INTRODUCTION

Phytochemical evaluation is one of the tools for quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques. Use of chromatography for standardization of plant products was introduced by the WHO (World Health Organization) and is accepted as a strategy for identification and evaluation of the quality of plant medicines [1]. HPLC and HPTLC both emerged as an efficient tool for the phytochemical evaluation. HPTLC is a widely accepted technique for its high accuracy, precision and reproducibility of results. In addition, HPTLC has many advantages because of high sample throughput at low operating cost, easy sample preparation, short analysis time and analytical assurance [2-4].

Secondary metabolites are natural products that often have an ecological role in regulating the interactions between plants and their environment. They can be defensive substances, such as phytoalexins and phytoanticipins, antifeedants, attractants and pheromones [5]. The importance of plant secondary metabolites in medicine, agriculture and industry has led to numerous studies on the synthesis, biosynthesis and biological activity of these substances [6]. The terpenes are biosynthetically constructed from isoprene (2-methyl-6-(1-methyl ethyl) bicyclic heptan-3-one) units [7]. The C9H18 isoprenes polymerise and subsequently fix the number and position of the double bonds. The basic molecular formulae of terpenes are thus multiples of C9H18 [8]. Terpenes comprise a large number of different types of compounds which may be divided into more important chemical structure families. The main groups of terpenoids are represented by pentacyclic derivatives of lupeol [9]. The 3-O-acyl-derivatives of lupeol have anti-inflammatory properties and many of them are present in different medicinal plants, as are lupeol acetate and lupeol hexacosanoate in Willughbeia firma [10].

Andrographis echioides belongs to Acanthaceae family, used for various medicinal purposes in South Asia particularly India and China. Based on the literature, this plant possess pharmacological properties include antimicrobial activity, anti-inflammatory, diuretic, antihelmintic, analgesic, antiinflammatory and antioxidant effect. It contains plenty of phytochemical constituents such as flavonoids, flavones, steroids, tannins, carbohydrate, glycosides and alkaloids [11, 12]. The leaf juice of A. echioides is used to cure fevers. Genus of Andrographis family plants are used to cure various diseases like goiter, liver diseases, fertility problems, bacterial, malarial and fungal disorders [13, 14]. Andrographis echioides boiled with coconut oil is used to decrease the falling and graying of hair [15]. From the leaves extract of Andrographis echioides various chemical constituents were isolated dihydro echioidinin, skullcap avone 1 2-methyl ether, echioidin, echioidin, skullcap avone 1 and 2'-O-b-D-glucopyranoside [16]. Some of the other chemical constituents present in the A. echioides are more than 17 compounds such as borneol, cyclohexanol 2,4 dimethylphenol, 3,4 altruson, n-decononic acid, Squalene, vitamin E, Methoprene, 2-nonenol Oxiane-octyl-2, 2-cyclopentene-1-undecanoic acid, ketone, 1,5-methylbicyclo [2.1.0] pent-5-y methyl and 2, 5-cyclohexadien-1,4-dione, 2, 5-dihydroxy-3-methyl-6-(1-methyl ethyl) bicycle heptan-3-one [17]. However, no single method was found in the literature to our knowledge to detect lupeol in methanolic extract of leaves of Andrographis echioides. Therefore, the present study aimed to develop a HPTLC method for analysis of lupeol in Andrographis echioides leaves which have not yet been reported. The developed method was optimized and validated in accordance with International Conference on Harmonization (ICH) guidelines. The present study was designed to develop a new simple, precise, rapid and selective thin-layer chromatographic (HPTLC) method for the determination of lupeol in methanolic leaves extract of Andrographis echioides.

MATERIALS AND METHODS

Chemicals and reagents

Lupeol (purity 99%) was purchased from Sigma-Aldrich, New Delhi. All the chemicals, including solvents such as n-hexane, ethyl acetate, chloroform, methanol, anisaldehyde sulphuric acid reagents (0.5 ml p-anisaldehyde in 50 ml glacial acetic acid and 1 ml conc. sulphuric acid. Heat to 105 °C until maximum visualization of spots) were of analytical grade and were procured from E. Merck, India.
Collection of plant material

The leaves of Andrographis echioides were collected in the month of May from the mulkipatti, pudukkottai, Tamil Nadu, India. The plant was identified and leaves of Andrographis echioides were authenticated and confirmed from Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirapalli, and Tamil Nadu for identifying the plants. The voucher specimen number SGP001 (7.06.2017).

