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Pharmacognostic standardization and High Performance Thin-Layer Chromatographic Fingerprinting of *Plumeria rubraL*. Seed pods

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Abstract: *Plumeria rubra L.* is a deciduous plant belonging to genus Plumaria, Fam. Apocynaceae. Plant native to Mexico, south America also found in India and its tropical regions. Commonly known as Champa or Chafa and is a popular Temple tree, Garden and Park plant used for Ornamental purpose. Though indigenously it has traditional folkloric valued medicinally to treat kidney stone as Neproprotective yet literature lacks in its Pharmacognostic and Phytochemical approach also have not been explored therapeutically. In the present study *Plumeria rubra* seed pods are studied for its Pharmacognostic and Phytochemical investigation. Seed pods are 7-9 cm in length available in pairs, showed reddish brown colour, rough fracture and course texture. Microscopy showed the existence of lignified fibers , vascular bundle. The physicochemical analysis shows no foreign organic matter, 3.0% average moisture content, total ash value 12.03, 0.90 acid insoluble ash. Aqueous extract of plumeria rubra seed pods was prepared by using continuous hot extraction using Soxhlet extractor. Extracts showed the presence of tannins, alkaloids, balsam, cardiac glycosides, phenols, terpenes and steroids. HPTLC fingerprinting of aqueous extract of *Plumeria rubra* showed presence of Lupeol,Oleic acid and Luteolin as important phytoconstituents.

Introduction: *Plumeria rubra* has traditional folkloric values in cardiovascular disease as Hypotensive,also showed cardioprotective in adrenaline induced myocardial infarction (1-2) *P. Rubra* decoction is found effective in diarrhoea and vomiting. Bacterial infection, rheumatic pains can be treated by root decoction(3). Latex of *P.rubra* is found effective in ear and toothache as per Mexican tradition also used to cure venereal disease, as well as rheumatism, leprosy and fever in the indigenous system of medicine.(4) High-performance liquid chromatography (HPLC) analysis revealed many vital phytoconstituents, such as α-allamcidin, α-amyrin, β-allamcidin, β-amyrin acetate, β-sitosterol, allamandin, allamcin, arjunolic acid, lupeol, Plumericidine, rutin, quercetin, ursolic acid, oleanolic acid, Luteolin and oleic acid, Luteolin (5). Luteolin acts as nephroprotective by reducing oxidative stress on kidney, Lupeol is a tritepenoid acts as a Neuroprotective , Anti-inflammatory, cholesterol lowering agent(6). Pharmacological investigations of *Plumeria rubra* reported analgesic, antidiabetic, antipyretic, anti-obesity, antimicrobial, lipid-lowering, anticancer, antioxidant, insecticidal, and gastroprotective effects(7). It is found Kidney stone is common in all kinds of urolithiasis. The kidney stone develops due to decrease in volume of urine formation or increase in excretion of stone forming components like urate, cystine, xanthine, calcium oxalate and phosphate. Urolithiasis is a common health problem with increasing prevalence of up to 20% all over the globe. The increased prevalence of the disease occurs due to the lifestyle changes such as lower dietary intake of vegetables or fruit, higher consumption of animal proteins, salt, sweetened beverages, and inadequate fluid intake. Amongst the nephrolithiasis calcium oxalate stone occurs most commonly (8). In vitro evaluation using dissolution method or titrimetry method is necessary to evaluate effectiveness of herbal medicines.(9). Urolithiasis has been regarded as one of the eight most problematic disorders, and in Ayurveda urinary stones are called asmutraashamari (mutra-urine; ashma-stone; ari-enemy) (mahagad). Four different forms of urinary calculi, including phosphatic stones (sleshmaashmari), urate stones (pittaashmari), oxalate stones (vataashmari), and spermolith or seminal concretions (sukraashmari), have been recorded in Ayurvedic writings.Herbal remedies, alkaline drinks, and surgical techniques, Ayurvedic medicine to treat and manage urinary stones (10-13).

