



CHAPTER 6

RESULT AND DISCUSSION

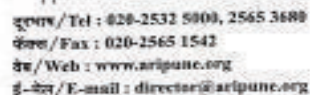


RESULT AND DISCUSSION

6.1 PHARMACOGNOSTICAL AND PHYTOPHYSICOCHEMICAL INVESTIGATIONS OF CRUDE DRUGS RESULTS

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

A



महाराष्ट्र जलसिंचन कॉरपोरेशन ऑफ इण्डिया
आधारकर अनुसंधान संस्थान
(विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार के अधिन स्थापित संस्थान)
पो. नं. आणकूर पथ, पुणे - ४११ ००४,
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संदर्भ: Reference: Your letter No. VJSM/VIPEER/2021-22 Res.06 dated 21/09/2021

समूह 4) -समूह Name of the sample: *Celastrus argenteus* (Kandit)

समं. वी. मास: Amount of sample; 1-4 fresh plant with flowers

ଅଫିସ୍ ଓ ଫିଲ୍ଡ : Date of the receipt : 22/09/2021

Report (AUFH 21-96): -

हाम्रै समुदाय वा वैज्ञानिक, आरोग्यकोषिक तथा तथा वैद्यकीयिक विधिहरूको माध्यमबाट विभिन्न प्रकारका रोगहरूको निदान र उपचार गर्न सक्नेछौं। हाम्रै समुदाय वा वैज्ञानिक, आरोग्यकोषिक तथा तथा वैद्यकीयिक विधिहरूको माध्यमबाट विभिन्न प्रकारका रोगहरूको निदान र उपचार गर्न सक्नेछौं।

The sample has been critically studied with the help of macroscopic, microscopic and Tasteronic characters. We hereby authenticate that the given sample belongs to the whole plant of *Cefissia argentea* L. (Family- Annonaceae).


दार्शनिक / Scientist
खोजीय दवायु प्रमाणित / Food & Drug Authentication
प्राणिकीय एवं पदार्थिकीय (प्राणिकीय एवं दवायु) समूह
Biochemistry and Pharmacology (Food & Drugs) Group

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The authors have no financial or personal relationships with other people or organizations that could inappropriately influence or bias the work reported.

B



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महाराष्ट्र अग्रोसिस्टम फॉर द कल्टिवेशन ऑफ सायन्स
आधारकर अनुसंधान संस्थान
(विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार के अधिन स्थापित संस्थान)
पो. ग. आगरकर रोड, पुणे - ४११ ००४.
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दिनांक / Date: 25/03/22

प्रमाणीकरण पत्र / AUTHENTICATION CERTIFICATE

नाम /Name: Ms. Jadhav Anuradha S.

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

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

नमूने का नाम /Name of the sample: Red Frangipani

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


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

Report (AUTH22/83):-

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

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

वैज्ञानिक / Scientist
औषधीय वनस्पति प्रमाणीकरण / Plant Drug Authentication
वैद्यकीय एवं द्रुगवैज्ञान (वनस्पति एवं अयुर्वेद) समूह
Biochem. & Dr. Pharmacology (Plants & Natural) Group


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

CONDITIONS AND REMARKS:

1. The party has submitted the sample as ARI 2. We ensure that party has obtained necessary permission from state/central government authority to use the plant material for research/academic purposes. 3. The sample has been obtained in careful analysis and examination of the sample only and in the enclosed material. 4. This report should be used only for academic and research purposes. It should not be used as an evidence of authenticity in any official government/academic/institutional or commercial. The institute shall not be bound to endorse the authenticity before any legal forum. 5. The contents of this report are confidential and being disclosed only to the party / supplier of sample. 6. The party must acknowledge the services (as provided/received) by ARI (software/report/publication/other) accordingly in.

C

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

6.1.2 Morphology and Specifications of *B. diffusa*, *P. rubra*, *C. argentea*Table No. 6.1: Morphology and physicochemical specifications of *B. diffusa*

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

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

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

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

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

6.1.3 Extraction

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

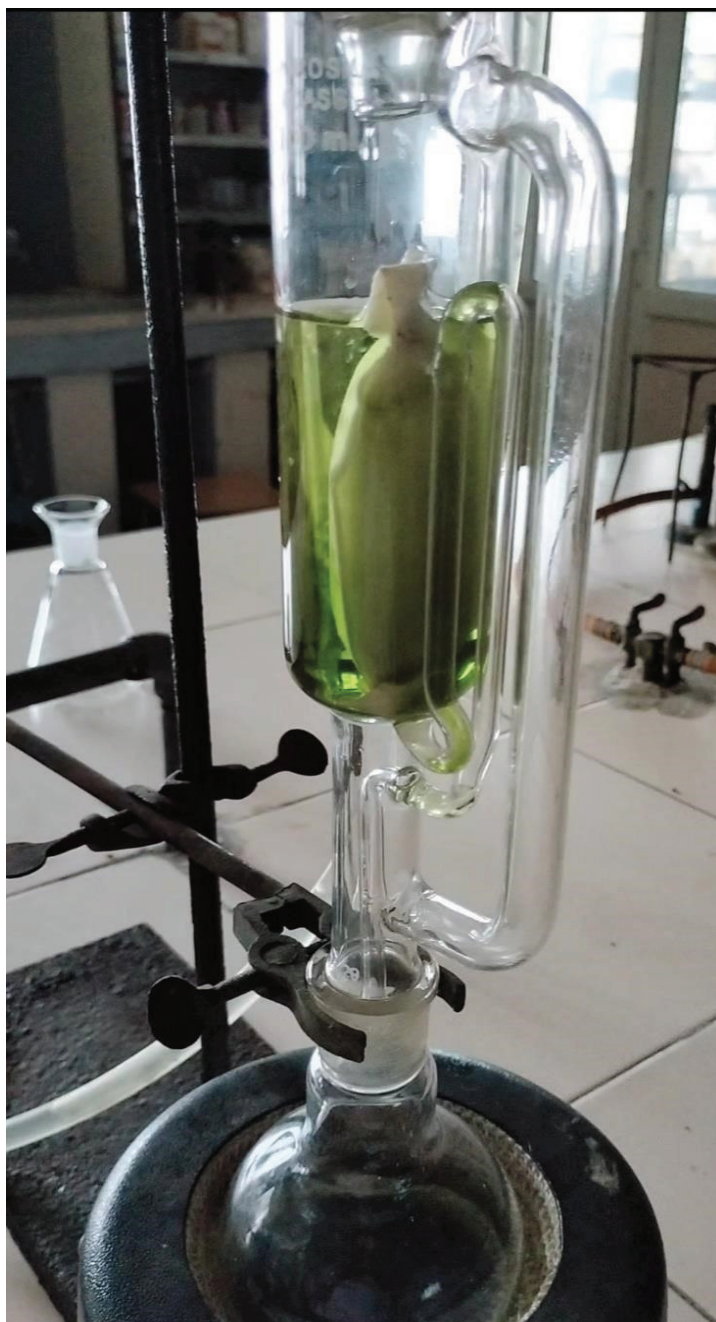


Figure No. 6.2: Soxhlet Extraction of *B. diffusa*



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



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

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

Sr. No.	Extracts	Colour	Consistency	% Yield
1.	<i>Boerhavia diffusa</i> leaves	Green	Semisolid	3.3
2.	<i>Plumeria Rubra</i> pods	Dark brown	Semisolid- Jelly like	24.66
3.	<i>Celosia argentea</i> seeds	Faint black	Sticky	4.96

Figure No. 6.5: Extract of *Boerhavia diffusa* leavesFigure No. 6.6 : Extract of *argentea* seedsFigure No. 6.7: Extract of *Plumeria Rubra* pods

6.1.4 Morphological Evaluation

A. *Boerhavia diffusa*

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



Figure No. 6.8: *Boerhavia diffusa* leaves

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

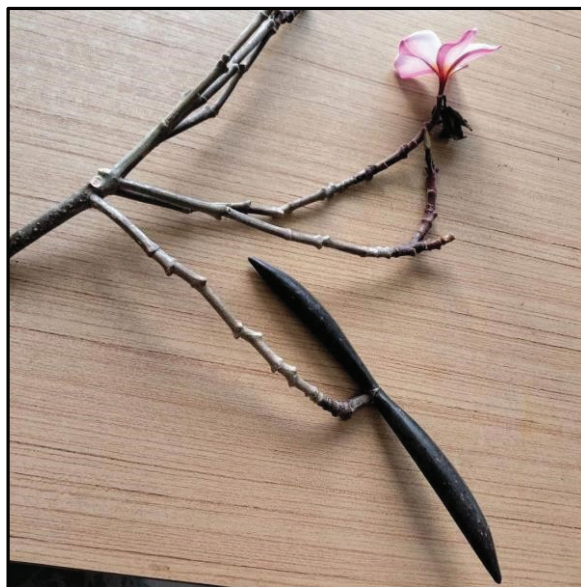


Figure No. 6.9: *Plumeria rubra* seed pods

C. Celosia argenteaseeds

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



Figure No. 6.10: *Celosia argentea* Linn seeds

6.1.5 Microscopic Evaluation

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

A. Boerhavia diffusa:

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

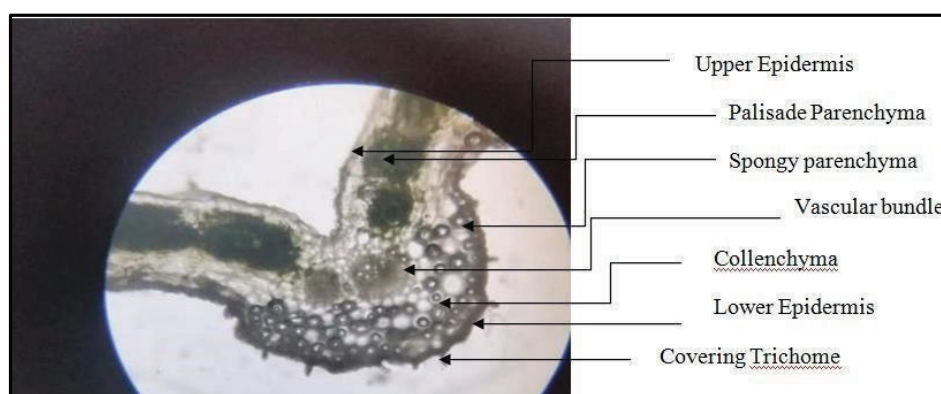
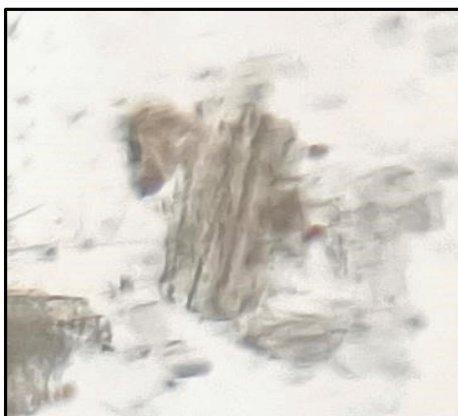
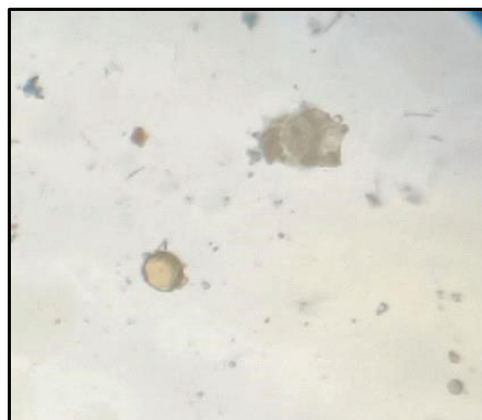
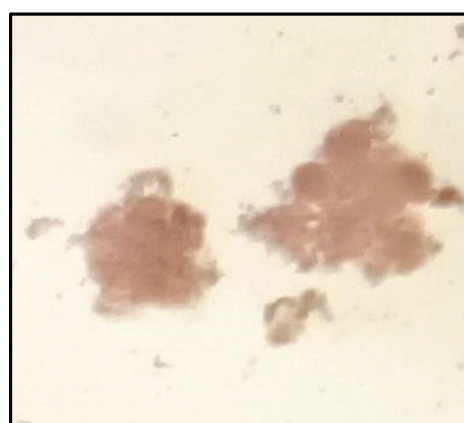
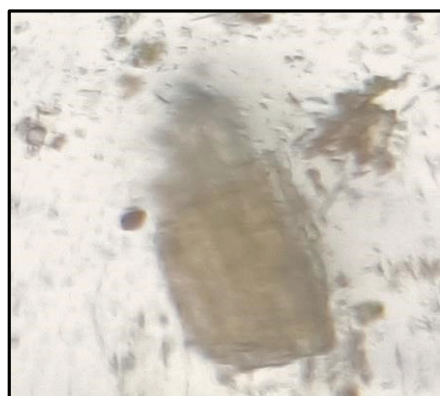


Figure No. 6.11: T. S. of *Boerhavia diffusa* leaf

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

**Vessel****Prismatic crystal****Covering Trichomes****Lignified parenchyma****Epidermal cells****Fibers****Figure No. 6.12: Powder Characteristics of *Boerhavia diffusa* whole plant**

