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Research Article

Method Validation and Quantification of Lupeol in *Hibiscus sabdariffa* (Malvaceae) calyx extracts by using High Performance Thin Layer Chromatography

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Abstract

The primary objective of the present investigation was to establish and validate a High-Performance Thin-Layer Chromatography (HPTLC) methodology for the precise determination of lupeol content in several extracts derived from the calyx of *Hibiscus sabdariffa* Linn extracts included ethanol, ethyl acetate, and aqueous extracts. The samples underwent analysis on a thin-layer chromatography (TLC) aluminium precoated plate (60 F254) using a mobile phase composed of toluene and methanol in a volumetric ratio of 8:2. The plate was subjected to derivatization using the Anisaldehyde – Sulphuric Acid reagent, followed by scanning at a wavelength of 550 nm. The procedure of validating the approach included the use of standards set out by the International Council of Harmonisation (ICH). The evaluation of several criteria, such as linearity, precision, accuracy, and robustness, was included by these recommendations. Lupeol was identified in the calyx ethanol extract at a concentration of 0.52% w/w, the ethyl acetate extract at a concentration of 0.47% w/w, and the aqueous extract at a concentration of 0.16% w/w. The ethanol extract exhibited a significant concentration of lupeol, above the levels reported in the other two extracts. As a result, the ethanolic extract was chosen for further validation criteria. The findings of the investigation demonstrated a direct correlation between the concentration of lupeol and the matching band intensity, spanning a range of 20 to 120 ng/band. The obtained correlation coefficient (r^2) value of 0.9959 suggests a robust positive relationship between the two variables. The developed technique exhibited a notable degree of precision, as seen by the relatively low values of the interday and intraday precision, with %RSD values of 1.33% and 1.20% respectively. The efficacy of the methodology was assessed by a series of recovery studies carried out at three distinct concentration levels, namely 80%, 100%, and 120%. The findings of the study revealed that the accuracy rates for lupeol were assessed to be 99.93%, 99.36%, and 99.58% respectively. The method used for the validation and quantification of lupeol in *Hibiscus sabdariffa* Linn was found to be simple, precise, accurate, and robust.

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Introduction

The many traditional medical systems use medicinal plants, which are abundant in bioactive substances, to treat a variety of diseases. Modern scientific methods are being used to study these medicinal plants as a result of technological advancement. Adopting these cutting-edge scientific methods will allow you to accurately assess a medicinal plant's therapeutic potential. *Hibiscus sabdariffa* L., a species of the Malvaceae family (fig. no. 1), is widely utilized in the traditional systems of medicine with distinct principles and practices (Ayurveda, Siddha, and Unani) due to its accepted medicinal benefits (1). Based on available studies, it has been observed that Roselle plants, categorized as annual shrubs, with the capacity to attain a height of around 3.5 meters, exhibiting erect stems with a crimson hue. In addition, they possess the characteristic of branching. The length of the leaves typically ranges from 7.5 cm to 12.5 cm, as seen in previous studies (2,3). These leaves exhibit an alternative arrangement along the stem. The diameter of the blossom varies between 8 and 10 cm, regardless of whether it is axillary or terminal. The presence of a robust calyx, characterized by its substantial thickness and fleshy composition, is seen. The calyx exhibits a basal width ranging from 1 to 2 cm, which subsequently expands to a diameter of 3-3.5 cm. As the flower matures, it undergoes a transformation, developing a fleshy texture and assuming a vibrant red colour[4]. The plant's ability to grow in tropical locations is facilitated by its specific requirements for water and sunshine, which it may get in sufficient quantities throughout the day (2).

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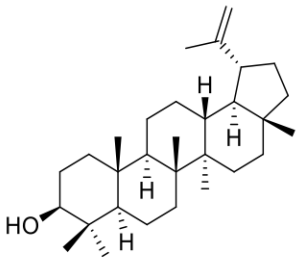
Roselle seedlings may be transplanted at an age ranging from 10 to 15 days. During the first growing period, it is necessary for Roselle plants to be exposed to a minimum nighttime temperature of 20 degrees Celsius and get at least 13 hours of sunlight every day. Around 130-250 mm of rainfall each month are needed, or enough watering is necessary. Approximately 4 to 5 months are required to harvest Roselle, and December to January is the best time of year to begin planting (5).

