Original Article

Isolation and high-performance thin layer chromatographic estimation of Lupeol from *Oxystelma esculentum*

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Abstract

Background: *Oxystelma esculentum* R. Br. (Family: Asclepiadaceae) is a perennial twiner growing in water-logged areas of the Indian sub-continent. It is used traditionally as a diuretic, laxative and an anti-ulcer agent. However, there is no method available for isolation and estimation of a marker compound from this plant. The present work deals with the isolation and structure elucidation of Lupeol from *O. esculentum* and high-performance thin layer chromatographic (HPTLC) method development for its estimation. **Materials and Methods:** The petroleum ether extract of the entire plant of *O. esculentum* was subjected to further fractionation followed by pilot thin layer chromatographic (TLC) experiments and column chromatography. This yielded a pure, white, crystalline solid which resolved at R_f 0.65 upon TLC of chloroform fraction of the petroleum ether extract using the mobile phase toluene: methanol (9:1). **Results:** This compound was subjected to ultraviolet (UV), infrared (IR), gas chromatography-mass spectroscopy (GC-MS) and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectral analysis and its structure elucidation revealed it to be Lupeol. A novel HPTLC method for the estimation of Lupeol from *O. esculentum* was developed, in which it was found to be 0.829 ± 0.09% w/w. **Conclusions:** The method developed was found to be easy, simple, precise, efficient, accurate, reproducible, specific and sensitive, and could serve as a suitable tool for routine analysis and phytochemical authentication of *O. esculentum.*

Key words: Asclepiadaceae, high-performance thin layer chromatographic, Lupeol, *Oxystelma esculentum*

INTRODUCTION

Oxystelma esculentum is a perennial twiner growing in the water-logged areas of the plains of India, Pakistan, Burma and Sri Lanka. It is used traditionally as a diuretic, laxative, anti-ulcer, galactogogue and anthelmintic. It is also used in throat infections, skin diseases and jaundice.[1,2] Recent pharmacological investigations reveal that it complies with its traditional claims as an anti-ulcer[3] and a diuretic[4] drug. It has been studied for its pharmacognostic characteristics.[5] However, negligible phytochemical investigations of this plant have been carried out so far and there is no method available for isolation and estimation of a marker compound from this plant. This work deals with the isolation of Lupeol from *O. esculentum* and high-performance thin layer chromatographic (HPTLC) method development and method validation for its estimation. This study could serve as a suitable tool for phytochemical study of *O. esculentum,* pave the way for isolation of other phytoconstituents and establish a link between the phytoconstituents and its pharmacological actions.

MATERIALS AND METHODS

Collection and authentication

O. esculentum in flowering and fruiting stage was collected from Barda Hills near Porbandar in October 2008. Herbarium of the collected sample was prepared

and deposited in Department of Pharmacognosy, R. K. College of Pharmacy (No. RKCP/COG/1/2008). Authentication was done by Dr. N. R. Sheth, Head of Department of Pharmaceutical Sciences, Saurashtra University. The entire plants were dried under shade, powdered to 60#, stored in airtight containers and used for further study.

Extraction and fractionation

Extraction of 1 kg powder of the entire plant was carried out in a round-bottom flask at a temperature <50°C using petroleum ether (Spectrochem Pvt. Ltd., Mumbai, India). The present study was carried out after the pharmacological activity (anti-ulcer, diuretic and laxative) using various extracts of *O. esculentum* suggested that petroleum ether extract had the best pharmacological action. So, petroleum ether extract was further investigated for its phytochemical constitution (thereby following bioactivity-guided fractionation).

The dried petroleum ether extract was further fractionated between *n*-hexane and water, carbon tetrachloride and water, toluene and water, diethyl ether and water, dichloromethane and water, *n*-butanol and water, chloroform and water, ethyl acetate and water in this order and finally between 80% methanol and water.

Chromatography

Each of the above organic solvent fractions of the petroleum ether extract were subjected to a series of pilot thin layer chromatographic (TLC) experiments using different proportions (0:10 to 10:0) of various solvents like petroleum ether, carbon tetrachloride, toluene, diethyl ether, dichloromethane, *n*-butanol, chloroform, ethyl acetate, acetone and methanol as the mobile phase.

