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Research Article

LUPEOL VALIDATION AND QUANTIFICATION IN *HETEROPOGON CONTORTUS* (L.) BEAUV. (SPEAR GRASS) THROUGH HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

Objective: This is aimed to study the chromatographic evaluation of triterpenoid, i.e., lupeol from methanolic extracts of leaves, stem, and inflorescence of *Heteropogon contortus*.

Methods: The high-performance thin-layer chromatography (HPTLC) densitometry determination of lupeol was performed using optimized mobile phase toluene:methanol:formic acid (7:3:0.3 v/v) with a derivatization of freshly prepared anisaldehyde-sulfuric acid. For densitometry measurements, the plates were scanned at 530 nm absorbance/reflectance wavelength. Quantification of lupeol marker compound in *H. contortus* leaves, stem, and inflorescence is estimated using 2-12 µg/spot.

Results: The appearance of light purple bands on the chromatograms confirmed the lupeol component in plant samples. Further, the confirmation of the compound is done from the densitometric scanning by comparing λ_{max} values. From this, it is reported that lupeol is present in leaf samples, i.e., 10 mg/g of dry wt., while in rest of the two samples, it is found absent.

Conclusion: The leaves of *H. contortus* (spear grass) are a good source of lupeol and can be used as an alternate natural source to synthesize herbal drugs to control cancer and other anti-inflammatory agents. The presently selected HPTLC is validated and most accurate for the quantification and identification of lupeol present in the selected plant. The leaves of the species which are rich in lupeol can be used in pharmaceutical industry.

Keywords: Lupeol, High-performance thin-layer chromatographic, Triterpenoid, *Heteropogon contortus.*

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INTRODUCTION

Triterpenoids are the secondary metabolites and have great importance in medicinal plants [1]. These are derived from vegetable oils, cereals, fruits, and vegetables and are consumed at an average of 250 µg/day [2]. Lupeol is an active triterpenoid. It exhibits important biological activities such as antiprotozoal, antitumor (antiprostate cancer, anti-melanoma, anti-head and neck squamous cell carcinoma, and anti-pancreatic cancer) [3-5], nutraceutical or chemopreventive agent [6-8], anti-inflammatory [9], and hepatoprotective [10].

Heteropogon contortus (L.) Beauv. (= *Andropogon contortus* L.), belonging to the family Poaceae (Gramineae), is commonly known as spear grass, black spear grass, bellary grass, kher (Hindi), gantegawata (Marathi). It is distributed in Southern Asia, Southern Africa, and Northern Australia. According to Blake and Richards [11], the grass is reported to have myo-inositol, galactinol, and raffinose. Polysaccharides were also observed in the plant species. It is medicinally important grass and is found useful in toothache, fever, atrophy, emaciation or cahexy, muscular pain, hematological disorders, dysentery, and scorpion sting [12,13]. Roots have diuretic and stimulant properties. The whole plant is used for asthma in the form of extracts or steam distillation product [14]. Oil distilled from the awns has been found to be useful in asthma.

Previously, Ghante *et al.* [15] obtained the results from the methanolic extract of *H. contortus* that it is used for the treatment of pathologies caused by mast cell destabilization, membrane destabilization, and free radical generation, which mainly include acute and chronic inflammatory response such as asthma, arthritis, cardiovascular, and neural diseases. Further, it was reported that *H. contortus* extract inhibits bronchoconstriction induced by histamine or acetylcholine [16]. Grasses are very important both economically as well as medicinally from the ancient time of herbal medicine use [17]. Compared the antimicrobial activities of ethanolic and hydroalcoholic extract of *Vetiveria zizanioides* root (a grass) and analyze the major bioactive compounds present in those extracts through high-performance thin-layer chromatography (HPTLC). It also inhibits inflammation induced by carrageenan and egg albumin. Some medicinally important grasses also show cytomorphological variations such as meiotic abnormalities and reduced pollen fertility [18]. Extraction-based studies were made on *Ormocarpum cochinchinense* and resulted that phenols in acetone and terpenoids in methanol extracts give better results for phytochemical analysis [19]. Thus, during the present study, methanol is used as a solvent for the quantification of lupeol from leaves, stem, and inflorescence of *H. contortus*.

