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:

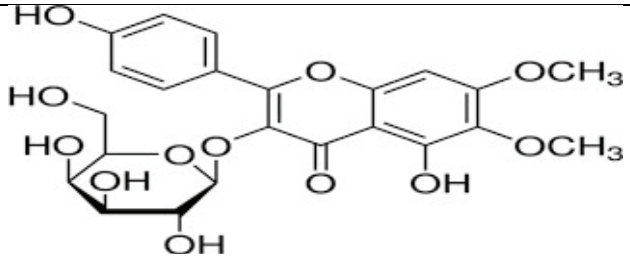
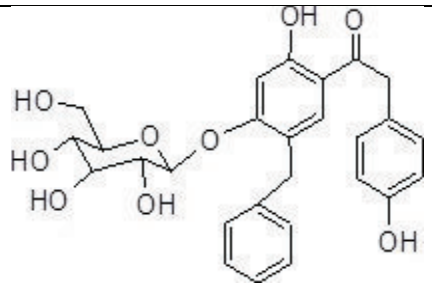
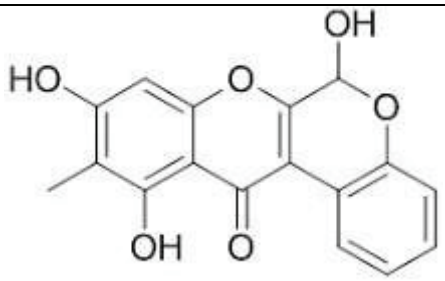
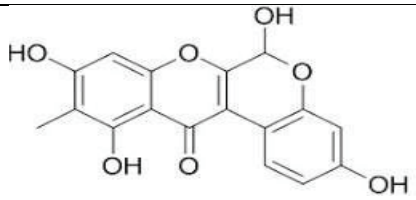
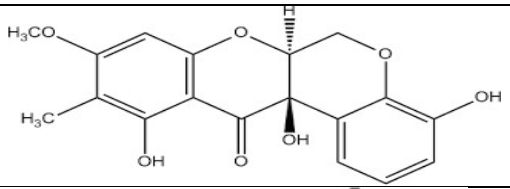
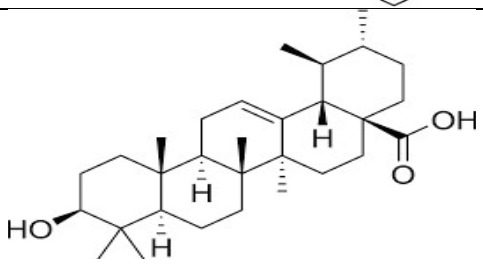
Boerhaavia diffusa contains numerous compounds including flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins. Specific compounds isolated and studied for their biological activities include punarnavine ($C_{17}H_{22}N_2O$, melting point 236–237°C), boeravinone A-F, hypoxanthine 9-L-arabinofuranoside, ursolic acid, punarnavoside, liirodendrin, and a glycoprotein with a molecular weight of kDa.[149]

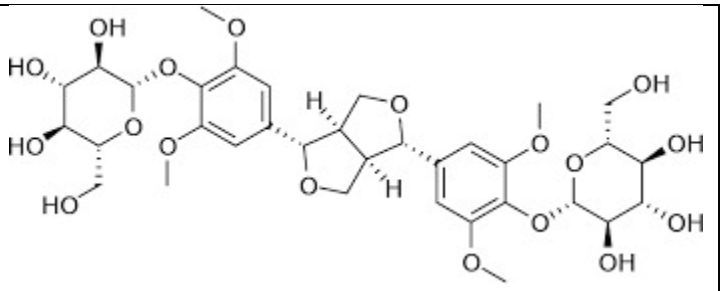
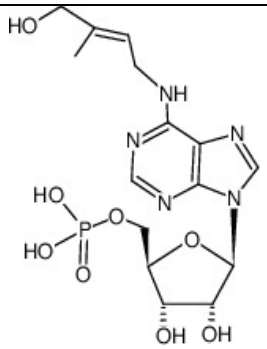
Punarnava (*Boerhavia diffusa*) is known for its rich phytochemical composition. Analysis has revealed the presence of β-Sitosterol, α-2-sitosterol, palmitic acid, ester of β-sitosterol, tetracosanoic acid, hexacosanoic acid, stearic acid, arachidic acid, urosilic acid, Hentriacontane, β-Ecdysone, and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