B. Plumeria Rubra

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

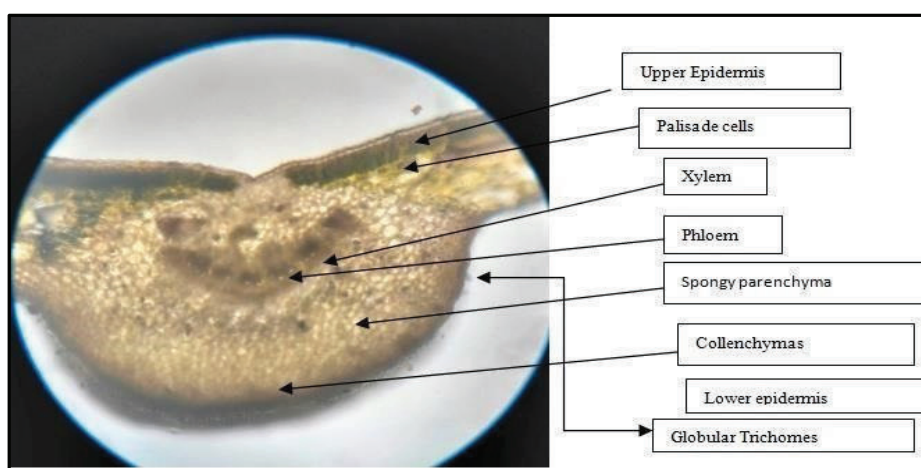


Figure No. 6.13: T. S. *Plumeria Rubra* leaf

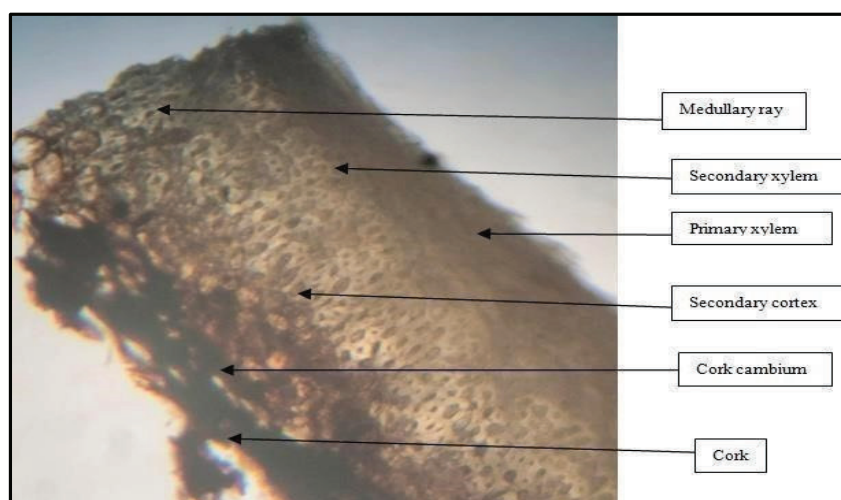


Figure No. 6.14: T. S. *Plumeria Rubra* seed pod shell

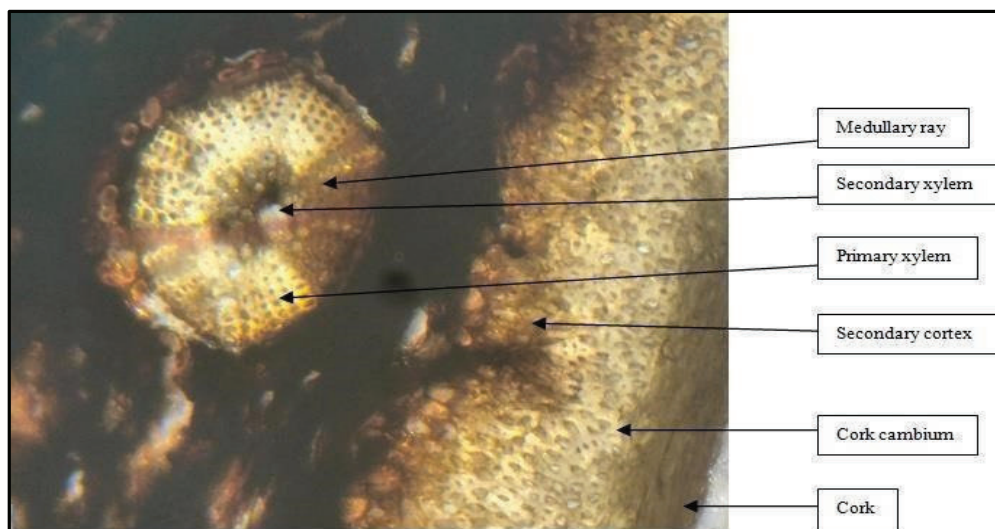


Figure No. 6.15: T. S. *Plumeria Rubra* seed pod showing medullary rays

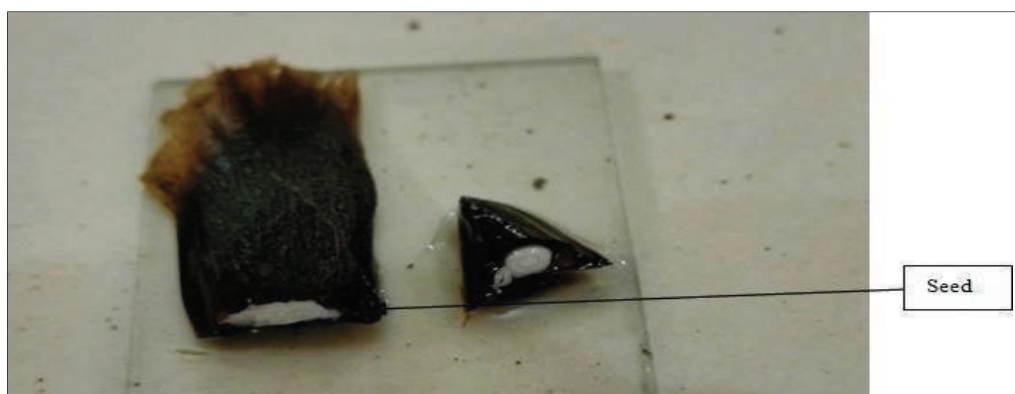
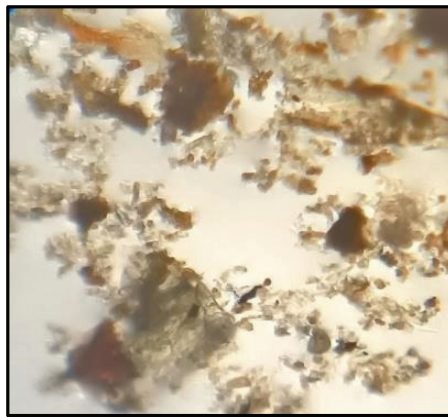


Figure No. 6.16: Images showing seed of *Plumeria Rubra* showing monocotyledon and embryo

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



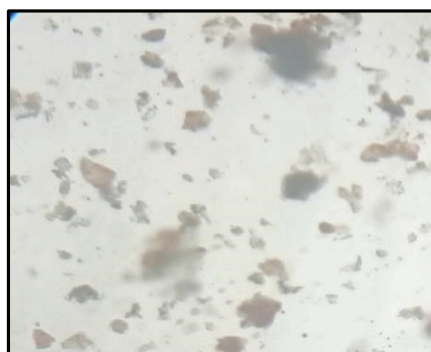
Unicellular trichomes



Lignified cells



Vascular bundle



Starch grains

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

C. *Celosia argentea*

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



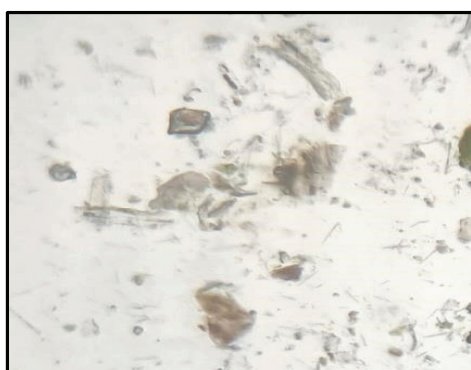
Figure No. 6.18: T. S. of *Celosia argentea* Linn seed cotyledon showing reticulate parenchyma with starch grains

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



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

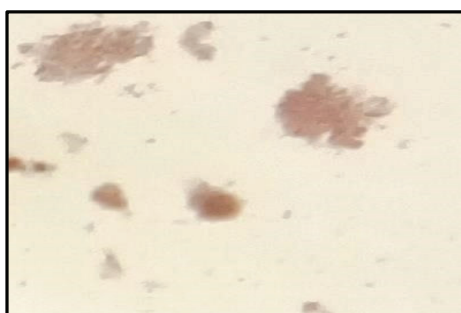
The microscopic examination of *Celosia argentea* Linn seed powder revealed various distinct features including starch grains, aleurone grains, cotyledon cells of testa, parenchymatous cells of cotyledons, and oil glands.



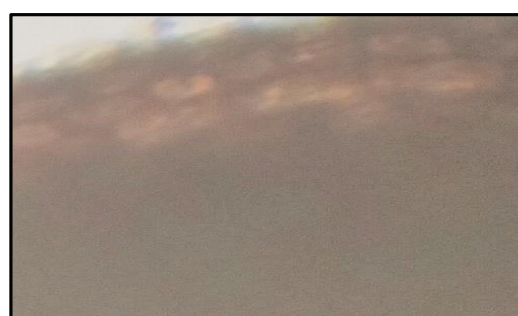
Starch grains



Aleurone grains



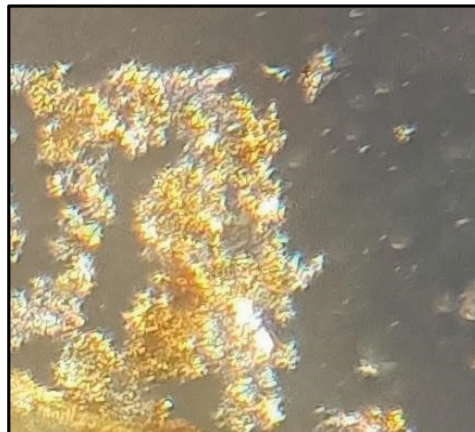
Lignified cells



Epidermal cells of testa



Parenchymatous cells of cotyledons



Oil glands

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

6.1.6 Physicochemical Evaluation

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

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

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

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

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

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

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

6.1.7 Fluorescence Analysis

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

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



Figure No. 6.21: UV Chamber for TLC visualization

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

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

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

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

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

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

6.1.8 Phytochemical Evaluation

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

- A. *Boerhavia diffusa*** Preliminary phytochemical screening of the aqueous extract of *Boerhavia diffusa* leaves revealed the presence of alkaloids, glycosides, saponins, phytosterols, tannins, and flavonoids. Carbohydrates, proteins & amino acids, fixed oils & fats, gums & mucilage, and volatile oil were absent.
- B. *Plumeria Rubra*** Preliminary phytochemical screening of the aqueous extract of *Plumeria Rubra* pods indicated the presence of alkaloids, carbohydrates, glycosides, phytosterols, fixed oils & fats, flavonoids, and gums & mucilage. Saponins, proteins & amino acids, tannins, and volatile oil were absent.
- C. *Celosia argentea*** Preliminary phytochemical screening of the aqueous extract of *Celosia argentea* seeds demonstrated the presence of alkaloids, carbohydrates, glycosides, saponins, proteins & amino acids, phytosterols, tannins, flavonoids, and gums & mucilage. Fixed oils & fats and volatile oil were absent.
- D.** Phytochemical constituents detected in crude extracts of *Boerhavia diffusa* leaves, *Plumeria Rubra* pods and *Celosia argentea* seeds are shown in table 6.9.

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

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

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

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

6.1.9 pH of Extracts:

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

Table No. 6.10: pH of Extracts

Sr. No.	Extract	pH
1.	<i>Boerhavia diffusa</i> leaves	7.0
2.	<i>Plumeria Rubra</i> pods	7.5
3.	<i>Celosia argentea</i> seeds	7.5

6.1.10 Optimization of TLC Solvent System

6.1.10.1. *Boerhavia diffusa*

Best solvent system for *Boerhavia diffusa*

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

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

High Performance Thin Layer Chromatography for Eupalitin

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

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

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

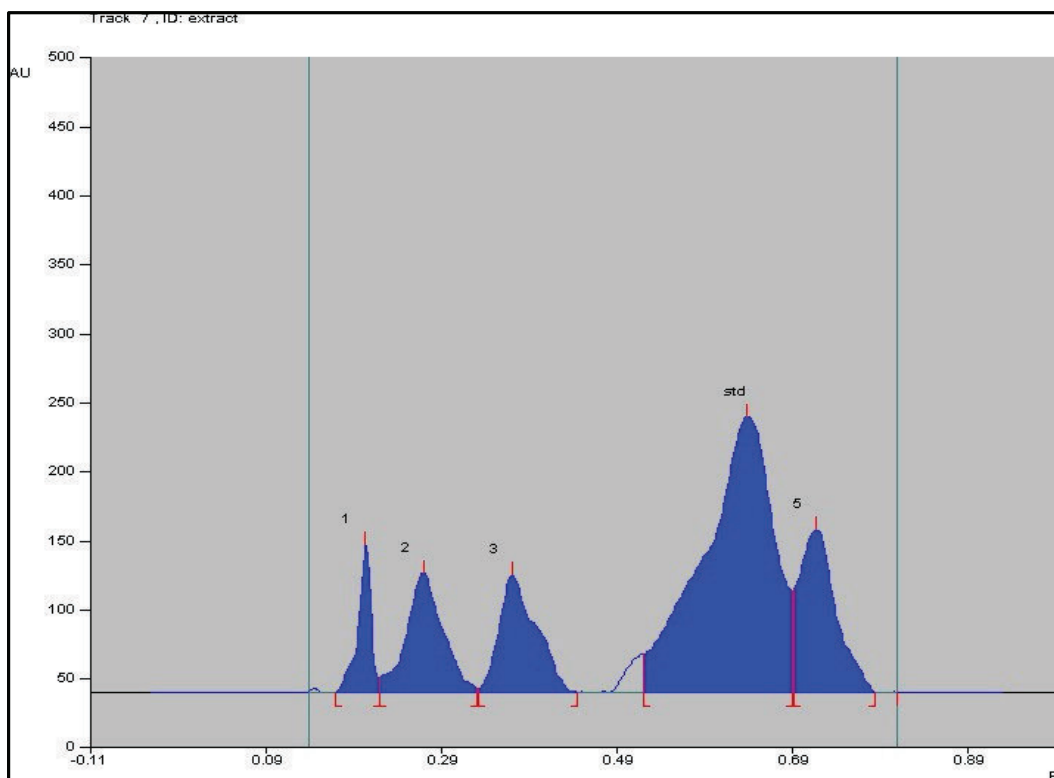
Figure No. 6.22: Densitogram of *B. diffusa*

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

Track 7, ID: extract										
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.17 Rf	0.2 AU	0.20 Rf	107.3 AU	17.94 %	0.22 Rf	11.3 AU	1315.3 AU	5.31 %	unknown *
2	0.22 Rf	11.4 AU	0.27 Rf	86.9 AU	14.54 %	0.33 Rf	2.6 AU	2996.7 AU	12.10 %	unknown *
3	0.33 Rf	2.8 AU	0.37 Rf	85.7 AU	14.34 %	0.44 Rf	0.2 AU	2996.1 AU	12.10 %	unknown *
4	0.52 Rf	27.8 AU	0.64 Rf	200.0 AU	33.46 %	0.69 Rf	74.0 AU	13278.6 AU	53.63 %	std
5	0.69 Rf	74.4 AU	0.72 Rf	117.9 AU	19.71 %	0.78 Rf	0.0 AU	4173.0 AU	16.85 %	unknown *

