU	0.40 Rf	72.2 AU	5.30 %	0.42 Rf	54.7 AU	2210.2 AU	7.07 %	AutoGenerated10
6	0.46 Rf	57.2 AU	0.48 Rf	73.2 AU	5.37 %	0.56 Rf	1.3 AU	3344.2 AU	10.70 %	QURCETIN+LUTELIN
7	0.66 Rf	13.8 AU	0.69 Rf	64.6 AU	4.74 %	0.76 Rf	1.0 AU	1994.6 AU	6.38 %	AutoGenerated8

Figure No. 6.60: Chromatogram of Galic acid, Rutin, Quarcetin and Luteolinin Polyherbal Formulation at 254nm

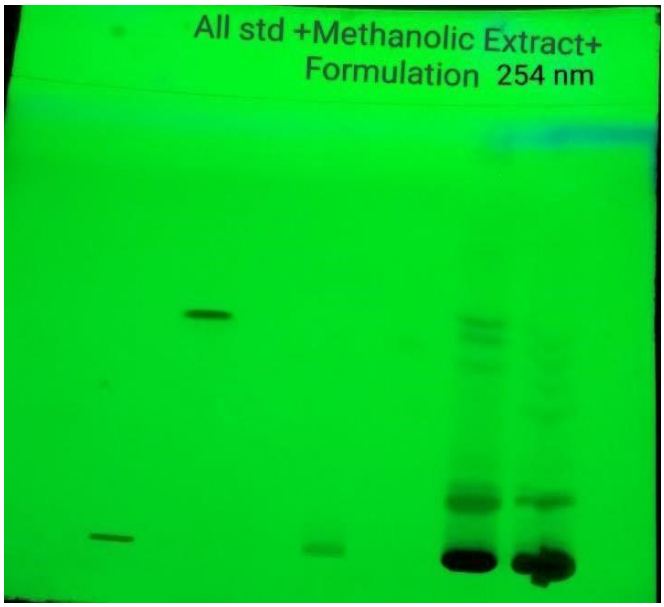


Figure No. 6.61: HPTLC finger print profile of Galic acid, Rutin, Quarcetine, Ursolic acid and Luteolin, Aqueous extract & Polyherbal Formulation at 254nm

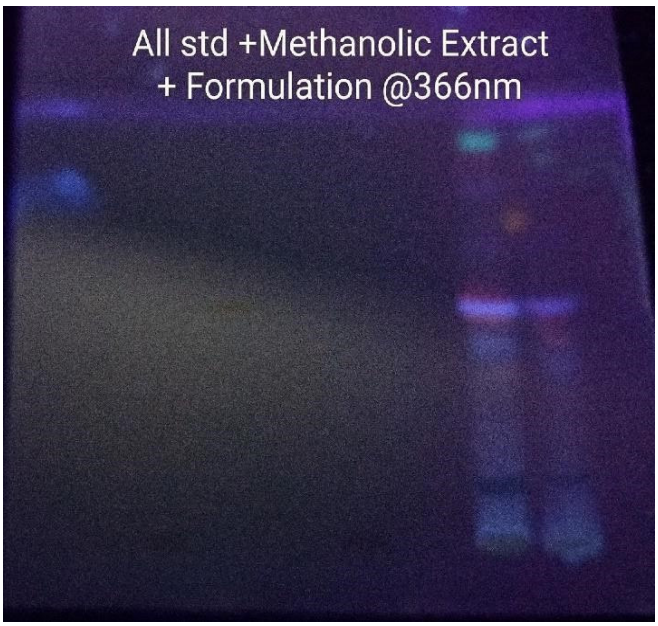


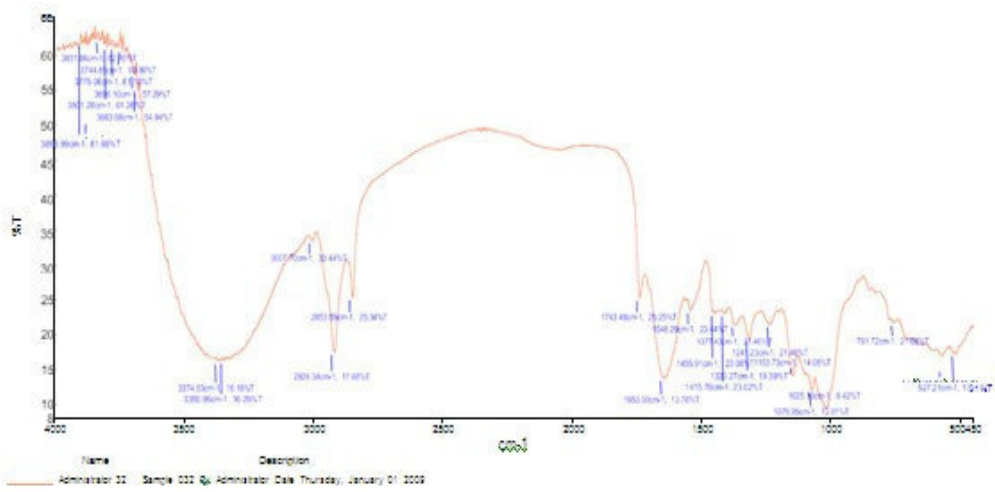
Figure No.6.62: HPTLC fingerprint profile of Galic acid, Rutin, Quarcetine, Ursolic acid and Luteolin, aqueous extract &Polyherbal Formulation at 366 nm

Conclusion of HPTLC Fingerprinting:

Quercetin is a metabolite of rutin, causes release of nitric oxide, leading to increased kidney filtration by renal vasorelaxation leading to its Diuretic effect .[177] Nephropathy, most encountered pathogenesis, occurs due to different physiological and metabolic differences occurring in kidney stone. In the study, Quercetin and rutin found in polyherbal combination demonstrated protective effect against ethylene glycol induced kidney stone and urolithiasis.[178] Administration of polyherbal extract in wistar rats significantly lowered the elevated levels of calcium, phosphorus and oxalate when compared with group II (diseased control) animals, and restore the magnesium level when compared with normal animals. Histopatholgical estimation revealed that, Rutin, Quercetin, Ursolic acid, Gallic acid and Luteolin present in polyherbal extract and formulation protected kidney from renal damage due to ethylene glycol induced nephropathy. [179-184]

6.7 FTIR Study:

KBR pellets of extracts of all the three drugs were prepared and analyzed using perkinelmer software. When three drugs are given in combination, they were not showing any incompatibility as well as all peaks of functional group are seen showing three drug combination is compatible with each other. Also, IR spectroscopy of Polyherbal formulation was seen compatible with excipients.



A

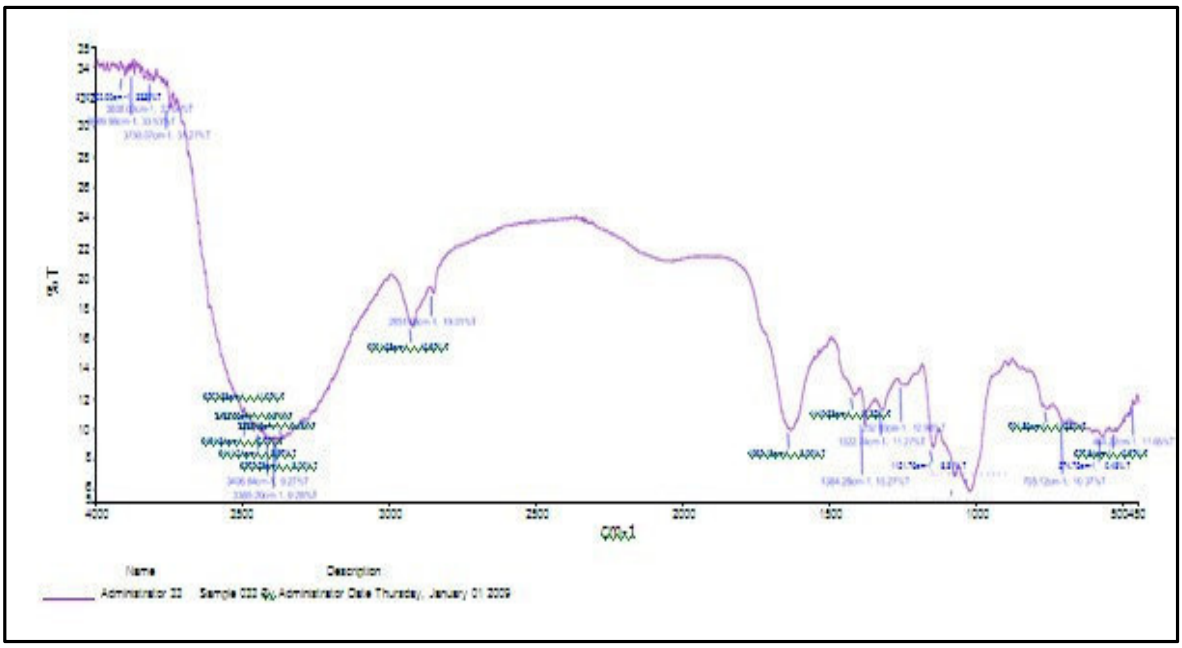


Figure No. 6.63: A: FTIR of extracts of three drugs; B: FTIR of extracts of three drugs with excipients Stability studies