Figure 1: *Hibiscus sabdariffa* L. Plant

Figure 2: Chemical Structure of Lupeol



A significant number of vitamins, minerals, flavonoids, protein, lipids, carbohydrates, and other nutrients have been found to be contained in the Roselle calyx, which is used for making a variety of beverages. Antioxidant, antihypertensive, hepatoprotective, nutritional, and antihyperlipemic characteristics of the plants have been identified (6). Triterpenoids represent the most extensive category of phytochemicals within the realm of natural substances. Triterpenoids are composed of around thirty carbon atoms, and their structural formation is believed to originate from the acyclic precursor known as squalene. There are many distinct types of triterpenes in nature, and over 20,000 of them have been isolated, including squalene, lanostane, dammarane, lupeol, oleanane, ursane, hopane, etc. Due to its wide range of biological actions, lupeol has attracted the most attention among these triterpenoids (7,8,9).

Lupeol (fig. no. 2) comes under the class of pentacyclic triterpenoids. The compound in question is present in several edible fruits and vegetables and has a broad distribution across the plant world. Research has shown the notable efficacy of this substance as an anti-allergic, anti-microbial, anti-protozoal, anti-proliferative, anti-cancer, and cholesterol-lowering agent. Additionally, it has undergone tests to see how well it works biologically against a variety of ailments, such as arthritis, diabetes, renal illness, and wound healing (10,11)

Materials and Methods

Plant materials and Chemicals

Lupeol marker chemical was purchased from Yucca Enterprises in Mumbai.

Hibiscus sabdariffa L. dried calyx was purchased from Manakarnika Aushadhalaya, Chinchwad, Pune-411033.

Extract and standard Preparation

Extract preparation

To remove any moisture, 10 gm. of dried *Hibiscus sabdariffa* calyx were placed in a hot air oven. Create a coarse powder from the dried calyx and macerate it on an orbital shaker with 100 ml of ethanol, ethyl acetate and water respectively for one hour. To get a good yield, keep it for 24 hours, then sonicate it. After sonication, filter it with Whatman filter paper 41. Keep the extract at a cool temperature till further analysis. Standard Solution was prepared by dissolving 10 mg of Lupeol into 100 ml of methanol, we were able to create a solution with a concentration of 100 µg/ml. Between 20 and 120 ng per band, we used the calibrating normal solution to apply to the HPTLC plate. Anisaldehyde – Sulphuric Acid derivatizing agent use for chromatographic analysis (12, 13).

HPTLC instrumentation and experimental conditions

The plates were first cleaned with the methanol. After that, chromatography plates were activated by being heated for ten minutes at 100 degrees Celsius. The drug standard and samples were distributed in bands that were 6 mm wide the bands were applied to silica gel-coated aluminium plates measuring 60 RP-18 F254 S using the Camag Linomat 5 applicator and a Camag microliter syringe. The scanning speed was set at a rate of 20 millimeters per second. The volumetric ratio of the mobile phase components was 8 parts toluene to 2 parts methanol. A linear ascending growth was conducted inside a twin-trough glass box (Camag, Muttenz, Switzerland) measuring 20 x 10 cm, which was filled with the mobile phase. The optimal duration for achieving mobile phase saturation in the optimization chamber at ambient temperature was determined to be 15 minutes. The chromatogram run lasted for almost 80 millimetres. After the developing step was over, the HPTLC plates were subjected to desiccation using an air dryer and a perfectly controlled airflow. The Anisaldehyde-Sulfuric Acid Reagent was administered onto the plate that had been produced, followed by drying it in a hot air oven set at a temperature of 100°C. Win CATS software (Version 1.3.0) was used to carry out the densitometric scanning operation using the Camag TLC scanner 3 at 550 nm. Based on a review of the relevant literature, we determined that the best mobile phase for detecting lupeol in *Hibiscus sabdariffa* Linn. calyx extract is toluene: methanol (8:2 v/v) (13-23).