Upon observing the results of TLC, 1 g dried chloroform fraction of the petroleum ether extract was subjected to column chromatography and loaded on a glass column (60 × 3 cm) packed with silica gel G (40 g, 60–120#, Spectrochem Pvt. Ltd.) as the stationary phase. Gradient elution was performed using toluene: methanol (10:0, 9.5:0.5, 9:1 up to 0:10) as the mobile phase. A total of 200 fractions were collected in test tubes. Upon evaporation of the mobile phase from the test tubes, pure, white crystals of a compound were obtained in test tubes of toluene: methanol (9:1) fraction. A single spot resolved at R_f 0.65 using the mobile phase toluene: methanol (9:1).

Spectral analysis and structure elucidation

This compound was subjected to spectral analysis: ultraviolet (UV; Labtronic, RKCP), infrared (IR; KBr; CSMCRI, Bhavnagar), gas chromatography-mass spectroscopy (GC-MS; CSMCRI, Bhavnagar) and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) ($CDCl_3$; CSMCRI, Bhavnagar). Upon the analysis of melting point and spectral data, the compound was suspected to be a sterol and triterpenoid. This was confirmed when the compound gave Salkowski test and Liebermann–Burchard test positive. The structure of the compound was elucidated on the basis of the spectra.

Method development for estimation by HPTLC

A novel HPTLC method for estimation of the isolated compound was developed**.** The instrument used was Camag Linomat V (semi-automatic spotting device) with Hamilton 100 µl HPTLC syringe, Camag twin trough chambers (20 × 10 cm), Camag TLC Scanner 3, Camag CATS 4 Integration software and Camag Reprostar-3.

Stationary phase used was pre-coated silica gel 60 F_{254} plate (E. Merck; methanol-washed, thickness 0.2 mm, 20 × 20 cm) and the mobile phase used was toluene: methanol (9:1).

The spotting parameters included start position of 15 mm from bottom edge, band width of 6 mm, space between two bands 12 mm and spraying rate of 6 sec/µl. The chromatographic conditions included ascending separation technique, twin trough chamber for plate development, chamber saturation time 4 min and migration distance 10 cm at a temperature of 25 ± 2°C. Detection was done in UV–visible range.

The spotting volume for calibration curve was 4–20 µl and for chloroform fraction of petroleum ether extract was 40 µl. The amount sprayed for standard curve was 160–800 ng. The mobile phase used was toluene: methanol (9:1).

Densitometric scanning was carried out in absorbance/reflectance mode at 254 nm using mercury lamp and slit dimension of 4 × 3 mm.

For calibration curve, accurately weighed 4 mg of standard isolated compound was taken in a 10 ml volumetric flask. Crystals were dissolved in methanol and the volume was adjusted to 10 ml with methanol (0.4 mg/ml). From this, 1 ml was diluted to 10 ml with methanol in a volumetric flask to give a final

concentration of the standard solution (40 µg/ml). Graded concentrations of the standard solution (40 µg/ml) in 4, 8, 12, 16 and 20 µl volume were applied on a pre-coated TLC silica gel 60 F_{254} plate (E. Merck, Darmstadt, Germany) using Camag Linomat IV automatic spotter. The concentration of the compound was 160, 320, 480, 640 and 800 ng/spot. The plate was developed in a mobile phase, toluene: methanol (9:1). Data of peak area of each of the compound spots was recorded. The calibration curve was obtained by plotting area versus concentration of each peak corresponding to the respective spot.

50 mg chloroform fraction of petroleum ether extract was dissolved in chloroform and the volume adjusted to 5 ml in a volumetric flask to get 10 mg/ml concentration. 40 µl of this test sample of chloroform fraction of petroleum ether extract was spotted along with standard solution of the compound (4–20 µl) on a pre-coated silica gel 60 F_{254} plate. The plate was developed in mobile phase and scanned at 254 nm. Peak area was noted and concentration was determined by comparing the area of standard solution from the calibration curve.

