

Pharmacognostic specifications and quantification of oleanolic acid and lupeol in *Mollugo oppositifolia* Linn.

Karuna Modi and Mamta Shah*

Department of Pharmacognosy, L. M. College of Pharmacy, Navrangpura, Ahmedabad

ABSTRACT

Background: *Mollugo oppositifolia*, is one of the plants commonly used as, 'Parpata' by Ayurvedic practitioners. It is indicated as a bitter tonic, antiseptic and febrifuge. **Aim:** To generate and ensemble data of physical parameters for ascertaining the identification and to develop validated HPTLC method for quantification of oleanolic acid and lupeol in *M. oppositifolia*. **Materials and Methods:** *M. oppositifolia* was studied for establishing pharmacognostic standards including macro and microscopical characters, physico-chemical analysis and quantification of oleanolic acid and lupeol by HPTLC method. **Results:** It is an annual, prostrate herb with linear-lanceolate leaf and white coloured flower. Microscopically root can be characterized by crescent shaped phloem associated with continuous or discontinuous rings of xylem; stem by epidermis bearing multi-cellular simple and glandular trichomes, and sclerenchymatous pericycle; and leaf by continuous band of a palisade cells and rosettes and prisms of calcium oxalate throughout parenchyma. Powdered drug can be typified by multi-cellular trichomes, fragments of epidermis of leaf in surface view, epidermis of corolla and entire or broken seeds. Saponins and flavanoids were found be the major components. HPTLC method was developed for quantification of oleanolic acid and lupeol using precoated silica gel plates as a stationary phase, and toluene: methanol (9.4: 0.6) as a mobile phase and scanning the plate at 545 nm. The amount of oleanolic acid and lupeol were found to be 0.027-0.029% w/w and 0.015-0.016% w/w respectively. **Conclusion:** The quality parameters and HPTLC method developed would serve as useful gauge in standardization of *Mollugo oppositifolia*.

Key words: HPTLC, Lupeol, *Mollugo oppositifolia*, Oleanolic acid.

INTRODUCTION

Mollugo oppositifolia Linn. (Syn.: *M. spergula* Linn., *Glinus oppositifolius* (Linn.) A. DC.; Family: Aizoaceae) is an indigenous plant, commonly known as, 'parpata', throughout South India. It is a diffuse, ascending or prostrate, annual herb, found to be growing in Assam, West Bengal, Delhi, Gujarat and South India.^{1,2} The plant is highly valued in traditional medicine in the treatment of liver disorders, earache and skin diseases.^{3,4} The pharmacognostical study on powdered whole plant was reported in brief.⁵ Flavanoids reported in plant include vitexin, vitexin-7-glucoside, 2''-p-coumaroyl

vitexin-7-glucoside, apigenin-8-C-glucoside and naringenin-7-rhamnoglucoside.^{6,7} Triterpenoids reported are oleanolic acid, spergulagenic acid, spergulagenin-A, spergulagenol, spergulacin and spergulatriol.⁸⁻¹⁰ Oleanolic acid and lupeol both are reported to be having multiple biological activities such as anti-inflammatory, hepatoprotective, antitumour etc.¹¹⁻¹⁶ Literature survey indicated that plant is not yet studied for physico-chemical parameters as well as no phytoconstituents are analysed by modern techniques. Hence, we here propose data for establishing a complete monograph required for quality development. Further, the HPTLC method for estimation of oleanolic acid and lupeol has been validated for linearity, interday precision, intraday precision, repeatability, accuracy, and specificity, limit of detection and limit of quantification.

*Corresponding author

Dr. Mamta B. Shah

Head, Department of Pharmacognosy, L. M. College of Pharmacy,
Navrangpura, Ahmedabad: 380 009
E-mail: mbshah2007@rediffmail.com

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MATERIALS AND METHODS

Plant Material

Fresh, fully-grown, flowering plants of *M. oppositifolia* were collected from Kerala in the month of March 2011. The plants collected were authenticated by taxonomist of Gujarat University, Ahmadabad, Gujarat. Voucher specimen sample (LM 631) was deposited at the Department of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad, Gujarat. The plant material was cleaned, dried, powdered to 60 # and used for the present study.

Chemicals and Reagents

Standards oleanolic acid and lupeol were procured from Sigma Aldrich, India. All the solvents used were of chromatography grade and other chemicals used were of analytical (AR) grade.

Pharmacognostical Studies

The whole plant was studied for morphological characters. Microscopical study was performed for both entire (free hand transverse sections) and powdered material. Quantitative microscopy was carried out for leaf. Moisture content,¹⁷ ash values and extractive values were determined.¹⁸

Phytochemical Studies

Phytochemical screening was performed¹⁹ and saponins²⁰ flavanoids, phenolics²¹ and alkaloids²² were estimated.