Method Validation

The guidelines set by the International Council for Harmonisation were followed for verifying the analytical method. Assessments were made on linearity, precision, accuracy, specificity, LOD, and LOQ.

To test for linearity, we used the experimental method to a range of concentrations of reference lupeol. Using calibration curves, we determined how much of the marker was present in each sample. We determined the LOD and LOQ by using the SD and slope (S) of the calibration curve. For this purpose, we used the chemical formulae LOD = 3.3(SD/S) and LOQ = 10(SD/S) to determine the former and the latter, respectively. The assessment of system precision and repeatability is included in investigations relevant to precision. By spiking pre-analyzed samples with a standard solution at a given concentration, accuracy by recovery investigations were conducted. The robustness was evaluated by applying minor adjustments to the optimised method parameters, such as changing the mobile phase or the duration of chamber saturation, among other factors. Specificity was established by comparing retention factor (R^f) values and UV spectra of component peaks in sample and reference chromatograms (24-25)

Preparation of nano suspension of *Hibiscus sabdariffa* L. (calyx)

Initially, 0.1% chitosan was prepared by dissolving 0.1g in 100mL water. Then 1.5% polyvinyl alcohol was prepared by dissolving

1.5g PVA in 100mL water. Ethanolic Extract of *Hibiscus sabdariffa* L solution was prepared by dissolving 1g extract in 1mL of ethanol. PVA and extract solutions were mixed in a beaker with stirring. The resultant mixture was kept in probe sonication for 30 minutes and chitosan was added dropwise in a beaker.

Results and Discussion

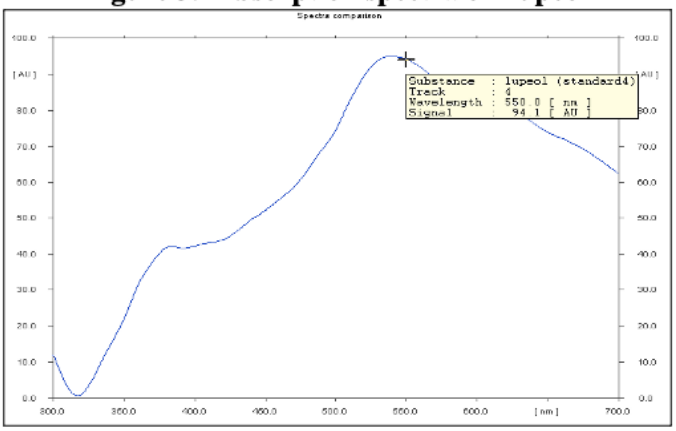
Quantification of Lupeol in calyx of *Hibiscus sabdariffa* L plant

A volume of 10 µl of the *Hibiscus sabdariffa* linn calyx extracts, namely the ethanol, ethyl acetate, and aqueous extracts, were carefully placed on the HPTLC plate. After that, a calibration curve produced from a standard was used to regulate the amount of lupeol existing in the samples. The present analytical methodology ascertains the presence of lupeol in several extracts, namely ethanol, ethyl acetate, and aqueous, at concentrations of 0.52% w/w, 0.47% w/w, and 0.16% w/w, respectively. The optimal concentration of lupeol was detected in the ethanol extract, therefore for further validation parameters ethanol extract was used. (Fig. 5)

Selection of Wavelength

ʎ max for lupeol was found to be 550 nm (fig. no. 3). It was verified by comparing the result to the lupeol value that has been published before. Therefore, the 550 nm wavelength was used for all subsequent measurements.

Figure 3. Absorption spectra of Lupeol



Method Validation

Linearity

The linearity range of the standard lupeol was determined by preparing 20-120 ng of standard per band using a range of spot volumes (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 l). The linearity was tested three times. A curve was produced by graphing the area versus the number of spots per area on the plate during scanning at a wavelength of 550 nm (Fig. no. 4). Lupeol had a regression of equation Y= 37.687x-115.290 and correlation coefficient (r2) of 0.9959, indicating a very strong linear relationship (Table no. 1).