Plumeria rubra shown pink or red blooms throughout the year (Fig 1), but seed pods can be seen only in plants after aging of at least 9-10 years and can only be seen in winter

season only. Many people are unaware of availability of seed pods to Champa plant as they look like bark of the plant, thus it has not been critically studied pharmacognostically as well as phytochemically. Seed pods are dark reddish brown in colour and available in pair (Fig 2)

Fig 1 : Natural habitat of *Plumeria rubra*and seed pods

Fig2 : Fresh seed pods of *Plumeria rubra*

Material and Methods :

Collection and authention of Plant :

Plumeria rubra seed pods were collected from Rajuri, Junnar, Pune,Maharastra. Seed pods were cleaned by wiping them with clean cotton cloth. Fresh seed pods with flowering twig and leaves are authenticated from Agharkar Research Institute Pune, Maharashra.Matured seed pods were kept as it is as they open up after drying, such seed pods are kept for shade

drying . Dried pods containg seeds inside are ground in herbal grinding mill, passed through seive and stored in air tight container for futher tests and activity.

Pharmacognostic Studies:

Morphology and Microscopy :

Morphology of*Plumeria rubra* leaves, bark and seed pods and seed were studied and their phographs were taken using light binocular microscope. Transverse sections (T.S.) of leaves, bark and seed pod were taken by free hand section. T.S. of leave and seed pod were treated with phloroglucinol-HCl solution to observe lignified cells in plant parts. For powder characterstic fine powder of seed pod was prepared by using herbal grinding mill. The powder was treated with phloroglucinol-HCl solution, Iodine and Potassium Iodide solution.(14-15)

Physicochemical Estimation:

It is very important to evaluate physicochemical parameter to detect whether material used is pure or adultrated. It involves estimation of ash value including total ash value, acid insoluble ash, water soluble ash, extractive values such as water soluble and alcohol soluble extractive values, also involves moisture content analysis.(15)

Phytochemical Estimation :

Plumeria rubra seed pods were screened for preliminary phytochemical estimation by using various reagents for the presence of Phytochemicals like flavonoids, saponins, alkaloids, glycosides, tannins, carbohydrates, steroids, and terpenoids.(16)

High performance Thin Layer Chromatography (HPTLC) (16, 19) :

HPTLC was performed using CAMAG TLC SCANNER 3, CHF 47150, for sample injection Linomet 5 sampler was used.,twin trough chamber,winCATS software (CAMAG Ver.1.4.1). TLC plates precoated with silica gel 60 F254 (2020 cm) of 0.2 mm thickness procured from Ajinkya Enterprise, Pune were used as the stationary phase. All reagents used are of analytical grade.

Preparation of sample

Aqueous extract of *Plumeria seed* pods were prepared by using 1 gm of powdered drug . Extraction was performed by using continuous hot extraction using 100ml of water for 48 hours. Before extraction defatting with250 ml98% Petrolium ether is to be done. The extract obtained is to be evaporated at 40 °C till we get 10ml extract .

Preparation of Standard solutions

Stock solution of Luteolin, Oleicacid and Lupeol were prepared by dissolving 0.1mg/ml in HPTLC grade methanol.

Optimised Solvent system :

Table 1: Solvent system

Toluene	Ethyl acetate	Methanol	Formic acid
6	4	0.5	0.5

Development of HPTLC Fingerprinting of Luteolin and Oleic acid

Chromatographic conditions

Chromatographic separation was done using HPTLC plates (10×10 cm) pre-coated with silica gel 60 F_{254} of 0.2 mm thickness and supported by an aluminium sheet. Using a CAMAG (Muttenz, Switzerland) Linomat 5 sample applicator fitted with a 100l Hamilton syringe, standard solutions of markers and extracts were applied to the plates as bands 6.0 mm wide, 10.0 mm from the bottom border of the same chromatographic plate. Ascending development to a distance of 80 mm was carried out with mobile phase at room temperature (24°C) in a Camag glass twin-trough chamber that had previously been saturated with mobile phase vapour for 30 minutes. Following development, the plates were dried and scanned at 500 nm can be visualize by using Derivatizing agent Anisaldehyde-Sulfuric acid reagent
with a Camag TLC Scanner 3 equipped with a deuterium lamp and winCATS.