6.8 PHYSICO-CHEMICAL PARAMETERS

Table No. 6.41: Physico-chemical parameters of Polyherbal granule Formulation subjected to stability studies

Parameter	Condition	0 day	30th day	60th day	90th day
Colour	40ºC/75% RH	Buff Greenish	Buff Greenish	Buff Greenish	Buff Greenish
	30ºC/65% RH	Buff Greenish	Buff Greenish	Buff Greenish	Buff Greenish
Odour	40ºC/75% RH	Characteristic	Characteristic	Characteristic	Characteristic
	30ºC/65% RH	Characteristic	Characteristic	Characteristic	Characteristic
Taste	40ºC/75% RH	Particular	Particular	Particular	Particular
	30ºC/65% RH	Particular	Particular	Particular	Particular

pH	40ºC/75% RH	7.8	7.8	7.5	7.5
	30ºC/65% RH	7.8	7.8	7.5	7.5
Tap Desity	40ºC/75% RH	0.44	0.44	0.44	0.44
	30ºC/65% RH	0.44	0.44	0.44	0.44
Bulk Density	40ºC/75% RH	1.434	1.433	1.436	1.436
	30ºC/65% RH	1.434	1.433	1.437	1.435
Size of granules	40ºC/75% RH	1.9	1.89	1.897	1.899
	30ºC/65% RH	1.9	1.88	1.892	1.895
Water content	40ºC/75% RH	258	257.2	258	257.5
	30ºC/65% RH	258	257.8	257.2	258.2
Microbial content	40ºC/75% RH	10.62	10.61	10.70	10.75
	30ºC/65% RH	10.62	10.62	10.75	10.75

The results of the stability studies indicated that the formulation had reasonable stability.

CHAPTER 7

SUMMARY AND CONCLUSION

In chapter 5, we summarized Pharmacognostic characteristics of selected herbs reveals their nature and their powder characteristics showed presence of lignified tissues, starch, aleurone grains etc. Phytochemical investigation of selected herbs by HPTLC method indicated presence of Polyphenol flavonoids like Quercetin and Luteolin, Triterpenoids like Ursolic acid, Lupeol and Oleanolic acid, Phenolic acid like Gallic acid, Fixed oil like Oleic acid etc. in aqueous extract of *Boerhavia diffusa*, *Plumeria rubra* and *Celosia argentea* contributing for its effect Antilithiatic activity against Ethylene glycol induced urolithiasis.

Two combinations of *B. diffusa*, *P. rubra* pods and *C. argentea* seeds were prepared containing 2:1:1 and 4:1:1 ratios out of these two combinations 4:1:1 was proved more effective through invitro and in vivo study. Preliminary study of plant extracts on kidney stone shows each plant has stone dissolution potential recommending their further in vitro and in vivo study of the plant extracts.

The in vivo study utilized two concentrations (200 mg/kg and 400 mg/kg) for the experimental groups. Ethylene glycol served as the stone-forming agent, and the study spanned 28 days with urine collection every 24 hours using metabolic cages. Urine analysis from the disease control animals indicated a significant increase in calcium, phosphorus, and oxalate excretion compared to group I animals, alongside a decrease in magnesium levels. Treatment with the plant extract notably reduced the elevated levels of calcium, phosphorus, and oxalate compared to group II animals, and restored magnesium levels comparable to those in normal animals.

Serum analysis involved blood collection from the retro-orbital plexus. It revealed that treatment with Extracts 1 and 2 (at 200 mg/kg and 400 mg/kg doses) and cystone (750 mg/kg) significantly increased serum calcium, serum phosphorus, blood urea nitrogen (BUN), serum creatinine, and serum uric acid levels compared to DC rats ($p < 0.05$; $p < 0.01$; $p < 0.001$). The polyherbal extract at 400 mg/kg from all three plants showed effectiveness in treating kidney stones. The exact mechanism underlying the antiurolithiatic activity of extracts from *B. diffusa*, *P. rubra* seed pods, and *C. argentea* seeds remains unexplained in the present study, though several potential mechanisms are proposed to account for their antilithiatic and prophylactic effects.

- Diuretic potential- *B. diffusa* and *P.rubra* seed pods are seen as potent diuretic, while *C.argenta* was mild diuretic, also various researcher studied and proved this property

in their studies. Kidney stone is generally associated with less urination. Many times hydrotherapy is employed by giving normal saline solution by IV route, as it dilutes the concentration of stone forming agents in urinary system. Diuretics accelerate the dissolution of preformed stone also can avoid reformation of stone. Therefore diuretic property of extrac of these herbal drugs may avoid supersaturation and thereby formation and reoccurrence of kidney stone.

- Antimicrobial activity- Any kidney disease like kidney stone, Urinary tract infection, anuria is associated with presence of microbial infection characterised by burning urination, itching, etc. Seed pod of *P. rubra* consists of antimicrobial property as many researched revealed the same through their studies. Antimicrobial assay proved the presence of antimicrobial property of *P. rubra* as plants containing latex shows antimicrobial potential and can be applied on open wound This can be beneficial in treating patient with such symptoms.
- Herbs used in present study were chosen from the history of their use for the treatment of kidney stone on the basis of their folklore value and used by local peoples. Some of the people showed very instantaneous result in dislodging of stone and non-reoccurrence of the stone has been reported. This study advocated the same by ascertaining the stone dissolution study by invitromodel and in vivo study of urine microscopy also revealed the stone dissolution potential of extract of drug combination as compared to normal and diseased control group.
- Conclusion drawn from this research activity is that, aqueous and methanolic extract of *B. diffusa*, *P. rubra* seed pod, *C. argentea* seeds if used in human in appropriate doses (on the basis of animal doses in the present study) might be very beneficial to dissolveand expel the kidney stones. Also may be prove beneficial to avoid reoccurrence of stone either by changing levels of parathyroid hormone and calcitonin or by avoiding nucleation process by facilitating increased excretion of magnesium, a potent inhibitor of stone formation and decreased excretion of stone forming constituents like calcium, oxalate and phosphoro This study is to flash light on such herbs available near us but as we don’t know the exact activity and combinations of such herbs in appropriate concentration. Many time we focus on main species of plants but we don’t know that allied species are also equally or more important. Also,

appropriate concentration of such herbs should be known for their effective and efficacious use.

Further research is recommended to identify the pharmacologically active phytoconstituents responsible for the antilithiatic activity observed in selected herbs. It is essential to evaluate these constituents' benefits in treating kidney stones through clinical research to confirm their efficacy in humans. Many plants produce seed pods or seeds that are only available during specific seasons. The current study also explores pharmaceutical dosage forms containing extracts from these plant seeds or seed pods, ensuring availability year-round for the treatment of kidney stones as needed.

So, finally, as the results are obliged for the use of three plant extract in suitable dosage form, we close the study with high delight and we excavate the avenue for transferring benefits to researcher as well as pateints suffering from kidney stone.

FUTURE SCOPE

The present investigations future scope may be aimed at:

1. The present investigation recommends exhaustive work work on various other novel drug delivery system dosage forms.
    - The present investigation needs determination pharmacokinetic parameters on human volunteer, aiming towards availability of one of the effective formulation in the market, to treat needy patients with less side effect due to use of polyherbal ingredients.

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Appendix

Published Research Article

AFJBS African Journal of Biological Sciences

Acceptance Letter

Date: 20.05.2024.

Manuscript ID. AJBS-137-2024

TITLE: "PHARMACOGNOSTIC STANDARDIZATION AND HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHIC FINGERPRINTING OF *PLUMERIA RUBRAL*. SEED PODS"

Dear Author/s: Jadhav A.S[1*], Dr. Rajesh Khathuriya[2], Dr. Jadhav S.L[3]

We are pleased to inform that above-mentioned manuscript has been reviewed and accepted forpublication in the upcoming issue (2024) of the African Journal of Biological Sciences, **ISSN 2663- 2187**. This letter of acceptance is to be considered as the official acceptance of your manuscript with no further amendments required. Author/s are requested to follow ethical and privacy guidelines mentioned in the journal homepage.

https://www.afjbs.com/ https://www.afjbs.com/

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https://doi.org/10.33472/AFJBS.6.Si2.2024.3096-3110

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Pharmacognostic standardization and High Performance Thin-Layer Chromatographic Fingerprinting of *Plumeria rubraL.* Seed pods

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Volume 6, Issue Si2, 2024

Received: 09 March 2024

Accepted: 10 April 2024

Published: 20 May 2024

doi:10.33472/AFJBS.6.Si2.2024.3096-3110

Abstract: *Plumeria rubra L.* is a deciduous plant belonging to genus Plumaria, Fam. Apocynaceae. Plant native to Mexico, south America also found in India and its tropical regions. Commonly known as Champa or Chafa and is a popular Temple tree, Garden and Park plant used for Ornamental purpose. Though indigenously it has traditional folkloric valued medicinally to treat kidney stone as Neproprotective yet literature lacks in its Pharmacognostic and Phytochemical approach also have not been explored therapeutically. In the present study *Plumeria rubra* seed pods are studied for its Pharmacognostic and Phytochemical investigation. Seed pods are 7-9 cm in length available in pairs, showed reddish brown colour, rough fracture and course texture. Microscopy showed the existence of lignified fibers , vascular bundle. The physicochemical analysis shows no foreign organic matter, 3.0% average moisture content, total ash value 12.03, 0.90 acid insoluble ash. Aqueous extract of plumeria rubra seed pods was prepared by using continuous hot extraction using Soxhlet extractor. Extracts showed the presence of tannins, alkaloids, balsam, cardiac glycosides, phenols, terpenes and steroids. HPTLC fingerprinting of aqueous extract of *Plumeria rubra* showed presence of Lupeol,Oleic acid and Luteolin as important phytoconstituents.