METHODS

Standard markers and chemical compounds

Reference standard, i.e., lupeol was purchased from Himedia. Chemicals such as toulene, formic acid, and methanol were obtained from S.D. Fine Chemicals, Mumbai, India. Pre-coated silica gel 60 F_{254} HPTLC aluminum plates (20×20 cm^2 layer thickness-0.2 mm, 5-6 µm particle size; E. Merck, Darmstadt, Germany) was obtained from E. Merck Ltd. (Mumbai, India).

Collection of plant material and sample preparation

The material for the present study was collected from different localities of Udaipur district of Rajasthan, India. The plant is authenticated from Botanical Survey of India, Arid Zone Regional Center, Jodhpur, Rajasthan. The leaves, stem, and inflorescence (with awns) were excised from the plant. The collected material is then washed under running tap water, air-dried, and grinded to form a fine powder. 5 g dried powder of each plant part was extracted with 150 ml methanol using Soxhlet extractor for 8 hrs. The obtained extracts

were then filtered and concentrated using rotary vacuum evaporator and then lyophilized with Allied Frost Lyophilizer-FD-3. The obtained lyophilized powder of samples was accurately weighed and then dissolved in methanol (1 mg/1 ml). These solutions were then used as test solutions for HPTLC analysis.

Equipment

- Spotting device-CAMAG Linomat V sample applicator (CAMAG, Switzerland)
- Syringe-100 µl Hamilton syringe
- TLC Chamber-CAMAG twin trough chamber.
- Scanner-CAMAG TLC scanner with D_2 and Hg lamp, Reprostar and win CATS planar chromatography manager and CAMAG integration software and TLC viewing cabinet (all from CAMAG, Muttenz, Switzerland).

Preparation of standard stock solution

Accurately weighed reference standard lupeol (5 mg) was transferred to 10 ml volumetric flask, dissolved in 5 ml methanol (1 mg/1 ml). The stock solution was ready to use for HPTLC.

Chromatographic analysis

The HPTLC analysis was performed using precoated silica gel 60 F_{254} aluminum plates. Linomat V autosampler was used for spotting of standard lupeol (2, 4, 6, 8, 10, and 12 µl each) and sample solutions (10 µl each) operated with 6 mm band length, distance between the tracks 10 mm, distance from the bottom of the plate, 8.0 mm. The linear ascending developments were carried out up to a distance of 75 mm in a CAMAG twin trough chamber presaturated with mobile phase toluene:methanol:formic acid (7:3:0.3 v/v) for 25 minutes, and anisaldehyde is used as derivatizing reagent. The plates were then scanned in the CAMAG TLC scanner V, operated by win CATS software in the absorbance mode at 530 nm. The images of the plates were then taken in the visible mode.

Calibration curve of lupeol

The standard stock solutions (1 mg/1 ml) of lupeol (2-10 µg spot^{-1}) were applied in triplicate on an HPTLC plate. These plates were developed with the mobile phase toluene:methanol:formic acid (7:3:0.3 v/v) for 25 minutes. After development, the plates were air-dried and scanned at 530 nm absorbance using deuterium lamp. The resolved peak areas were recorded for all the standards separately. The calibration curve of lupeol was plotted by taking peak area versus concentrations of standards (Fig. 1).

Method validation

The HPTLC method was validated using the ICH guidelines for specificity, linearity, limits of detection and quantification, precision and accuracy, robustness, and stability [20].

Specificity

The specificity was revealed by analyzing the standard marker compound and plant samples. The band of lupeol was confirmed by comparing the R_f and ultraviolet (UV) spectra of the spots to those of the standard.

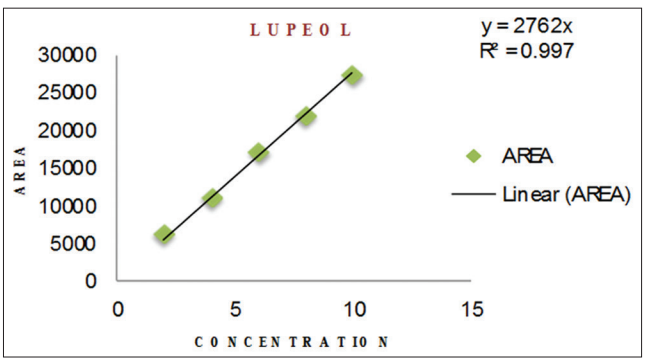

Fig. 1: Calibration curve of lupeol

Linearity

The different concentrations (2, 4, 6, 8, 10, and 12 ng spot^{-1}) of lupeol were applied on the HPTLC plate in triplicates, separately. The plate was developed as prescribed above. The calibration curve was drawn by plotting the standard concentrations ranges from 2 to 12 µg spot^{-1} versus peak area. The linear calibration curve was obtained from which linear regression equation and correlation coefficients were obtained.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for the present marker compound, i.e., lupeol was calculated using the following equations:

LOD=3.3 SD/S

LOQ=10 SD/S

Where SD stands for the standard deviation of replicates under the same conditions, and S is the slope of the calibration curve.

Robustness

Robustness was studied in triplicate at 800 ng spot^{-1} by making small changes to the volume of the mobile phase, the composition of the mobile phase, and saturation time of development chamber. The effects on the results were examined by calculation of relative SD (RSD) (%) and R_f values.

Precision and accuracy

Instrumental precision was checked by repeated scanning (n=6) of same spot for each standard separately (10 µg spot^{-1}) and expressed as % RSD. The accuracy of the present method was checked using a recovery study by spiking the sample with two levels of standards (6 and 12 µg band^{-1}). The % recovery was calculated using the formula given by the [21].

Quantitative determination of lupeol

The samples prepared from leaves, stem, and inflorescence of *H. contortus* were analyzed by using the validated HPTLC method, and the amount of the referred standard was calculated from the standard calibration curve of lupeol.

RESULTS AND DISCUSSION

During the present investigation, chromatographic conditions were optimized for the detection of lupeol using modified mobile phase toluene:methanol:formic acid (7:3:0.3 v/v) which gave the better

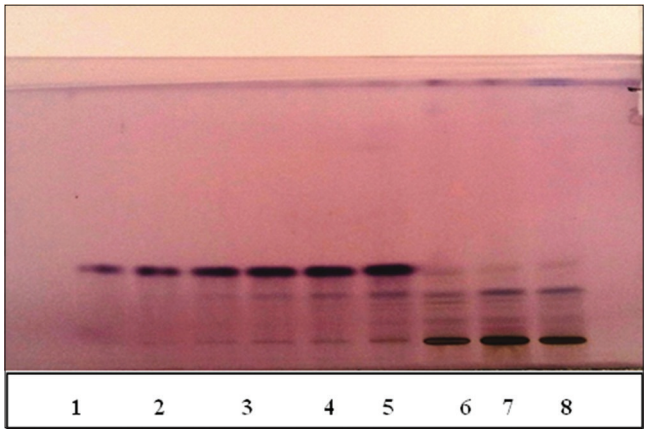

Fig. 2: High-performance thin-layer chromatography profile of lupeol (track 1-6) and methanolic plant samples of *Heteropogon contortus* (racks 7-9); 7(HCLM), 8(HCSM), 9(HCIM)

results, i.e., sharp and well-resolved bands of lupeol at R_f value of 0.21 (Fig. 2). Besides, the plates were derivatized with freshly prepared anisaldehyde-sulfuric acid and were heated at 110-120°C and scanned for densitometry measurements at an absorbing wavelength of 530 nm (Fig. 3). The identity and purity of the bands of selected sterols resolved in the plant samples (leaves, stem, and inflorescence)

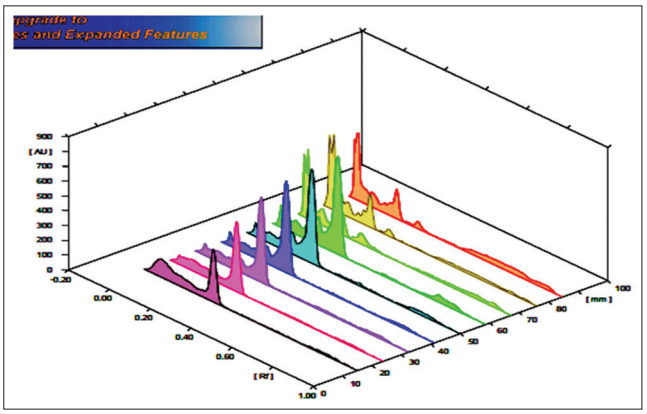

Fig. 3: Densitometric chromatogram of lupeol (track A-F); and plant samples of *Heteropogon contortus* (track G-I) HCLM, HCSM, and HCIM (λ_{max} 520 nm) (three-dimensional view)