Calibration curve of Luteolin and Oleic acid :

Stock solution of Luteolin and Oleic acid containing 0.1mg/ml in methanol were used. Apply 1µg to 6 µg spots per band of standard solution using Linomet 5 semiautomatic sampler. The plot of linearity is plotted of area against corresponding concentration to get the correlation coefficient (R^2) and equation of the line.

Fingerprinting of Luteolin and Oleic acid :

Standard solution of Luteolin and oleic acid given in linearity followed byaqueous extract of *Plumeria rubra*seed pod to check presence of these chemical constituents in seed pod. The plates are run separately in solvent system of Toluene: Ethyl Acetate: Methanol: Formic acid (6:4:0.5:0.5) and the Rf values are to be checked accordingly.

Standard solution of Lupeol is also given in linearity followed by aqueous extract of *Plumeria rubra* seed pod for checking presence Lupeol as Phytoconstituent in seed pod of *P.rubra*.

Result &Discussion :

Pharmacognostic Studies:

Morphology and Microscopy :

Morphology of *Plumeria rubra* leaves shown presence of epidermal cells followed by single layer of palisade cells and lignified vasular bundle as shown in Fig3.Seed pods shown presence of cutical, medullary rays Fig 4and 5. Seed from seed pod was boiled and cut into two pieces showed white monocotyledon and embryo shown in Fig. 6. For powder characterstic fine powder was treated with phloroglucinol-HCl solution shown presence of lignified cells, when powder was treated with Iodine and Potassium Iodide solution shown blue colour indicating presence of starch grains .

MorpholgicalCharacteristics of *Plumeria rubra* seed pod: Seed pods of *plumeria rubra* were obtained in the month of January from Rajuri Pune, Maharashtra. The pods are elongated in shape, dark reddish brown in color available in pair approximately 12-15cm containing pink to white latex inside. Rough in fracture and having course texture.

Table 2 :Morphological characteristics of *P. rubra* Seed pod

Sr. No.	Morphological Characters	Observations of seed pod
1	Colour	Dark Reddish Brown
2	Odour	Mild
3	Taste	Pungent
4	Shape	Elongated
5	Size	12-15 cm