Introduction: *Plumeria rubra* has traditional folkloric values in cardiovascular disease as Hypotensive,also showed cardioprotective in adrenaline induced myocardial infarction (1-2) *P. Rubra* decoction is found effective in diarrhoea and vomiting. Bacterial infection, rheumatic pains can be treated by root decoction(3). Latex of *P.rubra* is found effective in ear and toothache as per Mexican tradition also used to cure venereal disease, as well as rheumatism, leprosy and fever in the indigenous system of medicine.(4) High-performance liquid chromatography (HPLC) analysis revealed many vital phytoconstituents, such as α-allamcidin, α-amyrin, β-allamcidin, β-amyrin acetate, β-sitosterol, allamandin, allamcin, arjunolic acid, lupeol, Plumericidine, rutin, quercetin, ursolic acid, oleanolic acid, Luteolin and oleic acid, Luteolin (5). Luteolin acts as nephroprotective by reducing oxidative stress on kidney, Lupeol is a tritepenoid acts as a Neuroprotective , Anti-inflammatory, cholesterol lowering agent(6). Pharmacological investigations of *Plumeria rubra* reported analgesic, antidiabetic, antipyretic, anti-obesity, antimicrobial, lipid-lowering, anticancer, antioxidant, insecticidal, and gastroprotective effects(7). It is found Kidney stone is common in all kinds of urolithiasis. The kidney stone develops due to decrease in volume of urine formation or increase in excretion of stone forming components like urate, cystine, xanthine, calcium oxalate and phosphate. Urolithiasis is a common health problem with increasing prevalence of up to 20% all over the globe. The increased prevalence of the disease occurs due to the lifestyle changes such as lower dietary intake of vegetables or fruit, higher consumption of animal proteins, salt, sweetened beverages, and inadequate fluid intake. Amongst the nephrolithiasis calcium oxalate stone occurs most commonly (8). In vitro evaluation using dissolution method or titrimetry method is necessary to evaluate effectiveness of herbal medicines.(9). Urolithiasis has been regarded as one of the eight most problematic disorders, and in Ayurveda urinary stones are called asmutraashamari (mutra-urine; ashma-stone; ari-enemy) (mahagad). Four different forms of urinary calculi, including phosphatic stones (sleshmaashmari), urate stones (pittaashmari), oxalate stones (vataashmari), and spermolith or seminal concretions (sukraashmari), have been recorded in Ayurvedic writings.Herbal remedies, alkaline drinks, and surgical techniques, Ayurvedic medicine to treat and manage urinary stones (10-13).

Plumeria rubra shown pink or red blooms throughout the year (Fig 1), but seed pods can be seen only in plants after aging of at least 9-10 years and can only be seen in winter

season only. Many people are unaware of availability of seed pods to Champa plant as they look like bark of the plant, thus it has not been critically studied pharmacognostically as well as phytochemically. Seed pods are dark reddish brown in colour and available in pair (Fig 2)

Fig 1 : Natural habitat of *Plumeria rubra*and seed pods

Fig2 : Fresh seed pods of *Plumeria rubra*

Material and Methods :

Collection and authention of Plant :

Plumeria rubra seed pods were collected from Rajuri, Junnar, Pune,Maharastra. Seed pods were cleaned by wiping them with clean cotton cloth. Fresh seed pods with flowering twig and leaves are authenticated from Agharkar Research Institute Pune, Maharashra.Matured seed pods were kept as it is as they open up after drying, such seed pods are kept for shade

drying . Dried pods containg seeds inside are ground in herbal grinding mill, passed through seive and stored in air tight container for futher tests and activity.

Pharmacognostic Studies:

Morphology and Microscopy :

Morphology of*Plumeria rubra* leaves, bark and seed pods and seed were studied and their phographs were taken using light binocular microscope. Transverse sections (T.S.) of leaves, bark and seed pod were taken by free hand section. T.S. of leave and seed pod were treated with phloroglucinol-HCl solution to observe lignified cells in plant parts. For powder characterstic fine powder of seed pod was prepared by using herbal grinding mill. The powder was treated with phloroglucinol-HCl solution, Iodine and Potassium Iodide solution.(14-15)

Physicochemical Estimation:

It is very important to evaluate physicochemical parameter to detect whether material used is pure or adultrated. It involves estimation of ash value including total ash value, acid insoluble ash, water soluble ash, extractive values such as water soluble and alcohol soluble extractive values, also involves moisture content analysis.(15)

Phytochemical Estimation :

Plumeria rubra seed pods were screened for preliminary phytochemical estimation by using various reagents for the presence of Phytochemicals like flavonoids, saponins, alkaloids, glycosides, tannins, carbohydrates, steroids, and terpenoids.(16)

High performance Thin Layer Chromatography (HPTLC) (16, 19) :

HPTLC was performed using CAMAG TLC SCANNER 3, CHF 47150, for sample injection Linomet 5 sampler was used.,twin trough chamber,winCATS software (CAMAG Ver.1.4.1). TLC plates precoated with silica gel 60 F254 (2020 cm) of 0.2 mm thickness procured from Ajinkya Enterprise, Pune were used as the stationary phase. All reagents used are of analytical grade.

Preparation of sample

Aqueous extract of *Plumeria seed* pods were prepared by using 1 gm of powdered drug . Extraction was performed by using continuous hot extraction using 100ml of water for 48 hours. Before extraction defatting with250 ml98% Petrolium ether is to be done. The extract obtained is to be evaporated at 40 °C till we get 10ml extract .

Preparation of Standard solutions

Stock solution of Luteolin, Oleicacid and Lupeol were prepared by dissolving 0.1mg/ml in HPTLC grade methanol.

Optimised Solvent system :

Table 1: Solvent system

Toluene	Ethyl acetate	Methanol	Formic acid
6	4	0.5	0.5

Development of HPTLC Fingerprinting of Luteolin and Oleic acid

Chromatographic conditions

Chromatographic separation was done using HPTLC plates (10×10 cm) pre-coated with silica gel 60 F_{254} of 0.2 mm thickness and supported by an aluminium sheet. Using a CAMAG (Muttenz, Switzerland) Linomat 5 sample applicator fitted with a 100l Hamilton syringe, standard solutions of markers and extracts were applied to the plates as bands 6.0 mm wide, 10.0 mm from the bottom border of the same chromatographic plate. Ascending development to a distance of 80 mm was carried out with mobile phase at room temperature (24°C) in a Camag glass twin-trough chamber that had previously been saturated with mobile phase vapour for 30 minutes. Following development, the plates were dried and scanned at 500 nm can be visualize by using Derivatizing agent Anisaldehyde-Sulfuric acid reagent
with a Camag TLC Scanner 3 equipped with a deuterium lamp and winCATS.

Calibration curve of Luteolin and Oleic acid :

Stock solution of Luteolin and Oleic acid containing 0.1mg/ml in methanol were used. Apply 1µg to 6 µg spots per band of standard solution using Linomet 5 semiautomatic sampler. The plot of linearity is plotted of area against corresponding concentration to get the correlation coefficient (R^2) and equation of the line.

Fingerprinting of Luteolin and Oleic acid :

Standard solution of Luteolin and oleic acid given in linearity followed byaqueous extract of *Plumeria rubra*seed pod to check presence of these chemical constituents in seed pod. The plates are run separately in solvent system of Toluene: Ethyl Acetate: Methanol: Formic acid (6:4:0.5:0.5) and the Rf values are to be checked accordingly.

Standard solution of Lupeol is also given in linearity followed by aqueous extract of *Plumeria rubra* seed pod for checking presence Lupeol as Phytoconstituent in seed pod of *P.rubra*.