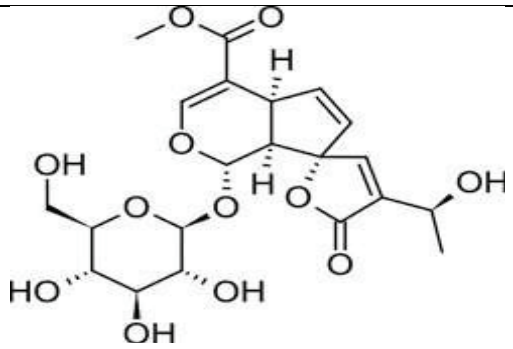
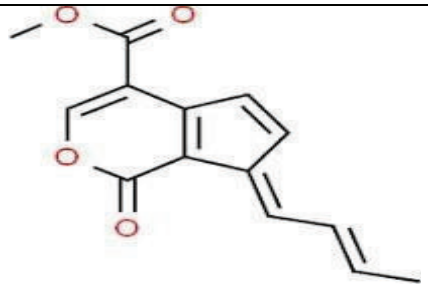
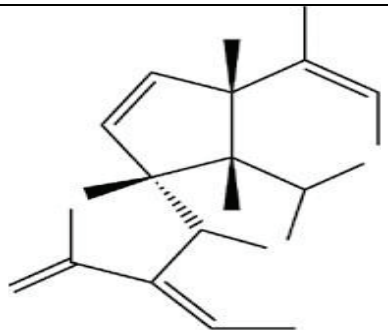
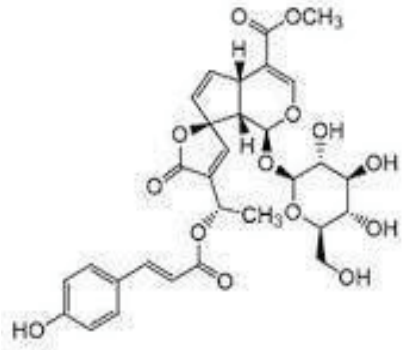
The essential oil of *Plumeria Rubra* was found to contain a diverse array of compounds, including 13 hydrocarbons, 17 alcohols, 13 esters, 9 aldehydes, and 15 miscellaneous compounds. Further analysis of the alcoholic extract from the leaves isolated five specific compounds: lupeol nanoate, lupeol heptanoate, 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

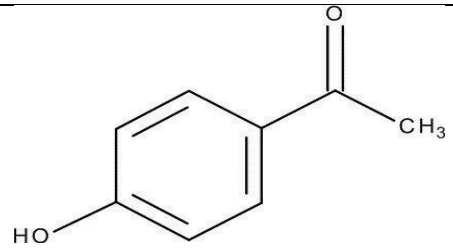
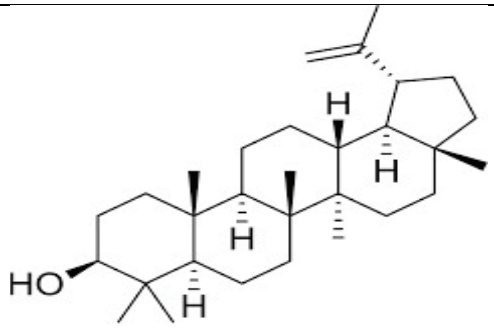
been identified from *Plumeria Rubra*, with the oil composition consisting of 10 esters (43.3%), 11 hydrocarbons (8.1%), 10 alcohols (29.8%), 4 carboxylic acids (8.5%), 2 ethers (1.0%), 3 aldehydes (2.5%), and benzyl salicylate (20.9%). *Plumeria Rubra* is notably rich in ursolic acid, a natural pentacyclic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