6	Fracture	Rough
7	Texture	Slightly coarse

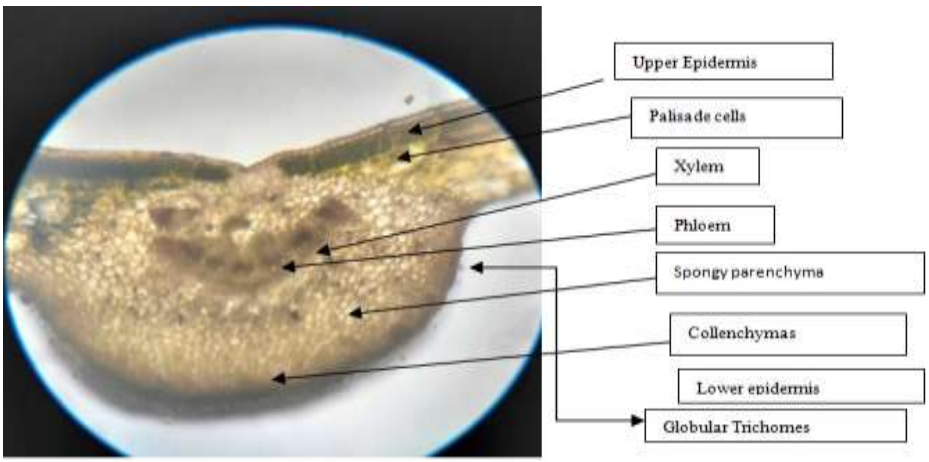

Fig3. T.S. of *Plumeria rubra* leaf

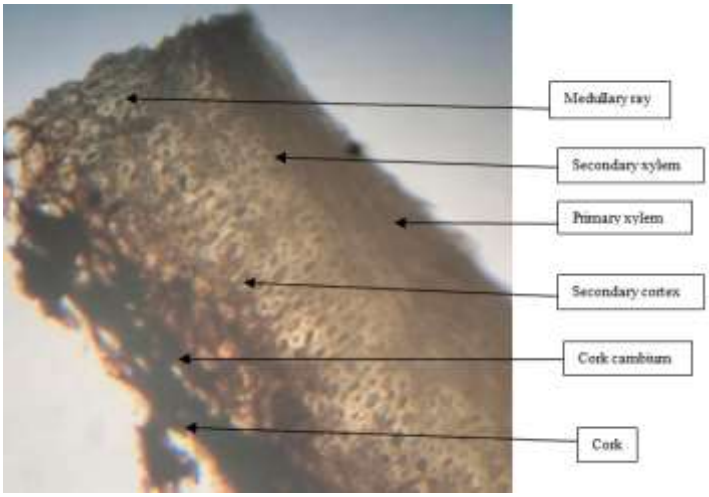

Fig.4. T.S. of *Plumeria rubra* Seed pod shell

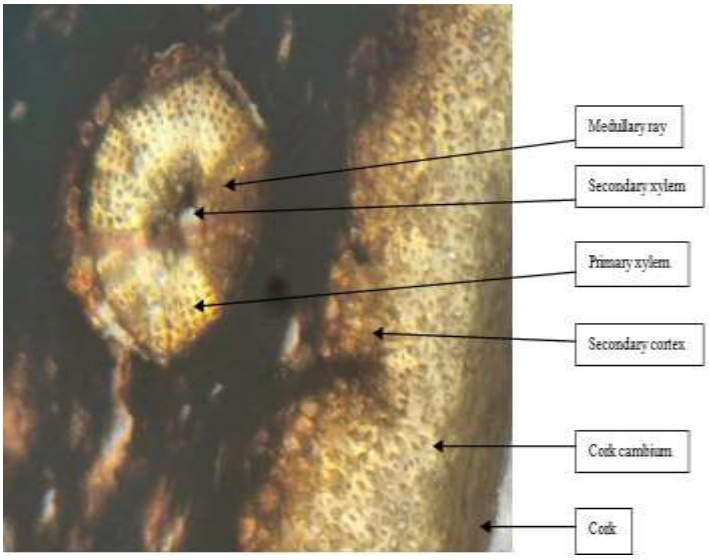

Fig. 5 T.S. of *Plumeria rubra*seed pod showing medullary rays

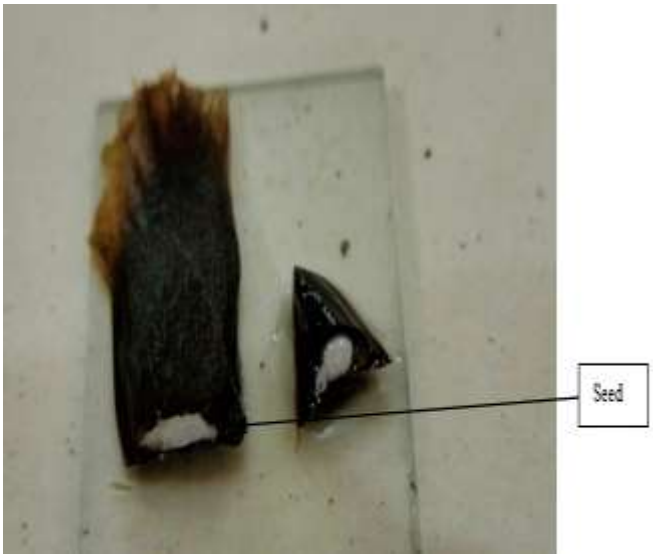

Fig. 6 Images showing seed of *plumeria rubra*

Fig.7 Plumeria seed pod powder + Phloroglucinol

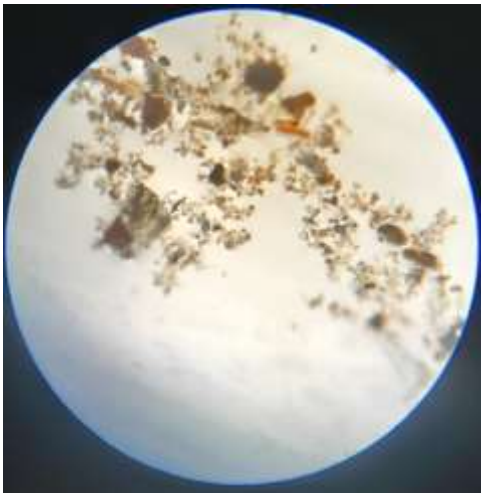

Fig.8 Plumeria seed pod powder+ Iodine

Fig.9 Plumeria seed pod powder + Potassium Iodide

Phytochemical Estimation :

Observations of *Plumeria rubra* seed pods phytochemical estimation by using various reagents for the presence of Phytochemicals like flavonoids, saponins, alkaloids, glycosides, tannins, carbohydrates, steroids, and terpenoids are shown in table no 3.

Table 3. :Phytochemical Parameter observations

Phytochemical Parameters	Observations in methanolic extract
Flavanoids	++
Saponins	-

Alkaloids	++
Glycoside	+
Tannins	++
Carbohydrates	+
Steroids	+
Terpenoids	+
Fixed Oil	-

Abbrevation+ : presence , - : Absence

Development of HPTLC Fingerprinting of Luteolin, Oleic acid and Lupeol:

Calibration curve of Luteolin and Oleic acid :

Stock solution of Luteolin and Oleic acid containing 0.1mg/ml in methanol were used for sample spot application from 1µg to 6 µg spots per band of standard solution using Linomet 5 semiautomatic sampler shown linear plot. The plot shown value of correlation coefficient (R^2) is 0.996 .

Table 4 : Linearity of **Luteolin and Oleic acid**

Sr. No.	Concentration (µg)	Area
1	1	1089.54
2	2	2839.75
3	3	4985.97
4	4	6273.8
5	5	8235.27
6	6	9331.94

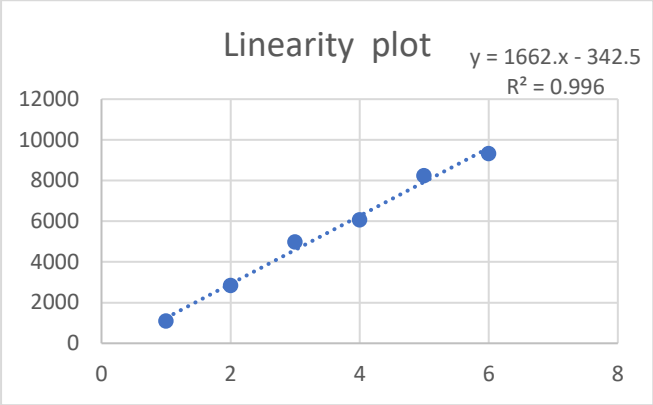

Fig 10. Linearity plot of standard Luteolin and Oleic acid

Fingerprinting of Luteolin and Oleic acid :

When Aqueous extract of *Plumeria rubra* seed pod run in solvent system of Toluene: Ethyl Acetate: Methanol: Formic acid (6:4:0.5:0.5) shown separations, visualized after derivatization with Anisaldehyde-Sulphuric at 540nm. In Fig11. The 3D spectra and Densiogram of extract shown presence of Luteolin and Oleic acid at corresponding Rf of standard Luteolin and Oleic acid i.e. for Luteolin it shown peak at Rf 0.59 and for Oleic acid it shown peak at Rf 0.80 in correspond to standard proves presence of these chemical constituents in extract of *Plumeria rubra* seed pod.