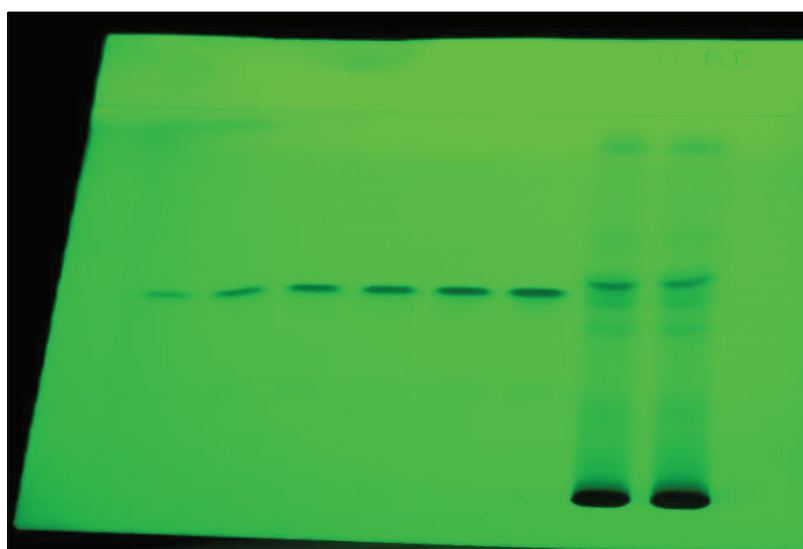


Figure No. 6.23: Image of *Boerhavia diffusa* leaves extract at 254 nm

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

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

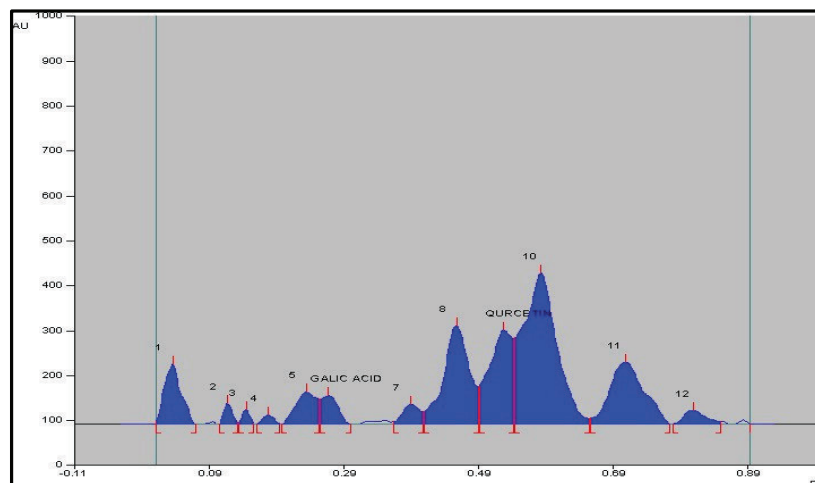


Figure No. 6.24: Densitogram *B. diffusa* of gallic acid and Quercetin

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

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

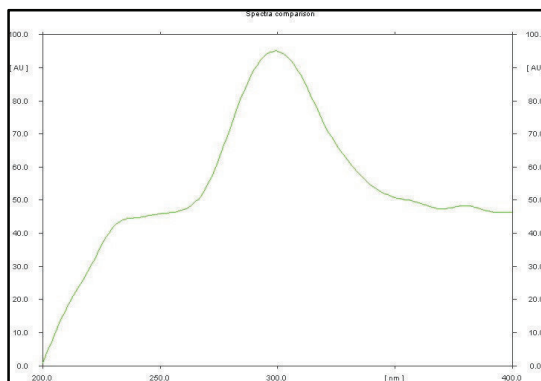
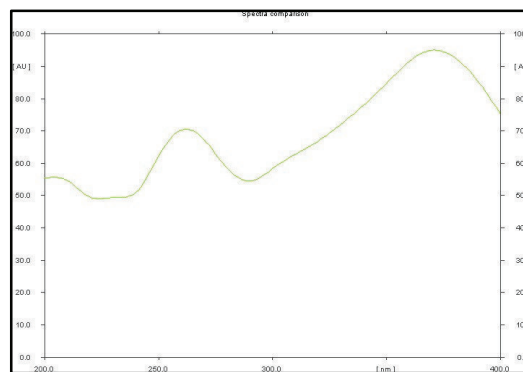
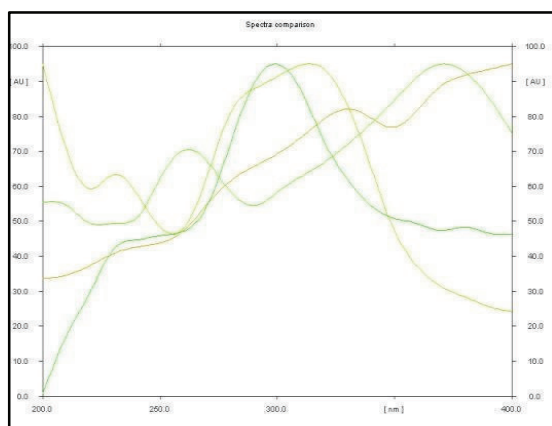
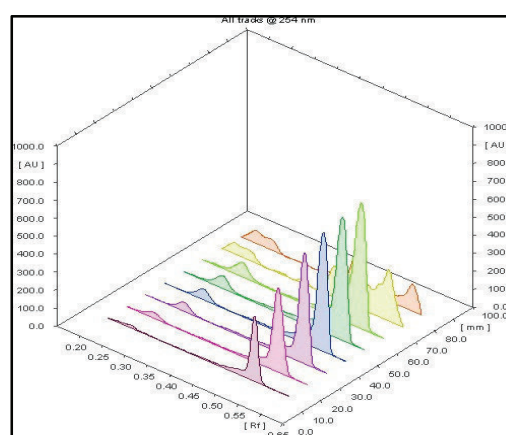
**A: Galic Acid****B: Quercetin****C: Gallic acid and Quercetin with Extract****D: 3D spectra of Standard and extract**

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

6.1.10.2 *Plumeria Rubra***Table No. 6.14: Phytochemicals of *P.rubra***

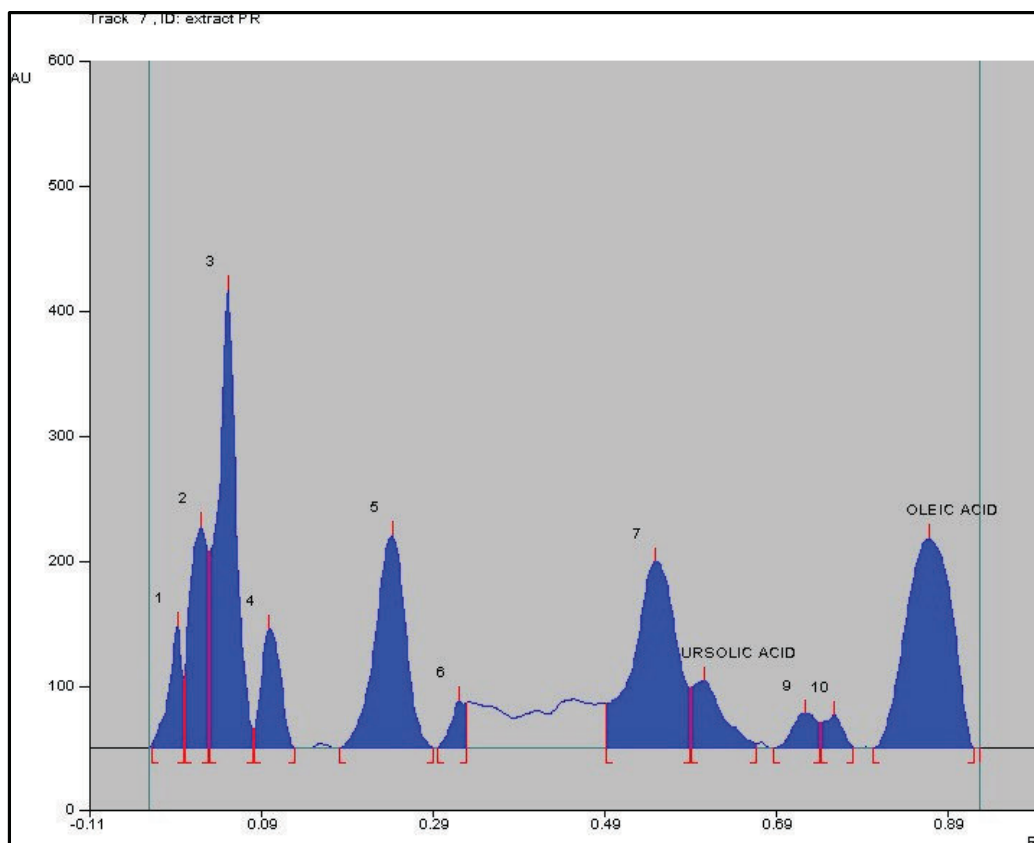
Sr. No.	Name Phyto constituent	Rf	Amount of phytoconstituents (%)
1.	Ursolic acid	0.61	4.60
2.	Oleic acid	0.57	21.71
3.	Lupeol	0.68	14

Plumeria Rubra**Best solvent system for *Plumeria Rubra***

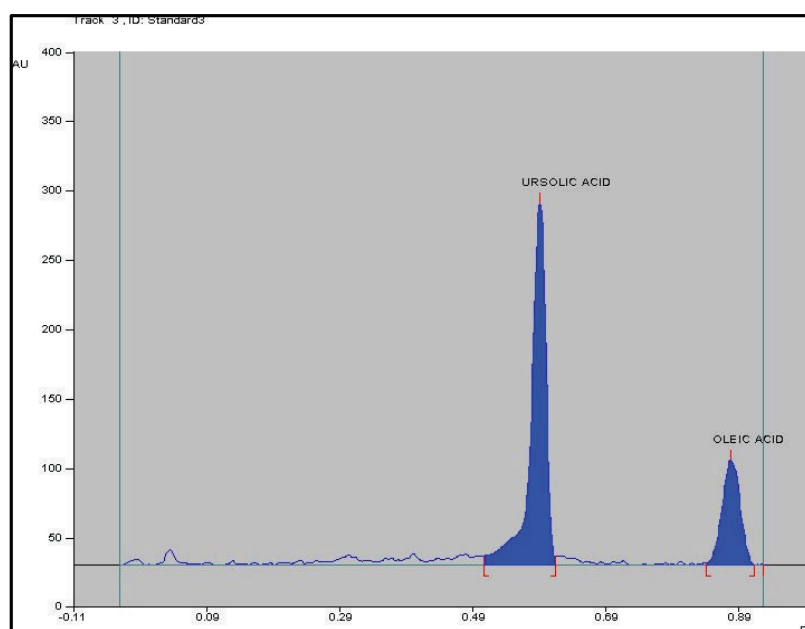
The TLC studies of aqueous extract show best separation using Toluene: Ethyl acetate: Formic acid (8:2:0.1 v/v) as a mobile phase.

High Performance Thin Layer Chromatography

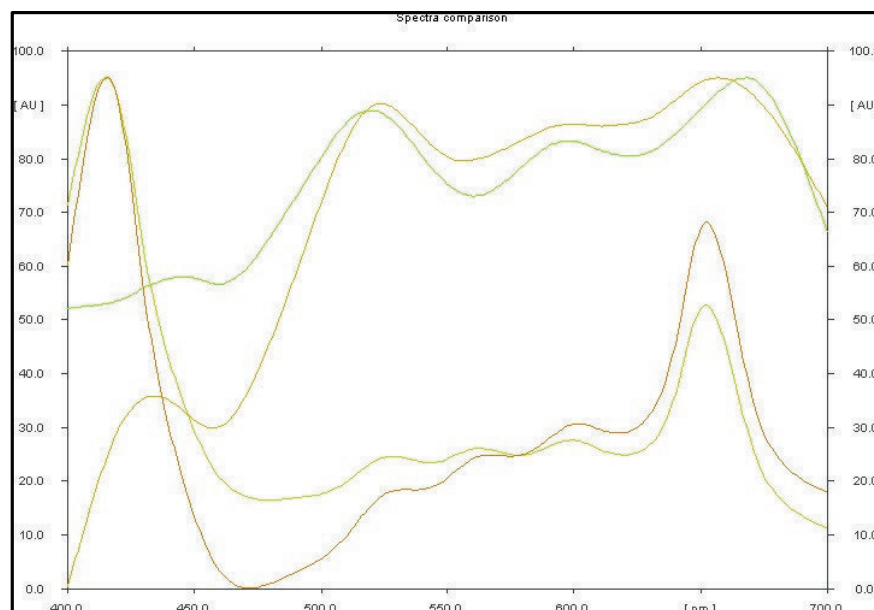
The obtained densitogram showed 10 spots at Rf 0.02, 0.05, 0.10, 0.24, 0.32, 0.55, 0.61, 0.72, 0.76 and 0.87 in UV light at 500 nm. The spot at Rf 0.61 was dark brown and Rf 0.87 was yellowish cream fluorescence under UV radiation (366 nm). Ursolic acid and oleic acid has been reported the main active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



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



B: Densitogram of standard Ursolic acid and Oleic acid



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

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

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

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

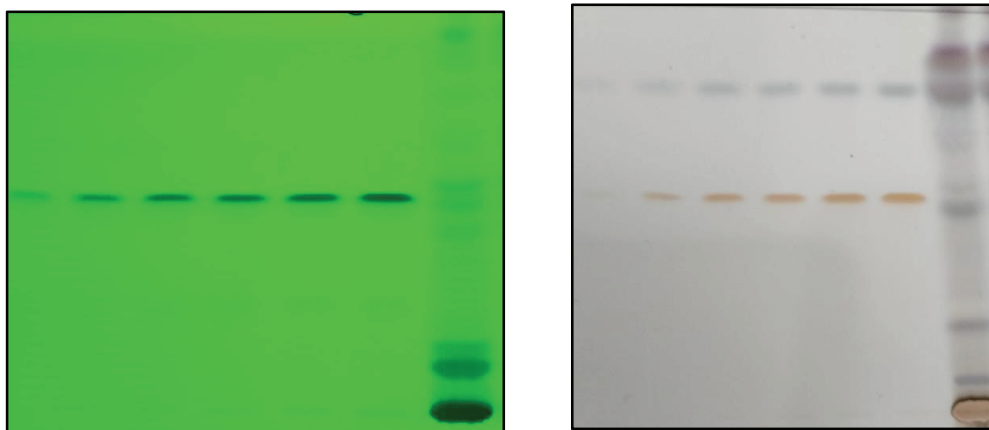


Figure No. 6.27: Image of *Plumeria Rubra* seed pod extract at 254 nm and after derivatization

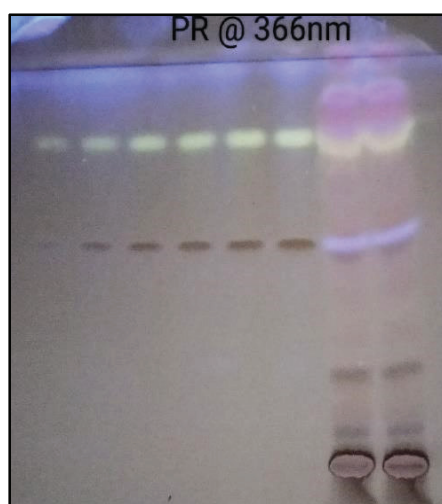


Figure No. 6.28: Image of *Plumeria Rubra* seed pod extract at 366 nm

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

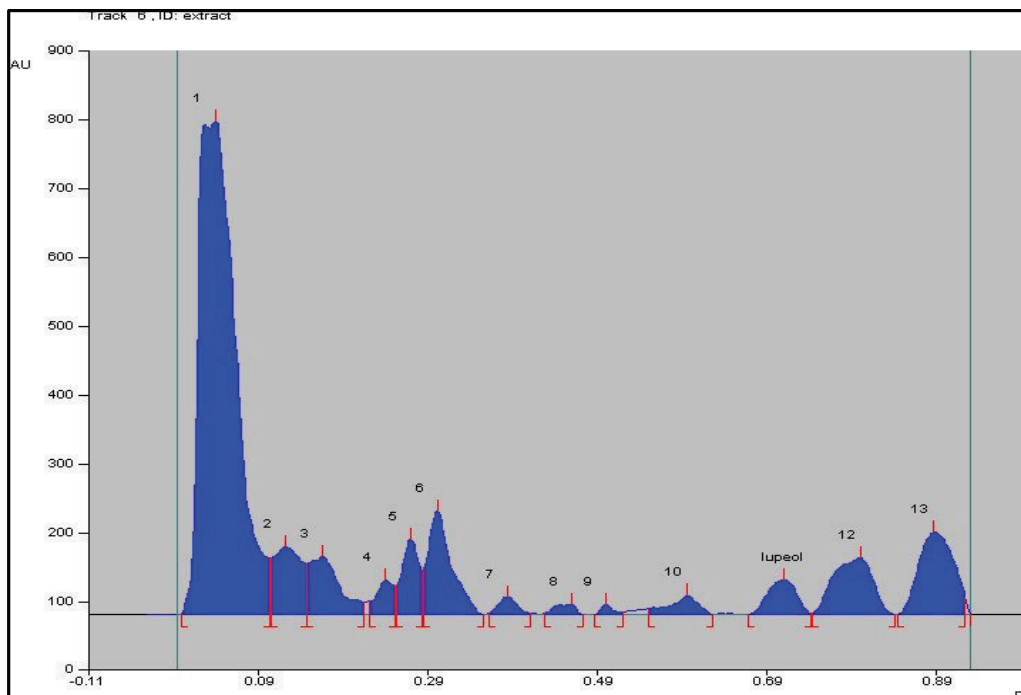


Figure 6.29: Densitogram of *Plumeria Rubra* showing presence of Lupeol

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

Track 6, ID: extract										
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.00 Rf	1.5 AU	0.04 Rf	716.7 AU	46.38 %	0.10 Rf	33.1 AU	26370.1 AU	54.58 %	unknown *
2	0.10 Rf	82.9 AU	0.12 Rf	98.8 AU	6.39 %	0.15 Rf	73.9 AU	2727.3 AU	5.65 %	unknown *
3	0.15 Rf	74.1 AU	0.17 Rf	84.7 AU	5.48 %	0.21 Rf	18.3 AU	2520.8 AU	5.22 %	unknown *
4	0.22 Rf	18.8 AU	0.24 Rf	50.2 AU	3.25 %	0.25 Rf	40.9 AU	858.7 AU	1.78 %	unknown *
5	0.25 Rf	42.2 AU	0.27 Rf	109.0 AU	7.05 %	0.28 Rf	34.0 AU	1846.4 AU	3.82 %	unknown *
6	0.28 Rf	66.1 AU	0.30 Rf	151.0 AU	9.77 %	0.35 Rf	0.2 AU	3468.7 AU	7.18 %	unknown *
7	0.36 Rf	2.2 AU	0.38 Rf	25.2 AU	1.63 %	0.41 Rf	1.7 AU	470.6 AU	0.97 %	unknown *
8	0.43 Rf	2.0 AU	0.46 Rf	14.4 AU	0.93 %	0.47 Rf	0.5 AU	312.5 AU	0.65 %	unknown *
9	0.49 Rf	0.2 AU	0.50 Rf	15.1 AU	0.98 %	0.52 Rf	2.8 AU	170.1 AU	0.35 %	unknown *
10	0.55 Rf	9.0 AU	0.59 Rf	26.3 AU	1.83 %	0.63 Rf	0.1 AU	768.4 AU	1.59 %	unknown *
11	0.67 Rf	0.1 AU	0.71 Rf	50.2 AU	3.25 %	0.74 Rf	2.0 AU	1442.8 AU	2.99 %	lupeol
12	0.74 Rf	2.2 AU	0.80 Rf	82.0 AU	5.31 %	0.84 Rf	0.1 AU	3219.4 AU	6.66 %	unknown *
13	0.84 Rf	0.3 AU	0.89 Rf	119.6 AU	7.74 %	0.92 Rf	25.9 AU	4134.4 AU	8.56 %	unknown *

