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Research Article

ISOLATION AND CHARACTERIZATION OF LUPEOL FROM METHANOLIC EXTRACT OF LEAVES OF ANDROGRAPHIS ECHIOIDES

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A B S T R A C T

In the present study was to isolate and characterize the lupeol from the methanolic extract of leaves of *Andrographis echioides*. The isolation was done using column chromatography using gradient elution with different mobile phases. The isolated compound was subjected to spectral analysis. Structure elucidation was carried out on basis of spectral analysis. Purity was checked by high performance thin-layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) technique. The infra-red spectra showed specific absorption bands for lupeol viz. 3432.72 cm-1 (Hydrogen bonded OH Stretch), 2943.72 cm-1 and 2356.85 cm-1 (C-H Stretch in CH_2 and CH_3), 2104.73 cm-1 (C≡C Stretch), 1661.92 cm-1 (C=C Symmetric Stretch), 1562.63 (C=C Asymmetric stretch), 1416.23 cm-1 (C-H deformation in CH_2 and CH_3), 1035.38 cm-1 (C-O Stretch of secondary alcohol), 887.84 cm-1 (=C-H bending exocyclic CH_2). Structural elucidation of lupeol was done by spectrum analysis such as ^{13}C and ^{1}H depth nuclear magnetic resources. Mass spectra of lupeol showed a parent molecular ion [M^+] peak at m/z 426 which corresponds to molecular formula $C_{30}H_{50}O$. From the spectral characteristics, the isolated compound from the methanolic extract of leaves of *Andrographis echioides* was confirmed to be lupeol.

INTRODUCTION

Medicinal plants used for traditional medicine to treat chronic as well as infectious diseases (Sasidharan *et al*., 2011). According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Farnsworth *et al*., 1985). 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, anti-feedants, attractants and pheromones (Hanson, 2003). 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 (Gershenzon and Kreis, 1999). The terpenes are biosynthetically constructed from isoprene (2-methylbutadiene) units (Ruzicka, 1953). The C_5H_8 isoprenes polymerise and subsequently fix the number and position of the double bonds. The basic molecular formulae of terpenes are thus multiples of C_5H_8 (Gershenzon and Dudareva, 2007). Triterpenes comprise a large number of different types of compounds which may be divided into more important chemical structure families.

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The main groups of triterpenoids are represented by pentacyclic derivatives of lupeol (Patocka, 2003). The 3-O-acyl-derivatives of lupeol have anti-inflammatory properities and many of them are present in different medicinal plants, as are lupeol acetate and lupeol docosanoylate in *Willughbeia firma* (Subha dhirasakul, 2000).

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, anthelmintic, analgesic, antipyretic, hepato-protective activities and antioxidant effect. It contains plenty of phytochemical constituents such as flavonoids, flavones, steroids, tannins, carbohydrate, glycosides and alkaloids (Ankita and Handique, 2010; Shanker *et al*., 2008). The leaf juice of *Andrographis echiodies* 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 (Zulfkar *et al*., 2009). *Andrographis echioides* boiled with coconut oil is used to decrease the falling and graying of hair (Kanchana and Rubalakshmi, 2014). From the leaves extract of *Andrographis echioides* various chemical constituents were isolated dihydro echioidinin, skullcap avone 1 2'-methyl ether, echioidinin, echioidin, skullcap avone 1 and 2'-O-bD-glucopyranoside (Jayaprakasam and Gunasekara, 1999). Some of the other