Preparation of methanol extracts

The leaves of Andrographis echioides were washed in running water, cut into small pieces and then shade dried for a week at 35-40°C, after which it was grinded to a uniform powder of 40 mesh size. The methanol extracts were prepared by soaking 100 g each of the dried powder plant materials in 1 L of methanol using a soxhlet extractor continuously for 10 hr. The extracts were filtered through whatmann filter paper No. 42 (125 mm) to remove all unextractable matter, including cellular materials and other Constitutions that are insoluble in the extraction solvent. The entire extracts were concentrated to dryness using a rotary evaporator under reduced pressure. The final dried samples were stored in labeled sterile bottles and kept at-20°C. The filtrate obtained was used as sample solution for the further HPTLC analysis [18].

Identification and quantification of lupeol from leaves of Andrographis echioides by HPTLC

Sample preparation

The HPTLC plates Si 60F254 (20 cmX10 cm) were purchased from E. Merck (India). Standards of lupeol (99% purity) were purchased from sigma (New Delhi, India). 100 mg/ml of methanolic extracts of leaves of Andrographis echioides was taken for analysis. The extracts were filtered and vacuum dried at 45°C. The dried extracts were separately redissolved in 1 ml of methanol and sample of varying concentration (1-5 µl) for lupeol were spotted for quantification. 1 mg of standard lupeol was prepared in 1 ml of chloroform, and different amounts of (5000-10000 ng) lupeol were loaded onto a TLC plate to get the calibration curve [19-22].

Thin layer chromatography

A Camag HPTLC system equipped with an automatic TLC sampler ATS, TLC scanner 3 and integrated software Win CATS version 3 was used for the analysis. Samples were washed on a pre-coated silica gel HPTLC plates Si 60F254 (20 cm x 10 cm) plate of 200 µm- layer thickness, for quantification of lupeol in leaves of Andrographis echioides. The samples and standards were applied on the plate as 8 mm wide bands with a constant application rate of 15ONl s⁻¹ with an automatic TLC sampler (ATS) under a flow of N₂ gas, 15 mm from the bottom, 15 mm from the side, and the space between two spots was 6 mm in the plate.

Detection and estimation of lupeol

The linear ascending development was carried out in a camag twin through chamber (20 cm x 10 cm), which was pre-saturicated with a 25 ml mobile phase, n-Hexane: ethyl acetate (80:20v/v) for lupeol for 30 min, at room temperature (25°C±2°C) and 50±5% relative humidity. The length of the chromatogram run was up to 90 mm. Subsequent to the development; the TLC plate was dried in a current of air, with the help of air dryer, in a wooden chamber with adequate ventilation. The dried plate was dipped into freshly prepared anisaldehyde sulphuric acid reagents. Quantitative estimation of the plate was performed in the absorption-reflection mode at 538 nm, using a slit width 6.00 x 0.45 mm, with data resolution 100 µm/step and scanning speed 20 mm/sec. The source of radiation utilized was a tungsten lamp emitting continuous visible spectra of 366 nm. Determination of lupeol in methanolic extracts of Andrographis echioides was performed by the external standard method using pure standards. Each was carried out in triplicate [23, 24].

Method validation

This method was validated as per the ICH guidelines (International Conference on Harmonization in 1994, 1996 and 2005), the method validation parameters checked were linearity, precision, accuracy and recovery, limit of detection, limit of quantification, specificity, robustness and ruggedness. All measurements were performed in triplicates [25-27].

Calibration curve and linearity

The calibration was performed by analysis of working standard solutions of lupeol (5000-10000 ng for leaves of Andrographis echioides) were spotted on precoated TLC plate, using semi-automatic spotter under a nitrogen stream. The TLC plates were developed, dried by hot air and photometrically analyzed as described earlier. The calibration curves were prepared by plotting peak area versus concentration (ng/spot) corresponding to each spot.

Recovery

To determine the recovery, known concentrations of standards were added to a pre-analyzed sample of leaves of Andrographis echioides. The spiked samples were then analyzed by the proposed HPTLC method and the analysis was carried out in triplicate.

Precision

A stock solution containing lupeol compound was prepared in chloroform and six 10 µl (1000 ng/spot) bands were applied and analyzed by the developed method to determine instrument precision. Six different volumes of same concentration were spotted on a plate and analyzed by the developed method to determine variation arising from the method itself. To evaluate intra-day precision, six samples at three different concentrations (1000, 2000 and 3000 ng/spot) for lupeol were analyzed on the same day. The inter-day precision was studied by comparing assays performed on three different days.

