Pharmacogn J. 2017; 9(2): 135-141
A multifaceted peer reviewed journal in the field of Pharmacognosy
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Original Article

Pharmacognostic Standardization and Chromatographic Fingerprint Analysis on Triterpenoids Constituents of the Medicinally Important Plant *Plumeria rubra f. rubra* by HPTLC technique

Gunja Srivastava[1], Abhishek Gupta[2], Manjul Pratap Singh[3], Anurag Mishra[3*]

ABSTRACT

Introduction: *Plumeria rubra f.* rubra commonly known as Lal Gulachin has wide horizon of medicinal possessions. Plant is found in India and in its tropical regions. Though the plant and its extracts have been indigenously valued as folklore medicine diversely in India, yet literature lacks somewhere in reverse pharmacognostical approach of this plant which reflects that plant have not been evidently explored therapeutically. There are several forms of *Plumeria rubra* among which *P. rubra* f. rubra is much appraised in India than its other forms. **Method:** In Present study the anticipated potential of this plant has been validated by laying down its pharmacognostical standards along with measurement of its active therapeutic constituent Ursolic acid and Lupeol via. HPTLC, information from organized search of published literature remarks that Ursolic acid and lupeol is ubiquitous to this plant. **Results:** Microscopic features revealed the presence of paracytic type of stomata, crescent bicollateral vascular bundle, calcium oxalate crystal and clothing trichomes in leaves whereas bark showed the presence of distinct periderm with cork and phellogen, sclereids, bast tissue with parenchymatous cells. Methanolic extract of both parts of plant was subjected to HPTLC. In HPTLC studies the Ursolic acid content in leaves was found to be 0.96% whereas in bark was detected as 0.051%, lupeol content in leaves and stem was found to be 0.014% and 0.018%. **Conclusion:** The data generated could be significantly used as reference for the standardization and quality control of Plumeria. rubra f. *rubra*, as no such work has been reported yet.

Key words: HPTLC, Lupeol, *Plumeria rubra f. rubra*, Pharmacognosy, Standardization, Ursolic acid.

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History
- Submission Date: 02-10-2016;
- Review completed: 08-11-2016;
- Accepted Date: 24-11-2016.

DOI : 10.5530/pj.2017.2.23

Article Available online
http://www.phcogj.com/v9/i2

INTRODUCTION

Plumeria rubra f. *rubra* (Apocynaceae) is known by different names in different languages. Hindi: Lal Gulachin,[1] English: True Frangipani.[2] *Plumeria* is native to tropical America in Hawaii; it is grown as an ornamental and is not found in the wild,[3] various species are now found widely and distributed in the warmer regions of the world. Different parts of the plant are used traditionally in medicine.[4] Frangipani is well-known for its intensely fragrant, lovely, spiral-shaped, reddish blooms which appear at branch tips in November.[5]

Phytochemical studies of *P. rubra* shows it contains β-sitosterol-β-D- glucoside, lupeol nanoate, lupeol heptanoate, rubrinolglucoside, plumeiride coumarate, three irroides- fulvoplumierin, allamcin, and allamandin.[6] Root of the plant contains plumericine, β- dihydroplumericin, isoplumericin, β- dihydroplumericinic acid, fulvoplumerin and plumeride. Bark consists of Rubrinol: an antibacterial tritripenoid together with teraxasteryl acetate, lupeol, stigmasterol, oleanolic acid.[7] Stem bark also consist of four new iridoids viz., plumeridoids A, B, and C and epiplumeridoid C along with isoplumericin, plumericin, dihydroplumericin, allamcin, fulvoplumerin, allamandin, plumieride, P- E-coumaric acid, 2,6-dimethoxy- P-benzoquinone, scopoletin, ajunolic acid, ursolic acid, oleanolic acid, beta-amyrin acetate, betulinic acid, lupeol and its acetate, glucoside of beta-sitosterol, and a mixture stigmasterol and beta-sitosterol.[8] The latex of the plant contain alkaloids and saponins.[9] Fresh leaves of Lal Gulachin consist of essential oils like, (Z)-β-farnesene- patchoulene, limonene, (E)-β-farnesene, α- copaene and phytol, while flowers consist of (E)-non-2- en-1-ol, limonene, phenyl acetaldehyde and n-tetradecanal.[10] It has been reported that distinct parts of Fringpiani have diverse biological properties. Pod has abortifacient,[11] hepatoprotective[12] effects; bark is antinociceptive and anti-inflammatory.[13] Leaves are found to have antiulcer activity,[14] whereas flowers have profound antioxidant effects.[15]