Result &Discussion :

Pharmacognostic Studies:

Morphology and Microscopy :

Morphology of *Plumeria rubra* leaves shown presence of epidermal cells followed by single layer of palisade cells and lignified vasular bundle as shown in Fig3.Seed pods shown presence of cutical, medullary rays Fig 4and 5. Seed from seed pod was boiled and cut into two pieces showed white monocotyledon and embryo shown in Fig. 6. For powder characterstic fine powder was treated with phloroglucinol-HCl solution shown presence of lignified cells, when powder was treated with Iodine and Potassium Iodide solution shown blue colour indicating presence of starch grains .

MorpholgicalCharacteristics of *Plumeria rubra* seed pod: Seed pods of *plumeria rubra* were obtained in the month of January from Rajuri Pune, Maharashtra. The pods are elongated in shape, dark reddish brown in color available in pair approximately 12-15cm containing pink to white latex inside. Rough in fracture and having course texture.

Table 2 :Morphological characteristics of *P. rubra* Seed pod

Sr. No.	Morphological Characters	Observations of seed pod
1	Colour	Dark Reddish Brown
2	Odour	Mild
3	Taste	Pungent
4	Shape	Elongated
5	Size	12-15 cm

6	Fracture	Rough
7	Texture	Slightly coarse

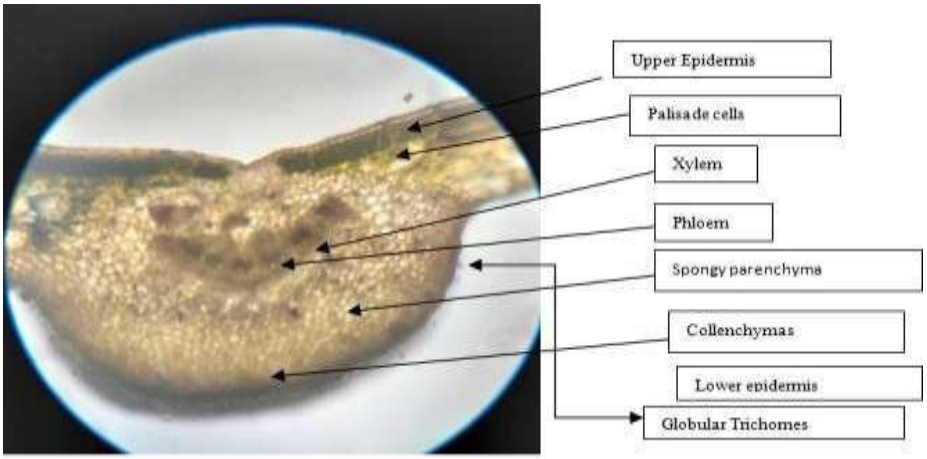

Fig3. T.S. of *Plumeria rubra* leaf

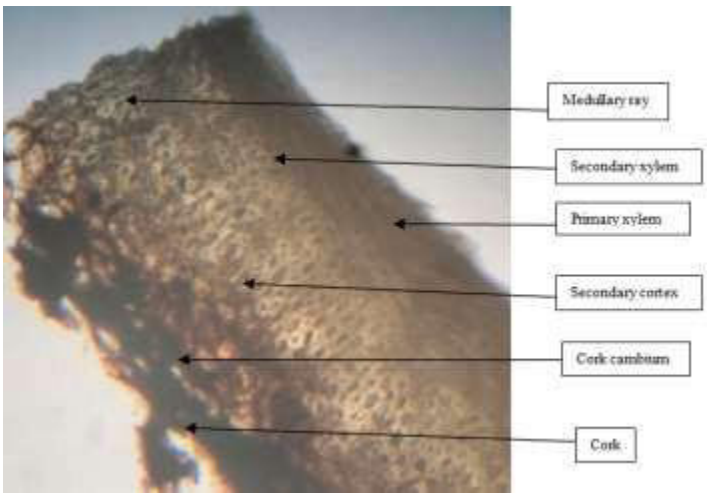

Fig.4. T.S. of *Plumeria rubra* Seed pod shell

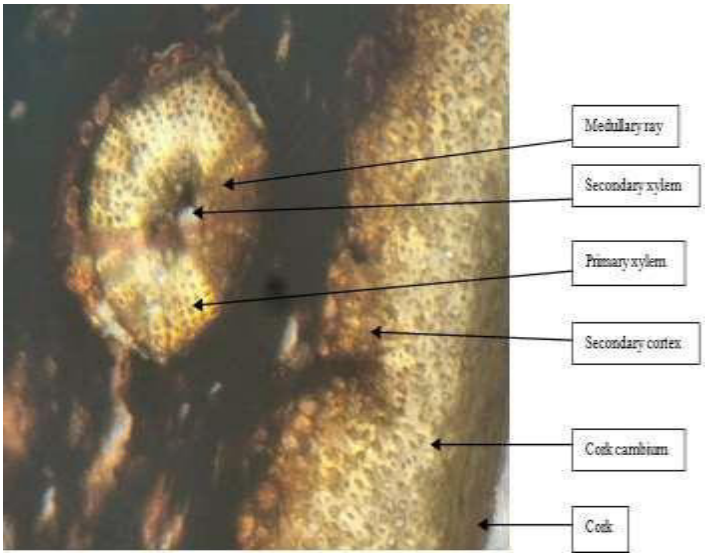

Fig. 5 T.S. of *Plumeria rubra*seed pod showing medullary rays

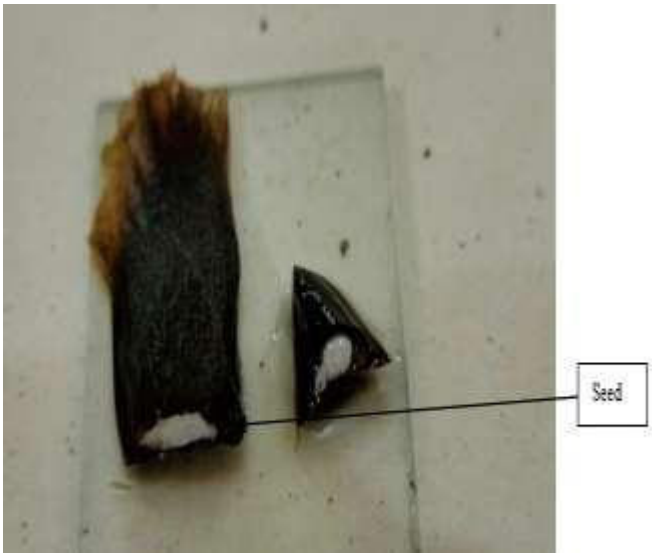

Fig. 6 Images showing seed of *plumeria rubra*

Fig.7 Plumeria seed pod powder + Phloroglucinol

Fig.8 Plumeria seed pod powder+ Iodine

Fig.9 Plumeria seed pod powder + Potassium Iodide

Phytochemical Estimation :

Observations of *Plumeria rubra* seed pods phytochemical estimation by using various reagents for the presence of Phytochemicals like flavonoids, saponins, alkaloids, glycosides, tannins, carbohydrates, steroids, and terpenoids are shown in table no 3.

Table 3. :Phytochemical Parameter observations

Phytochemical Parameters	Observations in methanolic extract
Flavanoids	++
Saponins	-

Alkaloids	++
Glycoside	+
Tannins	++
Carbohydrates	+
Steroids	+
Terpenoids	+
Fixed Oil	-

Abbrevation+ : presence , - : Absence

Development of HPTLC Fingerprinting of Luteolin, Oleic acid and Lupeol:

Calibration curve of Luteolin and Oleic acid :

Stock solution of Luteolin and Oleic acid containing 0.1mg/ml in methanol were used for sample spot application from 1µg to 6 µg spots per band of standard solution using Linomet 5 semiautomatic sampler shown linear plot. The plot shown value of correlation coefficient (R^2) is 0.996 .

Table 4 : Linearity of **Luteolin and Oleic acid**

Sr. No.	Concentration (µg)	Area
1	1	1089.54
2	2	2839.75
3	3	4985.97
4	4	6273.8
5	5	8235.27
6	6	9331.94

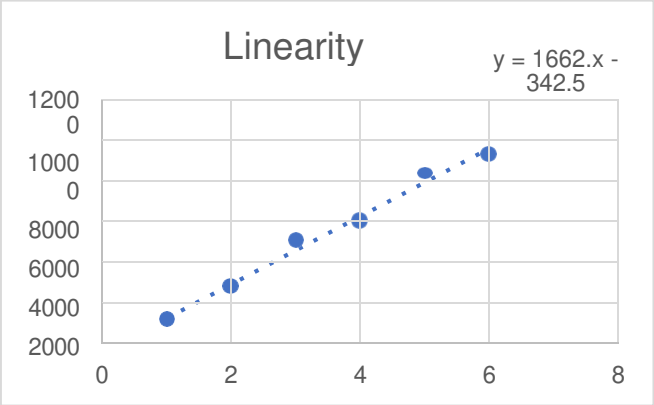

Fig 10. Linearity plot of standard Luteolin and Oleic acid

Fingerprinting of Luteolin and Oleic acid :

When Aqueous extract of *Plumeria rubra* seed pod run in solvent system of Toluene: Ethyl Acetate: Methanol: Formic acid (6:4:0.5:0.5) shown separations, visualized after derivatization with Anisaldehyde-Sulphuric at 540nm. In Fig11. The 3D spectra and Densiogram of extract shown presence of Luteolin and Oleic acid at corresponding Rf of standard Luteolin and Oleic acid i.e. for Luteolin it shown peak at Rf 0.59 and for Oleic acid it shown peak at Rf 0.80 in correspond to standard proves presence of these chemical constituents in extract of *Plumeria rubra* seed pod.

