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Original Article

Pharmacognostic Standardization and HPTLC Fingerprinting of *Prosopis cineraria*; An Ayurveda Mentioned Plant

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ABSTRACT

Background: *Prosopis cineraria (P. cineraria*) is an important mythological plant, popular in several indigenous systems of medicine including ayurveda, sidda and unani. Traditionally this plant is used for various ailments including anxiety, asthma, depression, epilepsy, hypertension, stress etc. **Object:** In the present study, macro and microscopic characters, physiochemical parameters, quantitave microscopy and phytochemical screening were done by using WHO guidelines. Anti-oxidant activity was assessed by DPPH method. HPTLC detection had been done to detect lupeol and urosolic acid and to quantify their relative levels. **Result:** Microscopy of the leaves showed the presence of different type of trichomes, stomata, calcium oxalate crystals, veins and epidermis. Total ash value, water soluble ash, acid soluble ash, water soluble extract, alcohol soluble extract, loss on drying, swelling index and foaming index were found to be 3.1±0.55, 2.1±0.43, 0.6±0.01 38.0±0.15, 37.8±1.50, 12.8±0.22, 1.4±0.02 and less than 100 respectively. Phytochemical analysis of different extracts showed the presence of alkaloids, glycosides, tannins, saponins, phenolic compounds etc. The IC_{50} values found to be 2.63 and 12.1for the ethanolic and aqueous extracts of *P. cineraria* respectively. HPTLC analysis showed the presence of both of the standards, the amount of ursolic acid and lupeol was found to be 0.017% and 0.02% for each extract respectively. **Conclusion:** The data generated would be useful for the preparation of monograph of the plant as no such work reported yet.

Key words: *Prosopis cineraria,* Fabaceae, Kalpavriksha, Ayurveda, Mythological plant, HPTLC.

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INTRODUCTION

Prosopis cineraria (*P. cineraria*),family- Fabaceae, an indigenous plant mentioned in Ayurveda with several clinical benefits.[1] In India, it is commonly called"Kalpavriksha"[2] and has an artistic and literary theme common to the Hindu Bhagavatas, the Jains and the Buddhists.[3] Mythologically, it is referred to as Shammi tree and is culturally important as a tree of lord Rama and Laxamana are said to have placed their swords and weapons on this tree.[4] The genus *Prosopis* comprises 44 species distributed mainly in Southwest Asia, Africa and in the Americas (from western North America to Patagonia).[5] The vernacular names are Khejri (Hindi and Sanskrit), Janti/Loong tree (Rajasthani), (Punjabi), Sami (Gujarat), Sumri (Tamil) and Jammi (Telugu) and in Sind it is known as Kandi.[6] The leaves contain campesterol, cholestrol, sitosterol, stigmasterol, actacosanol, hentriacontane, methyl docosanoate etc[7,8] and these compounds possessed potent antioxidant, hypoglycemic and thyroid inhibiting properties.[9] Fresh leaf juice mixed with lemon juice is used for dyspepsia; an extract of crushed pods is used for earache, toothache, pain relief from fractured bones while aqueous extracts of bark and leaves are useful in wounds if applied externally.[10,11] *P. cineraria* is reported to have various pharmacological activities such as analgesic, antipyretic, antihyperglycemic, antioxidant, antihypercholesterolemic, antitumor, nootropic, anticonvulsant etc. due to presence of various primary and secondary metabolites.[12-26]

There is no pharmacognostic data available on quantitation of urosolic acid and lupeol with respect to P. cineraria leaves to determine the physicochemical standards required for the future benefit of the crude drug. Urosolic acid and lupeol are the responsible for many of the pharmacological activities of this plant, so the main aim of the present investigation was to study the macroscopic, microscopic, physicochemical standards, antioxidant properties of this species. Phytochemical screening and high performance thin layer chromatography (HPTLC) studies has done as per WHO guidelines. These studies may be useful for pharmacognotic evaluations and for the development of formulations in future and for preparing a monograph of the plant

MATERIALS AND METHODS

Collection and authentication of plant material

The leaves of *P. cineraria* were collected from National Botanical Research Institute, Lucknow, India in July 2015 and authenticated by Dr. Sunita Garg, Botanist, NISCAIR, Delhi (Ref No. NISCAIR/RHMD/2015/2862/55-2).