In India the plant is traditionally acclaimed as a purgative, remedy for diarrhea and as an anti-itching agent. The milky juice has been used for the treatment of inflammation and rheumatism. The flowers are eaten with betel nut to cure ague.[16] The folklore information from Assam prompts that *Plumeria rubra* f. *rubra* is used for family planning and birth control.[17]

Bark of the plant is considered to be excellent source of ursolic acid, changes in the content of ursolic acid in different months throughout the year is being re-

Cite this Article: Srivastava G, Gupta A, Singh MP, Mishra A. Pharmacognostic Standardization and Chromatographic Fingerprint Analysis on Triterpenoids Constituents of the Medicinally Important Plant Plumeria rubra f. rubra by HPTLC technique. Pharmacogn J. 2017;9(2):135-41.

ported via HPLC, quantification showed that in the month of May the ursolic acid content in the bark is to its maximum level.[18] Present work is an effort to lay down the pharmacognostic standards of *Plumeria rubra* f. *rubra* which are still untouched and have not been explored yet. An attempt has also been made to generate the comparative fingerprint profile of the leaves and bark in respect to its ursolic acid and lupeol content via HPTLC.

MATERIAL AND METHODS

Pharmacognostic Studies

Collection and Authentication

Fresh leaves and bark of *Plumeria rubra* f. *rubra* were collected from NBRI, Lucknow in the month of October 2014 leaves and bark were washed and air-dried. The collected plant material was authenticated from National Institute of Science Communication And Information Resources (CSIR-NISCAIR), voucher specimen no (Ref. No. NISCAIR/RHMD/CONSULT/2015/2827/20) and voucher specimens were submitted in LWG herbarium. The air dried plant material was first washed with tap water, then again washed twice with double distilled water and then air dried. The air dried specimen (leaves and bark) were pulverized and sieved through 80# mesh size and stored in air-tight container at 25°C for future/further studies.

Macro and Microscopic Characteristics

The morphological characteristics of the specimen (leaves and bark) were studied and the photographs were taken with the help of Sony Corp. DSCS980, 12.1 megapixel camera. For microscopic studies transverse section (T.S) were preferred over longitudinal section (L.S). The fine sections of leaves were cut by free hand. The chlorophyll and the other pigments of the plant were removed by treating the sections with 5% potassium hydroxide (KOH) and 20% chloral hydrate as required. Photographs of different magnifications were taken with Olympus Microscope, Model Olympus (India), attached to YOKO CCD Camera.

Quantitative Microscopy

Quantitative microscopy of leaf such as stomatal number, stomatal index, veinislet, vein termination number, and palisade ratio were determined by using fresh leaves of the plant.[19,20]

Physiochemical Parameters

Evaluation of the physical constants of the drugs is an important parameter in detecting adulteration or improper handling of drugs. It includes ash values (total ash, acid insoluble ash, and water soluble ash), extractive values (alcohol soluble, water soluble), and moisture content.[21]

Phytochemical Screening

Preliminary phytochemical investigation of different extracts of leaves and bark of *Plumeria rubra* f. *rubra* was done by using several reagents assigned for the detection of several phytoconstituents like alkaloids, glycosides, flavonoids, saponins, tannins, carbohydrates, steroids and terpenoids.[22]

High Performance Thin Layer Chromatography (HPTLC)

Chemicals and Reagents

HPTLC analyses were performed on Merck 10×10 cm HPTLC silica gel 60F254 (0.25 mm) plates. Ursolic acid and lupeol was supplied by Sigma Aldrich, Germany. All the reagents used in the experiment were of analytical grade and were supplied by Merck, Darmstadt, Germany.

