# HPTLC Method Development and Validation for Identification and Quantification of Lupeol from Bark and Leaves of *Careya arborea* Roxb.

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***Abstract***

*Careya arborea Roxb. (Family: Lecythidaceae) is an ethno pharmacologically important medicinal plant. Among the complex mixture of biologically active compounds present in the stem bark and leaves of Careya arborea Roxb. lupeol, a constituent of the bark and leaves has been used as an analytical marker, indicative of the quality of the plant. In the present study, a simple and sensitive high-performance thin layer chromatography (HPTLC) method was developed and validated for the quantification of biomarker lupeol from C. arborea bark and leaves. Separation and quantification were carried out on silica gel 60 $F_{254}$ precoated TLC plates using toluene: methanol: glacial acetic acid [9: 1: 0.1 (v/v/v)] as mobile phase. After development, the plate was derivatized with anisaldehyde sulphuric acid, scanned and quantified at 540nm.*

*The response was linear over the range of 50 ng/band to 150 ng/band with correlation coefficient 0.998 for bark and 400 ng/band to 800 ng/band with correlation coefficient 0.999 for leaves. The estimated values obtained were 57.76 ± 2.3 and 1363.84 ± 2.3 µg/g for stem bark and leaves respectively. The method was validated using ICH guidelines in terms of linearity, precision, specificity, accuracy and robustness and can be adopted for routine quality control of raw material and formulations containing Careya arborea Roxb. bark and leaves.*

**Keywords:** *Careya arborea* Roxb., Stem bark, Leaves, Lupeol, HPTLC, Method validation.

## Introduction

Standardization of herbal drugs is important to ensure the quality and optimum levels of active principles for their bio potency. Recently, the concept of marker-based standardization of herbal drugs is gaining momentum.

Identification of phytocompounds mainly secondary metabolites in plant or plant parts as markers and development of analytical methods for monitoring them are the key steps involved in marker-based standardization[28]. Use of chromatography technique for standardization of plant products was introduced by the WHO and is accepted as a strategy for identification and evaluation of the quality of plant medicines[6].

Chromatographic methods play an important role in the pharmaceutical area; hence efficient method development and analytical validation by chromatography are of fundamental importance. According to The United States Pharmacopoeia (USP), validation of an analytical method is the process by which it is established for laboratory studies, so that the performance features of the method meet the requirements for the planned analytical applications. Therefore, validation is an important step in determining the reliability and reproducibility of the method because it could confirm that the method is suitable to be piloted on a particular system[26]. A properly developed and validated method by using modern analytical technique can be used for phytochemical profiling and marker compound analysis[22].

HPTLC is an excellent method for qualitative and quantitative analysis of a wide range of compounds in herbal drugs because of its high accuracy, precision and reproducibility of results. HPTLC is superior to other analytical techniques in terms of low operating cost and short analysis time. It can quantify the analytes at micro or even in nanogram levels. In addition, HPTLC method may help to minimize exposure risk of toxic organic effluents and significantly reduces its disposal problems, consequently reducing environmental pollution[20]. Furthermore, the colourful HPTLC image provides extra instinctive parameters of visible colour and fluorescence and it can analyse simultaneously different samples on the same plate using a small quantity of mobile phase[14].

Lupeol, an important marker compound, has not been quantified from the bark and leaves of *C. arborea* Roxb. until recently. It is reported to show anti-inflammatory and anti-arthritic[1], antioxidant[18], hepatoprotective[21], wound healing[9], anti-hypercholesterolemia[27], suppression of T-lymphocyte[4], antioxaluric and anticalciuric activity[2].

*Careya arborea* Roxb. is a species of deciduous tree in the Lecythidaceae family, native to the India, Sri Lanka, Peninsula and Malay[15]. It is known as Sthala kumbhi in Hindi and wild guava in English. It is planted in gardens and roadsides for its large conspicuous leaves and showy flowers and fruits[16,23]. This tree can be identified by its thick dark grey bark, large showy flowers and the leaves which turn red

in cold season. Leaves are simple, alternate, exstipulate and clustered at the tips of branchlets[16].