Limit of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were ascertained through computations employing the subsequent equations: LOD = 3.3 (SD/S) and LOQ = 10(SD/S). The observed values for lupeol were determined to be 0.238 and 0.722 ng spot^{-1}(Table 1).

Precision

Precision experiments were conducted to demonstrate the replicability of the approach that was devised. An intraday precision study was conducted using six replicates of the same concentration, which was 80 ng per band. The preciseness of the intraday analysis was assessed at three specific time points within a single day, while the precision over several days was determined by reproducing the same approach over a period of three consecutive days. The findings were presented in relation to the standard deviation, specifically in terms of the percent relative standard deviation. The interday accuracy was assessed and concluded to have a percentage coefficient of variation of 1.33%, as shown in Table 2. Similarly, the intraday accuracy was evaluated and found to have a percentage coefficient of variation of 1.20%, as provided in Table 2. The assessment of the correctness of the utilised technique for the study was conducted by considering the low relative standard deviation (%RSD).

Specificity

The assessment of method specificity was accomplished through the utilisation of standard pharmaceuticals and sample compounds. The verification of the existence of lupeol in the extracts was accomplished through the execution of a comparative examination of the retention factor (R^f) and ultraviolet-visible (UV-Vis) spectra of the samples with a standard reference. The analysis comprised evaluating the purity of the lupeol-associated sample spot by superimposing the spectra of standard and sample peaks (Fig. 6).

Recovery studies (Accuracy)

In order to assess the method's accuracy, a series of recovery tests were undertaken at three different levels of lupeol. To execute the analysis employing the prescribed HPTLC technique, the pre-analysed samples were fortified with standard lupeol at concentrations of 80%, 100%, and 120%. The experiment was replicated six times in order to ascertain the percentage recovery of lupeol at three discrete levels. The recorded values for the percentage recovery were 99.93%, 99.36%, and 99.58% accordingly, as shown in Table 3.

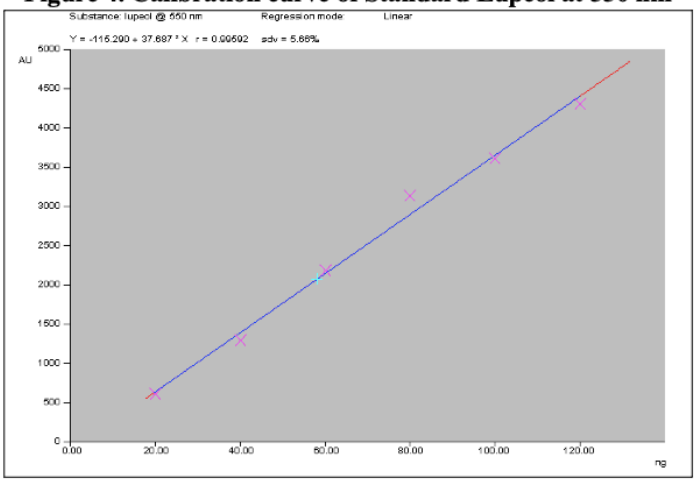
Robustness

The assessment of robustness was performed in triplicate at a concentration of 80 ng band-1 through slight modifications to the optimal method parameters, including variations in the mobile phase composition or adjustments to the duration of chamber saturation. The data analysis encompassed the utilisation of the relative standard deviation (% RSD) and the standard error of peak area. The mobile phase is prepared by employing a solvent solution, namely Toluene: Methanol, in diverse volumetric proportions, encompassing 9:1, 8:2, or 7:3, among other conceivable combinations. Table 4 presents the varying saturation times of 10, 15, and 20 minutes observed during the development of the chromatograph.