Preparation of Standard Solutions

Stock solutions of ursolic acid and lupeol were prepared by dissolving 0.1 mg/mL in methanol.

Sample Preparation

The fresh leaves and bark of *Plumeria rubra* f. *rubra* were collected, thoroughly washed with water to remove all debris. The plant materials were shade dried and powdered by using electric grinder at 60 mesh size. Extraction was performed by soxhlation method. Firstly the powdered plant material was defatted using soxhlet assembly with 250 mL of 98% petroleum ether for 24 hours. This was followed by 48 hours soxhlation of defatted powder by using 250 mL of methanol. The final methanolic fraction obtained was passed through Whatman No. 1 filter paper. The filtrate obtained was concentrated under vacuum in a rotary evaporator at 40°C and stored at 4°C for further use. The dried extracts were dissolved in 98% methanol to obtain a stock solution of 10 mg/mL, which is used for application of spots on HPTLC plates.

Development of HPTLC Fingerprinting of Ursolic acid and Lupeol

Instrumentation and Chromatographic Conditions

The following were the instruments and chromatographic conditions used. Spotting device: Linomat V automatic sample applicator; CAMAG (Muttenz, Switzerland), Syringe: 100 µL Hamilton (Bonaduz, Switzerland). TLC chamber: glass twin trough chamber (20×10×4 cm); CAMAG. Densitometer: TLC Scanner 3 linked to win CATS software V.4.06; CAMAG. HPTLC plates: 10×10 cm, 0.2 mm thickness precoated with silica gel 60 F_{254}; E. Merck (Darmstadt, Germany). Experimental conditions: temperature, 25 ± 2°C; relative humidity, 40%. Solvent system: toluene–ethyl acetate–formic acid (8:2:0.1). Detection wavelength: 500 nm. Visualization agent: Anisaldehyde -Sulphuric acid reagent. Slit dimension: 5.00×0.45 mm. Scanning speed: 10 mm s^{-1} and source of radiation: deuterium lamp.

Calibration Curve of Ursolic Acid and Lupeol

Stock solutions of ursolic acid and lupeol (100 µg mL^{-1}) were prepared in HPLC grade methanol. Different volumes of stock solution were spotted on the TLC plate to obtain concentrations of 100-600 ng per band of ursolic acid and lupeol respectively. The data of peak areas plotted against the corresponding concentrations were treated by least square regression analysis method validation.

RESULTS

Morphology and Microscopy

Leaf

Leaves are shortly stalked, pubescent, having characteristic odor and bitter taste, ventral surface of leaf has dark green color whereas the dorsal surface is light green in color. Leaf has average length of 31.35 cm and width of 0.2-10.2 cm with elliptical shape, entire margin, pinnate venation and acute apex (Figure 2).

Bark

Bark has adhering cork with small patches which are transparent as well as exfoliating, inner surface is smooth having cream yellow color whereas outer surface is rough and wrinkled with greyish brown color. Bark has average breadth of 1-2 cm with double quill (Figure 9).

Transverse section of midrib and lamina

Transverse section of the leaf shows typical dorsiventral structure with slightly wavy upper and lower epidermis that is covered with thin layer

of cuticle. Mesophyll shows the presence of single layer of highly compacted elongated palisade cells which is followed by 4-8 layers of spongy parenchyma (Figure 3, 4), lower epidermis has uniserriate blunt tip multicellular interwoven trichomes (Figure 5-6).

Vascular Bundle

The central portion of the midrib is occupied by prominent crescent bicollateral vascular bundle with xylem that is surrounded by inter-xyllary phloem towards upper epidermis and with outer phloem towards lower epidermis (Figure 7).

Stomata

Lower surface of the leaf contains more number of paracytic types of stomata than upper surface (Figure 8).

Transverse Section of Bark

Transverse section of bark at 4X magnification showing periderm with distinct cork (Figure 10). Periderm is followed by phloem that is divided by distinct medullary rays which are 3-4 cell wide (Figure 11), Periderm has outer layer of cork having alternating rectangular larger and smaller cells followed by 4-5 layers of phellogen having tubular horizontally elongated cells (Figure12-13), periderm is followed by phloem consist of calcium oxalate crystals (Figure 14), transverse section of periderm also shows presence of calcium oxalate crystals and sclereids as well (Figure 15), powder study showed cork cells, calcium oxalate crystals, asteosclerieds (type of sclereids) and stone cells (Figure 16-18).