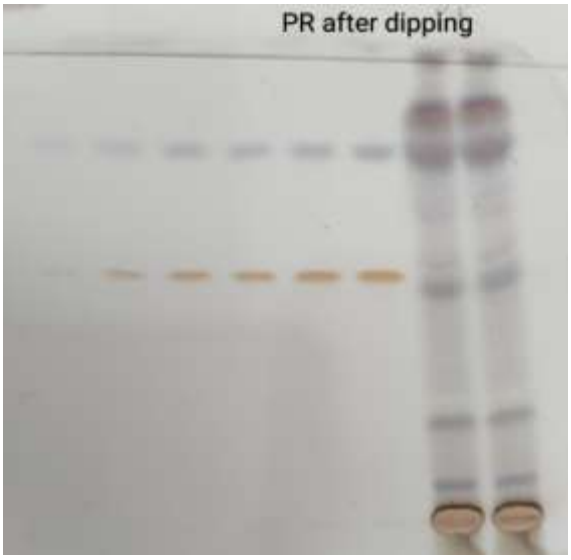

Fig 11 Slide of *Plumaria rubra* pod after derivatization

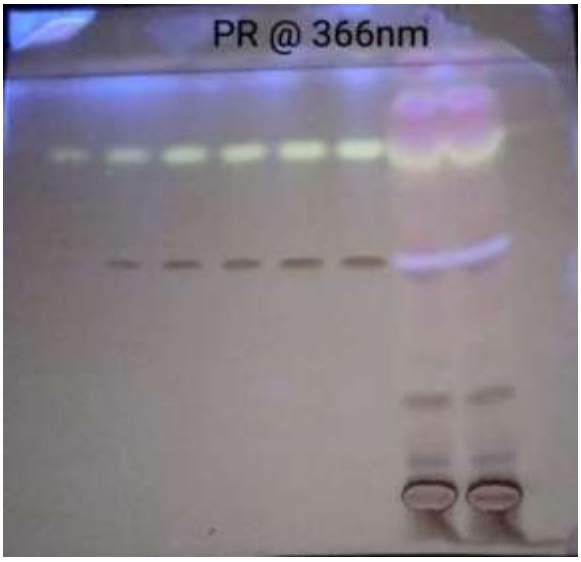

Fig 11 Slide of *Plumeria rubra*pod after derivatization at 366nm

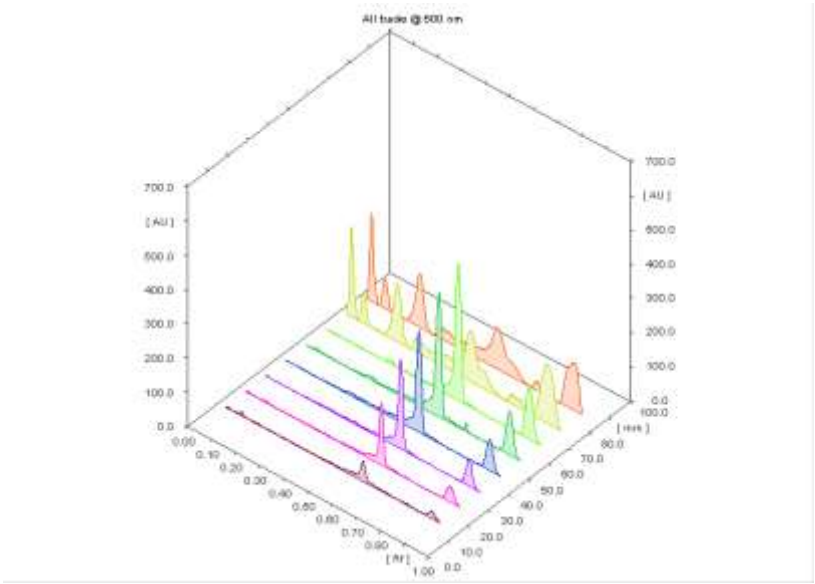

Fig.13:3D spectra of std Luteolin +Oelic acid+extract of *Plumeria rubra* Seed pod

