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METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF LUPEOL FROM BARK OF *CRATAEVA TAPIA* L. USING HPTLC

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ABSTRACT

The stem bark of Crataevatapia L. is diuretic, used in urinary disorders; including urolithiasis, prostatic hypertrophy, urinary infections, uterine and gastro intestinal problems. It exhibits anti-inflammatory activity, stimulates bile secretion, appetite and bowel movement. The bark is also useful in cases of fever, mild form of skin disease, relieves vomiting, symptoms of gastric irritation, promotes appetite and decreases secretion of bile and phlegm. Medicinal property of the plant is attributed to secondary metabolites it synthesizes. The principal phytoconstituent of stem bark is lupeol; pentacyclic triterpenoidal shown to exhibit various pharmacological activities under in vitro and in vivo conditions. In the present study, a method was developed and validated for quantification of phytochemical marker lupeol in methanolic bark extract of Crataevatapia L. by High Performance Thin Layer Chromatography (HPTLC) method as per ICH guidelines. Method was validated in terms of precision, specificity, ruggedness, recovery. Densitometric scanning of lupeol was carried out at 540nm. Linearity was obtained in the range of 0.1-0.6 µg/mland lupeol was found to be 1.99 mg/gm in bark. LOD and LOQ for lupeol was 0.013 and 0.043 µg/ml. This study represents simple, precise, rapid and selective HPTLC method for detection of lupeol from bark of Crataevatapia L.

Keywords: *Crateavatapia L., HPTLC, Lupeol, Method validation, Quantification.*

INTRODUCTION

*Crataevatapia*Linn.ssp.*odora* (Jacob.) Almedia (syn. *C. religiosa* var. *nurvula* Hook. f.) (henceforth written as *Crataevatapia* L.) belonging to family Capparaceae is a moderate, much branched deciduous tree, commonly called as 'Varuna'. *Crataevatapia* L. is globally distributed in India, Myanmar, Indonesia and China. In India it is found in Peninsular India, Western India, Gangetic Plains and Eastern India upto Tripura and Manipur [1]. It is often found along streams, but also in dry deep boulder formations in sub- Himalayan tract [2]. It is usually cultivated in vicinity of temples in Central India, Bengal and Assam [2, 3].

The stem bark is hot, bitter at first and then sweet sharp taste, easy to digest, stomachic, laxative, antilithic, vesicant, antihelminthic, detergent, bechic, expectorant; removes "vata", good in strangury, disease of chest, blood, tuberculous glands; causes biliousness. It is demulcent, antipyretic, sedative and tonic. The bark is useful in cases of urinary complaints, fever, mild form of skin disease, relieves vomiting, symptoms of gastric irritation, promotes appetite and decreases secretion of bile and phlegm [2]. The stem bark also exhibits anti-inflammatory activity, stimulates bile secretion, appetite and bowel movement. Bark is diuretic, finds application in urinary disorders; including urolithiasis, prostatic hypertrophy, urinary infections, uterine and gastro intestinal problems [4]. In North east of Brazil, *Crataevatapia* bark infusions have been used in popular medicine as hypoglycemic agent [5]. The bark of stem constitute a principal drug material which is used for calculous affections [6].

Lup-20(29)-en-3β-ol, generally known as lupeol, clerodol, fagarsterol and lupenol exhibits a broad spectrum of biological activities and can be used as chemopreventive agent to avoid several disease. The basic outline of biosynthetic pathway include series of reactions responsible for both triterpenes and steroids biosynthesis occurring in the cytosol and constitute the Mevalonate (MVA) pathway. Isopentenyl pyrophosphate isomerase (IPI) plays a decisive role towards terpenoid and steroid biosynthesis by MVA pathway [7-9].

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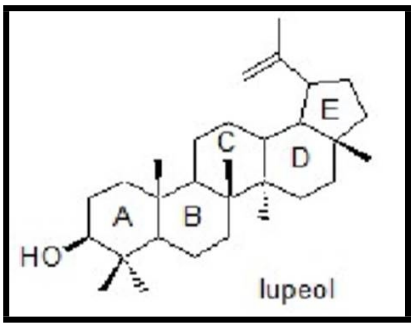

Fig. 1: Structure of Lupeol

Chemical formula of Lupeol is $C_{30}H_{50}O$ and its melting point is 215-216^{0}C. Lupeol is pentacyclictriterpenoidal type in nature. Lupeol has been shown to exhibit various pharmacological activities under *in vitro* and *in vivo* conditions. These include its beneficial activity against inflammation, cancer [10, 11], arthritis [12, 13], diabetes, heart diseases [14], renal toxicity, hepatic toxicity [15], microbes [16, 17] and protozoans [9, 18].The present study was carried out to quantify bioactive marker compound lupeol using methanolic extract of bark of *Crataevatapia* L.