Extraction and TLC Study

5 g of drug powder was exhaustively extracted with 100 ml methanol, filtered and dried. The methanolic extract was hydrolysed by refluxing with 70 ml 2 N HCl for 2 h. After neutralizing with sodium carbonate, it was extracted with toluene (3X 25 ml) and vacuum dried to yield 9.2% w/w of extract (Ext A).

The plant is reported to contain free oleanolic acid along with saponinins such as spergulagenic acid, methyl serpagulate that are representative of oxidative products of oleanolic acid. So, the Co-TLC of hydrolysate of methanolic extract is performed with reference standards oleanolic acid and lupeol, using precoated silica gel 60 F₂₅₄ plates and toluene: methanol, 9.4: 0.6 as a mobile phase.

Estimation of oleanolic Acid and lupeol by HPTLC method

Chromatographic conditions

HPTLC was performed on 10 cm × 10 cm precoated silica gel 60 F₂₅₄ plates (E. Merck, Germany). Before chromatography the plates were pre-washed by methanol and activated at 60°C for 5 min. Samples were applied to the plates as bands 6 mm wide and 12.2 mm apart using Camag Linomat V applicator (Muttenez, Switzerland) fitted with a 100 microlitre syringe (Camag, Switzerland). Linear ascending development was performed in Camag twin-trough glass chamber (10 × 10 cm) with mobile phase vapour [toluene: methanol, 9.4: 0.6] at room temperature (25±2°C). Plate was dried and derivatized using anisaldehyde sulfuric acid reagent in CAMAG derivatization chamber followed by heating at 110°C using Camag TLC plate heater. It was scanned in Camag TLC scanner using Win CATS software (version 1.4.3.6336) in absorption mode at 545 nm with slit dimensions 6.00 × 0.45 mm. The scanning speed was 20 mm/sec and source of radiation tungsten lamp.

The method was validated in terms of linearity, interday precision, intraday precision, repeatability, accuracy, and specificity, limit of detection and limit of quantification. International Conference on Harmonization (ICH) guideline was employed for validation of analytical method.²³

Calibration curve

A stock solution (100 µg ml⁻¹) of oleanolic acid was prepared by dissolving accurately weighed 5 mg in 50 ml methanol and that (200 µg ml⁻¹) of lupeol was prepared by dissolving accurately weighed 2 mg in 10 ml methanol in a volumetric flask. Standard solutions for calibration were prepared by dilution of the stock solution with methanol; the concentrations were such that amounts of oleanolic acid between 50 -1000 ng and that of lupeol between 100 -500 ng. The correlation coefficient, slope intercepts and regression equation were also calculated to provide mathematical estimate degree of linearity. A calibration curve was derived by plotting peak area (Y axis) versus concentration (X axis).

Quantification of oleanolic acid and lupeol in extract

10 mg of Ext A was dissolved in 2 ml methanol in a volumetric flask. 20 µl and 30 µl of this solution were used for estimation of oleanolic acid and lupeol respectively.

The peak area values of standards and sample were used to calculate the amount of oleanolic acid and lupeol in the plant.

RESULTS AND DISCUSSION

M. oppositifolia is a prostrate herb about 10-12 cm tall with simple linear-lanceolate leaf arranged in whorl of 4-5, 0.5-1 cm long, 0.2-0.4 cm wide with acute or rounded apex, entire margin and reticulate venation. Stem is cylindrical, glabrous about 0.5 cm in diameter and with long internodes (Figure 1). Root is thin, tapering and measures about 0.8 cm in diameter. Flowers are small white, borne in axillary fascicles. Fruit capsule, ellipsoid, 0.3-0.4 cm long and enclosed in persistent calyx. Seeds are minute, many, dark brown, reniform and tuberculate.

Microscopical Characters

Transverse section of the root is circular in outline and shows a narrow cork (ck) made up of 3-4 layers of tangentially elongated and radially arranged suberized cells; narrow thin walled parenchymatous cortex (ct); crescent shaped parenchymatous phloem (ph); 1 to 2 continuous

or discontinuous rings of lignified radially arranged xylem (xy) consisting of wide, thick walled vessels, fibres and uni to bi-seriate thin walled xylem rays (Figure 2).

Transverse section of the stem is wavy in outline and shows a layer of tangentially elongated, thick walled epidermis (e) with thick cuticle bearing simple triseriate and glandular trichomes with multi-cellular stalk and uni to bicellular head; parenchymatous cortex (ct); stele constituted of a layer of endodermis (en), sclerenchymatous pericycle (per), vascular bundle (vb) arranged in the form of a ring around parenchymatous pith (pi) (Figure 3).