Limit of detection and limit of quantification

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated using the following formula

\[ \text{LOD} = 3.3 \times \frac{\text{Standard deviation of they \ - \ intercept}}{\text{Slope of calibration curve}} \]

The quantification limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated using the following formula,

\[ \text{LOQ} = 10 \times \frac{\text{Standard deviation of they \ - \ intercept}}{\text{Slope of calibration curve}} \]

Specificity

The specificity of the method was ascertained by analyzing standard compound lupeol and the compound lupeol is present in the leaves of Andrographis echioides.

Method specifications

Silica gel 60 F254 precoated plates (20x 10 cm) were used with n-hexane: ethyl acetate (80:20 v/v) for lupeol as the solvent system. The sample was spotted on precoated TLC plates by using Linom at 5 applicator. Ascending mode was used for development of thin layer chromatography. TLC plates were developing up to 80 mm and scanned in fluorescence mode at 366 nm. The contents of lupeol in the leaves of Andrographis echioides were determined by comparing area of the chromatogram of standard lupeol with a calibration curve of the marker compound of leaves of Andrographis echioides considering the isolated compound to be 100% pure.

Robustness

For the determination of the robustness of method chromatographic parameters, such as mobile phase composition and detection wavelength, were intentionally varied to determine their influence on the retention time and quantitative analysis. Intraday variability...
was studied for the sample, by injecting the same concentration of the sample in triplicate and the standard error mean was calculated.

RESULTS

Optimization of HPTLC chromatographic conditions

HPTLC fingerprint patterns have been therefore evolved for methanolic extracts of leaves of *Andrographis echioides*. Lupeol standard was quantitated accurately using silica gel F$_{254}$, HPTLC pre-coated plates with the mobile phase n-hexane:ethyl acetate (80:20 v/v), the Rf value was about 0.55. The chromatographs of lupeol and methanolic extract of leaves of *Andrographis echioides* are shown in (fig. 1). The Rf value of lupeol was matched with the Rf value of extract was about 0.55 was shown in peak (fig. 2 (a) and (b)). A terpenoid compound lupeol were identified and isolated by HPTLC techniques.

Validation of HPTLC method

Calibration curve and linearity

The calibration curve was prepared by plotting peak area versus concentration (ng/spot) corresponding to each spot (fig. 3). The regression equation and correlation curves for lupeol in leaves of *Andrographis echioides* were, regression via height $y = 148.075x$ and $r = 0.99755$ sdev= 0.72 fig. 3 (a) and regression via area $y = 212.106 + 1730.405x$ and $r = 0.99917$ sdev= 0.72 fig. 3(b).

Accuracy and recovery

The results showed that the percentage recoveries after sample processing and application were in the range of 99.80 % to 100.10 % (lupeol) (table 1). The percentage of lupeol in leaves of *Andrographis echioides* (table 2).

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![Fig. 1: Quantitative estimation of lupeol in *Andrographis echioides* leaves](image1)

![Fig. 2: (a) HPTLC chromatogram of standard lupeol (b) HPTLC chromatogram of lupeol in *Andrographis echioides* leaves](image2)

![Fig. 3: Linear graph for lupeol in *Andrographis echioides* in all tracks (concentration vs. area)](image3)
Precision

The developed method was found to be precise as indicated by percent RSD (Relative Standard Deviation) not more than 1.5 (tables 3 and 4).

Specificity

It was observed that the other herbal constituents present in the formulations did not interfere with the peak of lupeol. Therefore the method was specific. The spectrum of standard compound lupeol and the corresponding spot present in leaves of *Andrographis echioides* matched exactly, indicating no interference by the other plant constituents and excipients. The peak purity of lupeol was assessed by comparing the spectra at three different levels like peak start (S), peak apex (M) and peak end (E) positions of the spot. Good correlation \( r = 0.99917 \) and SD = 0.72 for lupeol were obtained between the standard and sample overlain spectra of lupeol (fig. 4).

![Fig. 4: Spectral comparison of standard lupeol (green colour) and lupeol quantified from *Andrographis echioides* leaves (pink colour)](image)

| Compound | Amount of compound present in the plant material (mean, μg/100 mg) | Amount of standard added (μg) | Amount of standard found in the mixture (μg) | Recovery (%) |
|----------|---------------------------------------------------------------|-------------------------------|-------------------------------------------|--------------|
| Lupeol   | 265                                                           | 265                           | 520.00                                    | 100.10±1.14  |
|          | 520                                                           | 800.00                        |                                            | 99.80±1.93   |

\( n \) is number of determination, μg (microgram), mg (milligram)

| Compound | Quantity (mean) (mg/100 mg) | mean±SE   | CV (%) |
|----------|-----------------------------|-----------|--------|
| Lupeol   | 0.265                       | 0.265±0.004 | 0.72   |