Method validation

The HPTLC method was validated for various parameters. The range of concentration of the compound was determined for the linearity. The results were expressed in terms of correlation coefficient of the linear regression analysis. Intra-day precision was determined by analyzing the compound sample three times on the same day. Inter-day precision was determined by analyzing the compound sample daily for 5 days. Repeatability of measurement of peak area (RSD < 1% based on seven times measurement of same spot) and repeatability of sample application (RSD < 3% based on application of equal volume of seven spots) was performed using 40 µg/ml standard solution and 30 l of spotted volume. Same volume of standard solution was applied seven times and the plate was developed. Area was measured for the peaks. The accuracy of analytical method for estimation of the compound was determined by calculating the systemic error involved. Accuracy of the above method was ascertained by adding known concentration of compounds to the pre-quantified sample solution and then estimating the quantity of compound in each sample using the proposed method. Interference of other components present in the extract during analysis was studied to ascertain the specificity of the method. Limit of Detection was measured at a signal to noise ratio of 3:1 and Limit of Quantification was measured at a signal to noise ratio of 10:1. Minimum detectable concentration and minimum quantifiable concentration of the compound was ascertained during the HPTLC using different concentrations of test and standard solutions.

RESULTS

On TLC, the compound resolved at R_f 0.65 using the mobile phase toluene: methanol (9:1). Upon the analysis of melting point and spectral data, the compound was suspected to be a sterol and triterpenoid. This was confirmed when the compound gave Salkowski test and Liebermann–Burchard test positive. With the help of the data [Table 1] of melting point, UV λ_{max}, IR [Figure 1], NMR [Figure 2a and b], GC-MS [Figure 3a and b] and chemical tests, the structure of marker compound was elucidated and it was found to be Lupeol [Figure 4]. A total of 2.6 g Lupeol was isolated.

The HPTLC densitometric chromatogram [Figures 5 and 6] obtained by scanning at 254 nm showed super-