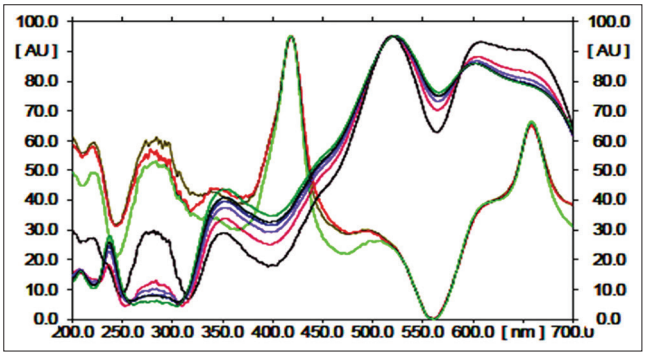

Fig 4: Absorption spectra of lupeol and plant samples of *H. contortus* (HCLM, HCSM, HCIM)

Table 1: Method validation parameters for the simultaneous quantification of lupeol

Parameters	Lupeol
Wavelength (nm)	530
R_f	0.28
Selectivity	Selective
Specificity	Specific
Linearity range (µg/spot)	2-12
Correlation coefficient (R^2)	0.997
Linear regression equation (y)	2762×
LOD (ng spot^{-1})	0.84
LOQ (ng spot^{-1})	0.25
Accuracy (average % recovery)	99.05

LOD: Limit of detection, LOQ: Limit of quantification

were confirmed by overlaying their UV-visible absorption spectra with those of the standard compound using TLC scanner V (Fig. 4). Line-to-line overlay spectra of leaf sample were obtained which shows that the identity and purity of the bands matches with those of the standard compound while stem and inflorescence show little bit variation in λ_{max}. It shows that lupeol is absent in leaf and inflorescence of *H. contortus*.

The method is validated as per the ICH guidelines in terms of precision, repeatability, and accuracy (Table 1). The linearity range for lupeol was found to be 2-12 µg spot^{-1} with correlation coefficient, i.e., 0.997. A linear calibration curve was obtained for the standard compound as described above. LOD value for a standard compound is 0.84 ng spot^{-1}, whereas LOQ value is 0.25 ng spot^{-1}. The average % recovery at 3 different levels of the referred marker compound was found to be 99.45 (Table 1). The accuracy of the present method was determined by spiking the sample along with known amounts of standard (100 µg band^{-1}). The present method showed a recovery of 99.05% for the addition of 100 µg band^{-1} of standard correspondingly (Table 2).

The results of quantification of lupeol are given in Table 3. During the present investigation, lupeol is only detected in leaf samples, whereas in rest of the two samples, i.e., stem and inflorescence, it is found to be absent. The amount of lupeol present in the leaf sample is 10±0.13 mg/g of dry wt.

CONCLUSION

The presently selected HPTLC is validated and most accurate for the quantification and identification of lupeol in medicinally important grass *H. contortus*. The developed method has been found to be sensitive, accurate, precise, specific, and robust for the screening and quantification of sterols. Although, HPTLC has a few limitations like limited developing distance and lower plate efficiency in comparison to HPLC and gas chromatography. HPTLC is still an effective tool for quality evaluation of medicinal plants due to its simplicity, low cost, and low requirements. Thus, leaves of the species which are rich in lupeol can be used in pharmaceutical industry.

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Table 3: Quantification of lupeol from different plant parts of *H. contortus*

Plant parts	Sample codes	Amount of lupeol in plant sample (% w/w)
Leaves	HCLM	10±0.13
Stem	HCSM	ND
Inflorescence	HCIM	ND

ND means not detected. *H. contortus: Heteropogon contortus*

Table 2: Recovery study of lupeol by the proposed HPTLC method

Marker compound	Amount present in sample (µg)	Amount added (µg)	Theoretical amount (µg)	Amount found (µg)	Recovery (%)	Average recovery (%)
Lupeol	100	75	175	172.62	98.64	99.05
	100	100	200	198.46	99.23	
	100	125	225	223.41	99.29	

HPTLC: High-performance thin-layer chromatography

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