Drugs and chemicals

Methanol, Toluene, Formic acid, Ethyl acetate, DPPH purchased from SDFCL, Mumbai. Ethanol was purchased from Changshu Yangyuan chemical, China. Ascorbic acid was purchased from Thomas Chemical Laboratory, Mumbai. Ursolic acid and lupeol was supplied by Sigma Aldrich, Germany. All the reagents used in the experiment were of analytical grade.

Macroscopic and microscopic analysis

The macroscopic features of color, size, shape etc of the leaves were studied followed the method of Evans (2009) with the help of digital camera of Sony with 10.1 megapixel. The microscopic features of the leaves were studied with the help of midrib. Very thin sections of leaves were treated with 5% potassium hydroxide (KOH) and 20% chloral hydrate for removed of chlorophyll and fatty substances. Images were taken with image analyzer (Olympus Microscope with YOKO CCD camera).[27]

Analysis by quantitative microscopy

The different parameters of fresh leaves including stomatal number, stomatal index, vein-islet number, vein termination number were performed according to WHO guideline.[28]

Analysis of powder material of leaves

Completely air dried plant material was first washed with distilled water, dried through air then powdered through a mechanical grinder and stored in an air tight container for pharmacognostical/pharmacological analysis or for the development of formulations.

Analysis of physical parameters of powder material

The physical parameters loss on drying, extractive values, ash value, swelling index, foaming index were performed by standard methods.[29-35]

Identification of secondary metabolites by phytochemical screening

Different primary and secondary metabolites including carbohydrates, alkloids, flavanoids, tannins, proteins, terpenoids etc were assayed for their presence in the leaf extracts.[36]

Antioxidant activity using DPPH (1, 1-diphenyl-2- picryl - hydrazyl) method[37]

DPPH (0.1 mM solution) in methanol was prepared. From this stock, 1.0 ml solution was added to 3.0 ml of extract solution in water at different concentrations (25, 50, 75, 100 µg/ml). The mixture was incubated at room temperature for 45 min. and absorbance was measured at 517 nm. Ascorbic acid was taken as reference. The percentage inhibition of DPPH free radical was calculated based on the control reading, which contain DPPH and distilled water without extract using the following equation:

DPPH scavenged (%) = Absorbance of control – Absorbance of test / Absorbance of control x100

High performance thin layer chromatography for urosolic acid and lupeol

Methanolic extract of *P. cineraria* (MEPC) showed the presence of triterpenoids. Hence, methanol fractions were used for HPTLC studies to detect and quantify the ursolic acid and lupeol.

Preparation of standard solutions

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

Sample preparation

A mass of 100 mg of air dried leaves powder was defatted with petroleum ether and then Soxhlet extracted with methanol for 16 h. The methanolic extracts were vacuum evaporated and concentrated, then 10 mg of the methanolic extracts were dissolved in 10 mL methanol to yield a test sample (1000 µg/mL).[38]

Instrumentation and Chromatographic Conditions

TLC plates (10 cm × 10 cm aluminum backed plates coated with Silicagel GF254; Merck, Mumbai, India) were used for HPTLC analysis. The standard solutions (ursolic acid and lupeol) and test solutions (MEPS) were applied to separate plates, maintaining a distance of 10 mm from the bottom and corner of the plate with the help of camag Linomat V sample applicator (Muttenz, Switzerland) with a 2 µL Hamilton (USA) syringe. Toluene: ethyl acetate: formic acid (8: 2: 0.1 v/v/v) was used as as mobile phase in a Camag glass twin trough chamber. The chamber was saturated with mobile phase for 30 min and all plates were dried in an oven after complete development then scannedat 500 nm by a Camag TLC Scanner with WINCAT software. Calibration curves of stock solutions of lupeol and ursolic acid (100 µg/ml) were prepared in HPLC grade methanol. Different volumes of stock solution were spotted on the TLC plate to obtain concentrations of 100–500 ng per band of lupeol and ursolic acid respectively.[39]

RESULTS AND DISCUSSION

Morphology

The fresh leaf of *P. cineraria* is compound, light green in color (Figure 1) and alternate in arrangement. Between 16 and 20 pairs of leaflets are present, margin is entire with acuminate apex. The ventral surface is smooth while the dorsal surface is rough. The leaves have reticulate venation, oblong shaped and petiole is 0.5–5 cm long.