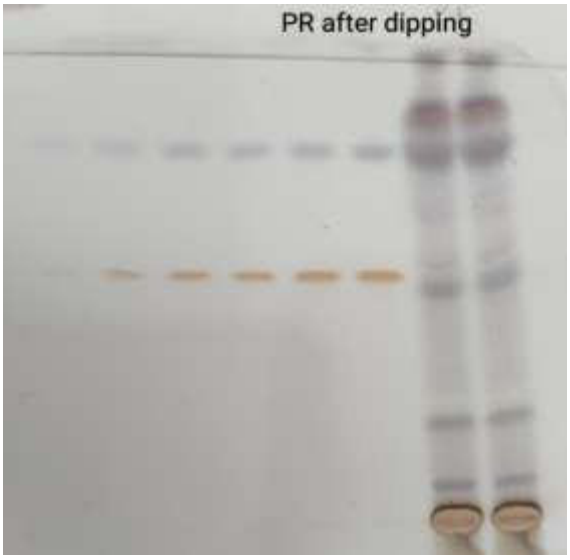

Fig 11 Slide of *Plumaria rubra* pod after derivatization

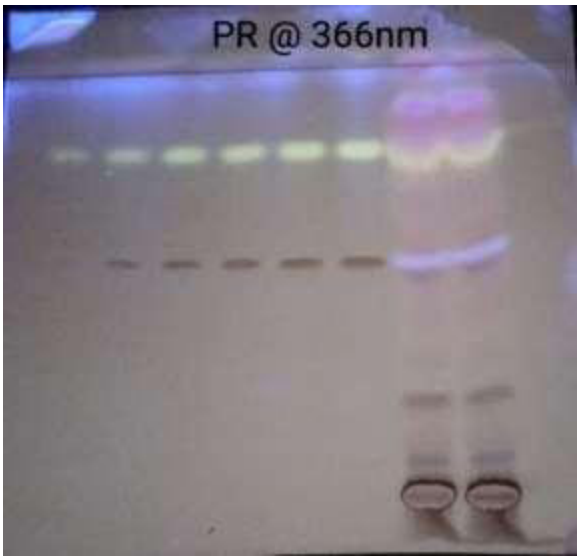

Fig 11 Slide of *Plumeria rubra*pod after derivatization at 366nm

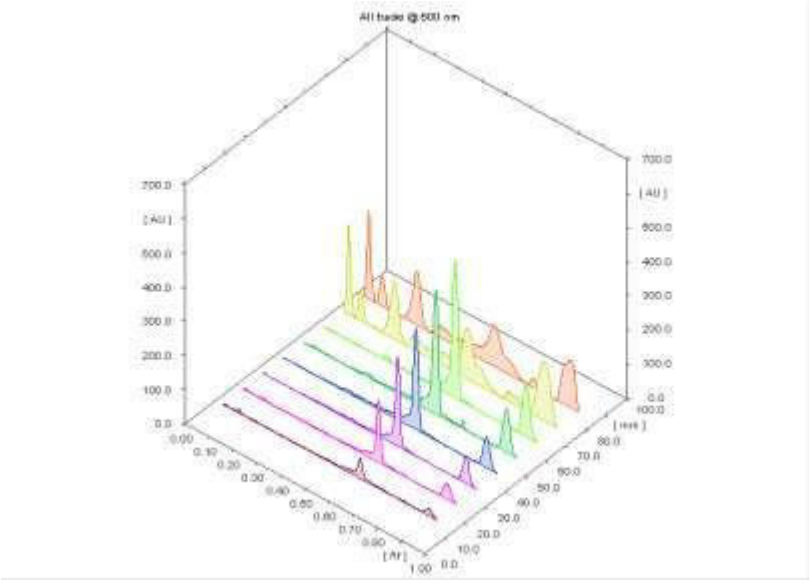

Fig.13:3D spectra of std Luteolin +Oelic acid+extract of *Plumeria rubra* Seed pod

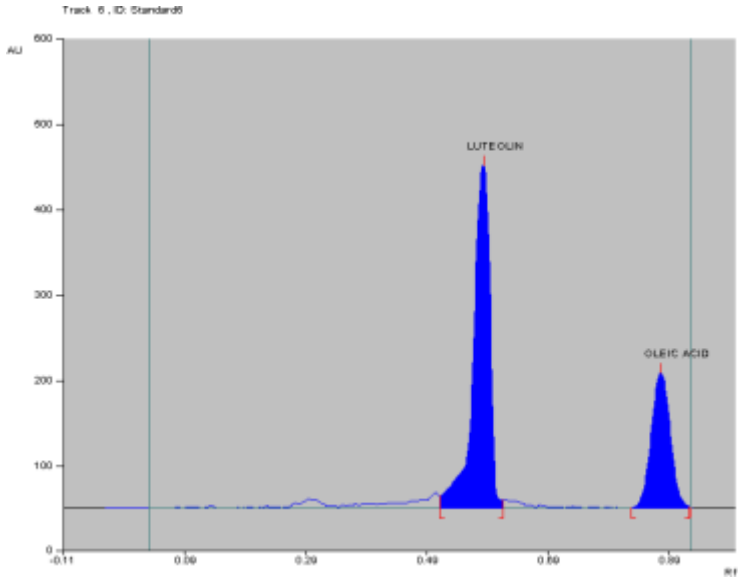

Fig 14 : Densiometric chromatogram of standard Luteolin and Oleic acid

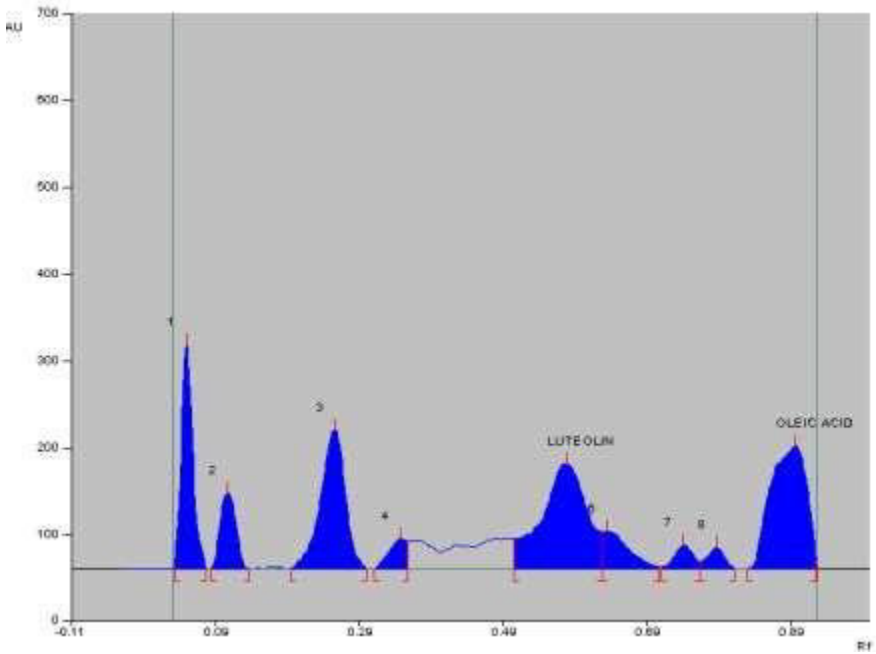

Fig 15: Densiometric chromatogram of Aqueous extract of *plumeria rubra* seed pod for Luteolin and Oleic acid

***Fingerprinting of Lupeol* from *plumeria rubra* seed pod:**

This is also found that *Plumaria rubra* seed pods showed presence of Lupeol. The obtained densitogram showed 13spots at Rf 0.04, 0.12, 0.17, 0.24, 0.27, 0.30, 0.38, 0.46, 0.50,0.59, 0.71, 0.80 and 0.89 in UV light at 500 nm. The spot at Rf 0.71 was Lupeol. Thus,Luteolin, Oleic acid and Lupeol are the phytoconstituents shown by HPTLC study of *Plumeria rubra* seed pod.