*Careya arborea* is reported to possess analgesic, antidiarrheal, hepatoprotective, antitumor, CNS-depressant, anticoagulant and *in vitro* cytotoxic activities[7]. Ethnobotanically, *C. arborea* stem bark is used in the treatment of tumours, bronchitis, skin diseases, epileptic fits, astringents, antidote to snake venom, abscesses, boil and ulcers[7] and the leaves paste and pulp are used as poultice to rapidly heal the obstinate ulcers. Leaves and flowers are also used in the form of paste to cure several skin diseases[5,17].

Available literature survey revealed that no method has been reported for quantification of lupeol, an important marker compound from bark and leaves of *C. arborea.* Hence, in the present research work, a simple, rapid, precise and accurate HPTLC method has been developed and validated using International Conference on Harmonization (ICH) guidelines.[11-13]

## Material and Methods

**Plant material:** Stem bark and leaves were collected from the forest area of Badlapur, Mumbai (Maharashtra) and authenticated from Agharkar Research Institute, Pune, India. Plant materials were washed with water to remove soil particles, air dried, ground into fine powder and stored in airtight container at room temperature for further studies.

**Reagents and Standards:** All chemicals and solvents used were of analytical grade and obtained from Hi-media (Mumbai, India). Reference standard lupeol (purity 99.7%) was procured from Sigma Aldrich Chemical Company (Steinheim, Germany). Derivatizing reagent anisaldehyde sulphuric acid was prepared as per the procedure described by Reich and Schibli. The precoated TLC silica gel 60 $F_{254}$ plates were obtained from E. Merck (India).

**Preparation of stock solution of lupeol:** A stock solution of lupeol was prepared by dissolving 10 mg in 5 ml methanol and making up the volume to 10 ml with methanol to get the final concentration of 1000µg/ml. This stock solution was further diluted with methanol to get 25µg/ml and 50 µg/ml of working standard solution of lupeol.

**Preparation of plant extracts:** 1g dried plant powder was added in 10ml methanol, sonicated for 15 minutes and left to stand overnight at room temperature. Sample was filtered through Whatmann filter paper no. 1 and the filtrate obtained was evaporated to dryness. The residue was dissolved in 10ml methanol.

**HPTLC Instrumentation**

**Chromatographic conditions:** Standards and samples were applied to the TLC aluminium precoated silica gel 60 $F_{254}$ plate as 8mm bands using the CAMAG Linomat 5 TLC sample applicator. After the application, plate was developed vertically ascending in a glass twin trough chamber pre-saturated for 20 mins at room temperature with mobile phase Toluene: Methanol: Glacial acetic acid [9:1:0.1 (v/v/v)]. The chromatographic run length was 70mm from the bottom edge of the plate.

After development, the plate was air dried for complete removal of mobile phase and derivatized by dipping the developed plate in anisaldehyde sulphuric acid reagent for 2 seconds. The plate was then air-dried and heated at 110ºC on TLC plate heater for 10 minutes. The plate was kept in photo documentation chamber and images were captured. Densitometric scanning was then performed at 540 nm using CAMAG TLC scanner 3 with winCATS software version 1.4.6. The slit dimension used was 6.0×0.45 mm with scanning speed of 20mm/sec throughout the analysis.

**Quantification**

**Calibration curve of lupeol:** 15 µl of bark and 5µl of leaves extract were applied in triplicate on TLC silica gel 60 $F_{254}$ plate. The plate was developed and scanned as mentioned above and the peak areas were recorded. The content of lupeol in bark and leaves was determined by using a calibration curve established with a standard lupeol concentration range.

**HPTLC method validation:** International Conference on Harmonization (ICH) guidelines were followed for the validation of the developed analytical method. The method was validated for linearity, precision, detection limit, quantification limit, specificity, ruggedness and accuracy.