chemical constituents present in the *Andrographis echioides* are more than 17 compounds such as borneol, cyclohexanol 2,4 dimethyl phenol, 3,4 altroson, ndeconoicacid, Squalene, vitamin E, Methoprene, 2-nonenlol Oxirane,octyl-, 2, 2-cyclopentene-1-undecanoic acid, ketone, 1,5-methylbicyclo [2.1.0] pent-5-ylmethyl and 2,5-cyclohexadiene-1,4- dione, 2, 5- dihydroxy-3-methyl -6- (1-methylethyl) bicycle heptan -3-one (Nirubama and Rubalakshmi, 2014). However, no single method was found in literature to our knowledge to detect lupeol in methanolic extract of leaves of *Andrographis echioides*. In the present study, first time we have isolated and characterized lupeol, a triterpenoids from the 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 (0.5ml p-anisaldehyde in 50ml glacial acetic acid and 1ml conc. sulfuric acid. Heat to 105°C until maximum visualization of spots) were of analytical grade and were procured from from E. Merck, India.

Collection of plant material

The leaves of *Andrographis echioides* were collected in the month of May from the mullipatti, 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 35-40ºC, after which it was grinded to a uniform powder of 40 mesh size. The methanol extracts were prepared by soaking 1.5kg each of the dried powder plant materials in 1.5 L of methanol using a soxhlet extractor continuously for 10 hr. The extracts were filtered through whatmann filter paper No. 42 (125mm) 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 -20ºC. The filtrate obtained was used as sample solution for the further HPTLC analysis (Deepti *et al*., 2015).

Isolation of Lupeol by column chromatography

The condensed methanol extract of leaves (986 g) of sample was subjected to column chromatography over TLC grade silica gel. Elution of the column first with n-hexane, increasing amount of ethyl acetate in n-hexane and finally with methanol yielded a number of fractions. The preparation of solvent systems used to obtain Lupeol (104 mg/786g) were n-hexane-ethyl acetate (30:70) from fraction 5. The compounds were detected on TLC plates by spraying with Libermann Burchard reagent and heated at 100°C for 10 minutes (Jain and Bari, 2010).

Purification of isolated compounds by HPTLC and High performance liquid chromatography

Preparative Thin-layer chromatography (TLC)

The isolated pure compound was dissolved in appropriate solvents. 5 µl of isolated compounds (lupeol) were applied to silica gel plates, Merck (Germany) 20×20 cm, 0.25 mm in thickness. Plates were developed using the solvent system n-Hexane: Ethyl acetate (80:20v\v) for lupeol. The separated zones were visualized with freshly prepared Libermann Burchard reagent and heated at 100°C for 10 minutes. Chromatograms were then examined under daylight within 10 minutes (Sarfaraj *et al*., 2014).

High-performance liquid chromatography (HPLC)

The analytical HPLC system (Shimadzu) was equipped with a diode array detector, a 20µl loop, 200 x 4.6 mm C18 column, methanol (HPLC grade, 0.2mm filtered) used as a mobile phase. The isolated Lupeol compounds were separated using a mobile phase of methanol: water (75:25 v/v) at a flow rate of 1.0 ml/min, column temperature 30 °C. Injection volume was 40 µl and detection was carried out at 346 nm (Suthar *et al*., 2001).

Structural elucidation study of isolated compound

Different spectroscopic methods including UV, FTIR, ^{1}H NMR, ^{13}C NMR and GC-MS were used to elucidate the structure of isolated compounds. The UV–visible spectrum of the isolated compounds in methanol was recorded using a Shimadzu 160A UV–visible spectrophotometer. The Fourier Transform Infrared (FTIR) spectra were recorded with a nominal resolution of 4 cm^{-1} and a wave number range from 400 to 4000 cm^{-1} using the KBr pellet technique. ^{1}H and ^{13}C NMR spectra were acquired on Bruker WP 200 SY and AM 200 SY instruments (^{1}H, 200.13 MHz; ^{13}C, 50.32 MHz) using TMS as internal standard and $CDCL_3$ as solvent. GC-MS analysis of the extract was performed using a Perkin-Elmer GC Clarus 500 system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with a Elite-I, fused silica capillary column (30mmX0.25mm 1D X 1 µMdf, composed of 100% Dimethyl poly siloxane) (Jain and Bari, 2010; Sarfaraj *et al*., 2014; Suthar *et al*., 2001).