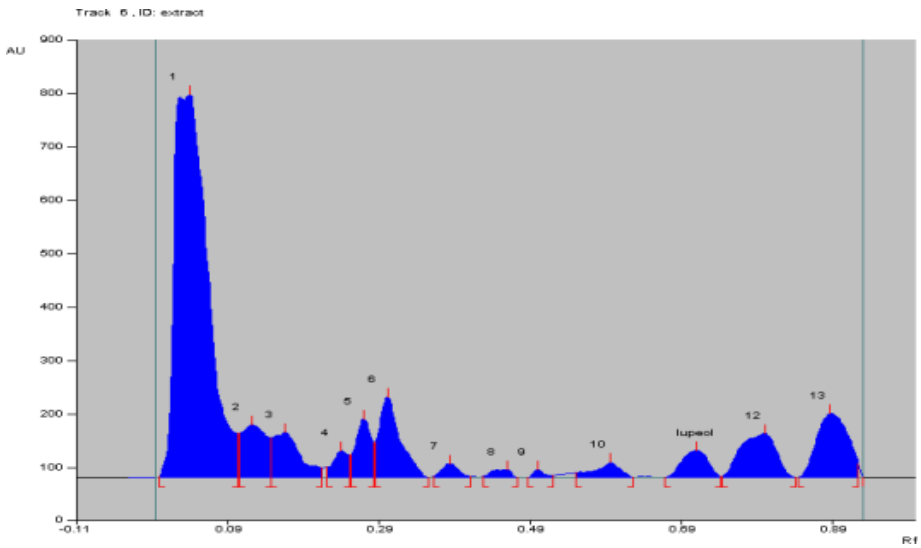

Fig 16: Densiometric chromatogram of *plumeria rubra* seed pod for Lupeol

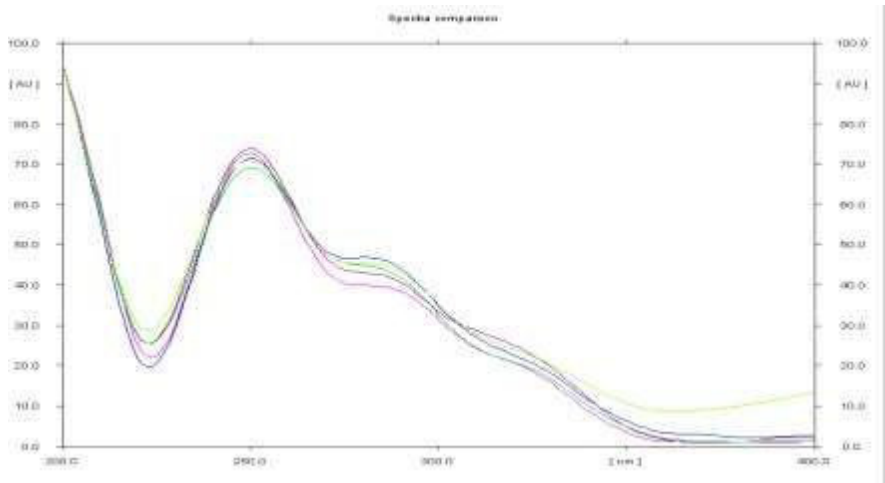

Fig. 17 :Overlain spectra for Lupeol+PR extract

Track 6, ID: extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.00 Rf	1.5 AU	0.04 Rf	716.7 AU	46.38 %	0.10 Rf	33.1 AU	26370.1 AU	54.58 %	unknown *
2	0.10 Rf	82.9 AU	0.12 Rf	98.8 AU	6.39 %	0.15 Rf	73.9 AU	2727.3 AU	5.65 %	unknown *
3	0.15 Rf	74.1 AU	0.17 Rf	84.7 AU	5.48 %	0.21 Rf	18.3 AU	2520.8 AU	5.22 %	unknown *
4	0.22 Rf	18.8 AU	0.24 Rf	50.2 AU	3.25 %	0.25 Rf	40.9 AU	858.7 AU	1.78 %	unknown *
5	0.25 Rf	42.2 AU	0.27 Rf	109.0 AU	7.05 %	0.28 Rf	34.0 AU	1846.4 AU	3.82 %	unknown *
6	0.28 Rf	66.1 AU	0.30 Rf	151.0 AU	9.77 %	0.35 Rf	0.2 AU	3468.7 AU	7.18 %	unknown *
7	0.36 Rf	2.2 AU	0.38 Rf	25.2 AU	1.63 %	0.41 Rf	1.7 AU	470.6 AU	0.97 %	unknown *
8	0.43 Rf	2.0 AU	0.46 Rf	14.4 AU	0.93 %	0.47 Rf	0.5 AU	312.5 AU	0.65 %	unknown *
9	0.49 Rf	0.2 AU	0.50 Rf	15.1 AU	0.98 %	0.52 Rf	2.8 AU	170.1 AU	0.35 %	unknown *
10	0.55 Rf	9.0 AU	0.59 Rf	28.3 AU	1.83 %	0.63 Rf	0.1 AU	768.4 AU	1.59 %	unknown *
11	0.67 Rf	0.1 AU	0.71 Rf	50.2 AU	3.25 %	0.74 Rf	2.0 AU	1442.8 AU	2.99 %	lupeol
12	0.74 Rf	2.2 AU	0.80 Rf	82.0 AU	5.31 %	0.84 Rf	0.1 AU	3219.4 AU	6.66 %	unknown *
13	0.84 Rf	0.3 AU	0.89 Rf	119.6 AU	7.74 %	0.92 Rf	25.9 AU	4134.4 AU	8.56 %	unknown *

Conclusion : Study reveals detailed Pharmacognostic, microscopic and phytochemical screening of *Plumeria rubra* Seed pod . Morphological characteristic of seed of pod showed identification characteristics of Seed pod also, Powder microscopy of Seed pod showed presence of lignified tissues, starch granules, etc. Seed pod showed presence of flavanoid, alkaloids, glycosides, carbohydrates, tannins, terpenoids, fixed oil.HPTLC fingerprinting analysis of *Plumeria rubra* seed pod showed presence of Luteolin,Oleic acid and Lupeol. This analytical investigation is helpful for researchers to find out it's Nephroprotective, Neuroprotective, Anti-inflammatory, Hepatoprotective properties of *plumeria rubra* seed pod.

Acknowledgement :

Author is thankful of Dr. D.Y. Patil Institute of Pharmaceuticals Science & Research, Pimpri, Pune for providing lab facility for giving permission to conduct HPTLC analysis.

Conflict of Interest :

We have no any conflict of interest

Abbreviations :

TS. :Transverse section, HPTLC: High Performance thin layer Chromatography, PR. : *Plumeria rubra.*

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INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS | ISSN: 2320 - 2882

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In recognition of the publication of the paper entitled

ANTILITHIATIC ACTIVITY OF POLYHERBAL EXTRACT ON ETHYLENE GLYCOL INDUCED UROLITHIASIS IN MALE WISTAR RATS

Published In IJCRT (www.ijcrt.org) & 7.97 Impact Factor by Google Scholar

Volume 12 Issue 2 February 2024 , Date of Publication: 04-February-2024

UGC Approved Journal No: 49023 (18)

PAPER ID : IJCRT2402054

Registration ID : 250777

ISSN 2320-2882 IJCRT

EDITOR IN CHIEF

Scholarly open access journals, Peer-reviewed, and Refereed Journals, Impact factor 7.97 (Calculate by google scholar and Semantic Scholar | AI-Powered Research Tool) , Multidisciplinary, Monthly Journal

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS | IJCRT

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Website: www.ijcrt.org | Email id: editor@ijcrt.org | ESTD: 2013

IJCRT | ISSN: 2320-2882 | IJCRT.ORG

IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

ANTILITHIATIC ACTIVITY OF POLYHERBAL EXTRACT ON ETHYLENE GLYCOL INDUCED UROLITHIASIS IN MALE WISTAR RATS

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ABSTRACT:

Kidney stones are common in all kinds of urolithiasis. The study focuses on Polyherbal extracts containing Boerhavia diffusa and Celosia argentea and Plumeria rubra seed pod was prepared and evaluated for antilithiatic byin vivo method. The traditional system of medicine includes the use of many herbal medicines for the treatment of kidney stone due to the presence of Flavonoids, triterpenoids, glycosides, and other chemicals having different mode of action. This study was conducted on Ethylene glycol-induced rats to examine the antilithiatic potential of selected herbs.

Objective:

To examine the Antilithiatic activity of Polyherbal extract on urine and serum parameters in Ethylene Glycol induced urolithiasis in male Wistar rats.

Method:

In this study Wistar rats were kept in metabolic cage separately from the day of starting the experiment until entire duration of 28 days of experiment, having six rats in each group. Urolithiasis was generated by administering ethylene glycol (0.75% v/v) in drinking water orally. Cystone 750mg/Kg is used as a standard for comparing the activity of plant extracts. Polyherbal extracts were used in two concentrations i.e. 200 and 400mg/Kg for 28 days.

Results:

It was found from urine analysis that, in disease control animals' calcium, phosphorus and oxalate excretion were significantly increased, while the magnesium level decreased when compared with Normal group animals. Also from serum analysis it was found that the treatment of rats with Extract 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$; $p<0.001$) increment in Serum Calcium, Serum Phosphorus, Serum BUN, Serum Creatinine and increment in Serum Uric acid when compared with DC rats. Thus polyherbal extract of 400mg/Kg of all three plants was found to be effective for treating Kidney stones.