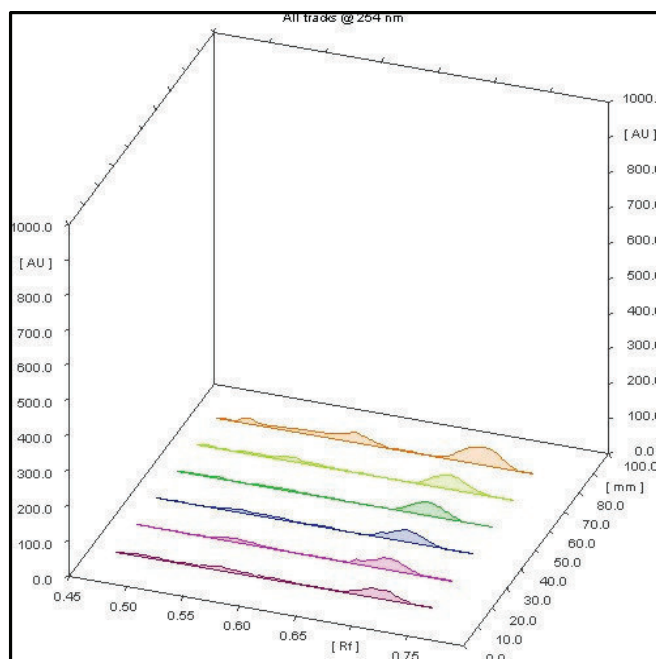


Figure 6.30: 3D spectra of Lupeol and *Plumeria Rubra* extract

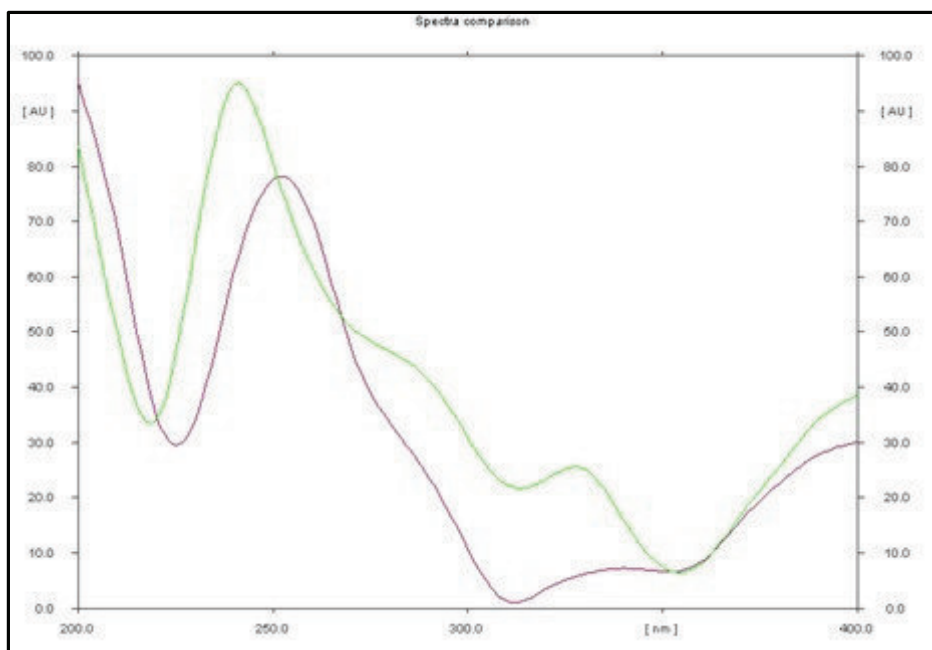


Figure 6.31: Overlain spectra for Lupeol and *Plumeria Rubra* Extract extract

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

<div>Show all of selected substance</div> <div>Clear all</div>						
Track	Rf	Assigned Substance	Max. Signal	Display	r(s,m)	r(m,e)
1	0.73	Lupeol	106 AU @ 200 nm	<input checked="" type="checkbox"/>	0.999928	1.000000
2	0.68	AutoGenerated9	66 AU @ 200 nm	<input type="checkbox"/>	0.999828	0.999990
2	0.71	Lupeol	84 AU @ 200 nm	<input type="checkbox"/>	0.999830	0.999921
3	0.68	AutoGenerated9	113 AU @ 200 nm	<input type="checkbox"/>	0.998130	0.998920
4	0.68	STD	139 AU @ 241 nm	<input checked="" type="checkbox"/>	0.999012	0.999935
5	0.67	STD	299 AU @ 400 nm	<input type="checkbox"/>	0.999874	0.999968
6	0.73	Lupeol	145 AU @ 200 nm	<input type="checkbox"/>	0.999946	0.999984

6.1.10.3. *Celosia argentea*

Best solvent system for *Celosia argentea*

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

Table No. 6.18: Phytochemicals present in *C. argentea*

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

High Performance Thin Layer Chromatography

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

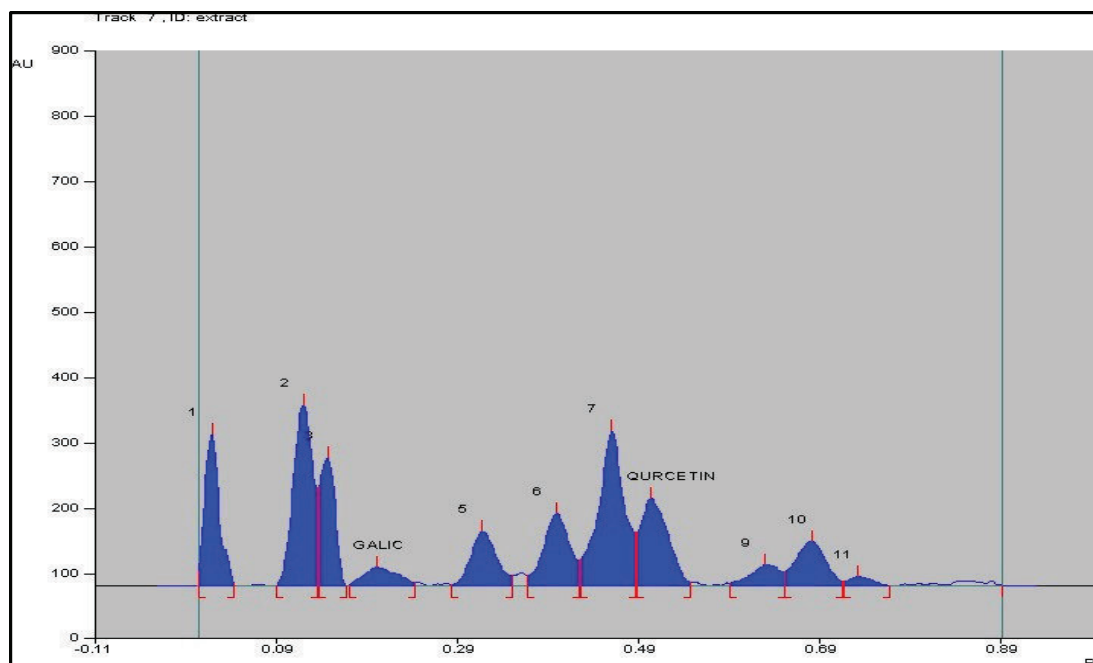


Figure No. 6.32: Densitogram *C. argentea*

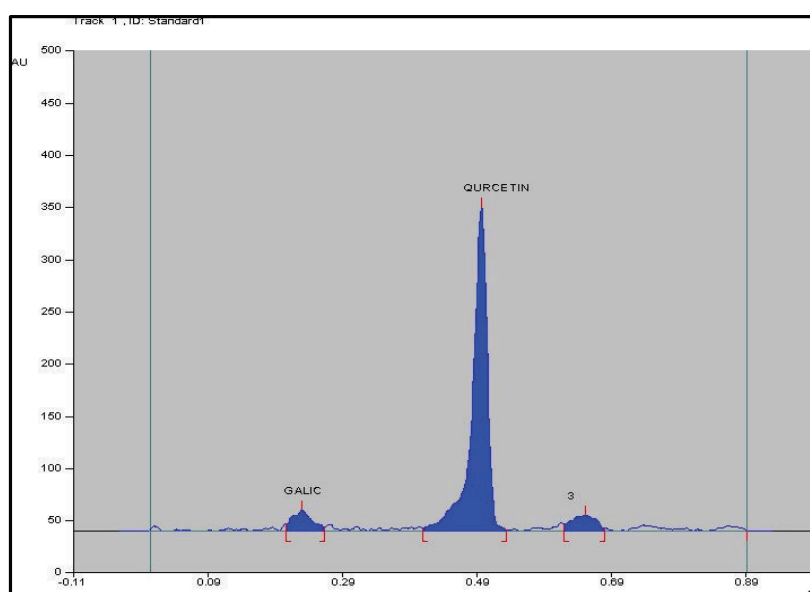
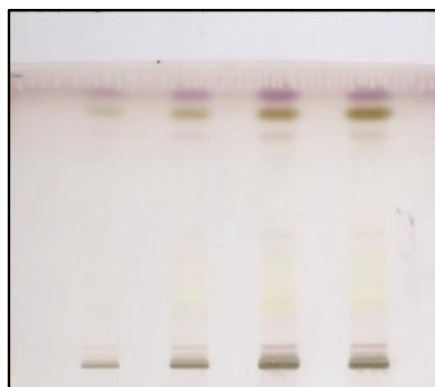


Figure No. 6.33: HPTLC densitogram of Aq. Extract of *Celosia argentea* seeds
extract at 430 nm

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

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

At Visible (Spraying reagent: Anisaldehyde sulphuric acid reagent)

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

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

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

At 366 nm (After Spraying reagent)



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

At Visible (Spraying reagent: Anisaldehyde sulphuric acid reagent)

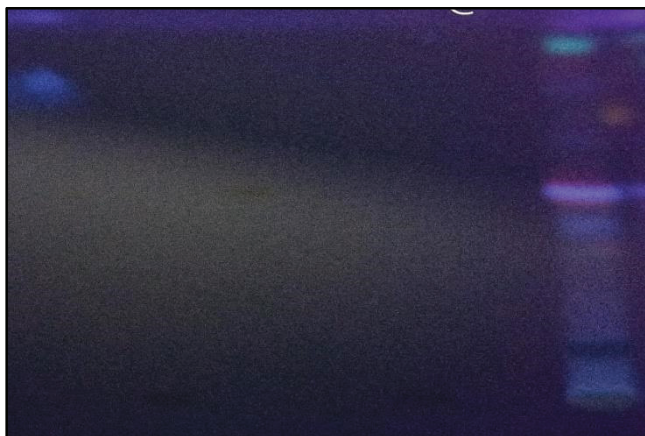


Figure No. 6.36: White Remission Image *Boerhavia diffusa* leaves, *Plumeria Rubra* pods and *Celosia argentea* seeds extract 366nm

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

6.1.11 Fourier-transform infrared spectroscopy (FTIR)

A. *Boerhavia diffusa*

The FTIR spectrum of aqueous extract of *Boerhavia diffusa* leaves is shown in Figure 6.37.

The interpretation of functional IR bands is shown in Table 6.20.