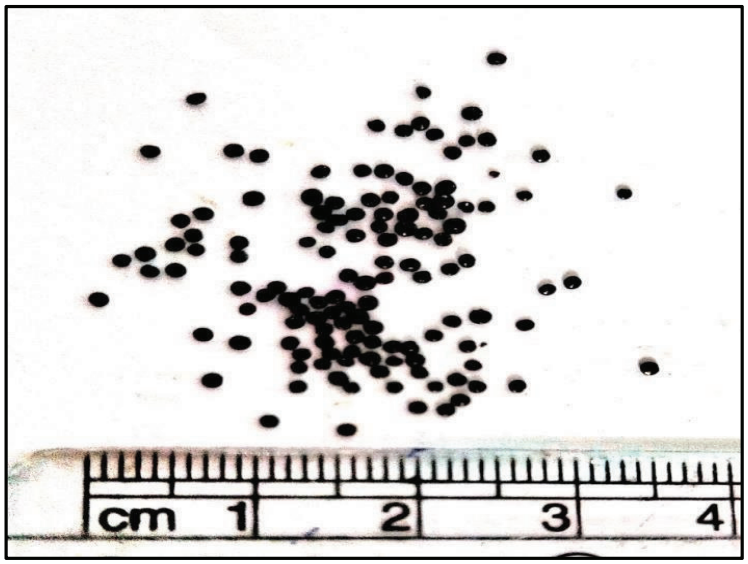


Figure No. 5.3: *Celosia argentea* Seeds

- **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.

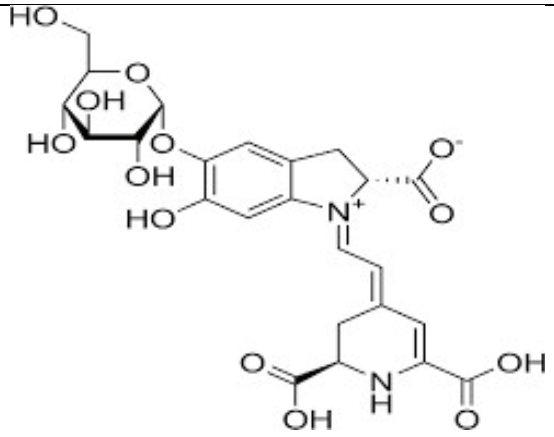
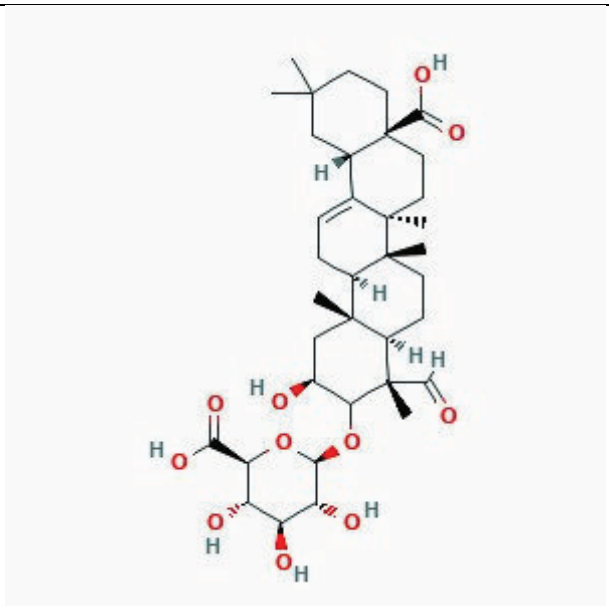
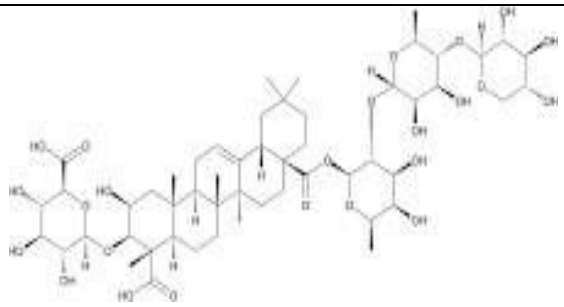
Leaf Characteristics: Apices: Acute Arrangement: Alternate in a spiral pattern Bases: Cuneate Margins: Entire Shapes: Elliptic Types: Simple