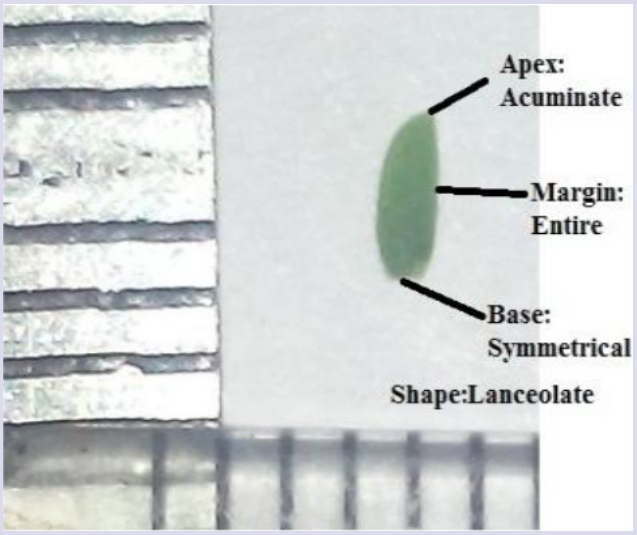

Figure 1: Morphology of *P. cineraria* leaf.

Microscopical characters

A transverse section of *P. cineraria* leaf (midrib and lamina) showed the presence of upper epidermis, lower epidermis and mesophyll (Figure 2a). The upper epidermis was single layered, slightly rectangular, with a distinct cuticle and simple trichomes. Just below the upper epidermis, a single palisade layer with 2 to 3 layers of spongy parenchyma is present.. The cells of upper epidermis and lower epidermis are similar in size. The central portion showed the presence of a bundle sheath. The leaf surface (upper and lower surface) study detected the presence of epidermal cells and paracytic stomata, which followed two subsidiary cells are parallel to that of stoma and simple trichomes on margin (Figures 2b and 2c).

Powder microscopical evidence

Powder characteristic of *P. cineraria* leaves revealed the presence of vessels, simple trichomes, calcium oxalate crystals, epidermal cells and paracytic stomata etc (Figure 3).

Quantitative parameters, physiochemical eveidence and phytochemical screening

The quantitative parameters and physicochemical properties of the leaves were performed according to Ayurvedic Pharmacopoeia of India. Phytochemical testing of different extracts shows the presence of alkaloids, glycosides, sterols, carbohydrates, tannins, terpenoids, saponins and flavonoids as shown in Tables 1-2 respectively.

Antioxidant activity using DPPH method

DPPH is a nitrogen based free radical which is converted to the stable molecule dipheny 1- picryl hydrazine.[40] The reduction of DPPH by the extracts is due to the transfer of a hydrogen atom or electrons, whereas phenolic compounds have better antioxidant property.[41] Table 3 shows the DPPH radical scavenging activity of different extracts compared to ascorbic acid. The best antioxidant activity was found for EEPC, with IC_{50} value of 2.63 (R^2=0.968) where asthe IC_{50} value of ascorbic acid is 1.96 (R^2=0.959).

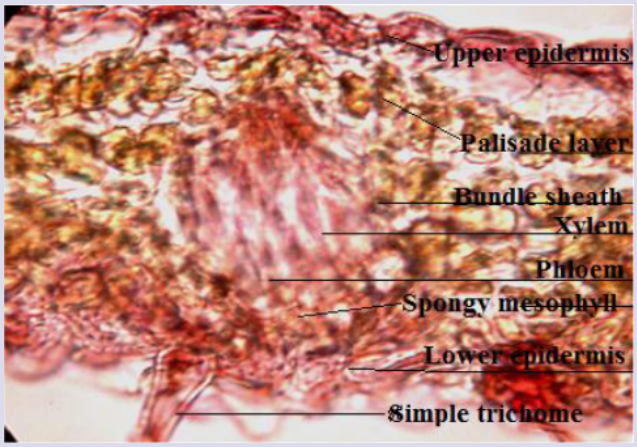

Figure 2a: Transverse section of *P. cineraria* leaf.