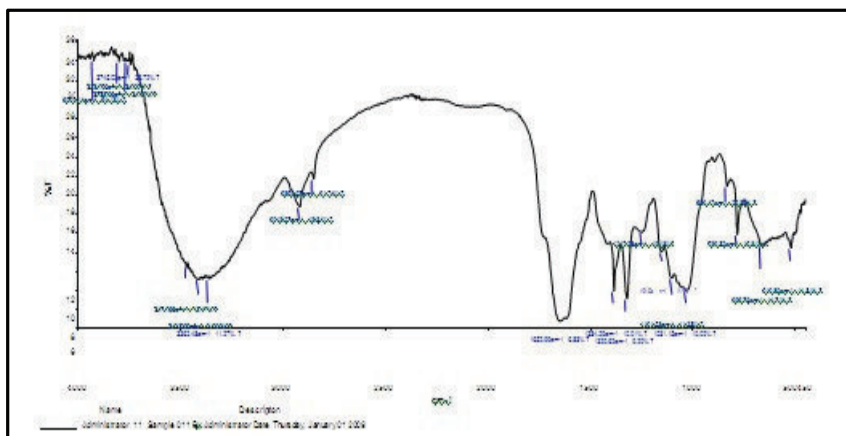


Figure 6.37: I.R. Spectra of aqueous extract of *Boerhavia diffusa* leaves

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

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

B. *Plumeria Rubra*

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

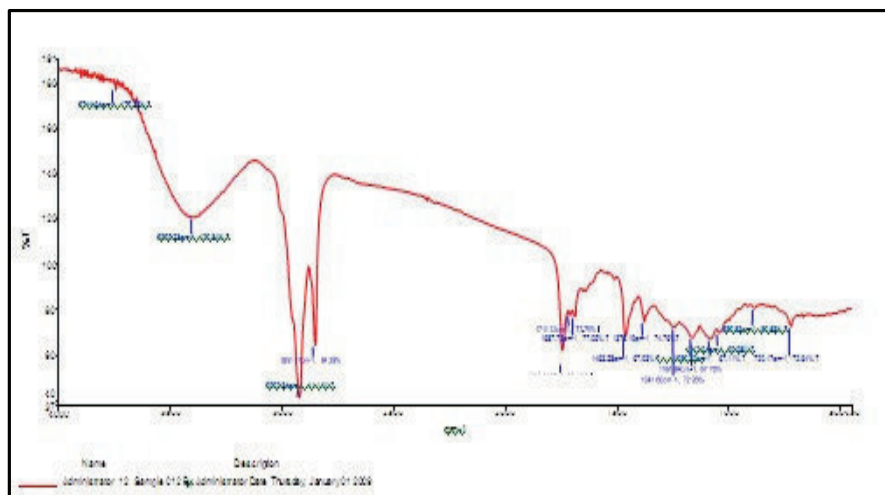


Figure No. 6.38: I.R. Spectra of aqueous extract of *Plumeria Rubra* pods

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

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

C. *Celosia argentea*

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

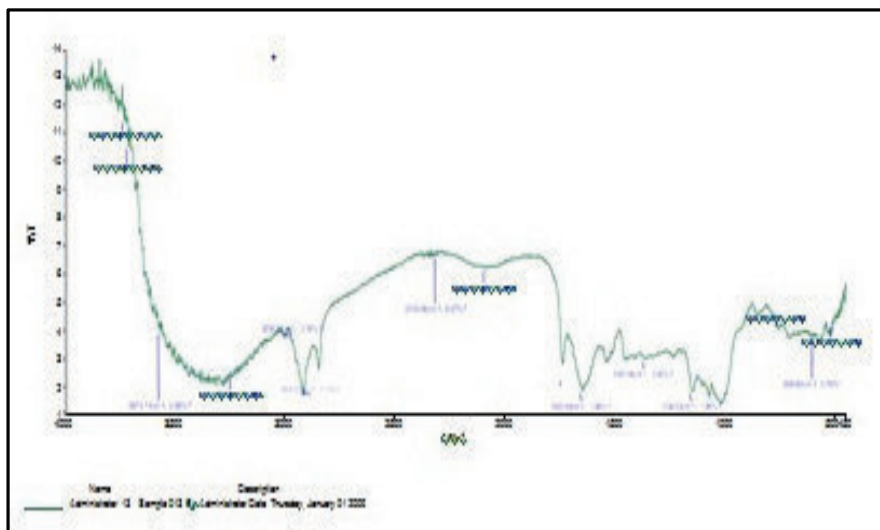


Figure No. 6.39: I.R. Spectra of aqueous extract of *Celosia argentea* seeds

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

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

Conclusion

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

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

6.1.12 B. HPTLC METHOD DEVELOPMENT AND VALIDATION

Results

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

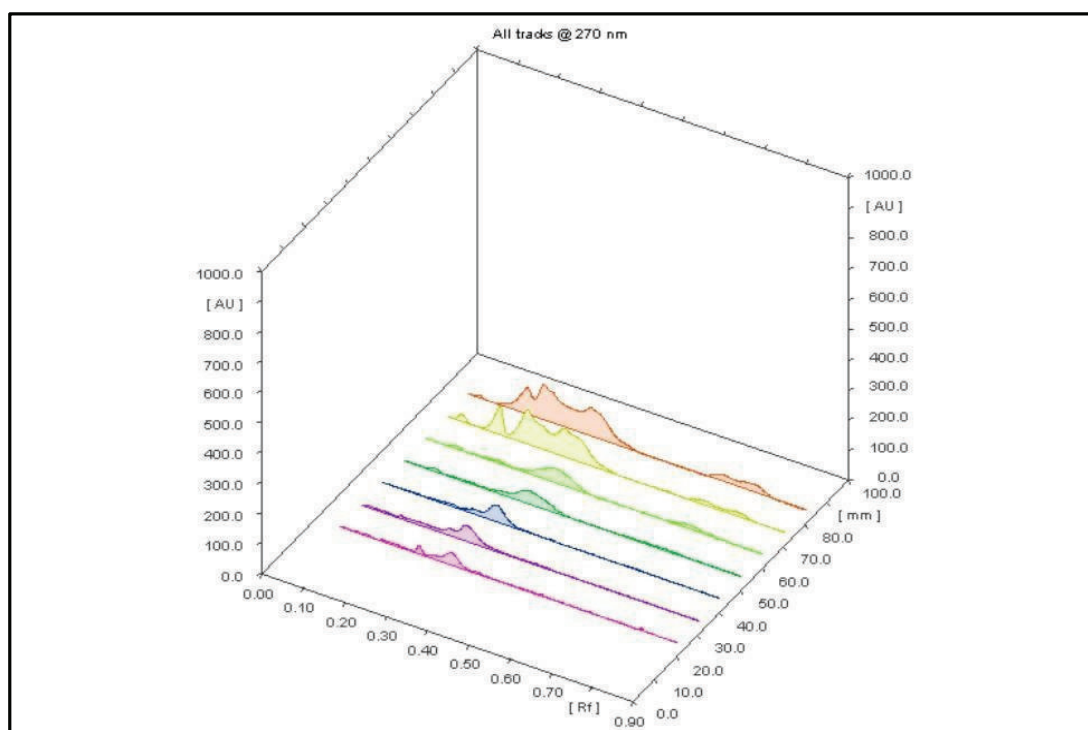


Figure No. 6.40: In situ HPTLC spectral of plant extract

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

HPTLC Method Validation

6.1.12.1 Linearity

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

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

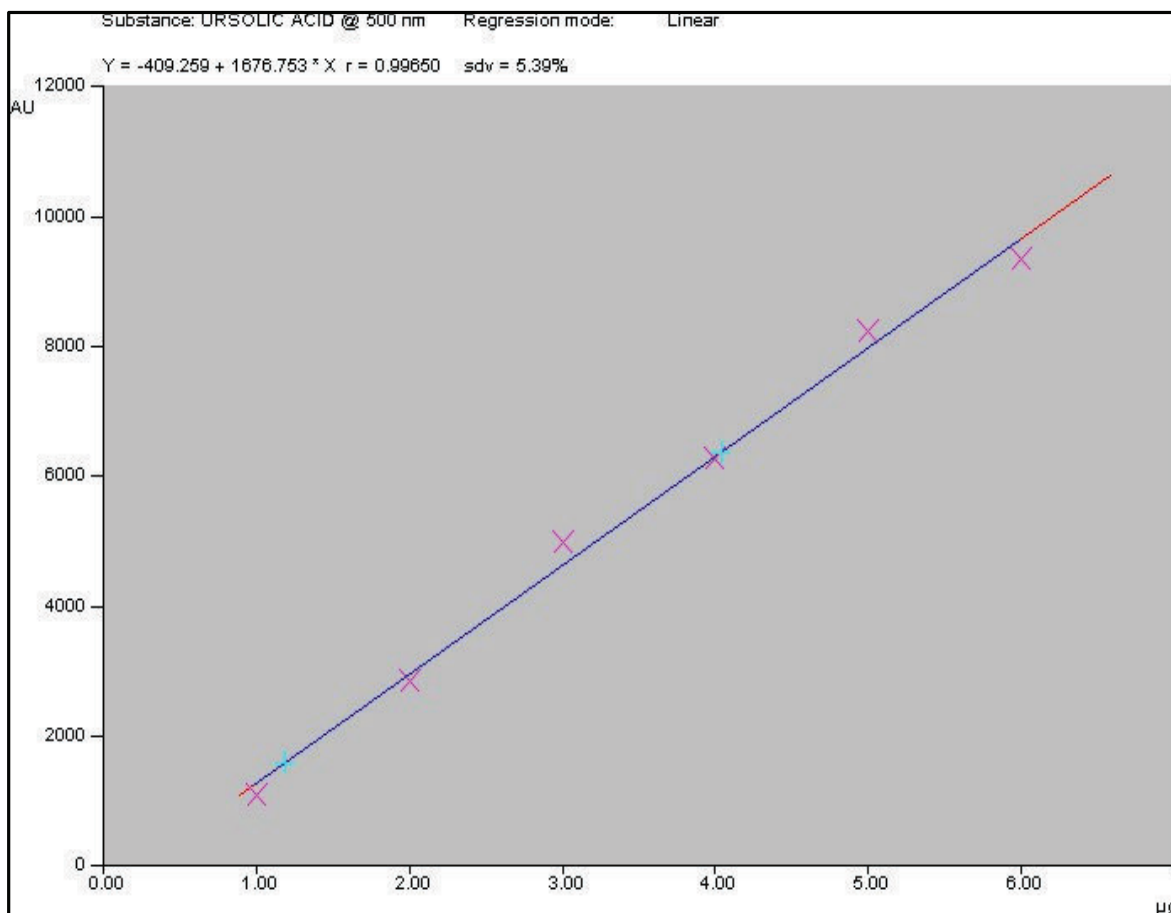


Figure No. 6.41: Linearity of plant extract

Table No. 6.23: Linear regression data for calibration plot

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

6.1.12.2 Specificity

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

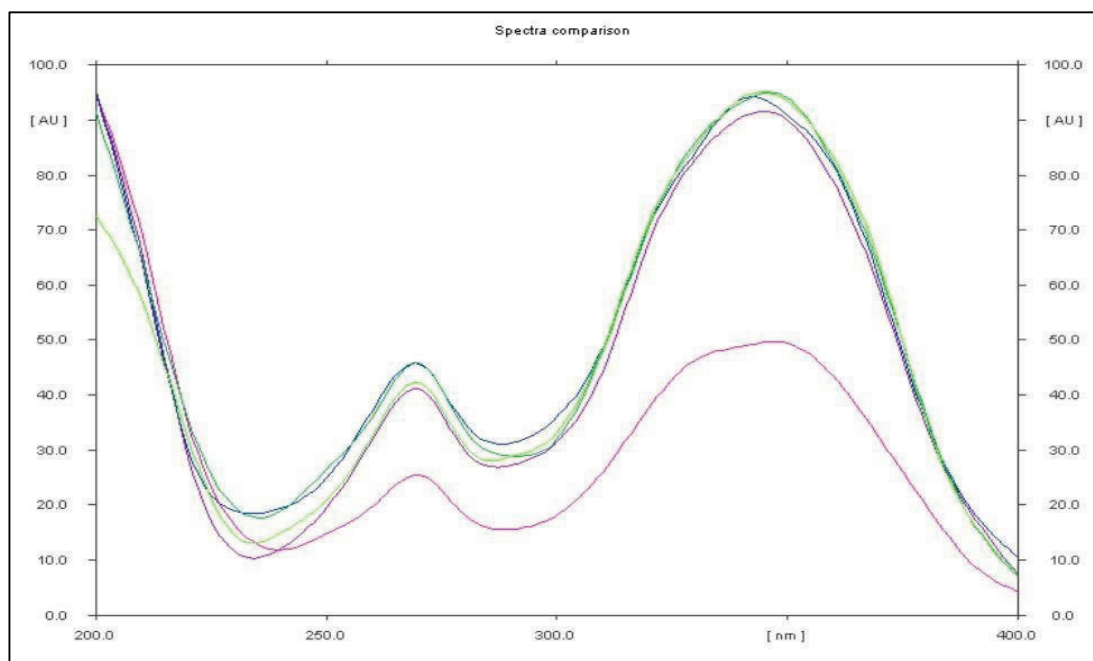


Figure No. 6.42: Overlay spectra of Standard and Extract Precision

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

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

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

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

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

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

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

6.1.12.4 Accuracy

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

Table No. 6.26: Results of Accuracy

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

6.1.12.5 Robustness

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

Table No. 6.27: Robustness

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

Conclusion

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

6.2 IN VITRO LITHOLYTIC ACTIVITY

Results:

6.2.1 Preliminary screening of Individual plant for their litholytic activity:

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

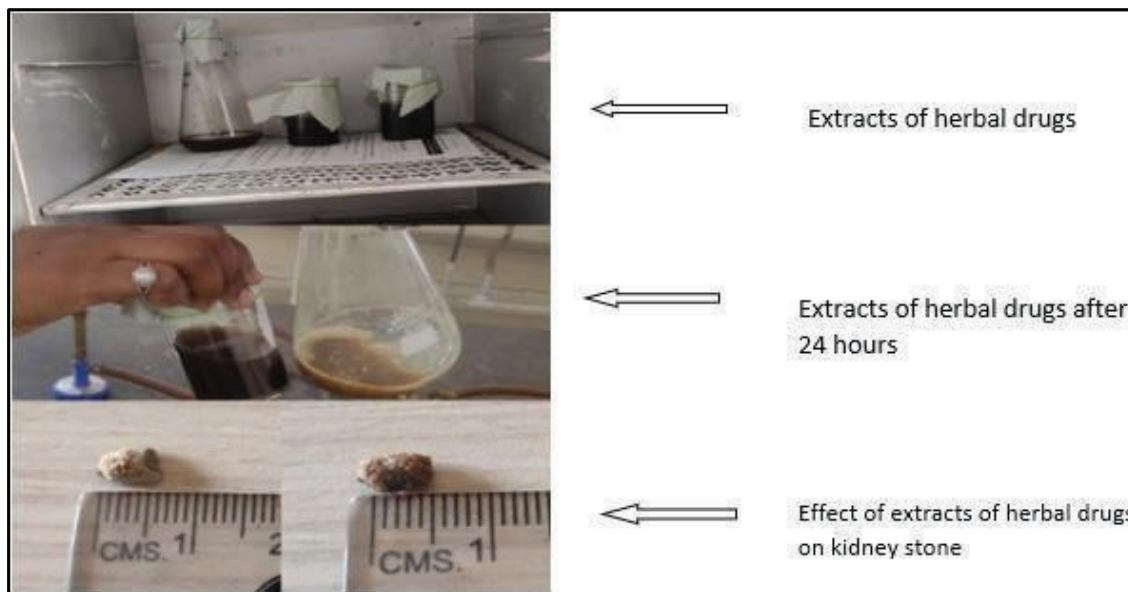


Figure No. 6.43: In vitro kidney stone dissolution method

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

Table No. 6.28: In vitro kidney stone dissolution

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

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

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

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

Table No. 6.29: Optimization of extract combination stone dissolution

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

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

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

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

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

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

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

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

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

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

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

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

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

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

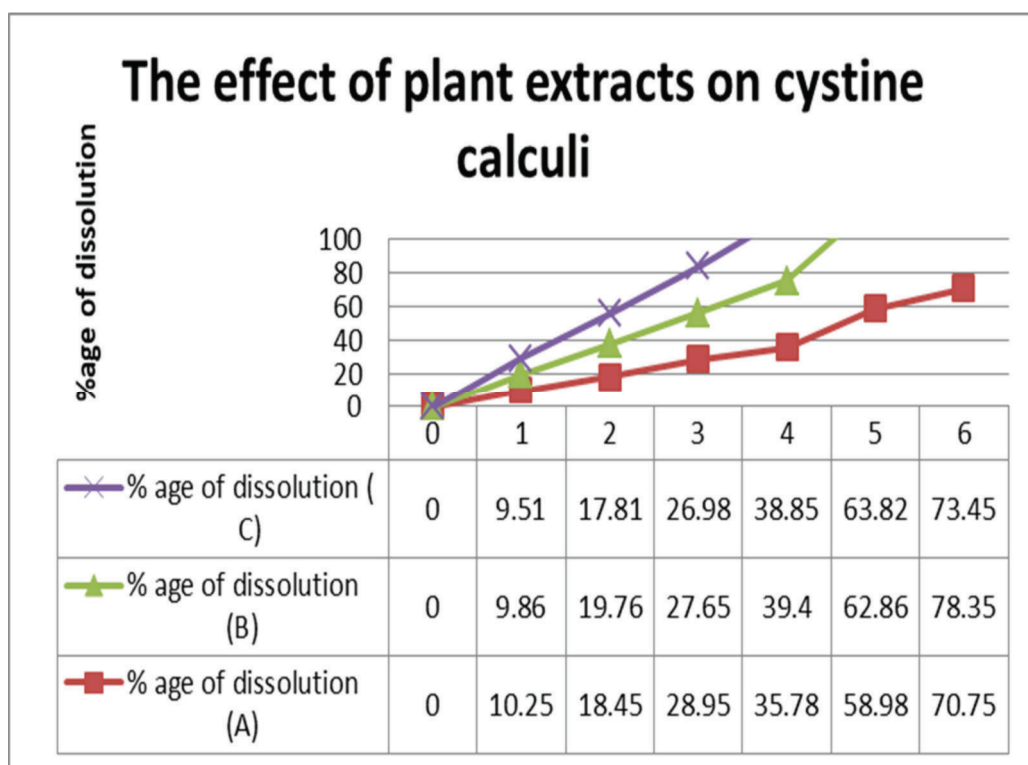


Figure No. 6.44: The effect of plant extracts on cystine calculi

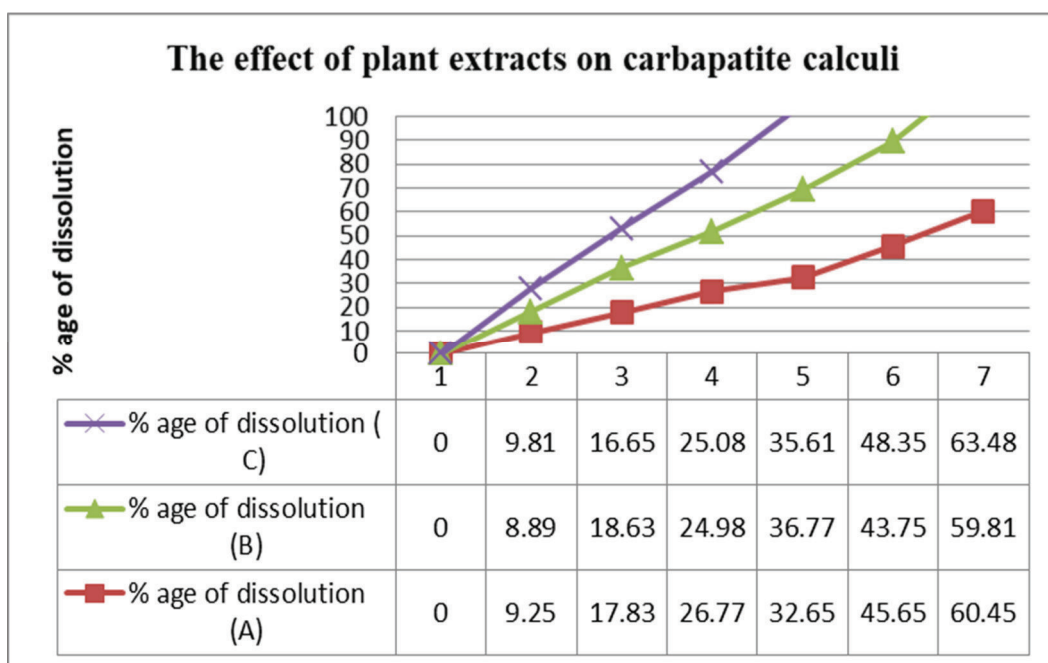


Figure No. 6.45: The effect of plant extracts on carbapatite calculi

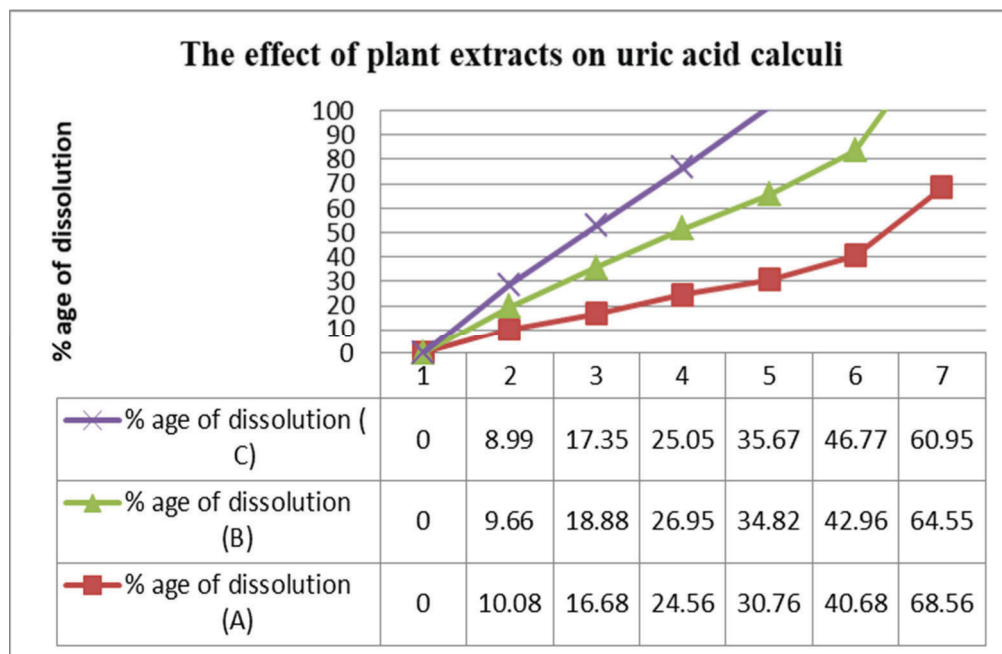


Figure No. 6.46: The effect of plant extracts on uric acid calculi

Conclusion

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

6.3 IN-VIVO STUDIES Results***In-Vivo* Anti-Urolithiatic Activity****6.3.1 Urine Analysis****i) Estimation of urine volume and pH****Table No. 6.33: Estimation of urine volume and pH**

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

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

*P ≤ 0.05 ,

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

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

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

6.3.2 Estimation of Calcium, Oxalate, phosphorus and magnesium in Urine**Table No. 6.34: Estimation of calcium, oxalate, phosphorus and magnesium in urine**

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

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

* $P \leq 0.05$,

** $P \leq 0.01$ and*** $P \leq 0.001$ compared with disease control

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

6.4 SERUM ANALYSIS:

6.4.1 Estimation of Creatinine and uric acid in serum

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

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

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

* $P \leq 0.05$,

** $P \leq 0.01$ and*** $P \leq 0.001$ compared with disease control

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

Serum Parameters

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

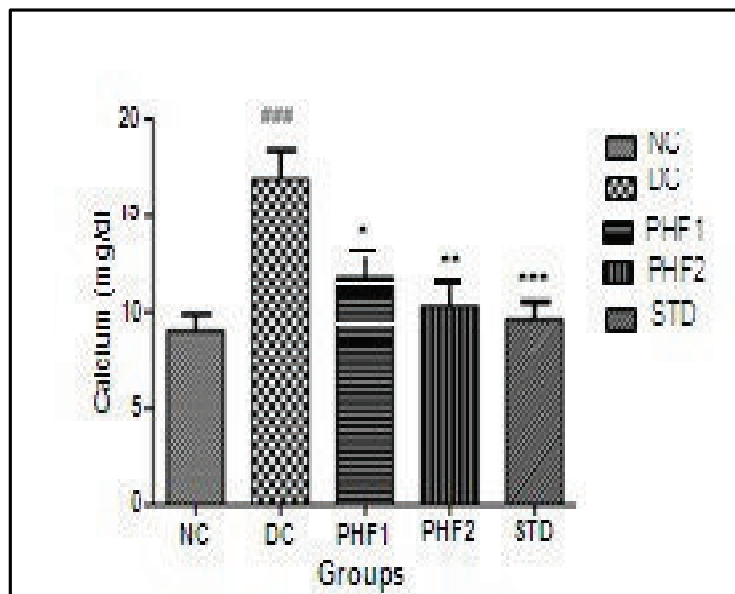


Figure No. 6.47: Effect of PHF 200 and PHF 400 on Serum Calcium (mg/dl) in Ethylene induced Urolithiasis in Rats

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

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

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

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

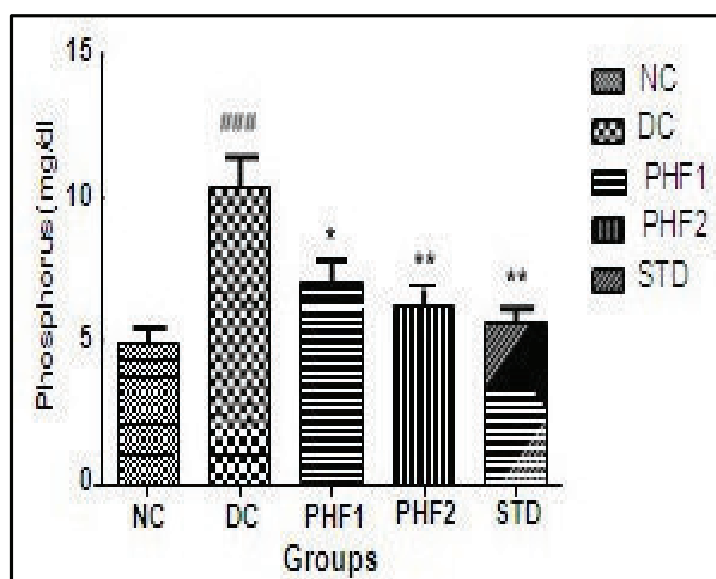


Figure No. 6.48: Effect of PHF 200 and PHF 400 on Serum Phosphorus (mg/dl) in Ethylene induced Urolithiasis in Rats

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

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

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

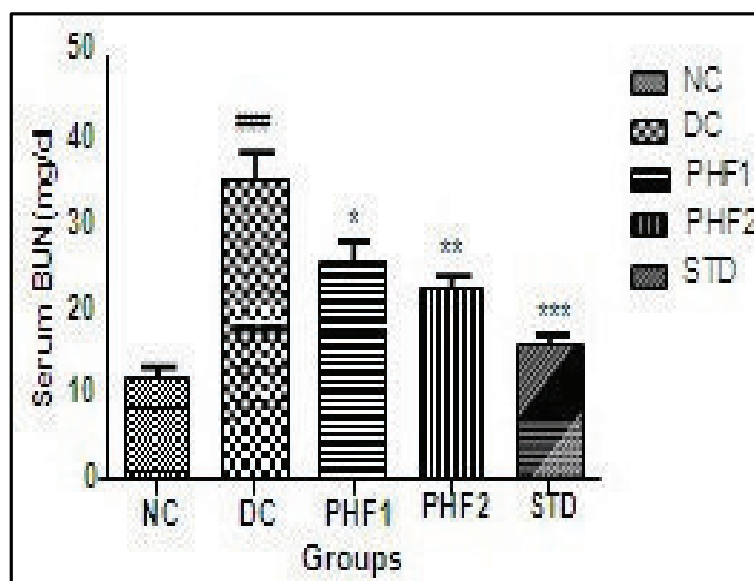


Figure No. 6.49: Effect of PHF 200 and PHF 400 on Serum BUN (mg/dl) in Ethylene induced Urolithiasis in Rats

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

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

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

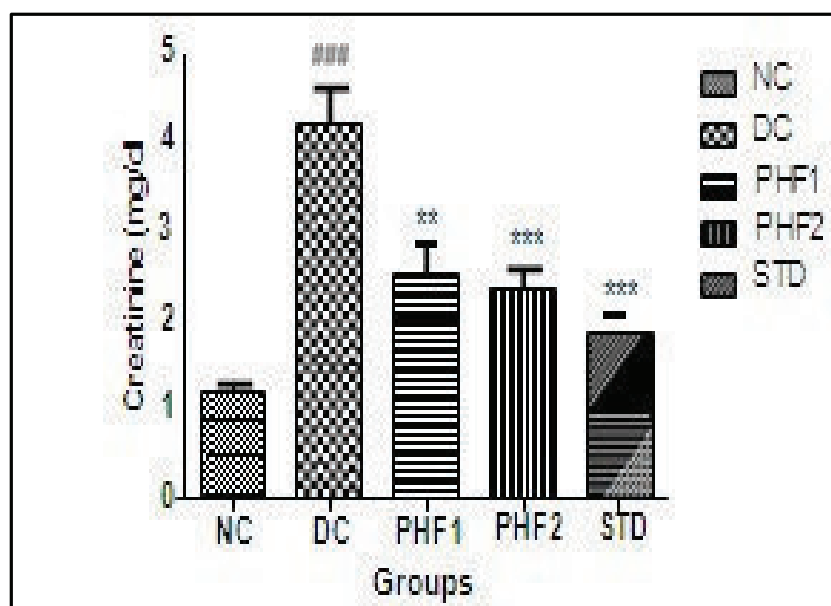


Figure No. 6.50: Effect of PHF 200 and PHF 400 on Serum Creatinine (mg/dl) in Ethylene induced Urolithiasis in Rats

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

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

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

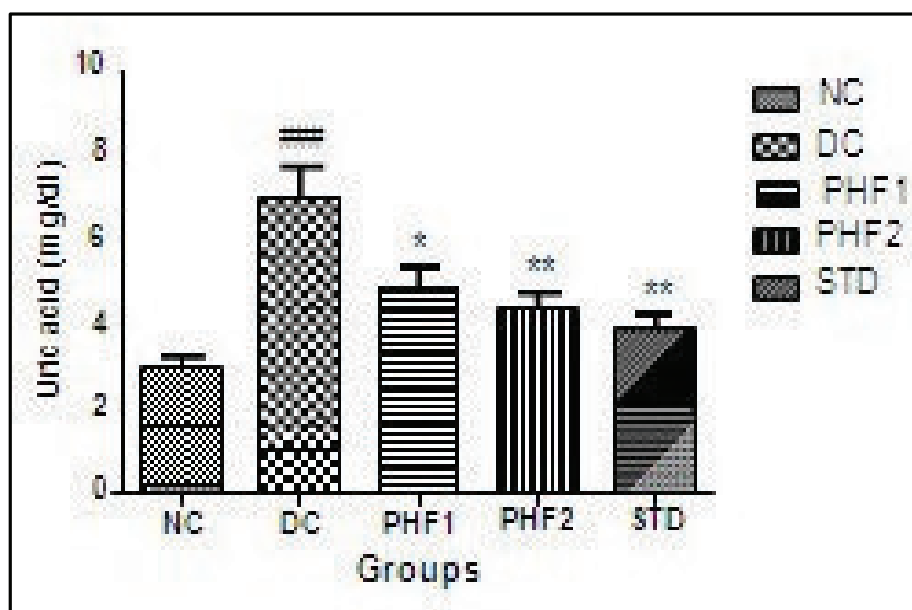


Figure No. 6.51: Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats

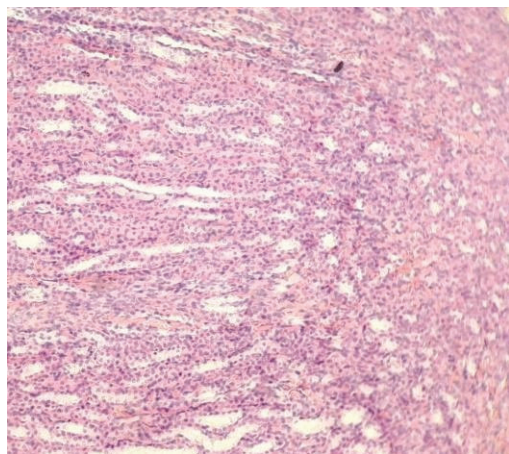
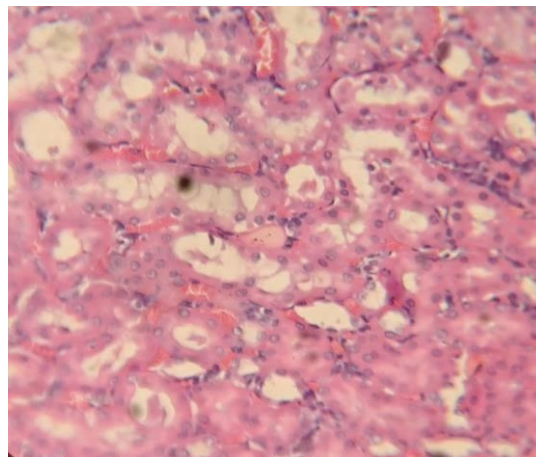
Figure 6.51: Effect of PHF 200 and PHF 400 on Serum Uric acid (mg/dl) in Ethylene induced Urolithiasis in Rats. Values are expressed as Mean \pm SEM (n = 6) & analyzed by One-way ANOVA followed by Tukey's Kramer test. $^{###}p < 0.001$ versus NC rats and $^{***}p < 0.001$ versus DC rats.