KEYWORDS: Polyherbal, Antilithiatic, Serum calcium, phosphorus, uric acid.

I. INTRODUCTION:

Kidney stone is common in all kinds of urolithiasis. The kidney stone develops due to decrease in volume of urine formation or increase in excretion of stone forming components like urate, cysteine, xanthine, calcium oxalate and phosphate. Urolithiasis is a common health problem with increasing prevalence of up to 20% all over the globe. The increased prevalence of the disease occurs due to the lifestyle changes such as lower dietary intake of vegetables or fruit, higher consumption of animal proteins, salt, sweetened beverages, and inadequate fluid intake. Amongst the nephrolithiasis calcium oxalate stone occurs most commnly.[1]For curing urinary stone disorders, numerous treatment plans have been established in recent decades. There are currently numerous techniques available within the NHS for removing lower pole kidney diseases like percutaneous nephrolithotomy (PCNL), extracorporeal shockwave lithotripsty (ESWL) and flexible urinoscopy (FURS) with laser lithotripsy. Commonly in about 35% cases stone develops in the lower pole of the kidney.[2] However, the majority of these treatments require surgery called Lithotripsy, making them costly and painful. Due to this, many individuals prefer using traditional herbal remedies to cure urinary stones. In vitro evaluation using dissolution method or titrimetry method is necessary to evaluate effectiveness of herbal medicines.[3, 4]Bladder calculi are usually a manifestation of an underlying pathological condition, including voiding dysfunction or presence of foreign body within the bladder. Vesical calculi are seen mostly in men, but in developing world, it is found in prepubescent boys. Only bladder calculus is commonly seen (75%) rather than multiple stones[5.] Ayurveda, Traditional Chinese medicine (TCM), Siddha, and Unani are having many such traditional medical systems that have described the use of various herbal treatments to treat urinary stone problems.[6-7] Urolithiasis has been regarded as one of the eight most problematic disorders, and in Ayurveda urinary stones are called as mutraashamari (mutra-urine; ashma-stone; ari-enemy) (mahagad). Four different forms of urinary calculi, including phosphatic stones (sleshmaashmari), urate stones (pittaashmari), oxalate stones (vataashmari), and spermolith or seminal concretions (sukraashmari), have been recorded in Ayurvedic writings. Herbal remedies, alkaline drinks, and surgical techniques, Ayurvedic medicine to treat and manage urinary stones.[8-9] Allopathic drugs are having many

side effects. As majority of drugs are excreted through kidney it will worsen the case, so it is better to prefer herbal medicines in treating such ailments as they are generally do not have side effects.

II. MATERIAL AND METHODS:

a. Procurement and Authentication of drugs:

The plants used in the present study consists of whole plant of Boerhavia diffusa Linn, were collected from local area of Khandala, Maharashtra, Matured pods of Plumeria rubra Linn was collected from Rajuri, Junnar Maharashtra. Seeds of Celosia argentea Linn, was purchased from Mankarnika Ayurvedic medical store, Chinchwad Pune. Plants and seeds were authenticated by Dr. R.K. Chaudhary, Scientist from Agharkar Research Institute, Autonomous body under DST, GOI, and Pune.

The collected plant material was washed thoroughly in running tap water, rinsed using distilled water and shade dried for seven days then dried and clean plants were grounded by using laboratory herbal grinding mill. Coarse powder (60#) of dried plants were stored in air tight container for preparing aqueous extract for further in vivo study.

Chemicals: Ethylene Glycol was procured from GS Lab, Pune Maharastra, India. Biochain assay Kit was procured from Ajinkya Enterprises, Pune.

Animals:

Wistar albino male rats weighing between 180 and 250 g were selected for the antiurolithiatic activity. The animals were allowed to stay in metabolic cages so as get accustomed to standard laboratory conditions. Animals were fed with standard rat diet and provided access to drinking water ad libitum. Animal care and animal experiments were conducted in accordance with CPCSEA/IAEC approval no: 1401/PO/RE/S/11/IAEC/2020-21/07/02. Rats used for the study were obtained from the animal house of an authorized local supplier Lachmi Biofarms Pvt. Ltd. Pune Maharastra, India. Rats were housed in groups of six in clean cages. The bedding material of the cages was changed twice weekly.

b. Preparation of extract of polyherbals.

Aqueous extract of Boerhavia diffusa, Plumeria rubra seed pods and Celosia argentea seeds was prepared using Soxhlet extraction method for the period of 48 Hrs. The extract was prepared in two concentrations 200 and 400mg/Kg for in vivo study.

c. Acute toxicity studies

Acute Toxicity Studies: Acute oral toxicity of the Polyherbal formulation was carried out as per the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423. The principle involves a stepwise procedure with the Use of a minimum number of animals per step to obtain sufficient information on the acute toxicity of the test substance to enable its classification. Healthy male Wistar

rats (3 animals/dose) were used for the experiment. Overnight fasted rats were orally fed with the Polyherbal formulation in increasing dose was 5mg, 50mg, 300 mg and 2000 mg/kg body weight, The animals were observed for their behavioral (alertness, restlessness, irritability, and fearfulness), neurological (spontaneous activity, Reactivity, touch response, pain response, and gait), and autonomic (defecation and urination) profiles continuously for 24 h. After a period of 24 h, the animals were observed for 14 days for mortality. [10]

d. Ethylene Glycol Induced Model of Urolithiasis in male Wistar Rats

The study was so designed to find out effect of aqueous Polyherbal extract of B. diffusa, P. rubra and C. argentea against ethylene glycol induced urolithiasis. Animals were divided into five groups each group containing six animals, kept in metabolic cages individually to collect urine. Group I is serves as Normal group, Group II to V receives ethylene glycol as stone inducer for 28 days. After administration of the same group III treated with cystone 750 mg/Kg as a standard antilithiatic drug (14, 15), Group IV and V were treated with 200 and 400mg/Kg aqueous extract of B. diffusa, P. rubra and C. argentea. After experimental period of 28 days, 24 hrs urine was collected from receivers of metabolic cage and further tested for urine parameters using biochain assay kit. Blood was collected from retroorbital plexus after making the animal unconscious.

Table No 1: Anti-urolithiasis in vivo experimental design

Group	Treatment	No. of Animals
I	Regular diet and water	6
II	Ethylene Glycol (0.75% v/v) for 28 days	6
III	Ethylene Glycol (0.75% v/v) for 28 days + standard drug 750mg/kg (15-28th day)	6
IV	Ethylene Glycol (0.75% v/v) for 28 days + Effective extract 200mg/kg (1-28th day)	6
V	Ethylene Glycol (0.75% v/v) for 28 days + Effective extract 400mg/kg (1-28th day)	6

III. RESULTS AND DISCUSSION:

a. Acute Toxicity Study

At a dose of 2000 mg/kg the extract of B. diffusa did not exhibited any toxic effects as shown by the experimental animals. Also, the dose of up to 2000 mg/kg body weight extract of C. argentea and P. rubra was well tolerated by experimental animals and therefore 200 mg/kg, 400mg/kg body weight (i.e. $1/10^{th}$ and $1/20^{th}$) doses were selected for the study. More research is needed to determine persistent toxic effects.

b. In-Vivo Anti-Urolithiaitic Activity

Urine Parameters

i) Estimation of urine volume and pH

Table No. 2: Estimation of urine volume and pH

PARAMETERS	GROUP-I	GROUP-II	GROUP-III	GROUP-IV	GROUP-V
Urine Volume	2.57±0.11	1.60±0.23###	5.40±0.14***	3.35±0.54***	5.19±0.32***
pH	7.25±0.04	5.7±0.13**	7.29±0.45***	6.30±0.24***	7.24±0.04***

Values are expressed as Mean ± SEM, n=6.

*P ≤ 0.05,

P ≤ 0.01 and*P ≤ 0.001 compared with disease control

From Table no. 02, it can be seen that Urolithiasis induced animals shows decreased in urine volume (Group II), whereas the treated groups (II, III, IV) shows increased in urine volume when compared with Group II rats.

In ethylene glycol induced rats pH was reduced when compared with normal control group. Treatment with standard drug 750 mg/kg and ethanolic extracts of plants were found to increase the urine pH in a dose dependent manner.

ii) Estimation of Calcium, Oxalate, phosphorus and magnesium in Urine

Table No. 3: Estimation of calcium, oxalate, phosphorus and magnesium in urine

PARAMETERS	GROUP-I	GROUP-II	GROUP-III	GROUP-IV	GROUP-V
Calcium	9.54±0.10	13.56±0.08###	10.24±0.11***	12.81±0.15**	10.36±0.169***
Oxalate	1.86±0.07	4.39±0.17###	2.11±0.07***	3.8±0.12**	2.55±0.10***
Phosphorous	5.15±0.20	7.13±0.17###	5.18±0.14***	6.33±0.14**	5.28±0.11***
Magnesium	4.16±0.08	1.73±0.12###	3.33±0.11***	2.11±0.09*	3.18±0.07***

Values are expressed as Mean ± SEM, n=6.