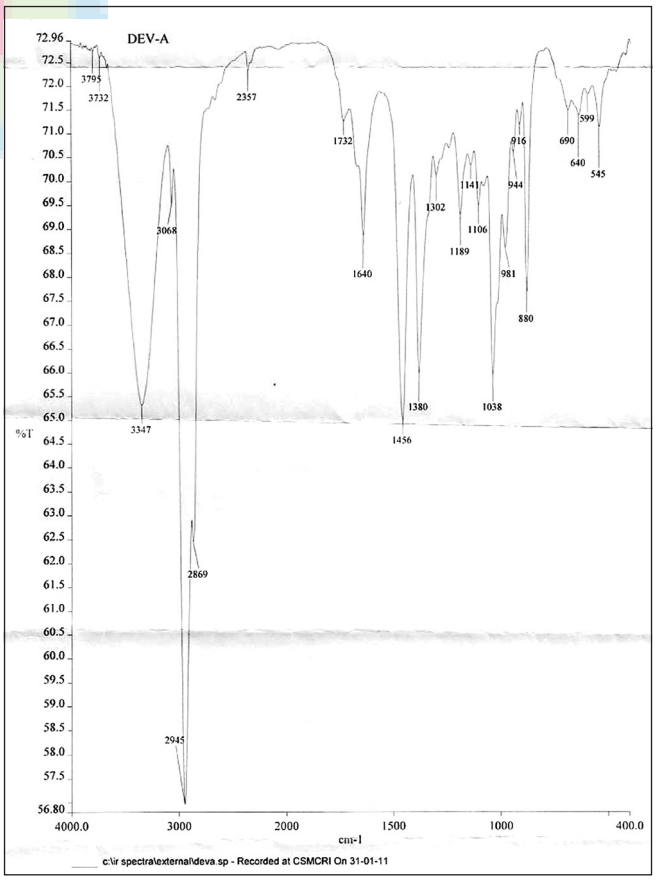

Figure 1: IR spectra of compound

Table 1: Data of melting point, UV λ_{max}, IR, NMR and GC-MS of the compound

Parameter	Experimental data
Melting point	215–219°C
UV (λ_{max})	235 nm and 270 nm in chloroform
IR peaks (cm^{-1})	3347, 3068, 2945, 2869, 1640, 1456, 1380, 1189, 1106, 1038, 880, 690, 640, 599, 545
NMR peaks	δ 4.69 and δ 4.57 (each 1H, m, 29), δ 3.18 (1H, tdd, 3), δ 2.39 and δ 1.92 (each 1H, m, 19), δ 1.68 (1H, t, 15), δ 1.66 (3H, s, 30), δ 1.60 (1H, d, 2), δ 1.59 (1H, q, 2), δ 1.42 (1H, d, 16), δ 1.39 (1H, q, 6), δ 1.36 (1H, t, 18), δ 1.33 (1H, m, 21), δ 1.20 (1H, m, 22), δ 1.03 (1H, q, 12), δ 0.99 (3H, s, 23), δ 0.97 (3H, s, 27), and δ 0.83, δ 0.79 (3H, s, 25, 28, 24)
GC-MS peaks (*m/z*)	M+ peak: 426 Base peak: 95 Other fragments: 411, 383, 370, 358, 315, 247, 218, 207, 189, 175, 161, 147, 135, 121, 109, 95, 81, 69, 55, 41