Transverse section passing through the midrib is flat on the upper and sinuous at the lower side and shows a centrally located collateral meristele (mer); continuous band of a palisade tissue (pal) occupying the major area of the mesophyll; narrow spongy tissue (spp) traversed by obliquely cut vascular bundles; rosettes and prisms of calcium oxalate scattered throughout lamina (Figure 4).

Powdered drug shows fragments of lamina embedded with prisms and rosettes of calcium oxalate (a); hemispherical seeds with seed coat having polyhedral and thick walled cells (b); epidermal cells of corolla in surface view with



Figure 1: Herb of *Mollugo oppositifolia* Linn.

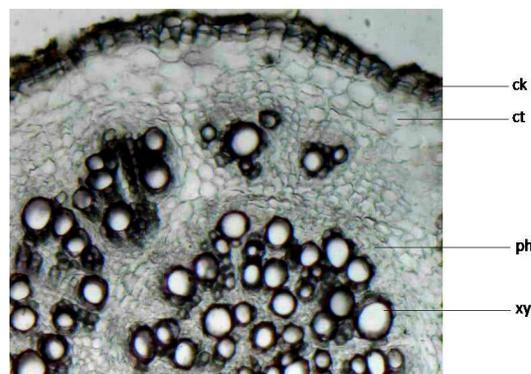


Figure 2 : TS of *M. oppositifolia* root

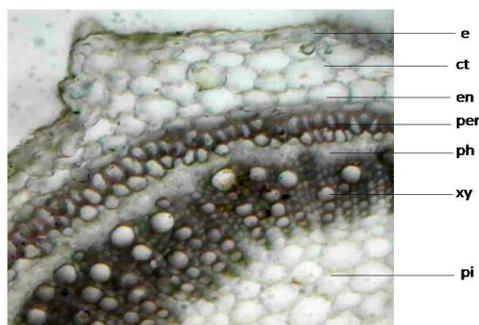


Figure 3 : TS of *M. oppositifolia* stem

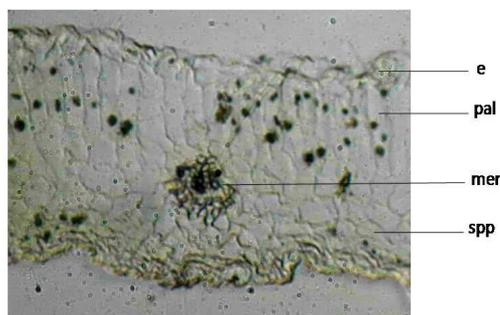


Figure 4 : TS of *M. oppositifolia* leaf

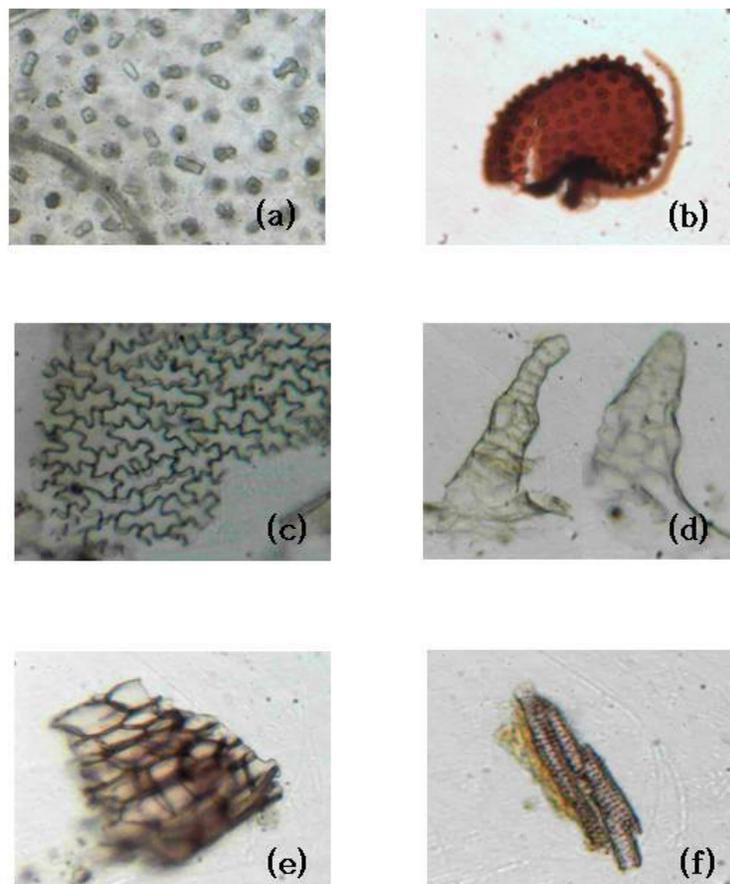


Figure 5: Powder characters of *M. oppositifolia* whole plant

thick and sinuous wall (c); simple triseriate and glandular trichome with multi cellular stalk and uni to bicellular head from stem (d); fragments of suberized cork with thick walled polygonal cells in surface view (e); fragments

of lignified bordered pitted vessel (f) and spherical pollen grains (Figure 5).