Table 1: Method validation parameters for the quantification of lupeol by HPTLC

Parameters	Results
Range of Linearity (ng/band)	20-120
Regression of equation	Y= 37.687x-115.290
Slope	37.687
Correlation Coefficient (r^2)	0.9959
LOD (ng/band)	0.238 ng
LOQ (ng/band)	0.722 ng

Figure 4. Calibration curve of Standard Lupeol at 550 nm



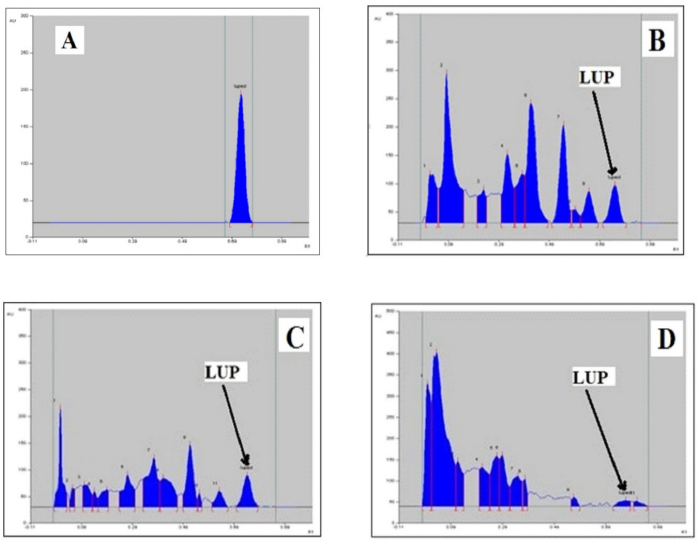


Figure 5. HPTLC Chromatogram at 550 nm A) Standard lupeol, B) Ethanolic extract, C) Ethyl acetate extract & D) Aqueous extract

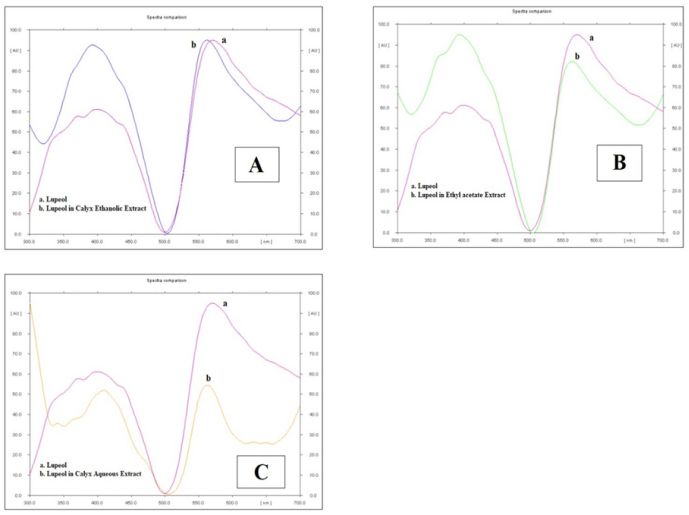


Figure 6. Overlay UV absorption spectra of Lupeol in peaks of Standard and extracts A) Ethanolic extract, B) Ethyl acetate extract C) Aqueous extract

Table 2: Interday and Intraday precision of HPTLC

Amount (ng/band)	Interday precision			Intraday precision		
Lupeol 80 ng	Mean[a] area	SD	%RSD	Mean[a] area	SD	%RSD
	3113.23	41.66	1.33	3329.79	40.12	1.20

[a] Mean of three determinations

Table 3: Result of accuracy study

Sr. No.	Level (%)	Amount taken (µg/ml)	Amount added (µg/ml)	Mean[a]± SD	Amount recovered Mean[a] ±SD	%Amount recovered Mean[a]± SD	%RSD
1	80	100	64	5995.16 ±1.22	63.96± 0.03	99.93±0.05	0.05
2	100	100	80	6580.54 ±0.7	79.49± 0.02	99.36±0.02	0.02
3	120	100	96	7187.54 ±1.93	95.6±0.05	99.58±0.05	0.05

[a] Mean of three determinations

Table 4: Robustness study for HPTLC method

Factors	Chromatographic changes			
	Level	Peak area	SD	%RSD
Mobile phase				
9:1	±1	4156.56	2.60	0.06
8:2	0	3363.26	3.83	0.11
7:3	±1	4896.11	3.54	0.07
Saturation time				
10	-5 min	2896.98	1.54	0.05
15	0	3247.99	4.84	0.14
20	+5 min	3287.19	21.76	0.66