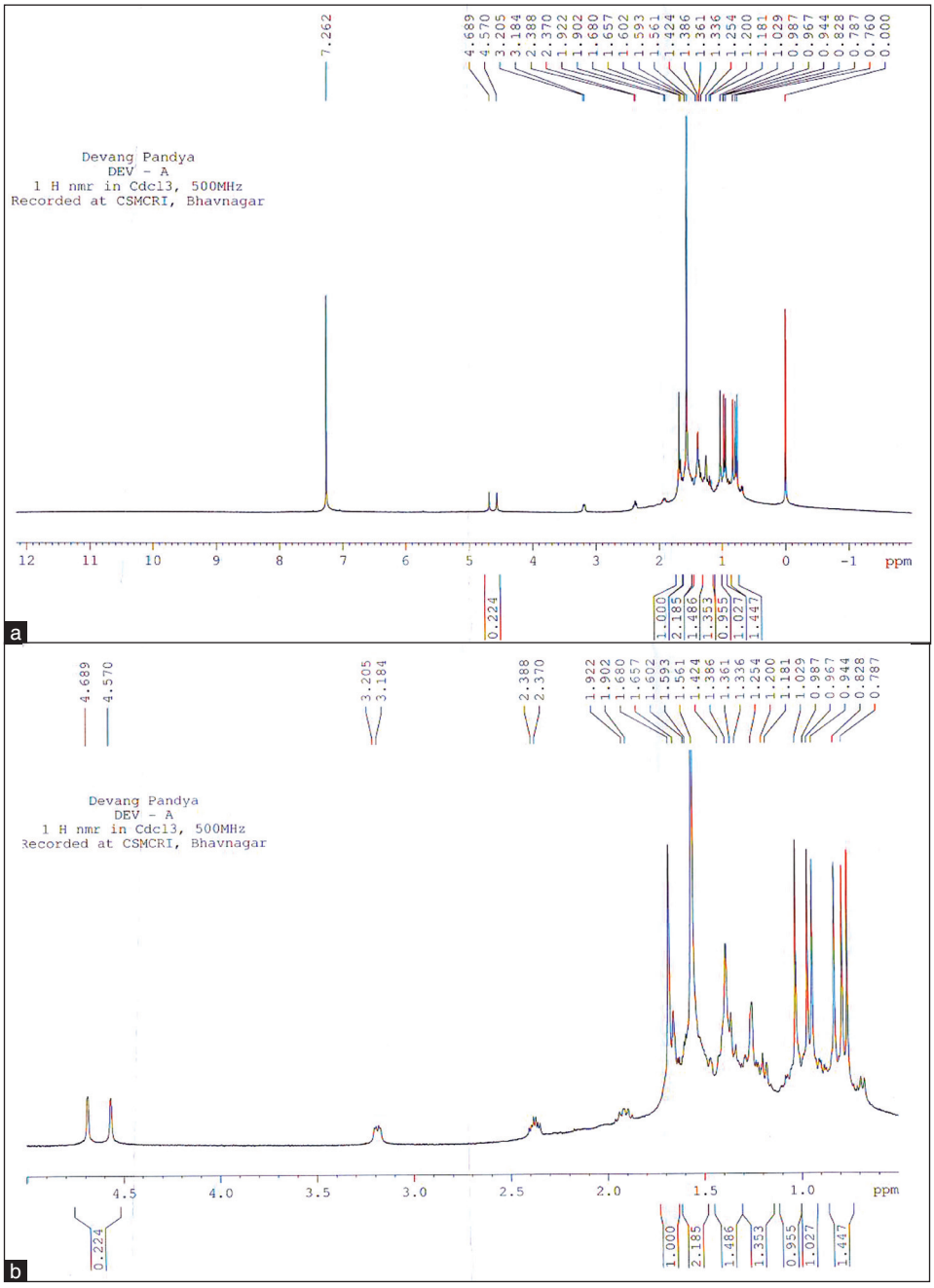

Figure 2: (a, b) NMR spectra of compound

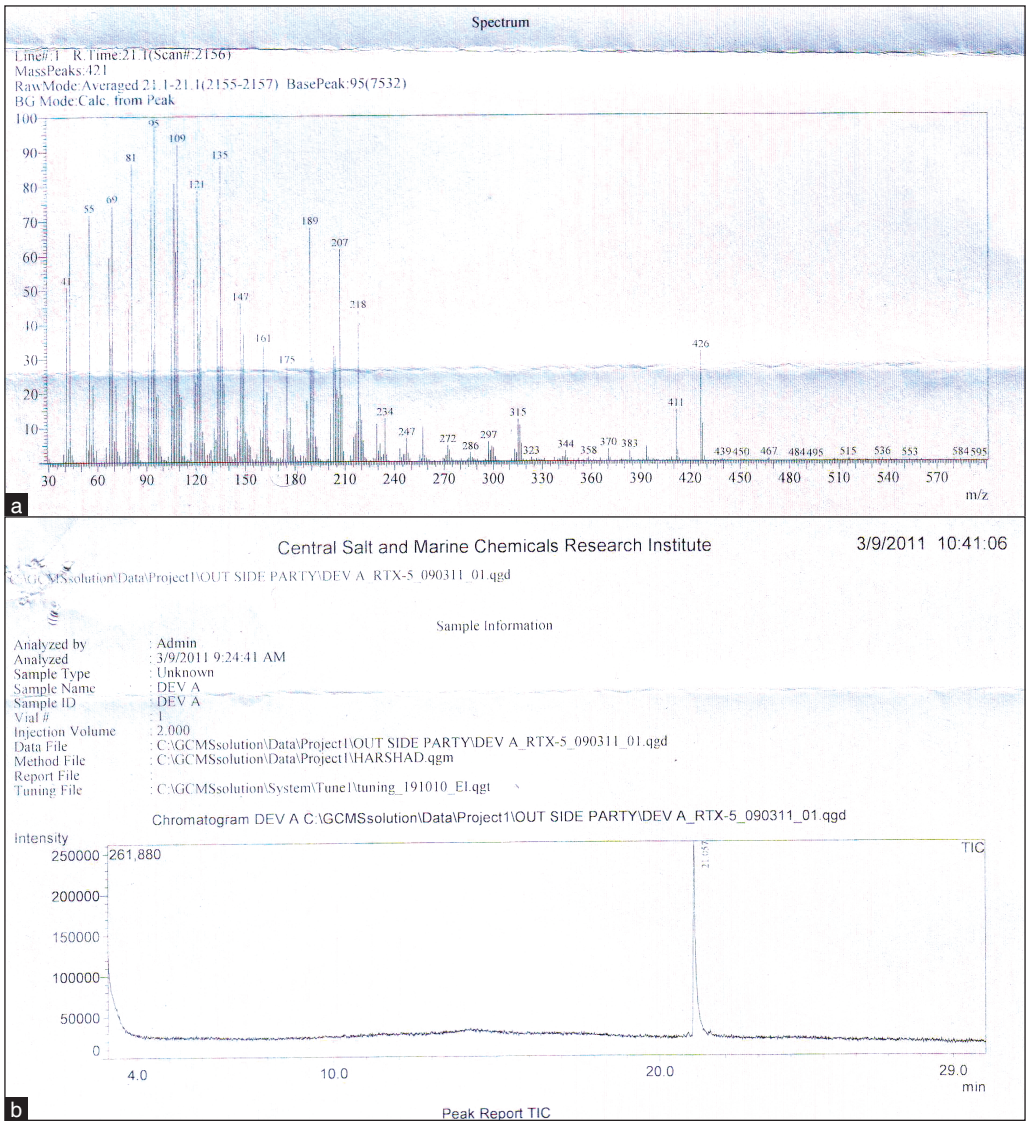

Figure 3: (a, b) GC-MS spectra of compound

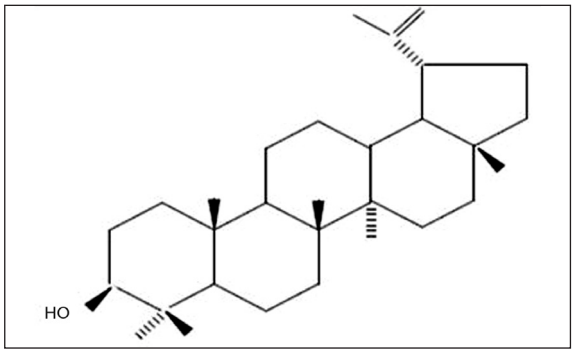

Figure 4: Lupeol

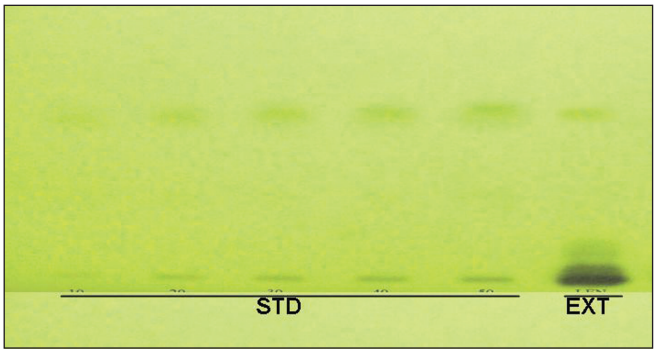

Figure 5: HPTLC chromatogram of Lupeol

imposable peaks of the standard concentrations of Lupeol at R_f 0.65. A calibration curve of Lupeol was obtained by plotting the peak area of Lupeol against the concentration of Lupeol. Correlation coefficient of this curve was found to be 0.9995, slope 3.968, *y*-intercept 1167.5 and mean peak area range 1776.2 ± 33.12 to 4356.5 ± 51.14. Lupeol was estimated to be 0.829 ± 0.09% w/w [Table 2]. The inter-day and intra-day coefficients of variation for Lupeol varied from 1.90 to 3.07% and from 1.65 to 3.14%, respectively