\( n \) is number of determination, SE is standard error, CV is cumulative value

| Compound | Amount (ng/spot) | Intra-day precision | Inter-day precision |
|----------|-----------------|---------------------|---------------------|
|          |                 | Mean area | SD | %RSD | Mean area | SD | %RSD |
| Lupeol   | 1000            | 2480.50    | 1.83 | 0.06 | 2490.30    | 3.57 | 0.14 |
|          | 2000            | 4900.45    | 2.81 | 0.05 | 4900.62    | 5.88 | 0.16 |
|          | 3000            | 7340.40    | 1.48 | 0.02 | 7338.02    | 4.80 | 0.06 |

\( n \) is number of determination, SD is standard deviation, RSD is relative standard deviation

| Parameters                                                                 | Lupeol |
|---------------------------------------------------------------------------|--------|
| Linearity \((i)\) Range                                                   | 5000-10000 ng |
| \((ii)\) Correlation coefficient                                          | 0.99755 |
| \((a)\) Height                                                            | 0.99917 |
| \((b)\) Area                                                              | 0.55   |
| \((iii)\) Rf value                                                       |        |
| Precision \((\%RSD)\) \((i)\) Instrument precision (CV%, n=6)            | 1.34   |
| \((ii)\) Method precision (CV%, n=6)                                     | 2.40   |
| LOD (ng/spot)                                                            | 120    |
| LOQ (ng/spot)                                                            | 435    |
| Specificity                                                              | Specific |
| Robustness                                                               | Robust |
| Ruggedness \((\%RSD)\)                                                   | 0.9512 |

\( n \) is a number of determination, RSD is a relative standard deviation, CV is cumulative value, LOD is Limit of detection, LOQ is Limit of quantification, Rf is retention factor.
Ruggedness of the method

It expresses the precision within laboratories variations like different days, different analyst, and different equipment. Ruggedness of the method was assessed by spiking the standard 6 times in two different days with the different analyst (table 4).

**CONCLUSION**

In conclusion, an HPTLC method has been developed with some modifications and it can be used for the quantitative determination of lupeol in methanolic extract leaves of *Andrographis echioides* its main advantages are its simplicity, accuracy and selectivity. The average recovery values of lupeol were found to be about 99.80%, which showed the reliability and suitability of the method. This method could also be used for the estimation of these compounds in other herbal preparations and might be useful for standardization purposes.

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**AUTHORS CONTRIBUTIONS**

All author contribute equally to this manuscript

**CONFLICTS OF INTERESTS**

The authors declare that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

**REFERENCES**

1. Dhalwal K, Shinde VM, Mahadik KR, Kakasahab RM. Optimization and validation of reverse phase HPLC and HPTLC method for simultaneous quantification of vasicine and vasicinone in Sida species. Med Plants Res 2010;4 Suppl 13:928-936.
2. Di X, Chan KKC, Leung HW, Hui CW. Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high-performance thin-layer chromatography. J Chromatogr A 2003;1018:85-9.
3. Larsen T, Axelsen J, Ravn HW. Simplified and rapid method for extraction of ergosterol from natural samples and detection with quantitative and semi-quantitative methods using thin layer chromatography. J Chromatogr A 2004;106:301-4.
4. Suthar AC, Banavaliker MM, Biyani MK, Priyadarshini Indira K, Sudarsan V, Mohan HA. High-performance thin layer chromatography method for qualitative estimation of lupeol in *Crataeva nurvala*. Indian Drugs 2001;38 Suppl 4:474-8.
5. Hansson JR. The biosynthesis of secondary metabolites. In Natural Products, the secondary Metabolites; 1999. p. 222-99.
16. aavanone from Jayaprakasam D, Gunasekara B, Bodo. Dihydroechioidinin, Specification, November; 2005. p. 5–10.

17. Antika K, Handique A. Brief overview on Andrographis Paniculata (Bur. f) Nees. A High valued medicinal plant: boon over synthetic drugs. Asian J Sci Technol2010;6:113–8.

18. Shanker AS, Lalit Kumar Tyagi, Mahendra S, Ch V Ra. Herbal medicine for market potential in india: an overview. Academic J Plant Sci 2008 1 Suppl 2:26–36.

19. Kirtikar. In: Indian Medicinal Plants. 3. New Delhi: Periodical Experts; 2007.

20. Zaibkar LQ, Beena J, Anandan R, Mohammed RJ. Antibacterial activity of ethanol extracts of Indoneesiella echioidees evaluated by the filter paper disc method. Pak J Pharm Sci 2009;22:123–5.