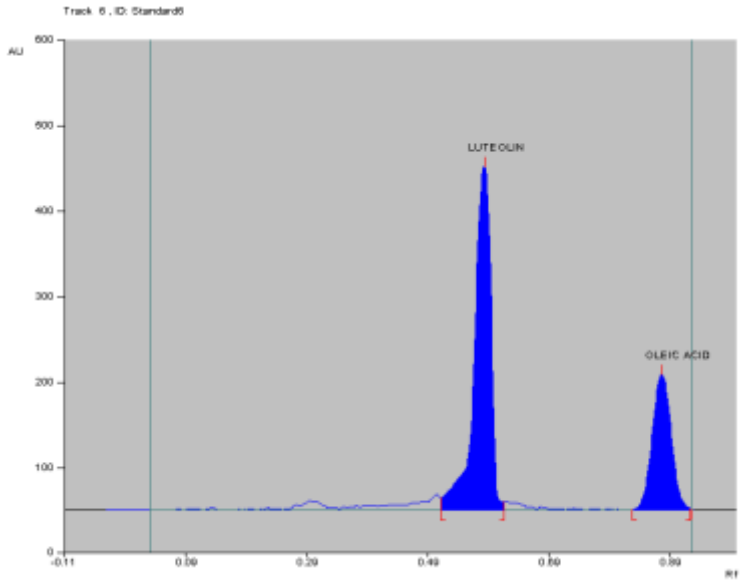

Fig 14 : Densiometric chromatogram of standard Luteolin and Oleic acid

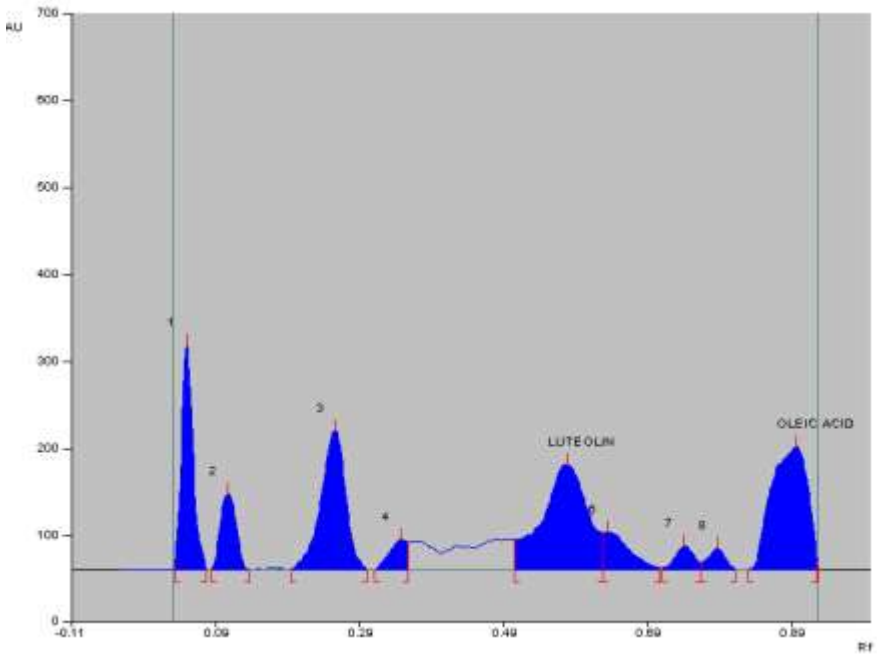

Fig 15: Densiometric chromatogram of Aqueous extract of *plumeria rubra* seed pod for Luteolin and Oleic acid

***Fingerprinting of Lupeol* from *plumeria rubra* seed pod:**

This is also found that *Plumaria rubra* seed pods showed presence of Lupeol. The obtained densitogram showed 13spots at Rf 0.04, 0.12, 0.17, 0.24, 0.27, 0.30, 0.38, 0.46, 0.50,0.59, 0.71, 0.80 and 0.89 in UV light at 500 nm. The spot at Rf 0.71 was Lupeol. Thus,Luteolin, Oleic acid and Lupeol are the phytoconstituents shown by HPTLC study of *Plumeria rubra* seed pod.