Quantitative microscopy, physiochemical parameters and phytochemical screening

These standardization parameters were performed as per the guidelines of Ayurvedic Pharmacopoeia of India. Preliminary phytochemical investigation revealed the presence of alkaloids, glycosides, sterols, carbohydrates, flavonoids, saponins and terpenoids. The results of quantitative microscopy and phytochemical screening are depicted in Table 1-2 whereas the result of physiochemical analysis is showed in Figure 1

High performance thin layer chromatography

In this study, several solvent systems were used for estimation of this triterpenoid and were investigated to evaluate the combinatorial separation of these compounds in a single solvent system and between different components of the extract. Among the different solvents systems investigated, mobile phase consisting of toluene: ethyl acetate: formic acid in the ratio of 8: 2: 0.1 *v*/*v*/*v* demonstrated good resolution between other peaks of the extract. The procedure for the separation and determination of different compounds in methanolic fraction of *Plumeria rubra* f. *rubra* leaves and bark using HPTLC-densitometry is reported at six point calibration curve in which ursolic acid and Lupeol were observed and quantified Table 3**.** The Rf value for ursolic was found to be 0.68±0.01 and that of lupeol was 0.46±0.01. HPTLC chromatogram and densitograms were obtained from standard compounds and methanolic fractions (Figure. 19-22), separation of all bands of plant samples and standard is shown in Figure 23. Both targeted compounds were identified by retention factor (Rf), peak purity 3D spectra and overlay UV-spectrum (Figure 24-26).

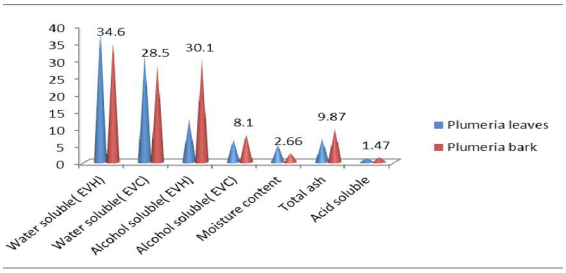

Figure 1: Physiochemical parameters of *Plumeria rubra f. rubra* leaf and bark EVH-Extractive Value Hot; EVC-Extractive Value Cold