**Linearity:** Various concentrations of working standard solution of lupeol were applied on TLC plate to get a range of 50ng/band to 150ng/band for bark and 400ng/band to 800ng/band for leaves. The peak areas obtained from densitograms for each applied concentration were noted. The graph of peak area against concentration of lupeol was plotted.

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** For the determination of LOD and LOQ, different concentrations of the standard lupeol were applied along with methanol as blank and determined based on signal to noise ratio. LOD was determined at 3:1 and LOQ at 10:1.

**Specificity:** The specificity of the proposed HPTLC method was ascertained by comparing the $R_f$ and chromatograms of standard lupeol compound with the standard compound found in samples.

**Robustness:** Robustness of the method was checked by varying the selected parameters (mobile phase composition, mobile phase volume and duration of mobile phase saturation) within certain limits to determine their influence on the retention factor and quantitative analysis.

**Accuracy:** The accuracy of the method was assessed by performing recovery studies using standard addition method

at three different levels (80%, 100% and 120% spiking of lupeol). The values of percent recovery as well as average percent recovery were calculated.

**Precision:** Instrument precision was checked by repeated scanning (n=12) of same band of lupeol. Interday precision was done by analysing the standard solution in triplicate on three different days. Intraday precision was done by analysing the standard solution in triplicate on the same day and the results were expressed as coefficient of variance (%CV).

## Results and Discussion

Precise sample application, standardized reproducible chromatogram development and software-controlled evaluation make HPTLC the method of choice. The possibility of visual inspection of separated samples on the plate is one of the most valued aspects of TLC. HPTLC is the most advanced form of TLC and comprises the use of chromatographic layers of extreme separation efficiency[10]. In the present study, Lupeol an important marker compound has been quantified from *Careya arborea* Roxb. bark and leaves using HPTLC. There are very few reports available on quantification of lupeol from bark and leaves from various plant species.

Shailajen et al[25] estimated the amount of lupeol in *Rhododendron arboreum* leaves (Garhwal region) and was found to be 14.26±1.82. Gurupriya et al[8] also detected 0.265±0.004mg/100 mg lupeol in *Andrographis echioides* leaves.

Patel et al[19] quantified the amount of lupeol in acetone extract and methanol extract of *Ougenia Dalbergioides* bark as 9.9 µg /mg of extract and 8.3 µg /mg of extract respectively. Badami et al[3] determined lupeol by HPTLC in *Grewia tiliaefolia* bark which was found to be 2.902-± 0.243 mg/g bark. Sharma et al[24] also reported 1.99mg/g lupeol in *Crataeva tapia* L. bark.

In the present HPTLC analysis, several different mobile phases were tried but mobile phase consisting of toluene: methanol: glacial acetic acid in the volume ratio of 9:1:0.1 gave good separation for lupeol. Since, lupeol does not show UV and visible sensitivity on plate, the plate was derivatized further with anisaldehyde sulphuric acid reagent. Lupeol was resolved at $R_f$ 0.52 from methanolic extract of bark and leaf sample when the plate was developed in mobile phase and derivatized as mentioned above (Figure 1).

Graph of peak areas versus concentrations of lupeol when plotted shows linear relationship. The linear regression equation is obtained from this graph. Using the regression equation, the amount of lupeol is calculated from bark and leaves and found to be 57.76 ± 2.3 and 1363.84 ± 2.3 µg/g respectively (Table 3). The presence of lupeol was confirmed by comparing $R_f$ and colour of the band with the standard solution of lupeol.