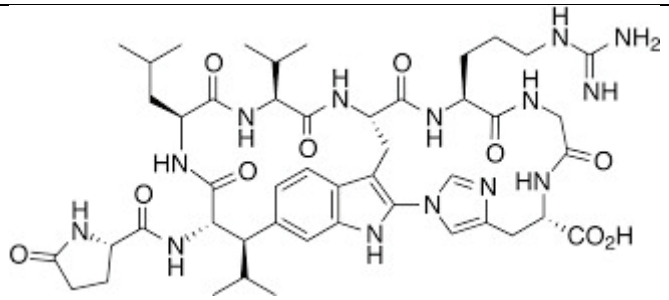
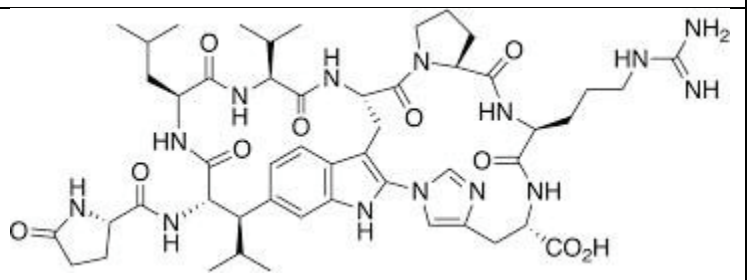
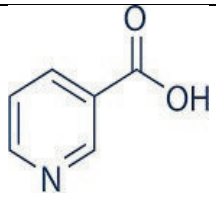
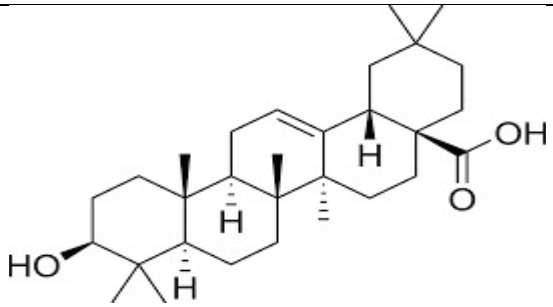
Habit: *Celosia argentea* is an erect, glabrous annual herb extensively branched with a profuse growth pattern.[169]

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.