Data of quantitative microscopy for leaf are entered in Table 1.

Table 1: Quantitative microscopy	
Parameters	<i>M. oppositifolia</i>
Stomatal index	
Upper surface	19.89 ± 0.20
Lower surface	23.26 ± 0.12
Vein islets no.	23.67 ± 0.08
Vein termination no.	18.0 ± 0.61

Table 2: Physico-chemical parameters		
Particulars	% w/w	SD ¹
Loss on Drying	68.4 ± 0.07	
Total ash	6.88 ± 0.48	
Water soluble ash	1.13 ± 0.75	
Acid insoluble ash	0.75 ± 0.27	
Water soluble extractive value	24.0 ± 0.20	
Alcohol soluble extractive value	9.0 ± 0.41	

¹SD=standard deviation, Number of readings=3

Physicochemical Evaluations

Data of Physico-chemical parameters including moisture content, ash and extractive values are given in Table 2. Water-soluble ash value was found to be more than acid insoluble ash value. The plant showed higher water-soluble components than alcohol soluble components. Phytochemical screening revealed presence of saponins,

Table 3: Content of phytoconstituents	
Phytoconstituents	% w/w
Phenolic substances	3.83 ± 0.11
Alkaloids	5.05 ± 0.18
Flavanoids	2.66 ± 0.42
Saponins	
Froth number	833.33 ± 0.21

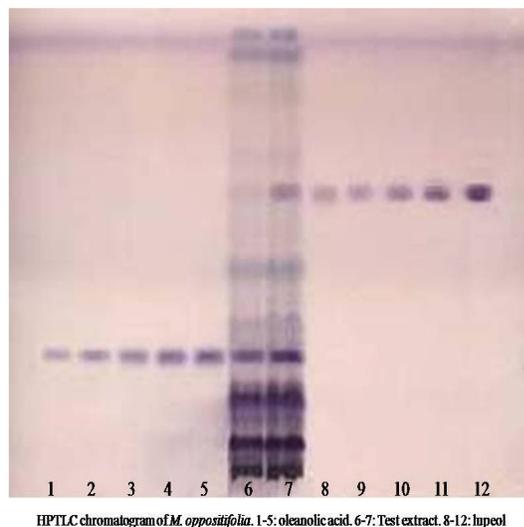


Figure 6: HPTLC chromatogram of *M. oppositifolia*
1-5 : oleanolic acid. 6-7 : Test extract. 8-12 : lupeol