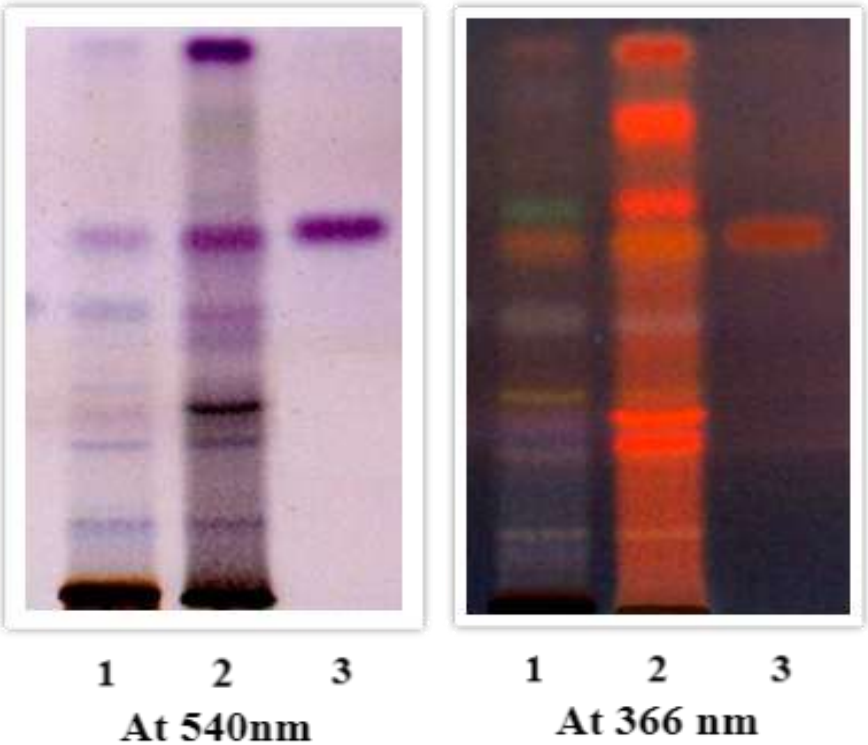


**Fig. 1: HPTLC profile of *Careya arborea* Roxb. bark and leaves with standard lupeol after derivatization under 540 nm and 366 nm. Key: Track 1- Methanolic extract of Stem bark**
**Track 2- Methanolic extract of Leaves**
**Track 3- Standard lupeol**

The developed method was found to be linear in a concentration range of 50-150 ng/band for bark and 400-800 ng/band for leaves with correlation coefficient of 0.998 and 0.999 respectively. Under the experimental conditions employed, the lowest amount of lupeol that could be detected was found to be 6.4 and 10.85 ng/band for bark and leaves respectively. The lowest amount of lupeol that could be quantified was found to be 19.61 and 328.96 ng/band for bark and leaves respectively.

The results for precision are expressed as % CV in table 1. % CV values were less than 5% confirming the precision of this method. The spectra of standard compound lupeol and the corresponding band present in bark and leaves of *Careya arborea* Roxb. matched exactly, indicating no interference by the other plant constituents and excipients, so the method is found to be specific.

Robustness tests examine the effect of the operational parameters on the analysis results by introducing small changes. No significant change of $R_f$ or response was observed indicating the robustness of the method. Results from recovery studies (Table 2) were in acceptable limits indicating the accuracy of the method was good.

## Conclusion

The developed HPTLC method provided simple, accurate and reproducible quantitative analysis for the determination of lupeol from bark and leaves of *Careya arborea* Roxb. The method was validated as per ICH guidelines and statistical tests proved that the proposed HPTLC method is sensitive, specific and repeatable and it can be conveniently employed for rapid routine quality control analysis and quantification of lupeol from *Careya arborea* Roxb. stem bark and leaves and its formulations without any interference from excipients.