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

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

6.5 HISTOPATHOLOGY STUDY INTERPRETATION:

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

**Group 1 : Normal****Group 2: Positive control**

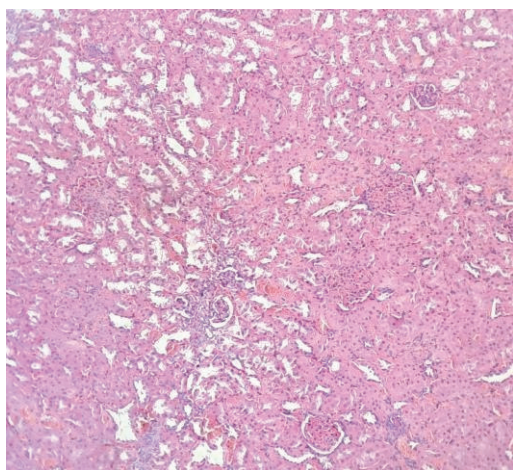
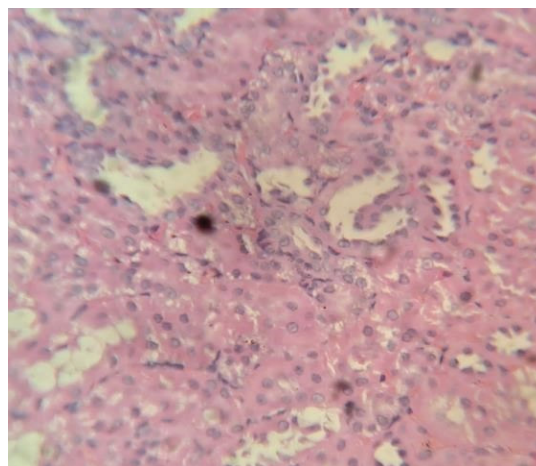
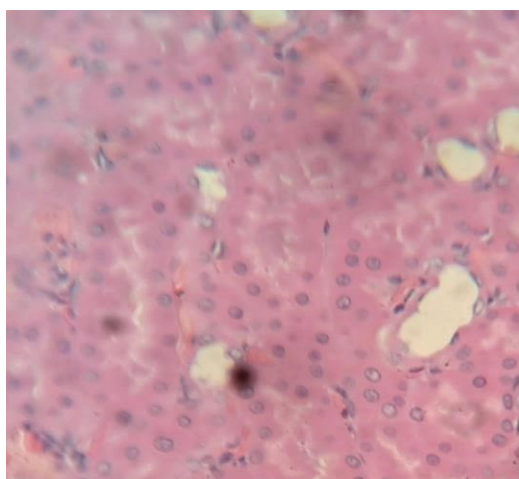
**Group 3: Cystone****Group 4: Extract 200 mg/Kg****Group 5: Extract 400mg/Kg**

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

Group - 1 - Normal control.

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

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

Group - 2 - Disease control - EG model.

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

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

Group - 3 - Standard drug administration.

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

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

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

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

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

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

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

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

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

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

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

6.5 FORMULATION AND EVALUATION OF POLYHERBAL GRANULES^[206]

Results

6.5.1 Macroscopic evaluation:

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

Table No. 6.36: Evaluation of Organoleptic Characteristics

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



Figure No. 6.53: Polyherbal Formulation

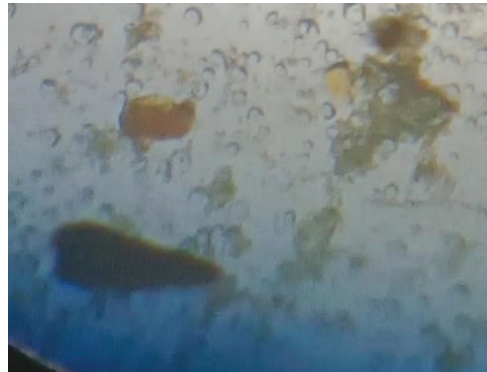


Figure No. 6.54: Polyherbal Granule

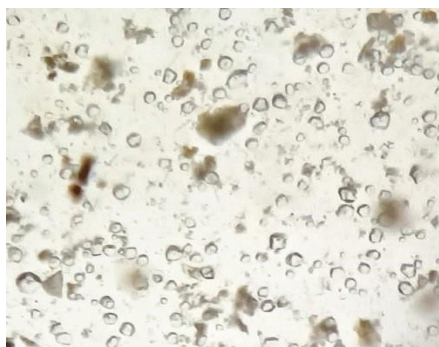
6.5.2 Microscopic evaluation: Powder characteristic of granules showed presence of lignified cells, starch grains, cuticle, contain xylem vessel, lignified sclerides, endosperm, starch grain and epidermis.



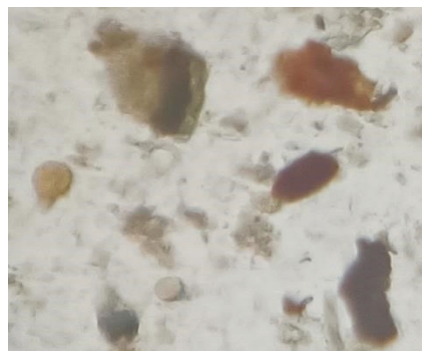
Lignified cells



Oil glands



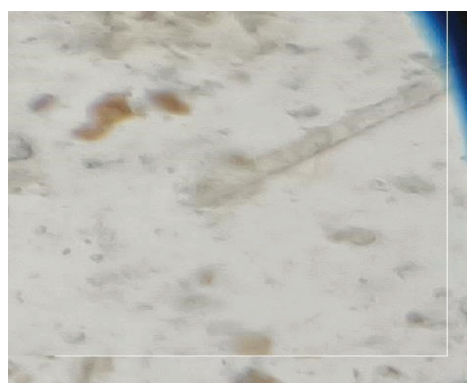
Starch grains



Cuticle cells



Xylem fiber



Fiber of plants

Figure No. 6.55: Powder microscopy of Granules

6.5.3 Determination of pH:

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

6.5.4 Loss on drying at 105°C:

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

6.5.5 Determination of Ash Values

A) The Polyherbal formulation were evaluated and the obtain total ash value is 6.254% w/w, water soluble ash value is 3.56% w/w and acid insoluble ash value is 0.94% w/w. As shown in Table 6.37.

6.5.6 Determination of Extractive Values:

The Polyherbal granule formulation were evaluated for extractive value determination and the obtain result for Water soluble extractive Value 12.26% w/w and Alcohol soluble extractive Value 3.36% w/w. As shown in Table 6.35.

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

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

6.5.7.1 Bulk Density:

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

6.5.7.2 Tap Density:

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

6.5.7.3 Angle of Repose:

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

Table No. 6.37: Physicochemical evaluation of Polyherbal Granule formulation

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

6.6 HPTLC FINGERPRINTING OF POLYHERBAL GRANULE FORMULATION:

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

6.6.1 Optimized Solvent system:

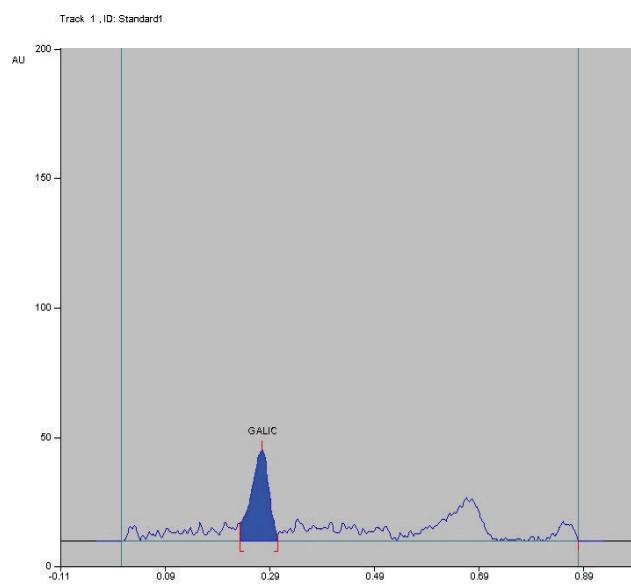
Table 6.38: Optimized solvent system

Toluene	Ethyl acetate	Methanol	Formic acid
6	4	0.5	0.5

6.6.2 Application of standards, Aqueous extract and formulation:

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

1. Galic Acid:



Track 1, ID: Standard1

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

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

2. Rutin:

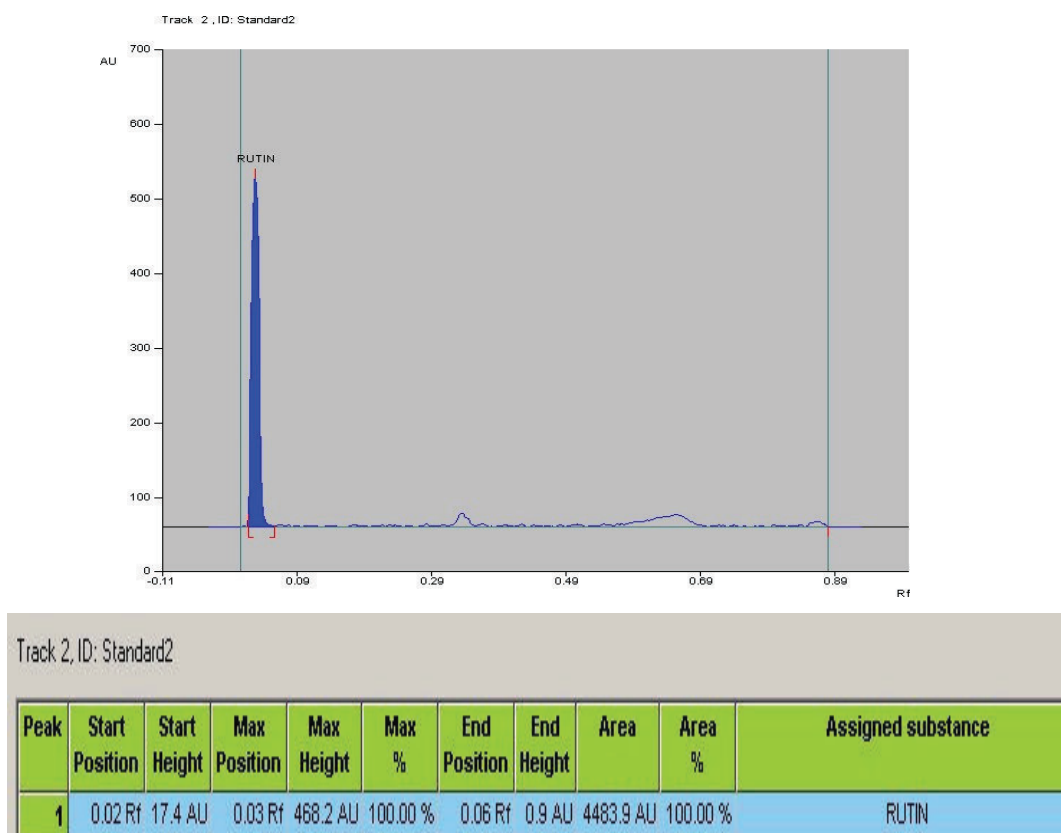


Figure No. 6.57: Chromatogram and Densitogram of Rutin marker

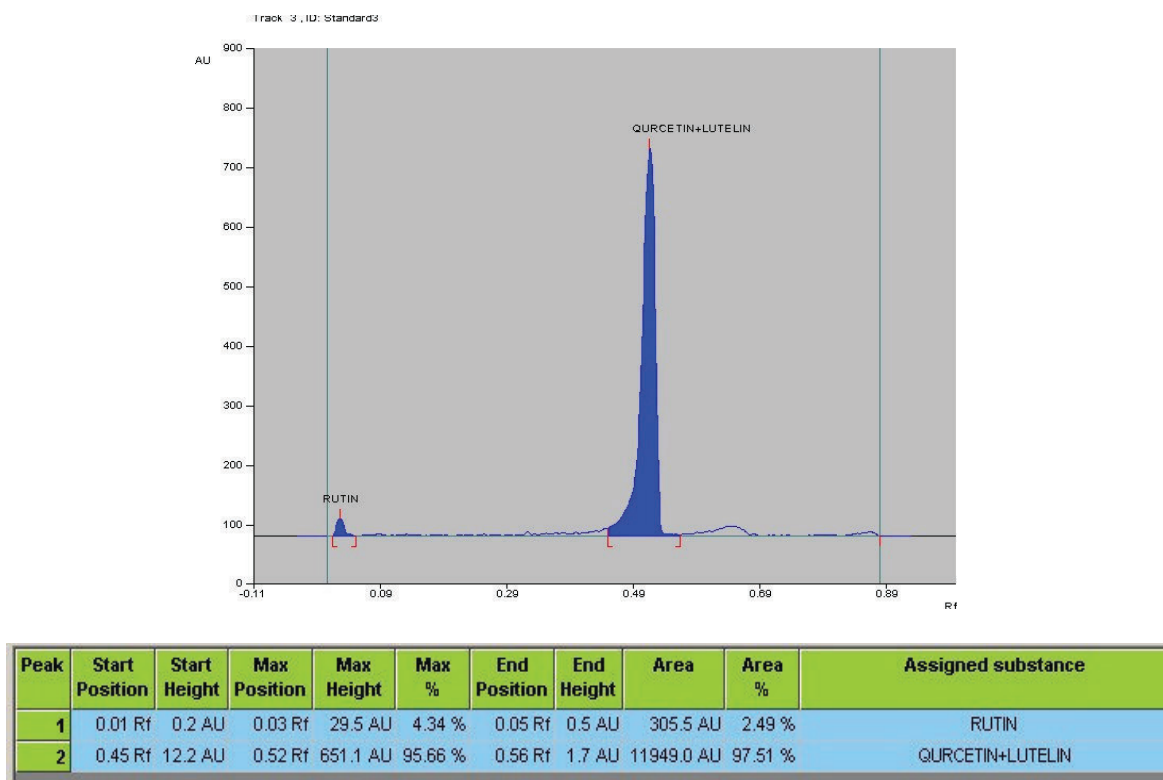


Figure No. 6.58: Chromatogram and densitogram of Quercetin and Luteolin marker compound