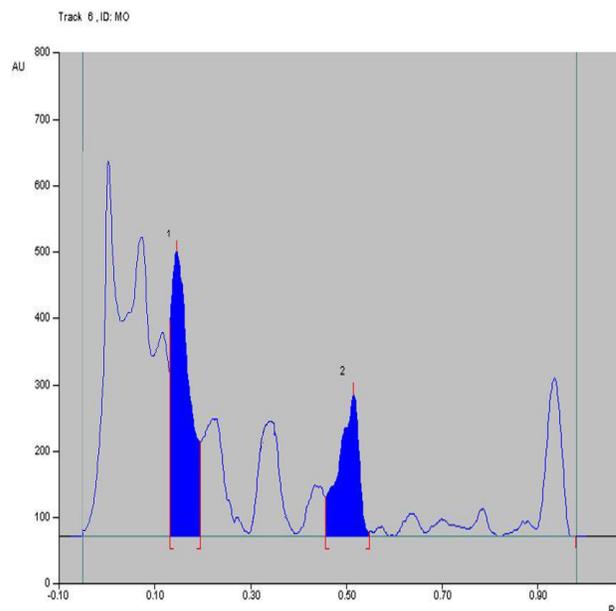


Figure 7: HPTLC densitometric chromatogram of *M. oppositifolia*
at 545 nm

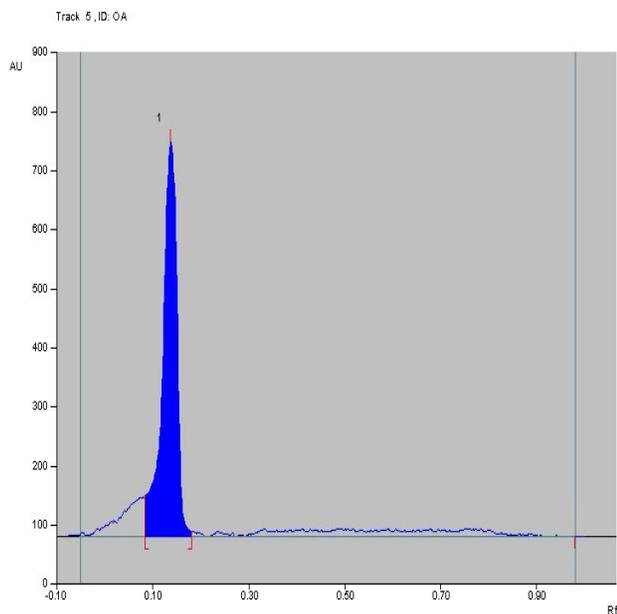


Figure 8: HPTLC densitometric chromatogram of oleanolic acid

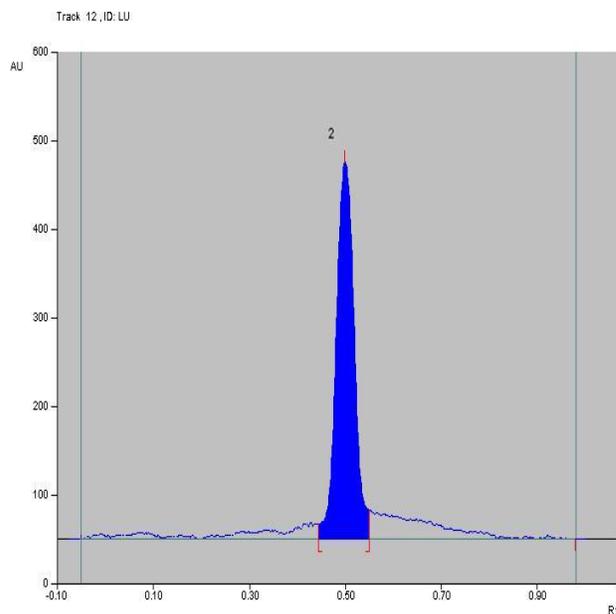


Figure 9: HPTLC densitometric chromatogram of lupeol

flavonoids, phenolics, alkaloids, steroids and triterpenoids, tannins and carbohydrate. Saponins and flavonoids were found to be the major constituents and data is entered in Table 3.

Estimation of oleanolic Acid and lupeol by HPTLC Analysis

TLC studies of extract indicated presence of both oleanolic

acid and lupeol at R_f 0.16 and R_f 0.51 respectively exactly matching with reference standards (Figure 6-9). The content of oleanolic acid and lupeol were found to be 0.027-0.029% w/w and 0.015-0.016% w/w respectively. The validation parameters are given in Table 4 and Table 5. The LOD and LOQ, for signal-to-noise ratios were 3:1 and 10:1, respectively.

Table 4: Summary of validation parameters of oleanolic acid

Parameters	Results
Linearity	0.986
Precision (% C.V.)	
• Repeatability of Measurement	0.608
• Repeatability of Application	1.264
• Interday	1.17-2.76 %
• Interday	1.25-1.65 %
Range	50-1000 ng band ⁻¹
Limit of Detection	24.15 ng band ⁻¹
Limit of Quantification	73.19 ng band ⁻¹
Accuracy	99.58 – 100.19 %
Specificity	Specific

CONCLUSION

This is the first report on the pharmacognostic study corroborated with HPTLC analysis for *Mollugo oppositifolia*. The ensemble of data on standard parameters is useful for the endorsement of quality control and for documenting a monograph on this crude drug. The proposed HPTLC method for estimation of oleanolic acid and lupeol was precise, accurate and selective. The method was rapid, sensitive, reproducible and economical. It does not suffer any positive or negative interference due to other common components present in the extract.

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Table 5: Summary of validation parameters of lupeol

Parameters	Results
Linearity	0.997
Precision (% C.V.)	
• Repeatability of Measurement	0.308
• Repeatability of Application	1.016
• Interday	2.03-3.18 %
• Intraday	1.58-1.89 %
Range	100-500 ng band ⁻¹
Limit of Detection	20.66 ng band ⁻¹
Limit of Quantification	62.60 ng band ⁻¹
Accuracy	99.28 – 99.93 %
Specificity	Specific

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