Figure 2: Leaf of *Plumeria rubra f. rubra*

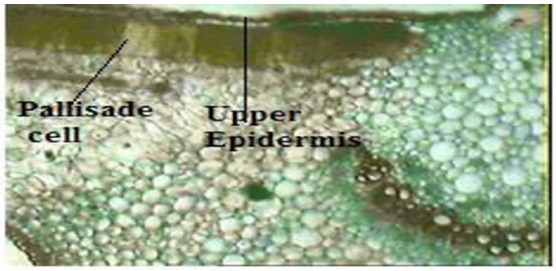

Figure 3: TS of upper epidermis and palisade cells

Figure 4: T.S of lower epidermis and spongy cells

Figure 5: TS of interwoven clothing trichome

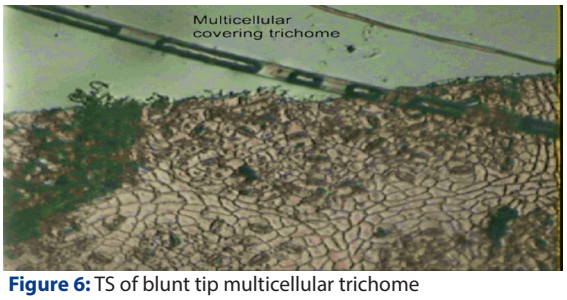

Figure 6: TS of blunt tip multicellular trichome

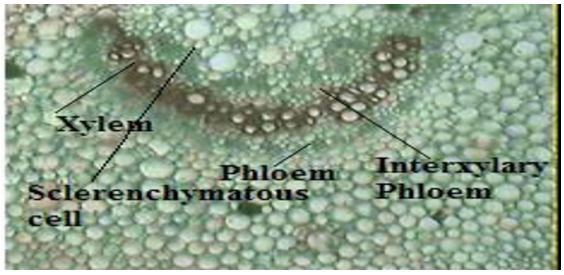

Figure 7: TS of midrib with bicollateral vascular bundle

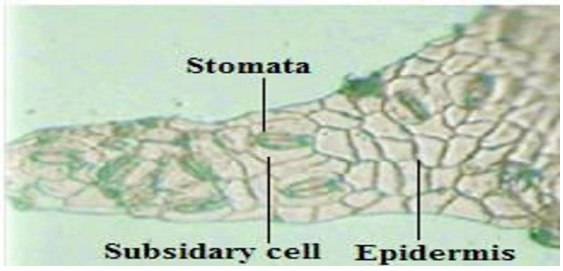

Figure 8: Surface study of leaf showing paracytic stomata at 10X

Figure 9: Fresh bark of *Plumeria rubra f. rubra*

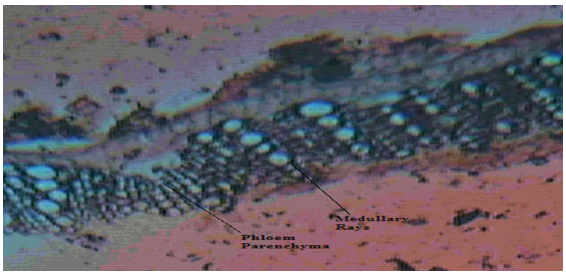

Figure10: TS of bark at 4 X

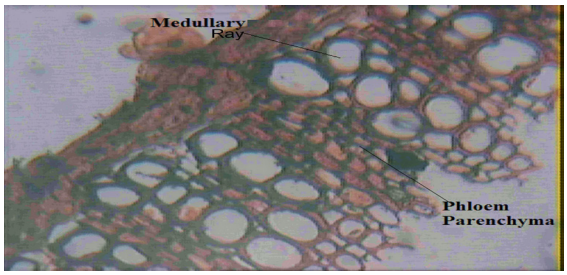

Figure 11: TS of bark at 10 X

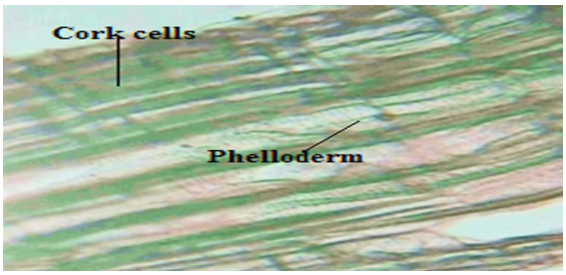

Figure 12: TS of Periderm

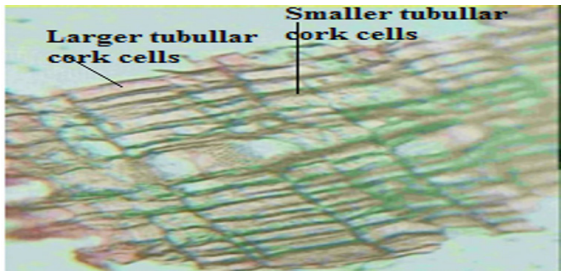

Figure 13: TS of Cork

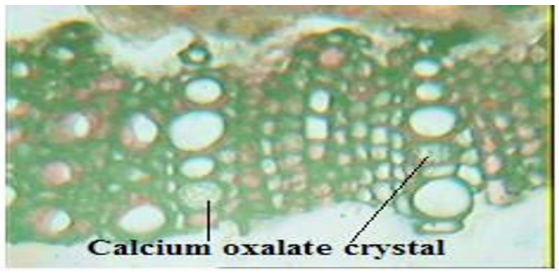

Figure 14: TS of Medullary rays showing calcium oxalate crystals

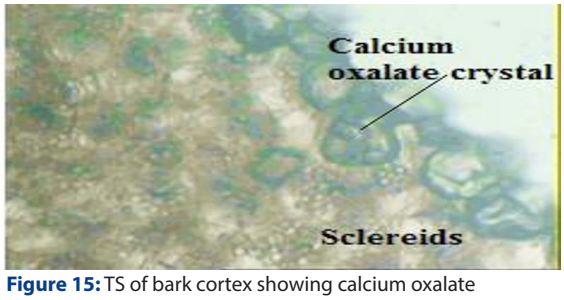

Figure 15: TS of bark cortex showing calcium oxalate

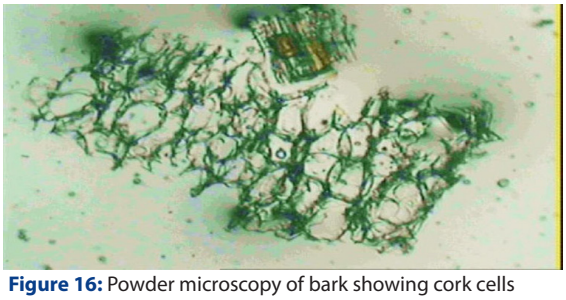

Figure 16: Powder microscopy of bark showing cork cells