Conclusion

Developing and testing a High-Performance Thin-Layer Chromatography (HPTLC) methodology for precise determination of lupeol content in several kinds of extracts done from the calyx of *Hibiscus sabdariffa* Linn extracts, including ethanol, ethyl acetate, and aqueous extracts, was the primary aim of the present investigation. The lupeol content in the calyx ethanol, ethyl acetate and aqueous extract was determined to be 0.52% w/w, 0.47% w/w, and 0.16% w/w, respectively. The content of lupeol was found to be significantly greater in the ethanol extract compared to the other two extracts. Consequently, additional validation criteria were used by the utilisation of an ethanolic extract. The approach that was suggested has been deemed to be accurate, exact, selective, and robust in terms of its ability to identify and assess the presence of lupeol in *Hibiscus sabdariffa* Linn. Lupeol has been shown to possess significant pharmacological efficacy, and the ethanolic extract derived from the calyx of *Hibiscus sabdariffa* Linn exhibits a substantial concentration of lupeol. Consequently, this extract has promise for utilisation in the development of diverse pharmaceutical preparations.

Conflict of Interest: There are no conflicts of interest.

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References

1. Pegu F, Paul A, Chetia A and Zaman K Md. An updated review on phytochemistry and therapeutic use of *Hibiscus sabdariffa* L., *CurrTrends Pharm Res,*2021; 8(1): 41-72.
2. Qi Y, Chin KL, Malekian F, Berhane M, Gager J. Biological characteristics, nutritional and medicinal value of roselle, Hibiscus sabdariffa. Circular-urban forestry natural resources and environment. 2005 Mar;604:1-2.
3. Nadlene R, Sapuan SM, Jawaid M, Ishak MR, Yusriah L. A review on roselle fiber and its composites. Journal of Natural Fibers. 2016 Jan 2;13(1):10-41. https://doi.org/10.1080/15440478.2014.984052
4. Riaz G, Chopra R. A review on phytochemistry and therapeutic uses of Hibiscus sabdariffa L. Biomedicine & Pharmacotherapy. 2018 Jun 1;102:575-86. https://doi.org/10.1016/j.biopha.2018.03.023
5. Hapsari BW, Setyaningsih W. Methodologies in the analysis of phenolic compounds in roselle (Hibiscus sabdariffa L.): Composition, biological activity, and beneficial effects on human health. Horticulturae. 2021 Feb 23;7(2):35. https://doi.org/10.3390/horticulturae7020035
6. Onyeka BO, Ogochukwu T. *Hibiscus sabdariffa* -uses, nutritional and therapeutic benefits -A review, Journal of Bioscience and Biotechnology Discovery Volume 8(2), pages 18-23, April 2023. DOI: 10.31248/JBBD2023.1
7. Vasudeva N, Sharma SK. Biologically Active Compounds from the Genus Hibiscus. Pharmaceutical Biology. 2008 Jan 1;46(3):145-53. https://doi.org/10.1080/13880200701575320
8. Rao SD, Rao BN, Devi PU, Rao AK. Isolation of lupeol, design and synthesis of lupeol derivatives and their biological activity. Oriental Journal of Chemistry. 2017 Feb 1;33(1):173-80. http://dx.doi.org/10.13005/ojc/330119
9. Connolly JD, Hill RA. Triterpenoids. Natural product reports. 1989;6(5):475-501. https://doi.org/10.1039/NP9890600475
10. Hill RA, Connolly JD. Triterpenoids. Natural product reports. 2012;29(7):780-818. https://doi.org/10.1039/C3NP70032A
11. Sharma N, Palia P, Chaudhary A, Verma K, Kumar I. A review on pharmacological activities of lupeol and its triterpene derivatives. Journal of Drug Delivery and Therapeutics. 2020 Sep 15;10(5):325-32. https://doi.org/10.22270/jddt.v10i5.4280