MATERIALS AND METHODS

Collection of plant material:

The flowering twig of *Crataevatapia* L. was collected from Kalyan, M.S., India. Herbarium was prepared and authenticated from Blatter herbarium, St. Xavier's College, Mumbai, M.S., India. Bark was collected and dried in oven at 40±2^{0}C for a week. After drying, the plant material was homogenized to fine powder, sieved through 100 microns mesh and stored till further use.

Reference Standard and reagents:

Standard Lupeol (≥94% purity) was procured from Sigma-Aldrich Chemie GmbH (Aldrich, Division, Steinbeim, Germany). Toluene, Methanol, Anisaldehyde and Sulphuric acid used in the present work were procured from S. D. Fine Chemicals, Mumbai, India.

Preparation of standard lupeol solution:

Stock solution of standard Lupeol (50 ppm) was prepared in methanol. It was diluted further to give concentration ranging from 0.1-0.6 µg/ml.

Preparation of Sample extract:

1g of dried bark samplewas weighed and phytoconstituents were extracted in 10 ml methanol by vortexing for 1-2 minutes, left to stand overnight at R.T. (26±2^{0}C). The extracts were filtered through Whatmann filter paper No. 41 (E. Merck, Mumbai, India) and the filtrate was used for quantification.

HPTLC Instrumentation:

Chromatographic conditions:

Chromatography was performed on precoated silica plate (silica gel G60 F_{254} - Merck). 10 µl of bark and Lupeol extracts were loaded on precoated silica plates (silica gel G60 F_{254} - Merck) as bands of 8 mm width using CAMAG LINOMAT 5 applicator at distance of 14 mmfrom the edge of the plates. The plate was developed to a distance of 85 mm in a CAMAG twin trough chamber (20 X 10 cm) presaturated for 25 minutes with mobile phase Toluene: Methanol: Glacial acetic acid (9:1:0.1 v/v). The chromatographic conditions had been previously optimized to achieve best resolution and peak shape.

The plates were further derivatized using Anissulphuric acid and heated at 105^{0}C for 5 minutes visualized using CAMAG TLC visualizer and scanned densitometrically at 550 nm in absorbance mode with Tungsten lamp using CAMAG TLC Scanner 3 in conjunction with winCATS software.

Method Validation:

The proposed HPTLC method was validated according to the International Conference on Harmonization guidelines [19-25].

Precision Studies:

Instrumental precision was checked by repeated scanning of (n=12) of same spot of lupeol (0.4µg/ml).Interday and intraday precision was studied by analyzing aliquots of standard lupeol containing 0.4 µg/ml on same day (Intraday) and on different days (Interday) in triplicates. The results were expressed as % RSD.

Linearity:

Stock solution of 50µg/ml was diluted to give a range of 0.1-0.6 µg/ml of lupeol in methanol. Each of these concentrations was loaded on plate, plate was developed and detector response for different concentration was measured. Graph of peak area against concentration of lupeol was plotted.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

For LOD, LOQ determinations, different concentrations of standard were applied along with methanol as blank and determined on basis of signal to noise ratio. LOD was considered as 3:1 and LOQ as 10:1.

Specificity:

Specificity was ascertained by analyzing standard compounds and samples. The bands for lupeol from sample solution were confirmed by comparing Rf to those of standard.

Ruggedness:

Ruggedness of the method was checked by introducing changes in chromatographic parameters, such as mobile phase composition, spotting volume to determine their influence on Rf.

Accuracy:

The accuracy of the method was assessed by performing recovery studies at three different levels (80, 100 and 120%, spiking of lupeol at these levels in plant matrix).

RESULTS AND DISCUSSION

Precision Studies:

Instrumental precision was checked by repeated scanning of the same band of lupeol for twelve times each. Standard lupeol was spotted both at intra-day (spotting three times within 24h) and inter-day (spotting three times within 3 days intervals) to check the precision. The results are expressed as %RSD (Table 1). The method was found to be precise with % RSD for instrument precision 0.07, intraday as 1.08 and interday as 1.13. The results indicated that the method is precise and reproducible (Table 1).