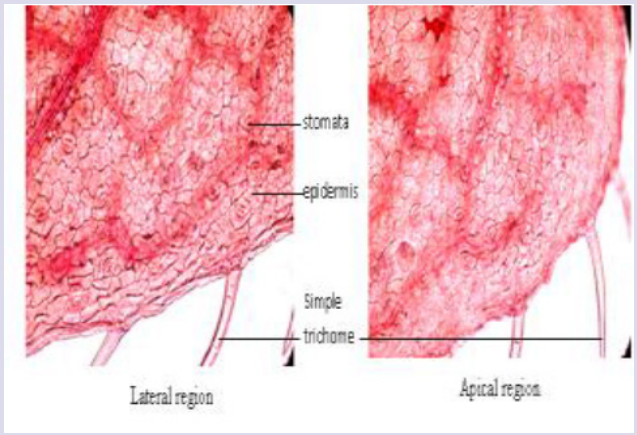

Figure 2b: upper leaf surface study of *P. cineraria* leaf.

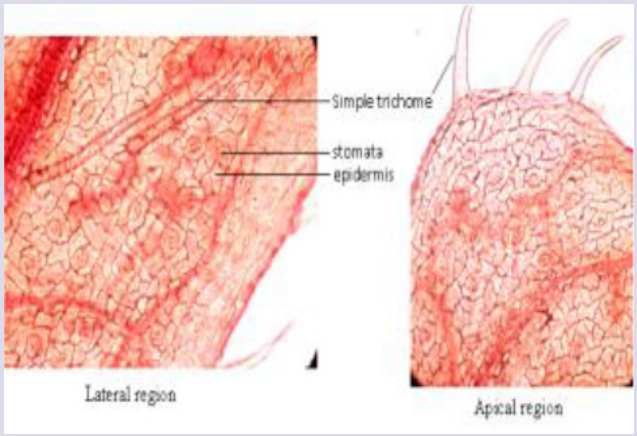

Figure 2c: lower leaf surface study of *P. cineraria* leaf.

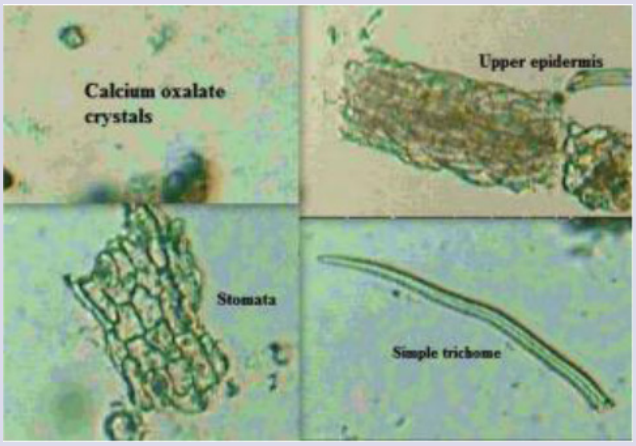

Figure 3: Powder microscopy.

Table 1: Quantitative microscopy and physiochemical analysis of *P. cineraria*.

Parameters	*P. cineraria*
Vein islet number (1 mm² leaf surface)	16.30±0.09
Vein termination number (1 mm² leaf surface)	24.28±0.16
Stomatal number (1 mm² leaf surface on lower epidermis)	19.36±0.63
Stomatal number (1 mm² leaf surface on upper epidermis)	24.39±0.22
Stomatal index	7.46±0.28
Palisade ratio	8.06±0.42
Total ash	3.1±0.55
Water soluble ash	2.1±0.43
Acid insoluble ash	0.6±0.01
Water extractive value	38.0±0.15
Ethanol extractive value	37.8±1.50
Loss on drying	12.8±0.22
Swelling index	1.4±0.02
Foaming index	<100

Table 2: Phytochemical analysis of different extracts of *P. cineraria*.

Phytoconstituents	AEPC	EEPC	MEPC
Alkaloids	+	++	+++
Glycosides	+	+	++
Tannins	++	+++	+++
Flavonoids	+++	+++	+++
Fats and oil	+	+	+
Carbohydrates	++	++	++
Reducing sugar	+	+	+
Proteins	+++	++	+++
Saponins	-	-	+
Terpenoids	++	++	+++

AEPC- Aqueous extract of *P. cineraria*, EEPE- Ethanolic extract of *P. cineraria* MEPC-Methanolic extract of *P. cineraria*, Positive sign indicate the presence of various phytoconstituents with respective to concentration like + (good), ++ (better), +++ (best) and negative sign indicate the absence of phytoconstituents.