21. Kanchana N, Rubalakshmi. Phytochemical screening and antimicrobial activity of Andrographis echioidees(L) Nees–an indigenous medicinal plant. World J Pharm Pharm Sci 2014;3 Suppl 5:702–10.

22. Jayaprakasam D, Gunasekara B, Bodo. Dihydroechioidinin, aavanone from Andrographis echioidees. Phytochemistry 1999;1 Suppl 3:92–7.

23. Nirubama K, Rubalakshmi. Bioactive compounds in Andrographis echioidees (L) Nees. leaves by GC-MS analysis. Int J Curr Res Biosci Plant Biol 2014;1 Suppl 3:92-7.

24. Deepit R, Sushila R, Permender R, Aakash D, Sheetal A, Dharmender R. HPTLC densitometric quantification of stigmasterol and lupeol from Ficus religiosa. Arab J Chem 2015; 8:366–71.

25. Suthar AC, Banavaliker MM, Biyani MK, Priyadarssini Indira K, Sudarsan V, Mohan HA. High-performance thin layer chromatography method for quantitative estimation of lupeol in Crataeva nurvula. Indian Drugs 2001;38 Suppl 9:474–8.

26. Badami S, Gupta MK, Ramaswamy S, Rai SR, Nanjaian M, Bendell DJ, Subban R, et al. Determination of betulin in Grewia titiaefolia by HPTLC. J Separ Sci 2004;27:129–31.

27. Purnima D, Hamrapurkar PK. HPTLC determination of stigmasterol and tocopherol acetate in Leptadenia reticulata and its formulation. J Plan Chromatogr 2007;20 Suppl 3:183–7.

28. Geneva. ICH, Q2A(R1), Validation of Analytical Procedure: Text and Methodology, International Conference on Harmonization Specification, November; 2005. p. 5–10.

29. Ravindra CS, Sanjay BK, Kalaichevan VK. Phytochemical profile studies on the steroids of methanolic leaf extract of medicinally important plant Holoptelea integrifolia (Roxb.) planch using high-performance thin layer chromatography. Asian J Pharm Clin Res 2014;7:4.

30. Lavanya MS, Gnanamani A, Ilavarasan R. Physico-chemical, phytochemical and high-performance thin layer chromatography analysis of the whole plant of Orthosiphon thymiflorus (Roth) sleesen. Asian J Pharm Clin Res 2015;8:1.

31. Geneva. ICH, Q2B, Guideline on Validation of Analytical Procedure: Methodology, International Conference on Harmonization; 1996. p. 1–8.

32. Geneva. ICH, Q2A, Text on Validation of Analytical Procedure, International Conference on Harmonization; 1994. p. 1–5.

33. Sarfaraj H, Sheeba F, Mohammad Ali, Sarfaraz Alam, Akhlaque R, Srivastava AK. Phytochemical investigation and simultaneous estimation of bioactive lupeol and stigmasterol in Abutilon indicum by validated HPTLC method. J Coastal Life Med 2014;2 Suppl 5:394–401.

34. Rozylo JK, Janicka M. Different planar techniques for prediction of solute retention in column liquid chromatography. J Planar Chromatogr 1996;9 Suppl 1:26–40.

35. Weins C, Hauck HE. Advances and developments in thin layer chromatography. LC-GC Int 1996;4 Suppl 1:455–71.

36. Poole CF, Poole SK. Instrumental thin-layer chromatography. Anal Chem 1994;66 Suppl 1:27A–37A.

37. Meenu S, Sharma RG. Identification, purification and quantification of andrographolide from Andrographis paniculata (Bur. F.) Nees. by validated HPTLC method at different stages of life cycle of the crop. J Curr Chem Pharm Sci 1994;3 Suppl 1:23–32.

38. Padma Sarojini D, Manjunatha P, Venkata R. Preliminary phytochemical screening and antimicrobial activity of Andrographis echioidees nees. J Pharma Res 2015;5 Suppl 9:4801–3.

39. Ramasubramania R. Pharmacognostical psychochemical and anti-ulcer activity of Andrographis echioidees (Acanthaceae). J Pharmacogn Phytochem 2014;3 Suppl 3:39–49.

40. Suthar AC, Banavaliker MM, Biyani MK, Priyadarssini Indira K, Sudarsan V, Mohan H. A high-performance thin layer chromatography method for quantitative estimation of lupeol in Crataeva nurvula. Indian Drugs 2001;38 Suppl 9:474–8.