12. Siddique HR, Saleem M. Beneficial health effects of lupeol triterpene: a review of preclinical studies. Life sciences. 2011 Feb 14;88(7-8):285-93. https://doi.org/10.1016/j.lfs.2010.11.020
13. Hildebert Wagner and Sabine Bladt, "Plant Drug Analysis, A Thin Layer Chromatogram Atlas", Published by Springer, Second Edition, 2009, Page no. 659.
14. Rathee D, Rathee S, Rathee P, Deep A, Anandjiwala S, Rathee D. HPTLC densitometric quantification of stigmasterol and lupeol from Ficus religiosa. Arabian Journal of Chemistry. 2015 May 1;8(3):366-71. https://doi.org/10.1016/j.arabjc.2011.01.021
15. Khatoon S, Irshad S, Pandey MM, Rastogi S, Rawat AK. A validated HPTLC densitometric method for determination of lupeol, β-sitosterol and rotenone in Tephrosia purpurea: A seasonal study. Journal of Chromatographic Science. 2019 Jul;57(8):688-96. https://doi.org/10.1093/chromsci/bmz041
16. Alam P, Al-Yousef HM, Siddiqui NA, Alhowiriny TA, Alqasoumi SI, Amina M, Hassan WH, Abdelaziz S, Abdalla RH. Anticancer activity and concurrent analysis of ursolic acid, β-sitosterol and lupeol in three different Hibiscus species (aerial parts) by validated HPTLC method. Saudi Pharmaceutical Journal. 2018 Nov 1;26(7):1060-7. https://doi.org/10.1016/j.jsps.2018.05.015
17. Yadav H, Mungara P, Jivrajani M, Nivsarkar M, Anandjiwala S. TLC-densitometric quantification of negundoside, ursolic acid, eugenol, lupeol, and β-sitosterol using HPTLC from vitex negundo leaves. Journal of liquid chromatography & related technologies. 2012 Jun 1;35(11):1565-84. https://doi.org/10.1080/10826076.2011.619044
18. Venkatachalapathi S, Subban RA. Isolation and quantification of lupeol in Strobilanthes ciliatus Nees by HPTLC method. Int J Pharm Pharm Sci. 2012;4(4):405-8.
19. Singh N, Khatoon S, Srivastava N, Rawat AK, Mehrotra S. Qualitative and quantitative standardization of Myrica esculenta Buch.-Ham. Stem bark by use of HPTLC. JPC–Journal of Planar Chromatography–Modern TLC. 2009 Aug;22:287-91. https://doi.org/10.1556/JPC.22.2009.4.9
20. Raja MM, Mishra SH. Quantification of l-dopa, lupeol and β-sitosterol from leaves of Clerodendrum phlomidis by TLC. Herba. Polonica. 2009;55:44-51.
21. Anandjiwala S, Srinivasa H, Rajani M. Isolation and TLC densitometric quantification of gallicin, gallic acid, lupeol and β-sitosterol from Bergia suffruticosa, a hitherto unexplored plant. Chromatographia. 2007 Nov;66:725-34. https://doi.org/10.1365/s10337-007-0389-1
22. Kaur N, Gupta RC. Lupeol validation and quantification in Heteropogon contortus (L.) Beauv.(Spear grass) through High-performance thin-layer chromatography. Asian journal of pharmaceutical and clinical research. 2017 Dec 1:392-5. http://dx.doi.org/10.22159/ajpcr.2017.v10i12.21431
23. Pandya DJ, Anand IS. Isolation and high-performance thin layer chromatographic estimation of Lupeol from Oxystelma esculentum. Pharmaceutical methods. 2011 Apr 1;2(2):99-105. https://doi.org/10.4103/2229-4708.84446
24. Shrinivas KK, Choudhary KC, Choudhary MR. Stability-indicating and analysis of quercetin, Rutin and lupeol in Benincasa hispida (Curcurbitaceae) fruit, leaves extracts and formulation by High Performance Thin Layer chromatographic method. Bulletin Environment Pharmacology Life Science. December 2021;11 (1):92-101.
25. Abhimanyu KK, Ravindra CS, Avanapu RS. A validated HPTLC method for the quantification of friedelinin Putranjivaroxburghii Wall extracts and in polyherbal formulations. Bulletin of Faculty of Pharmacy, Cairo University. 2017 Jun 1;55(1) https://doi.org/10.1016/j.bfopcu.2016.11.002.

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