**Table 1**
**Method validation parameters for quantification of lupeol in *Careya arborea* Roxb. bark and leaves by proposed HPTLC densitometric method**

| S. N. | Parameters | Lupeol | |
|---|---|---|---|
| | | Bark | Leaves |
| 1 | Linearity range (ng/band) | 50-150 | 400-800 |
| 2 | Correlation Coefficient | 0.998 | 0.999 |
| 3 | Slope | 11.8 | 8.78 |
| 4 | Intercept | 95.97 | 1874 |
| 5 | LOD (ng/band) | 6.4 | 10.85 |
| 6 | LOQ (ng/band) | 19.61 | 328.96 |
| 7 | Instrument precision (n=12, %CV) | 1.2 | 0.9 |
| 8 | Intraday precision (n=3, %CV) | 1.3 | 2.3 |
| 9 | Interday precision (n=3, %CV) | 3.05 | 4 |
| 10 | Specificity | Specific | Specific |
| 11 | Robustness | Robust | Robust |

**Table 2**
**Recovery studies of lupeol at 80%, 100% and 120% addition**

| Plant Extracts | Amount of lupeol added (µg) | Recovery (%) | Average recovery (%) ± SD |
|---|---|---|---|
| Bark | 0.06 | 96.65 | 97.28±1.18 |
| | 0.075 | 98.95 | |
| | 0.09 | 96.26 | |
| Leaves | 0.48 | 98.14 | 97.92±0.16 |
| | 0.6 | 97.86 | |
| | 0.72 | 97.75 | |

**Table 3**
**Amount of lupeol found in *Careya arborea* Roxb. bark and leaves**

| Samples | Lupeol (µg/g) (mean±SD) |
|---|---|
| Bark | 57.76 ± 2.3 |
| Leaves | 1363.84 ± 2.3 |

## Acknowledgement
The author and co-authors are thankful to Management, B. K. Birla college of Arts, Science and Commerce (Autonomous), Kalyan for providing essential facilities. We are also grateful to Anchrom laboratories, Mumbai for their help and technical support.

## References
1. Agarwal R.B. and Rangari V.D., Anti-Inflammatory and Anti-Arthritic Activities of Lupeol and Lupeol Isolated from *Strobilanthus callosus* and *Strobilanthus ixiocephala* Roots, *Indian J Pharmaco*, **35,** 384–387 **(2003)**

2. Anand R., Patnaik G.K., Roy K. and Bhaduri A.P., Antioxaluric and Anticalciuric Activity of Lupeol Derivatives, *Indian J Pharmacol,* **27,** 265–268 **(1995)**

3. Badami S., Gupta M., Mathew N., Meyyanathan S., Bhojraj S. and David B., HPTLC determination of lupeol in *Grewia tiliaefolia. J planar chromatogr-Mod TLC,* **15,** 114-118 **(2002)**

4. Bani S., Kaul A., Khan B., Ahmad S.F., Suri K.A., Gupta B.D., Satti N.K. and Qazi G.N., Suppression of T Lymphocyte Activity by Lupeol Isolated from *Crataeva religiosa, Phytothe Res,* **20**, 279–287 **(2006)**

5. Basak A., Banerjee R., Bose L. and Basu K., Chemical examination of the leaves of *Careya arborea*, *J Indian Chem Soc*, **53,** 639–640 **(1976)**

6. Dhalwal K., Shinde V.M. and Mahadik K.R., Optimization and validation of reverse phase HPLC and HPTLC method for simultaneous quantification of vasicine and vasicinone in Sida species, *J Med Plants Res*, **4,** 1289-1296 **(2010)**

7. Gupta P.C., Sharma N. and Rao C.V., Pharmacognostic studies of the leaves and stem of *Careya arborea* Roxb, *Asian Pac J Trop Biomed,* **2,** 404–408 **(2012)**

8. Gurupriya S., Cathrine P. and Pratheema P., HPTLC method for the determination of Lupeol from *Andrographis echioides* leaves, *Int J Pharm Pharmaceu Sci,* **10**, 102-107 **(2018)**

9. Harish B., Krishna V., Santosh H.K., Ahamed B.K., Sharath R. and Swamy H.K., Wound Healing Activity and Docking of Glycogen-synthase-kinase-3-b-protein with Isolated Triterpenoid Lupeol in Rats, *Phytomedicine,* **15,** 763–767 **(2008)**

10. https://www.camag.com/en/tlc_hptlc/what_is_tlchptlc.cfm

11. ICH, Guidance on analytical method validation. Proceedings of International Convention on Quality for the Pharmaceutical Industry, Toronto, Canada **(2002)**