*P ≤ 0.05,

P ≤ 0.01 and*P ≤ 0.001 compared with disease control

From table no. 03, in disease control animals calcium, phosphorus and oxalate

Excretion were significantly increased, while the magnesium level decreased when compared with group I animals. When supplement with plant extract significantly lowered the elevated levels of calcium, phosphorus and oxalate when compared with group II animals, and restore the magnesium level when compared with normal animals.

Serum Parameters

Effect of PHF 200 and PHF 400 on Serum Calcium (mg/dl) in Ethylene induced Urolithiasis in Rats

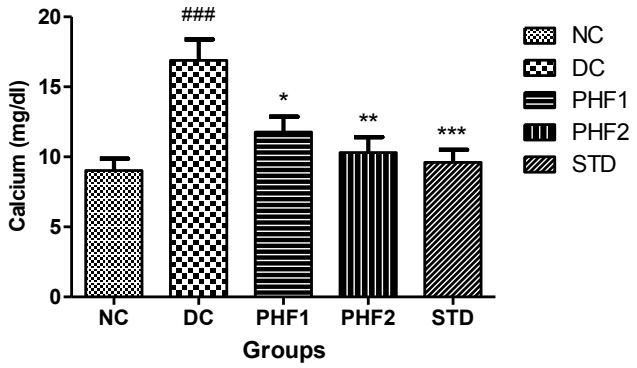

Figure 1: Effect of PHF 200 and PHF 400 on Serum Calcium (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and PHF 2 on Serum Calcium in EG induced Urolithiasis in Rats in Figure 1. The treatment of rats with EG (0.75 %, w/v) induced significant (p<0.001) elevation in Serum Calcium when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.05; p<0.01; p<0.001) increment in Serum Calcium when compared with DC rats.

Effect of PHF 200 and PHF 400 on Serum Phosphorus (mg/dl) in Ethylene induced Urolithiasis in Rats

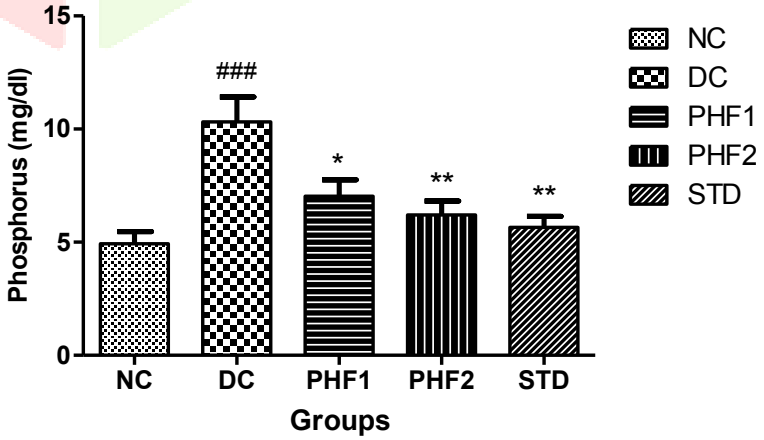

Figure 2: Effect of PHF 200 and PHF 400 on Serum Phosphorus (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and PHF 2 on Serum Phosphorus in EG induced Urolithiasis in Rats in Figure 2. The treatment of rats with EG (0.75 %, w/v) induced significant (p<0.001) elevation in Serum Phosphorus when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.05; p<0.01; p<0.01) increment in Serum Phosphorus when compared with DC rats.

Effect of PHF 200 and PHF 400 on Serum BUN (mg/dl) in Ethylene induced Urolithiasis in Rats

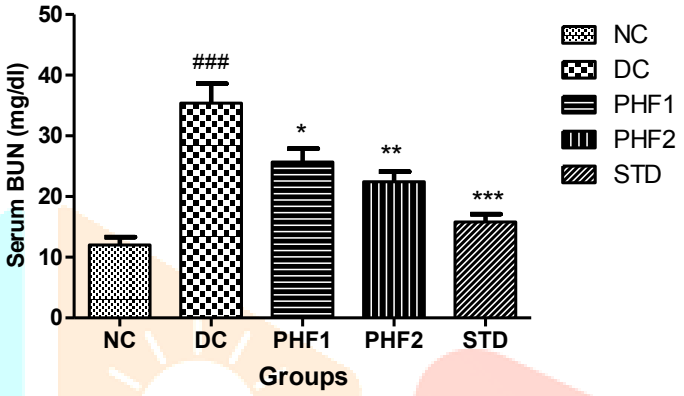

Figure 3: Effect of PHF 200 and PHF 400 on Serum BUN (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and 2 400 on Serum BUN (mg/dl) in EG induced Urolithiasis in Rats in Figure 3. The treatment of rats with EG (0.75 %, w/v) induced significant (p<0.001) elevation in Serum BUN when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.05; p<0.01; p<0.001) increment in Serum BUN when compared with DC rats.

Effect of PHF 200 and PHF 400 on Serum Creatinine (mg/dl) in Ethylene induced Urolithiasis in Rats

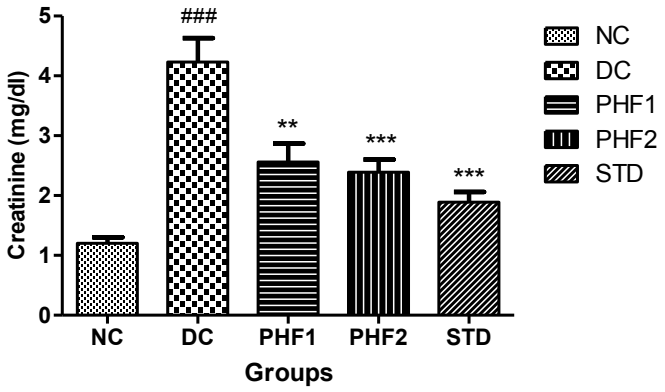

Figure 4: Effect of PHF 1 and PHF 2 on Serum Creatinine (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and 2 on Serum Creatinine in EG induced Urolithiasis in Rats in Figure 4. The treatment of rats with EG (0.75 %, w/v) induced significant ($p<0.001$) elevation in Serum Creatinine when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.01$; $p<0.001$) increment in Serum Creatinine when compared with DC rats.

Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats

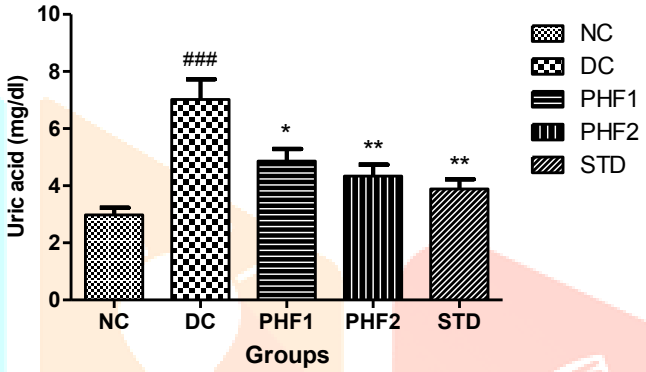

Figure 5: Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean ± SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p<0.001$ versus NC rats and $^{***}p<0.001$ versus DC rats.

The effects of PHF 1 and 2 on Serum Uric acid in EG induced Urolithiasis in Rats in Figure 5. The treatment of rats with EG (0.75 %, w/v) induced significant ($p<0.001$) elevation in Serum Uric acid when compared with DC rats. However, the treatment of rats with PHF 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant ($p<0.05$; $p<0.01$; $p<0.001$) increment in Serum Uric acid when compared with DC rats.

IV. CONCLUSION:

The effect three medicinal plants i.e. Boerhavia diffusa, celosia argentea and Plumeria rubra studied by in vivo method for their litholytic activity. The polyherbal extract of 400mg/Kg was shown better effect it was found from urine analysis that, in disease control animals' calcium, phosphorus and oxalate excretion were significantly increased, while the magnesium level decreased when compared with Normal group animals. Also from serum analysis it was found that the treatment of rats with Extract 1 and 2 (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) exhibited significant (p<0.05; p<0.01; p<0.001) increment in Serum Calcium, Serum Phosphorus, Serum BUN, Serum Creatinine and increment in Serum Uric acid when compared with DC rats. Thus poyherbal extract of 400mg/Kg of all the three plants was found to be effective for the treatment of Kidney stone.

FUTURE SCOPE: Role of these plants in preventing reoccurrence is remains to be assessed. Imbalance in the level of parathyroid and Calcitonin hormone may contribute in reoccurrence of stone is to be assessed.

AUTHOR CONTRIBUTIONS. Guide and Co-guide who have contributed by guiding and providing facilities to conduct this research.

V. ACKNOWLEDGEMENT: Author would like to thank Dr. Suresh L. Jadhav for providing all the equipments, Chemicals and Instruments needed for study**.**

CONFLICT OF INTEREST: There are no conflicts of interest in present study.

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