Linearity:

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was found to occur in the range of 0.1-0.6 µg/ml.The linearity range for lupeol was 0.1-0.6 µg/ml with correlation coefficient 0.999.LOD and LOQ value for lupeol was found to be 0.013 and 0.043µg/ml respectively (Table 1).

Specificity:

Specificity was ascertained by analyzing standard and sample. The bands for lupeol from sample solution were confirmed by comparing Rf to those of standard (Table 1).

Ruggedness:

The method was found rugged for the parameters like change in mobile phase composition, change in spotting volume and detection wavelength. No significant changes in Rf or response to lupeol was observed, indicating the ruggedness of the method.

Accuracy:

The accuracy of the method was established by performing recovery experiments, using the standard addition method, at three different levels (80%, 100% and 120% of standard lupeol solution). Value of percentage recovery for lupeol is found to be 98.36% (Table 2).

Quantification of lupeol in *Crataevatapia* L. bark:

Lupeol was quantified and amount in sample was found to be 1.99 mg/gm (Table 3).

After derivatisation with Anissulphuric acid

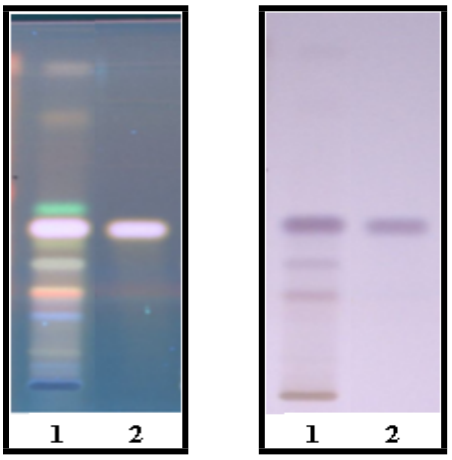

Plate 1a: At 366 nm Plate 1b: At 550 nm

Key: Track 1: Methanolic extract of *Crataevatapia* L. bark**;**
Track 2: Standard Lupeol

Fig. 2: HPTLC profile of methanolic extract of *Crataevatapia* L. bark

Table No. 1: Method validation parameters for quantification of lupeol

S. No.	Parameters	Lupeol
1	Instrument precision (% RSD, n=12)	0.07
2	Intraday precision (% RSD, n= 3)	1.08
3	Interday precision (% RSD, n= 3)	1.13
4	Linearity (correlation coefficient = r^2)	0.999
5	Limit of detection (LOD) µg/ml	0.013
6	Limit of quantification (LOQ) µg/ml	0.043
7	Linearity range	0.1-0.6 µg/ml
8	Specificity	Specific
9	Ruggedness	Rugged

Table No. 2: Recovery studies of lupeol at 80%, 100% and 120% addition

No	Marker	Amount of marker present in sample (µg)	Amount of marker added (µg)	Mean Recovery (%)
1	Lupeol	0.25	0.4	96.99
2			0.5	95.53
3			0.6	99.24

Table No. 3: Content of lupeol in *Crataevatapia* L. bark

Sample	Lupeol content (mg/gm)
Bark	1.99

Herbal medicines are composed of many constituents and are therefore capable of variation. Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active components of the herbal medicine. HPTLC fingerprinting profile is very important parameter of herbal drug standardization for the proper identification of medicinal plants[26].

A characteristic HPTLC profile was developed using methanolic extract of barkwhich may be used for quality evaluation and standardization. The solvent system of Toluene: Methanol: Glacial Acetic Acid (9:1:0.1 v/v) gave well resolved bands for different phytoconstituents. The bark extract was runalong with bioactive marker lupeol.

Dark pinkish color band was observed in bark sample under UV-366 nm and purple band was observed in visible light which matched with standard lupeol, confirming its presence in bark extract. Quantification of lupeol was carried out using methanolic bark extract (Plate 1a and 1b).

High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the evaluation of botanical materials. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. Additionally, numerous samples can be run in a single analysis thereby dramatically reducing analytical time. With HPTLC, the same analysis can be viewed collectively in different wavelengths of light thereby providing a more complete profile of the plant[27].

CONCLUSION

In the present study, a method was developed and validated for quantification of phytochemical marker lupeol in methanolic bark extract of *Crataevatapia* L. by High Performance Thin Layer Chromatography (HPTLC) method as per ICH guidelines.The method was found to be simple, precise, accuratewhich can be used in quality control and standardization. It can also be used as quality control of herbal materials as well as formulations containing lupeol.

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