DISCUSSION

Plumeria rubra f. *rubra* is one of the widely distributed plants in tropical part of India; despite of its diverse existence and equivalent ethno medicinal possessions the plant is yet not having a much proven evidences to justify its therapeutic efficacy in modern system of medicine. Author selected *Plumeria* genus as a plant of choice in a view of the fact that the *Plumeria* species ranges from 5-45,[25] thus the possibility of perplexity increases when it comes to selection(collection) of the related species of *Plumeria* as a drug of choice, similitude is the matter of concern as it not only exist within species but also prevalent among four forms of *Plumeria rubra* i.e. *Plumeria rubra* f. *acutifolia*, *Plumeria rubra* f. *lutea*, *Plumeria rubra* f. *rubra* and *Plumeria rubra* f. *tricolor*, all these forms can be differentiated mainly on the basis of color of its flowers

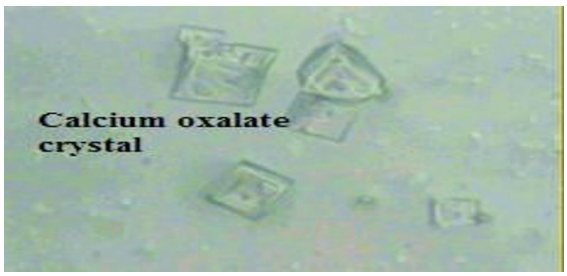

Figure 17: Powder microscopy of bark showing calcium oxalate crystals

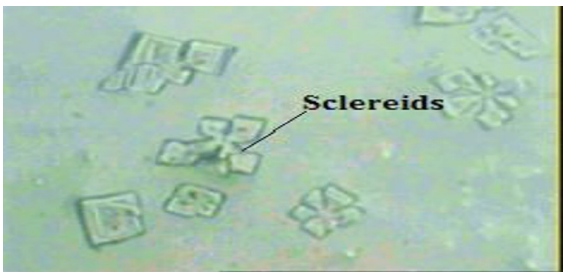

Figure 18: Powder microscopy of bark showing sclereids (asterosclereids)