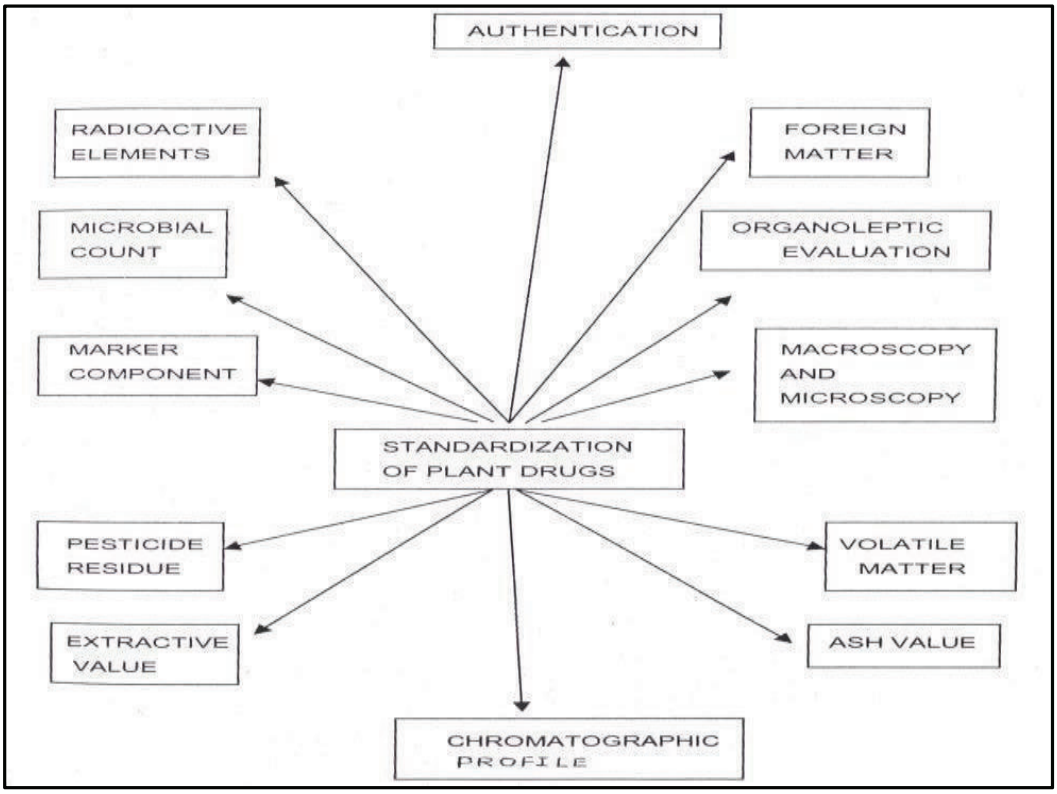


Figure No. 5.4: Standardization chart of herbal raw materials

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

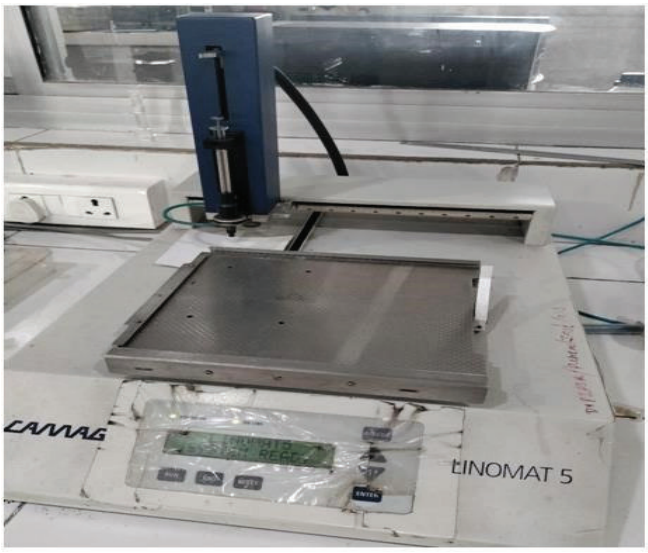


Figure No. 5.7: Linomat Semiautomatic sampler for HPTLC plates HPTLC Conditions

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.

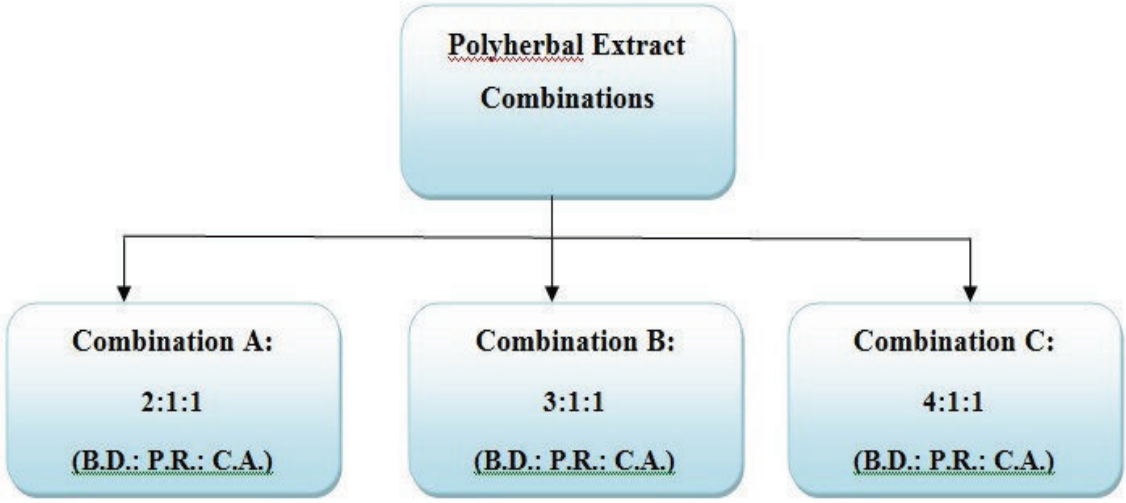


Figure No. 5.9: Composition of Extract

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

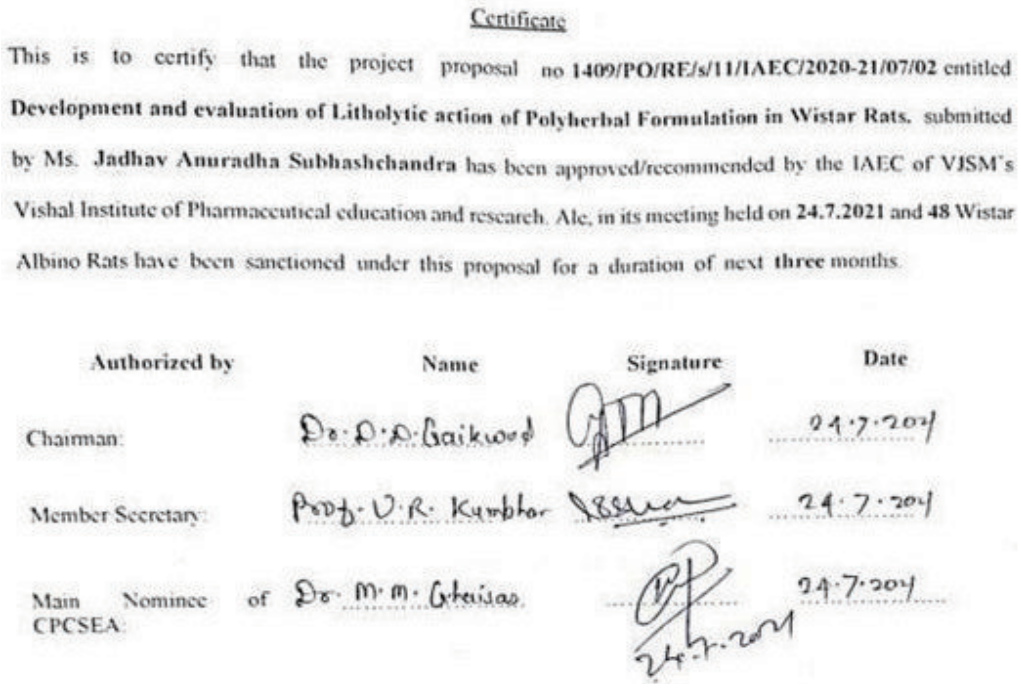
Objective:

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. A. Gaikwad		24.7.2021
Member Secretary	Prof. V. R. Kumbhar		24.7.2021
Main Nominee of CPCSEA	Dr. M. M. Ghaisas		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.

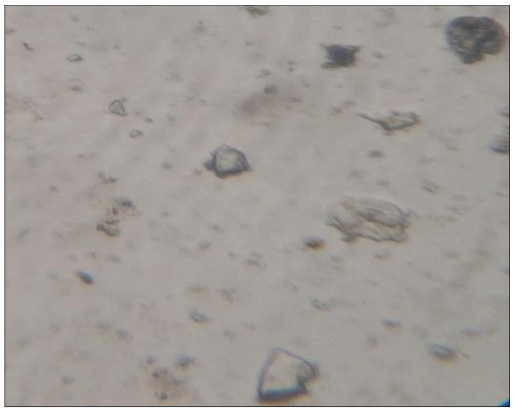
5.4.4.1 Analysis of Urine

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.

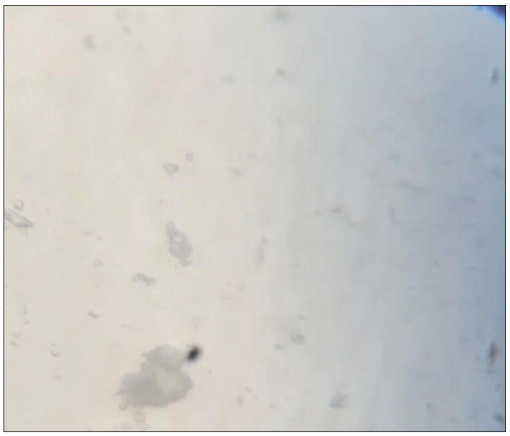
5.4.4.2 Microscopic observation

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.

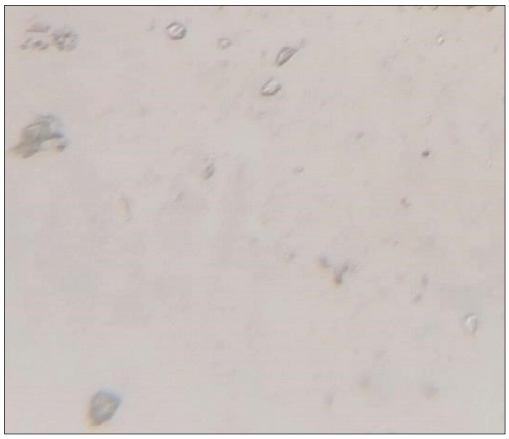
A: Normal Group



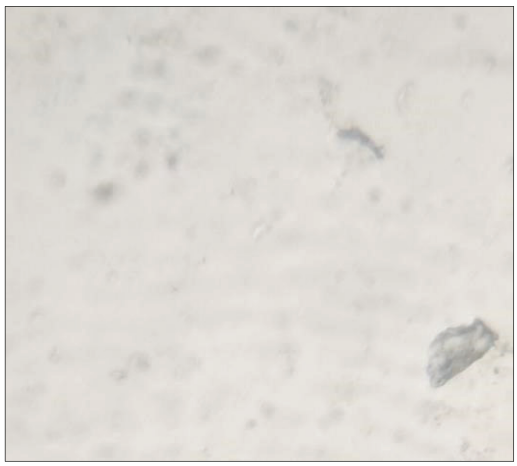
B: Diseased Control



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 + H_2O → Allantoin + H_2O_2 (catalyzed by Uricase) H_2O_2 + 4 Aminoantipyrine + Phenolic → Red Quinoneimine dye + H_2O (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.