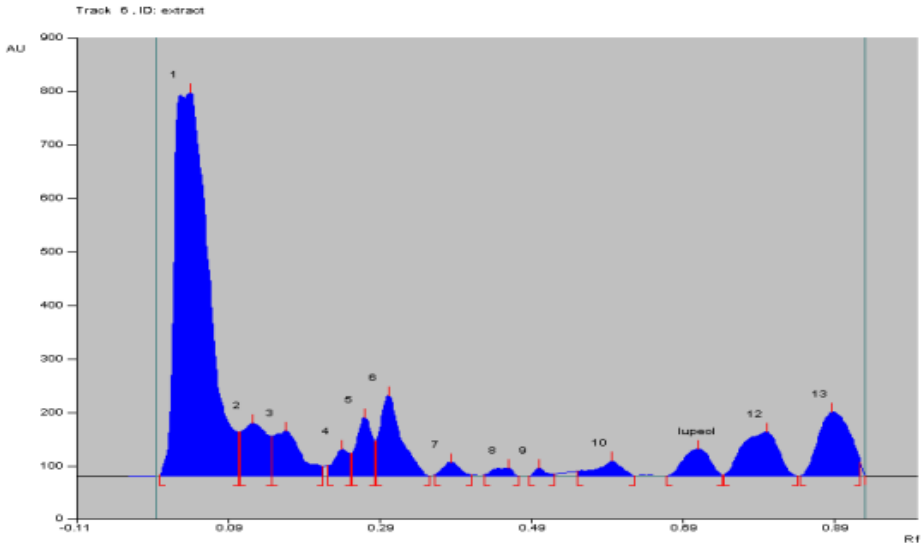

Fig 16: Densiometric chromatogram of *plumeria rubra* seed pod for Lupeol

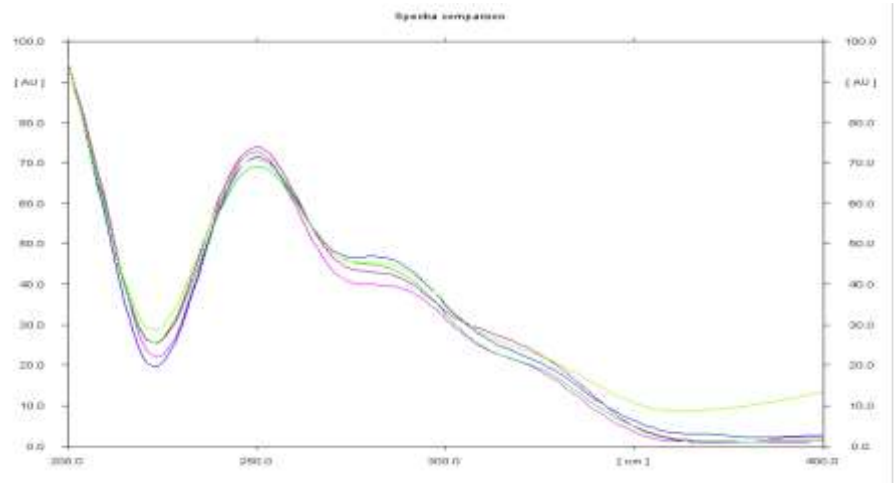

Fig. 17 :Overlain spectra for Lupeol+PR extract

Track 6, ID: extract

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

Conclusion : Study reveals detailed Pharmacognostic, microscopic and phytochemical screening of *Plumeria rubra* Seed pod . Morphological characteristic of seed of pod showed identification characteristics of Seed pod also, Powder microscopy of Seed pod showed presence of lignified tissues, starch granules, etc. Seed pod showed presence of flavanoid, alkaloids, glycosides, carbohydrates, tannins, terpenoids, fixed oil.HPTLC fingerprinting analysis of *Plumeria rubra* seed pod showed presence of Luteolin,Oleic acid and Lupeol. This analytical investigation is helpful for researchers to find out it's Nephroprotective, Neuroprotective, Anti-inflammatory, Hepatoprotective properties of *plumeria rubra* seed pod.

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Conflict of Interest :

We have no any conflict of interest

Abbreviations :

TS. :Transverse section, HPTLC: High Performance thin layer Chromatography, PR. : *Plumeria rubra.*

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