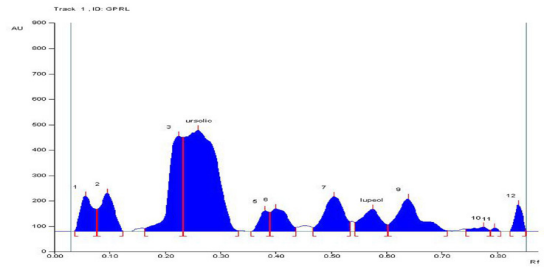

Figure 19: Densiometric chromatogram of *Plumeria rubra f. rubra* leaves

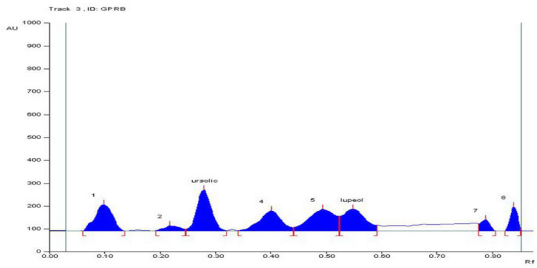

Figure 20: Densiometric chromatogram of *Plumeria rubra f. rubra* bark

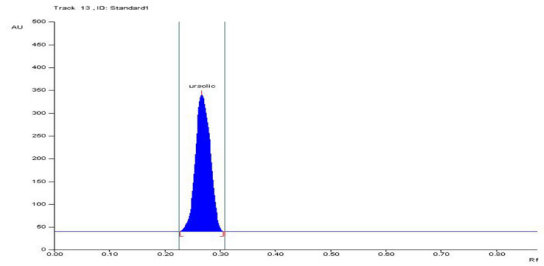

Figure 21: Densiometric chromatogram of standard Ursolic acid

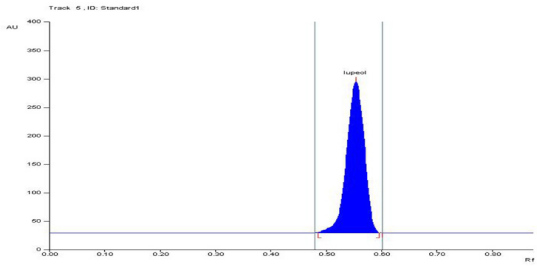

Figure 22: Densiometric chromatogram of standard Lupeol

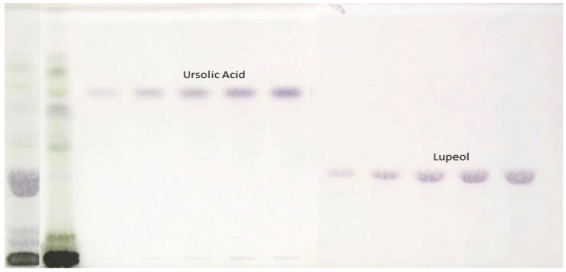

Figure 23: HPTLC Plate showing bands of *Plumeria rubra f rubra* leaves, bark, Ursolic acid and Lupeol

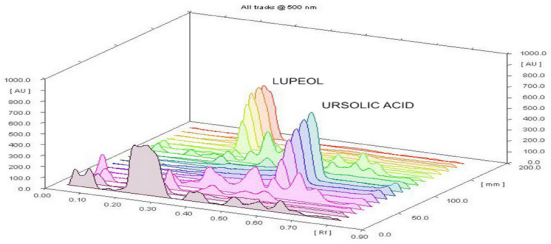

Figure 24: 3D spectra of all peaks at 500 nm

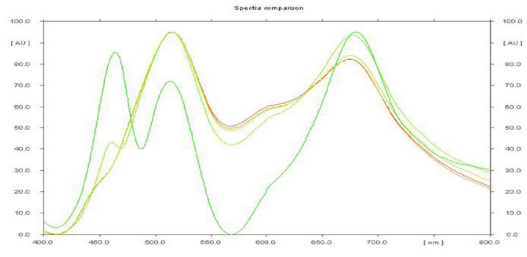

Figure 25: Spectral comparison of Ursolic acid

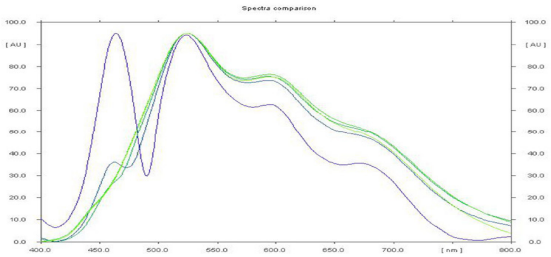

Figure 26: Spectral comparison of Lupeol

but still oodles of uncertainty prevails in establishing the identity of this strenuous plant as no pharmacognostic or anatomical work is on record to justify its authenticity. The literature survey narrate that the plant has abundant amount of ursolic acid and lupeol which in itself is a pronounced antihepatotoxic agent,[26] thus by detecting the presence and amount of these active constituents in both leaf and bark will pave a pathway for its exposure in several hepatic ailments. Also the plant is lacking in the standardizing parameters that can act as quality control principles to ensure the quality assurance that is much in demand when it comes to modern era of medicines. An attempt has been made to