6.6.3 Presence of Marker compounds in extract:

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

Table 6.39: Phytochemicals found in Combination Extract

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

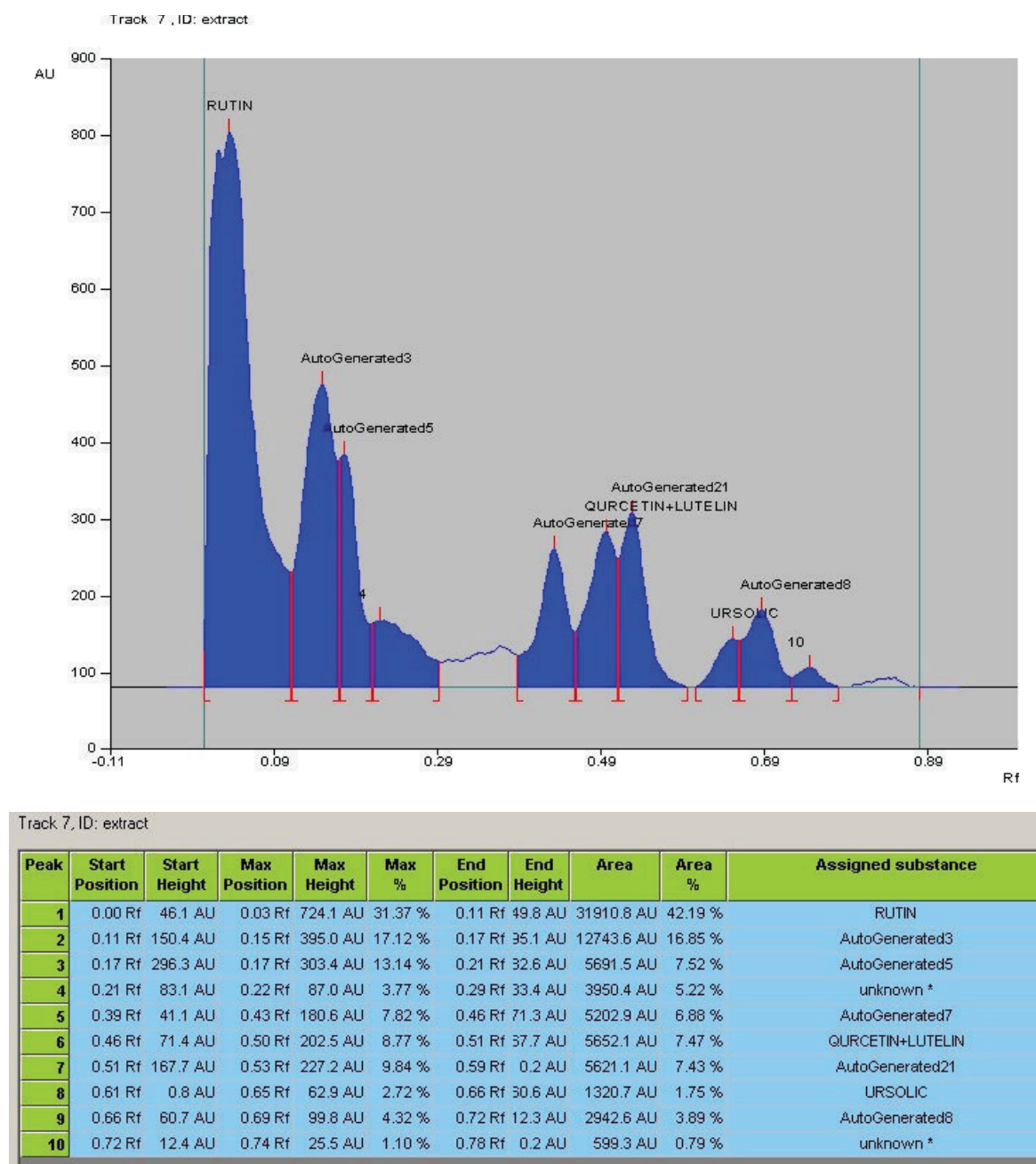


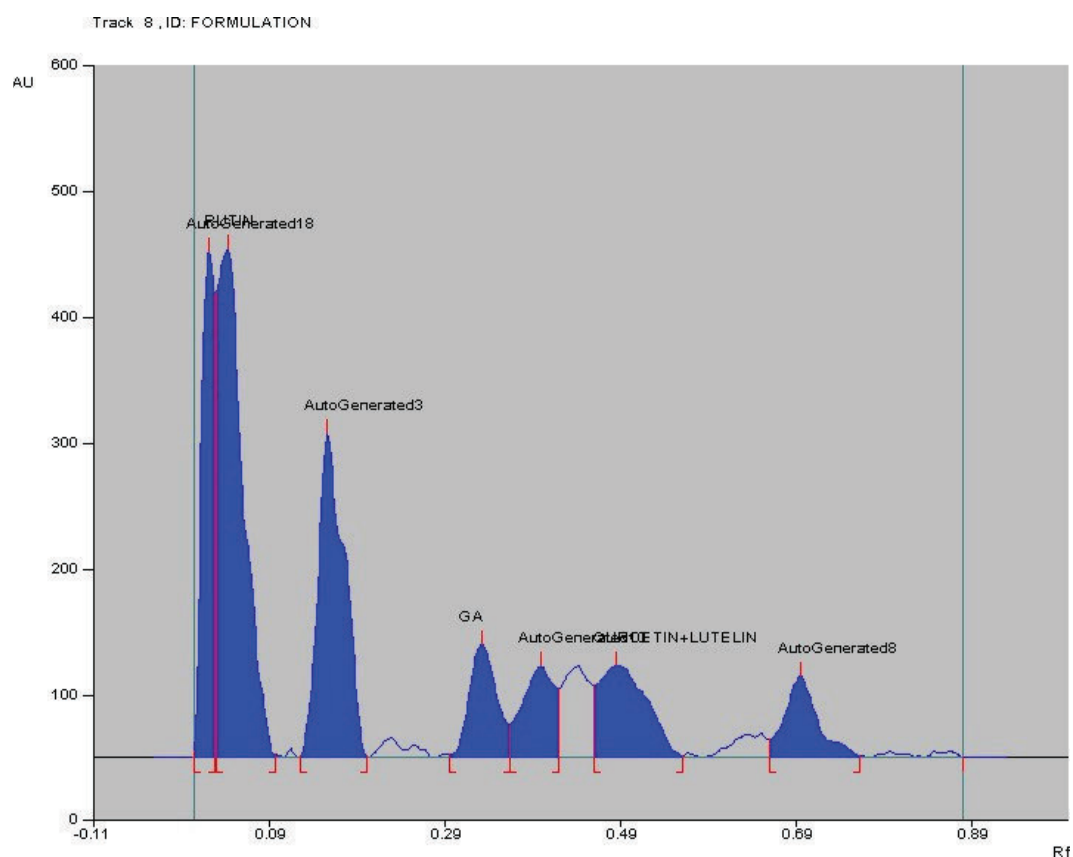
Figure No. 6.59: Densitogram and chromatogram of combination B extract

6.6.4 Presence of marker compounds in Polyherbal Formulation:

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

Table No. 6.40: Phytochemicals found in polyherbal granule formulation

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



Track 8, ID: FORMULATION

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

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

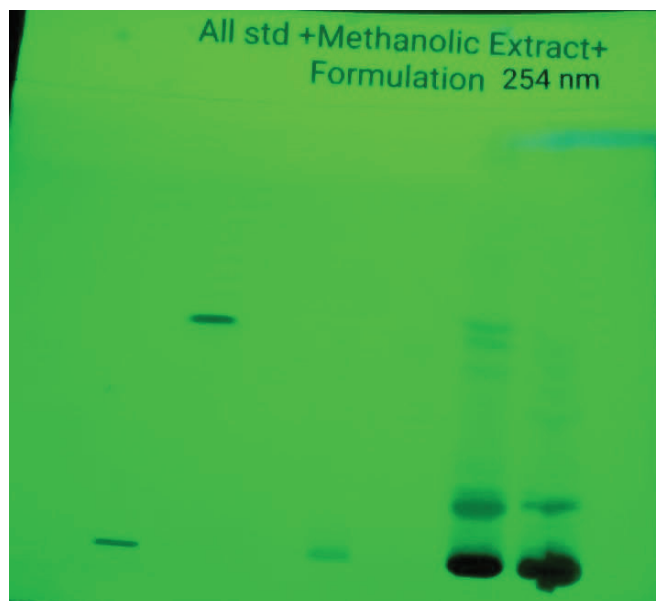


Figure No. 6.61: HPTLC finger print profile of Galic acid, Rutin, Quarcetine, Ursolic acid and Luteolin, Aqueous extract & Polyherbal Formulation at 254nm

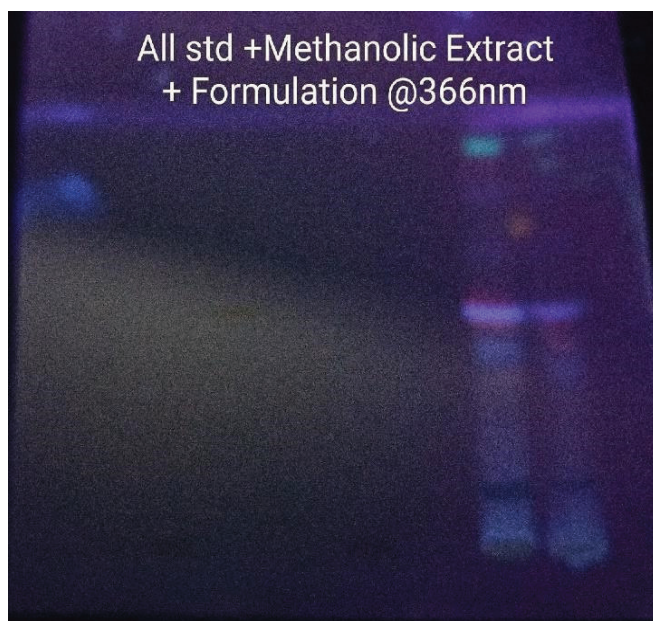


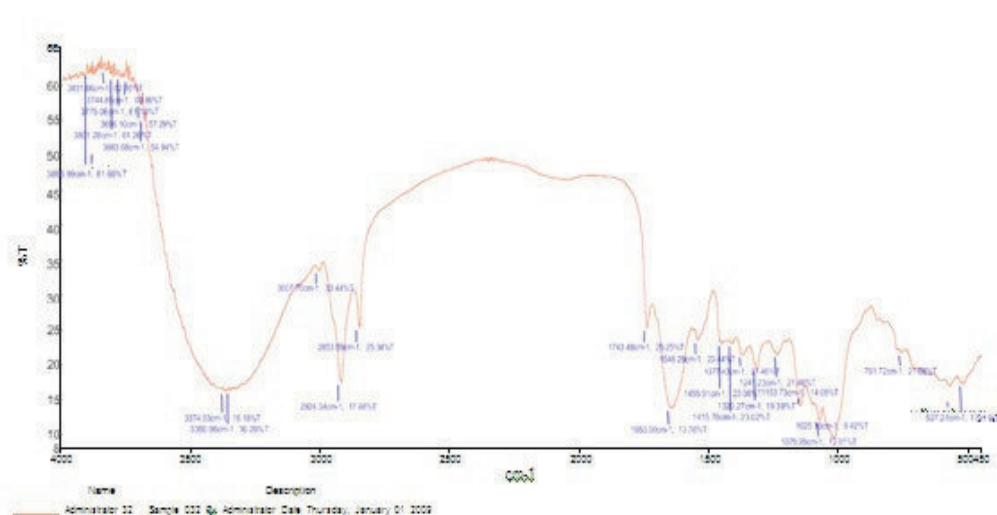
Figure No.6.62: HPTLC fingerprint profile of Galic acid, Rutin, Quarcetine, Ursolic acid and Luteolin, aqueous extract &Polyherbal Formulation at 366 nm

Conclusion of HPTLC Fingerprinting:

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

6.7 FTIR Study:

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



A

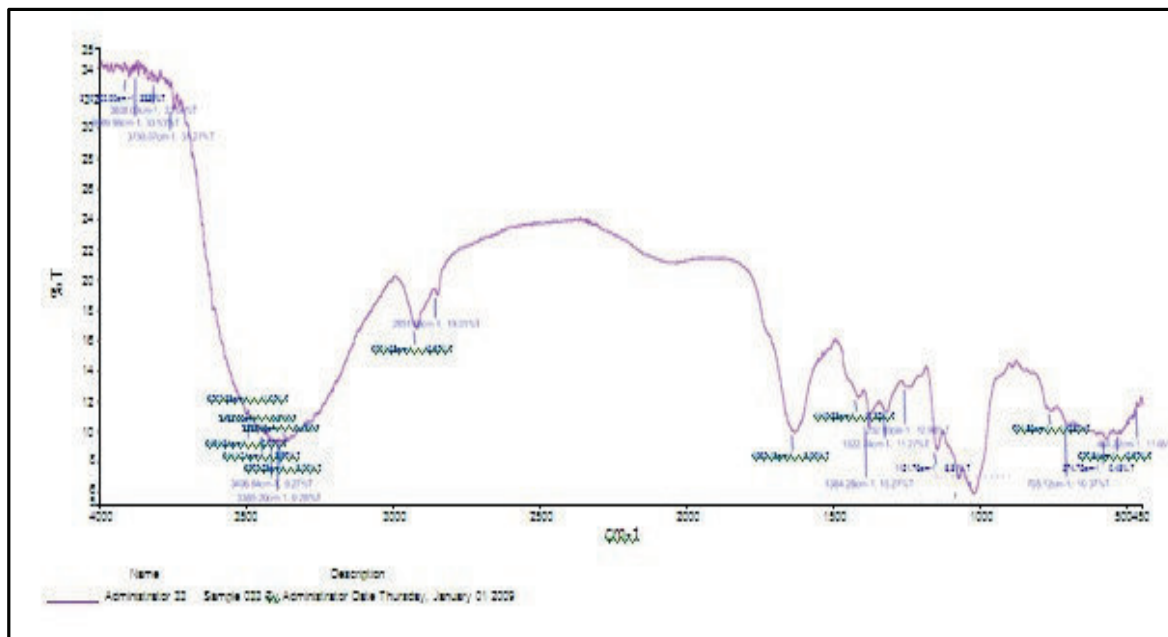


Figure No. 6.63: A: FTIR of extracts of three drugs; B: FTIR of extracts of three drugs with excipients Stability studies

6.8 PHYSICO-CHEMICAL PARAMETERS

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

Parameter	Condition	0 day	30 th day	60 th day	90 th day
Colour	40°C/75% RH	Buff Greenish	Buff Greenish	Buff Greenish	Buff Greenish
	30°C/65% RH	Buff Greenish	Buff Greenish	Buff Greenish	Buff Greenish
Odour	40°C/75% RH	Characteristic	Characteristic	Characteristic	Characteristic
	30°C/65% RH	Characteristic	Characteristic	Characteristic	Characteristic
Taste	40°C/75% RH	Particular	Particular	Particular	Particular
	30°C/65% RH	Particular	Particular	Particular	Particular

pH	40°C/75% RH	7.8	7.8	7.5	7.5
	30°C/65% RH	7.8	7.8	7.5	7.5
Tap Density	40°C/75% RH	0.44	0.44	0.44	0.44
	30°C/65% RH	0.44	0.44	0.44	0.44
Bulk Density	40°C/75% RH	1.434	1.433	1.436	1.436
	30°C/65% RH	1.434	1.433	1.437	1.435
Size of granules	40°C/75% RH	1.9	1.89	1.897	1.899
	30°C/65% RH	1.9	1.88	1.892	1.895
Water content	40°C/75% RH	258	257.2	258	257.5
	30°C/65% RH	258	257.8	257.2	258.2
Microbial content	40°C/75% RH	10.62	10.61	10.70	10.75
	30°C/65% RH	10.62	10.62	10.75	10.75

The results of the stability studies indicated that the formulation had reasonable stability.