12. ICH, Q2A, Text on Validation of Analytical Procedures, International conference on Harmonization of technical requirements for the registration of drugs for human use, Geneva, Switzerland **(1994)**

13. ICH, Q2B, Guideline on Validation of Analytical Procedures: Methodology, International conference on Harmonization of technical requirements for the registration of drugs for human use, Geneva, Switzerland **(1996)**

14. Kamboj A. and Saluja A.K., Development of validated HPTLC method for quantification of stigmasterol from leaf and stem of *Bryophyllum pinnatum*, *Arabian J Chem*, **10,** 644-650 **(2013)**

15. Kirtikar K.R. and Basu B.D., *Indian Medicinal Plants*, 2nd edition, Lalit Mohan Basu Publications, Dehradun, India, 894-895 **(1980)**

16. Kumar B.N.S., Swamy B.M.V., Kumar G.K. and Behera G.M., Review on *Careya arborea* Roxb, *Int J Res Ayur Pharma,* **1,** 306-315 **(2010)**

17. Nadkararni K.M., Medicinal plants of India, Dehradun: Reprint Prakashan **(2004)**

18. Nagaraj M., Sunitha S. and Varalakshmi P., Effect of Lupeol, a Pentacyclic Triterpene, on the Lipid Peroxidation and Antioxidant Status in Rat Kidney after Chronic Cadmium Exposure, *J Appl Toxicol*, **20,** 413–417 **(2000)**

19. Patel M.R., Rajput N., Panchal H.S. and Dalwadi H.B., Quantification of Lupeol and Betulin in *Ougenia Dalbergioides* Bark by Column Chromatography and TLC, *J Pharm Sci Biosci Res*, **7,** 114-120 **(2017)**

20. Patel R., Patel M., Dubey N., Dubey N. and Patel B., HPTLC method development and validation: Strategy to minimize methodological failures, *J Food Drug Anal*, **20,** 794-804 **(2012)**

21. Prasad S., Kalra N. and Shukla Y., Hepatoprotective Effects of Lupeol and Mango Pulp Extract of Carcinogen Induced Alteration in Swiss Albino *Mice, Mol Nutri Food Res*, **51,** 352–359 **(2007)**

22. Saraf A. and Samant A., HPTLC method for identification and quantification of β sitosterol in the roots of *Achyranthes aspera* Linn., *Int J Pharma Sci*, **5,** 1117-1122 **(2015)**

23. Sharma P.V., Classical use of medicinal plants, 1st edition, Chowkhambha Bharati Academy, Varanasi, India **(1996)**

24. Sharma P., Patil A. and Patil D., Method development and validation for quantification of lupeol from bark of *Crataeva Tapia* L. using HPTLC, *J. Pharm. Res.*, **5(10),** 217-219 **(2016)**

25. Shilajan S. and Swar G., Simultaneous estimation of three triterpenoids-ursolic acid, β-sitosterol and lupeol from flowers, leaves and formulations of *Rhododendron arboreum* Smith. using validated HPTLC method, *Int J Green Pharm*, **7,** 206-210 **(2013)**

26. Srivastava M.M., editor, High-Performance Thin-Layer Chromatography (HPTLC), Springer, New York, Heidelberg Dordrecht London **(2011)**

27. Sudhahar V., Kumar S.A., Sudharsan P.T. and Varalakshmi P., Protective Effect of Lupeol and Its Ester on Cardiac Abnormalities in Experimental Hypercholesterolemia, *Vasc Pharmacology*, **46,** 412–418 **(2007)**

28. Willy S., Kekare M.B. and Vaidya V., Development and validation of high-performance liquid Chromatographic method for the simultaneous determination of β sitosterol and lupeol in *Vernonia cinerea* linn, *Int J Pharma Bio Sci*, **1,** 1-5 **(2010)**.

(Received 25th February 2020, accepted 25th April 2020)