Table 3: Antioxidant activity of different extracts of *P. cineraria* using the DPPH method.

Extracts	Concentration (µg/ml)	Absorbance	Percentage inhibition	Regression equation	IC_{50}
AEPC	25 µg/ml	0.530	41.3	Y=0.7x + 41.5 (R^2=0.890)	12.1
	50 µg/ml	0.537	40.1		
	75 µg/ml	0.541	39.8		
	100 µg/ml	0.547	39.5		
EEPC	25 µg/ml	0.535	40.2	Y=3.6x + 40.5 (R^2=0.968)	2.63
	50 µg/ml	0.631	29.6		
	75 µg/ml	0.636	29.5		
	100 µg/ml	0.641	28.2		
Ascorbic acid	25 µg/ml	0.458	49.1	Y=0.33x + 49.35 (R^2=0.959)	1.96
	50 µg/ml	0.462	48.6		
	75 µg/ml	0.465	48.3		
	100 µg/ml	0.467	48.1		

High performance thin layer chromatography fingerprinting for ursolic acid and lupeol

HPTLC methods are commonly applied for the identification, assay or content uniformity of herbal raw materials and their formulations.[42-44] In this study, several solvent systems were used for individual estimation of these phenolics and flavonoids to evaluate the combinatorial separation of these compounds in a single solvent system and between different components of the extract. From the solvent system investigations, mobile phase consisting of toluene: ethyl acetate: formic acid in the ratio of 8: 2: 0.1 *v*/*v*/*v* demonstrated the best resolution between other peaks of the extract. The procedure for the separation and determination of different compounds in methanolic fraction of *P. cineraria* using HPTLC-densitometry is reported at six point calibration curve in which lupeol and ursolic acid were observed. The identification of lupeol and ursolic acid were confirmed by the bands which obtained from the test sample and standards. The peak of ursolic acid and lupeol from the test sample was very near to standard. The spectral comparison of ursolic acid and lupeol and test extracts is shown in Figures 4a and 4b. Three-dimension-spectra of standard ursolic acid and of ursolic acid present in the test samples scanned at 500 nm. The three-dimension-spectra of the standard lupeol and of the lupeol present in the test sample scanned at 500 nm (Figure 4c). The calibration curves of ursolic acid and lupeol are linear in the range of 100 – 500 µg/mL as illustrated in Figures 5a and 5b. The chromatograms of standard ursolic acid and lupeol are shown in Figure 6. The regression equations were found to be y = 4404+30.110x (R^2 =0.98697) for ursolic acid and y = 6189.130 +34.022x (R^2 =0.98553) for lupeol respectively. The estimated values of ursolic acid (0.017 %) and 0.02 % lupeol (0.02%) were calculated by linear regression.

CONCLUSION

Prosopis cineraria is a mentioned in Ayurveda with many clinical properties. The present work has performed according to Ayurveda pharmacopoeia of India and other official standards. Pharmacognostical features clearly explained the anatomy and physiology of plant. Phytochemical screening