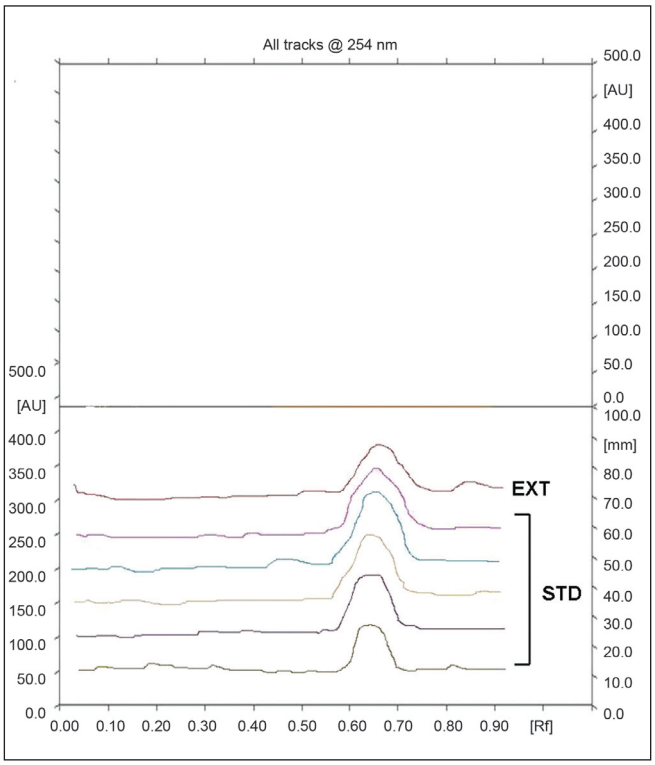

Figure 6: Densitometric chromatogram

Table 2: Estimation of Lupeol

Mean peak area (n = 5)	Avg. amount of Lupeol (µg/spot)	Avg. % w/w of Lupeol ± SD	% CV
2011.2	20	0.829 ± 0.09	2.5

CV = coefficient of variance; SD = standard deviation

[Table 3]. The coefficient of variance (%CV) for repeatability of measurement was found to be 0.312 and for repeatability of sample application was found to be 0.298. The % recovery of Lupeol was found to be from 99.11 to 99.44%, which was satisfactory [Table 4]. It was observed that the other constituents present did not interfere with the peak of Lupeol. Therefore, the method was specific. The Limit of Detection was found to be 150 ng/spot, whereas the Limit of Quantification was found to be 190 ng/spot.

DISCUSSION

The compound, a white crystalline solid, resolved at R_f 0.65 upon TLC of chloroform fraction of the petroleum ether extract using the mobile phase toluene: methanol (9:1). It also gave Salkowski test and Liebermann–Burchard test positive. The IR spectrum of this compound showed the presence of a hydroxyl function (OH) which showed intense bands in the spectrum at 3347 and 1189 cm^{-1}. The olefinic moiety showed its presence in the spectrum at 1640 cm^{-1}. The out of plane C–H vibrations of the unsaturated part was observed at 880 cm^{-1}. The stretching and bending of methyl group was observed as an intense band at 2940 cm^{-1} and as a medium intensity band at 1456 cm^{-1}. The methylenic group showed its presence at 2869 cm^{-1}. The corresponding C–C vibrations were visible as a weak band at 1038 cm^{-1}. The presence of seven methyl singlets and two olefinic protons (δ 4.69 and δ 4.57, multiplet) in the H-NMR spectrum revealed that the compound

Table 3: Data for inter-day and intra-day precision for Lupeol

Concentration (µg/spot)	Inter-day precision (n = 5)		Intra-day precision (n = 3)	
	Amount found (µg)	% Recovered	Amount found (µg)	% Recovered
10	9.93	99.30	9.92	99.20
20	19.86	99.45	19.84	99.65
30	29.79	99.02	29.76	99.29
40	39.72	99.11	39.68	99.47
50	49.65	99.22	49.60	99.73

Table 4: Data of accuracy for Lupeol

Concentration of Lupeol (ng/spot)		Amount of Lupeol found Mean ± SD (n = 3)	% Recovery (n = 3)
Isolated Lupeol taken	Standard Lupeol added		
200	0	198.06 ± 0.59	99.11
200	50	247.33 ± 1.11	99.19
200	100	296.11 ± 1.23	99.26
200	150	346.19 ± 1.24	99.44
200	200	395.26 ± 1.71	99.39

SD = standard deviation

may be a pentacylic triterpenoid. The GC-MS spectra indicated the molecular weight of the compound to be 426. The physical, chemical and spectral data and the comparison of H-NMR chemical shifts with that of the reported data of similar type of compounds led to the conclusion that the compound is Lupeol.

The HPTLC method developed for estimation of Lupeol was found to be easy, simple, precise, efficient, accurate, reproducible, specific and sensitive. This study could serve as a suitable tool for phytochemical authentication of *O. esculentum*, isolation of new bioactive phytochemicals, preparation of natural or semi-synthetic herbal products and exploration of its clinical aspects.

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