Table 1: Quantitative microscopy of *Plumeria rubra f. rubra* Leaf

Parameters	Results
Vein islet number	15.4/sq.mm
Vein termination number	29.4/sq.mm
Stomatal number	11.7/sq.mm
Stomatal index	21.70%

Table 2: Phytochemical Screening of *P. rubra f .rubra* Leaves and Bark

Phyto-constituents	n-Hexane Extract		Methanolic Extract		Aqueous Extract	
	Leaf	Bark	Leaf	Bark	Leaf	Bark
Carbohydrate	-	-	+	+	-	-
Alkaloids	-	-	++	++	+	+
Proteins & Amino acids	-	-	-	-	-	-
Flavonoids	-	-	+	-	-	-
Tannins	-	-	-	-	+	+
Saponins	-	-	-	-	+	+
Steroids	-	-	-	-	-	-
Glycoside	+	-	+	+	-	-
Terpenoids	-	+	+	++	-	+

Table 3: Quantification of Ursolic acid and lupeol in *Plumeria rubra f. rubra* leaves and bark methanolic fraction

Plant Sample	Ursolic Acid (%)	Lupeol (%)
P. rubra Leaves	0.96	0.014
P. rubra Bark	0.051	0.018

finger out several morpho-microscopic parameters that can be utilitarian in establishing the identity of this plant. In this background certain reliable exemplar in transverse section of the leaf can be the presence of crescent bicollateral vascular bundle that is surrounded by some cells that are sclerenchymatous in nature (Figure 7). Transverse section of the bark show wavy periderm which distinctively reveal presence of cork and phelloderm (Figure 12) the bast tissue consist of parenchymatous cells few of which has conspicuous calcium oxalate crystals, phloem is traversed longitudinally by 2-6 cell wide medullary rays (Figure 14), the sui generis feature of the bark powder is the presence of asterosclereids (sclereids) (Figure 18). HPTLC was accomplished to generate fingerprint profile of *Plumeria rubra* f. *rubra* in order to identify ursolic acid and lupeol and results revealed that it is present in appreciable amount, both of these biomarkers has corroborated itself in fortifying the liver against several adverse conditions. Thus this plant can be further explored for its antihepatotoxic potentials. Information's generated in this work are empirical in terms of standardization of the drug and also to fetch the attention of pharmacologist to explore this plant in the line of scientific research.

CONCLUSION

Author endeavored to bring out every relevant detail on macroscopic and microscopic characters of this plant. Phytochemical investigation of leaf and bark revealed presence of several phytoconstituents like alkaloid, glycoside, flavonoids, terpenoids and sterols which in itself reveal that this plant can be the center of several pharmacological activities. HPTLC analysis of *Plumeria rubra* f. *rubra* showed that it contain significant amount of ursolic acid and lupeol. Thus this analytical result opens several doors for the plant to build its identity as a hepatoprotective agent as hypothesized by author. Present study is an attempt to figure out basic needs necessary to generate scientific/technical standards so as to justify the herbal drug worth exploring for further research work and also to keep a check on intentional/unintentional adulteration also it lay downs the standards which could be used as the standardization parameters for the identification and authentication of plant *Plumeria rubra* f. *rubra.*

ACKNOWLEDGEMENT

Authors are thankful to Professor Rajiv Gupta, Dean, School of Pharmacy, BBD University, Lucknow for providing necessary laboratory conditions.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ABBREVIATION USED

TS: Transverse section; **HPTLC:** High Performance Thin Layer Chromatography; **HPLC:** High Performance Thin Layer Chromatography; **P. *rubra*:** *Plumeria rubra*

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Cite this Article: Srivastava G, Gupta A, Singh MP, Mishra A. Pharmacognostic Standardization and Chromatographic Fingerprint Analysis on Triterpenoids Constituents of the Medicinally Important Plant Plumeria rubra f. rubra by HPTLC technique. Pharmacogn J. 2017;9(2):135-41.