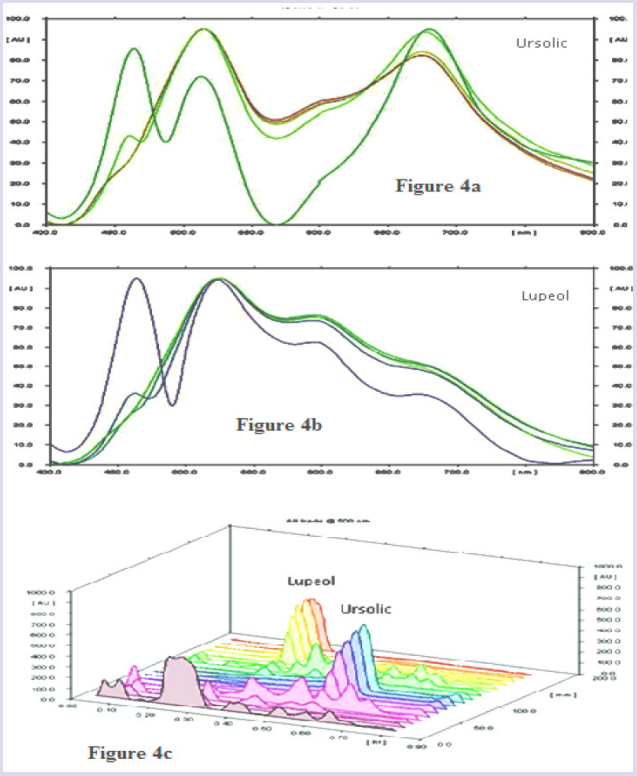

Figure 4a: Standard chromatogram of Ursolic acid, Figure 4b: Standard chromatogram of Lupeol, Figure 4c: Three dimensional spectra of ursolic acid with different concentration of test samples and lupeol with different concentration of test samples.

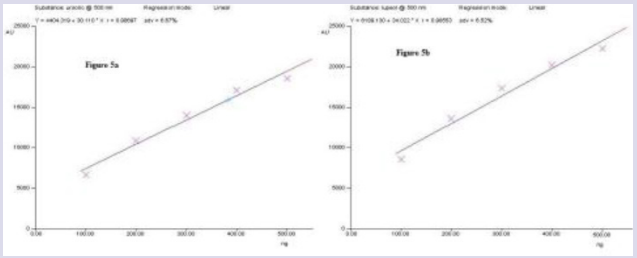

Figure 5a: Calibration curve of Ursolic acid, Figure 5b: Calibration curve of Lupeol.

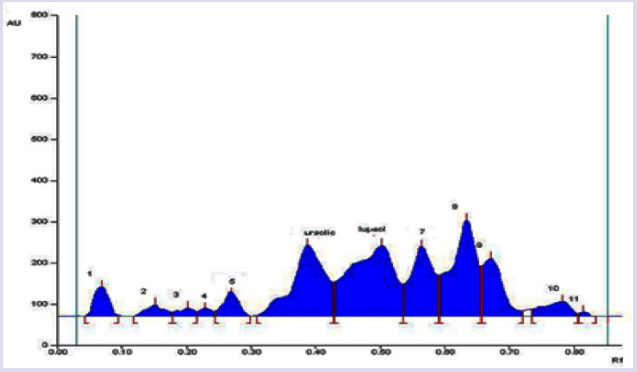

Figure 6: Chromatograms of ursolic acid and lupeol in *P. cineraria*.

revealed the various primary and secondary metabolites which are also useful to cure different diseases. HPTLC analysis of the shows the presence of substantial amounts of urosolic acid and lupeol.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

P. cineraria: *Prosopis cineraria*; **WHO:** World Health Organization; **HPTLC:** High performance thin layer chromatography; **DPPH:** 1, 1-diphenyl-2- picryl -hydrazyl; **AEPC:** Aqueous Extract of *P. cineraria;* **EEPE:** Ethanolic extract of *P. cineraria*; **MEPC:** Methanolic Extract of *P. cineraria.*

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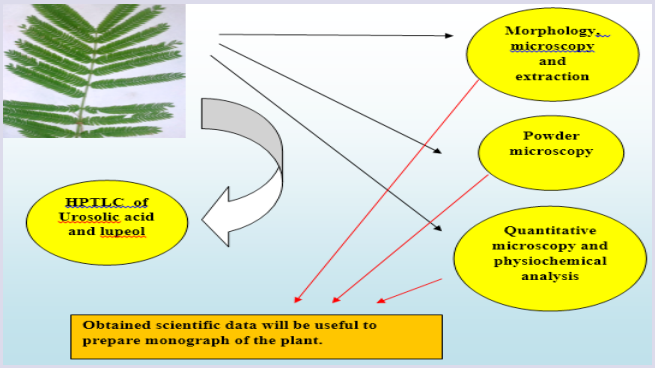
PICTORIAL ABSTRACT



SUMMARY

- It is most important or we can say very essential to study the pharmacognostical features of medicnal plants which are very useful or play a important role in human values and research. The current published research clearly concluded that the pharmacognostic specifications are very useful to prepare the plant data (monograph) and helpful for new researchers in coming future. The morphology and microscopy indicated the anatomy, plant features and may be indicated the location. Phytochemical testing shows the presence of various metabolites and these metabolites are useful to cure various diseases. HPTLC analysis indicated the presence of urosolic acid and lupeol. Futher research needed for the more exploitation of *Prosopis cineraria*.

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