RESULTS

Structural Elucidation of isolated compounds

The lupeol is white amorphous solid compound with melting point 213°C which corresponds to the molecular formulae $C_{30}H_{50}O$. The **UV** λ_{max} value of compound lupeol was 350 nm (fig 1).

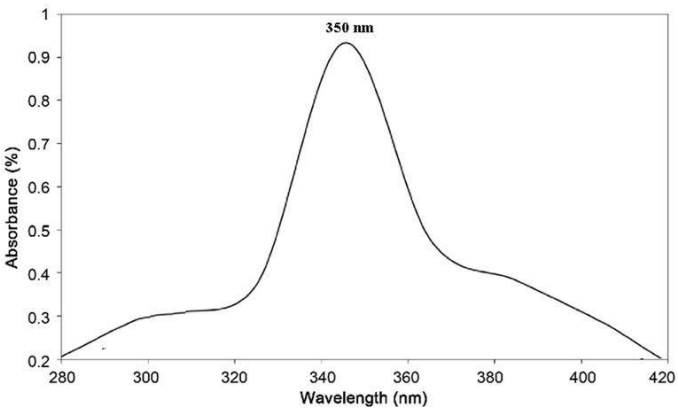

Fig 1 UV spectra of the isolated compound

In the IR spectrum of isolated compound (fig. 2) a very intensely broad peak at3432.72 cm-1 (Hydrogen bonded OH Stretch), 2943.72 cm-1 and 2356.85 cm-1 (C-H Stretch in CH_2 and CH_3), 2104.73 cm-1 (C≡C Stretch), 1661.92 cm-1 (C=C Symmetric Stretch), 1562.63 (C=C Asymmetric stretch), 1416.23 cm-1 (C-H deformation in CH_2 and CH_3), 1035.38 cm-1 (C-O Stretch of secondary alcohol), 887.84 cm-1 (=C-H bending exocyclic CH_2).

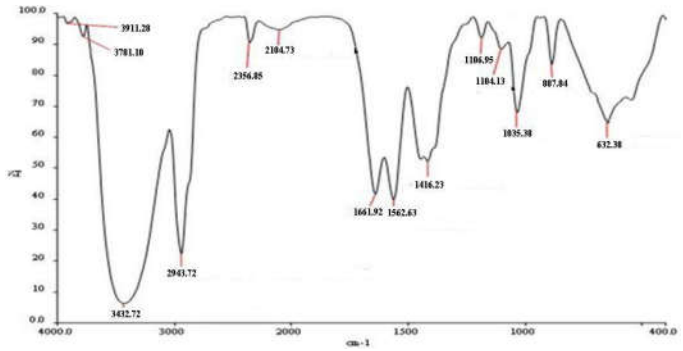

Fig 2 IR spectra of the isolated compound

In the proton ^{1}H NMR spectrum of lupeol (fig. 3) showed 7.19(CDCL3 peak), 4.62, 4.61, 4.5(H-29, d,d, 2H), 3.14-3.09 (H,3, d,d, 1H, 6 Hz, 5Hz), 2.33(H-19, m, 1H), 2.32 (H-21a, m, 1H), 2.26 (H-15A, t, 1H), 2.10 (H-30, s, 3H), 1.61 (H-12A, 1A, d, 2H), 1.58 (H-13, t, 1H), 1.57 (H-2A, d, 1H), 1.54 (H-2B, q, 1H), 1.53 (H-12A, q, 1H), 1.52 (H-23, s, 3H), 1.50 (H-15A, d, 1H), 1.49 (H-23,s, 3H), 1.46 (H-27, s, 3H), 1.45 (H-18, t, 6 Hz, 1H), 1.44 (H-28, s, 3H),1.43 (H-24, s, 3H), 1..34 (H-25, s, 3H), 1.31 (H-5, d, 1H).

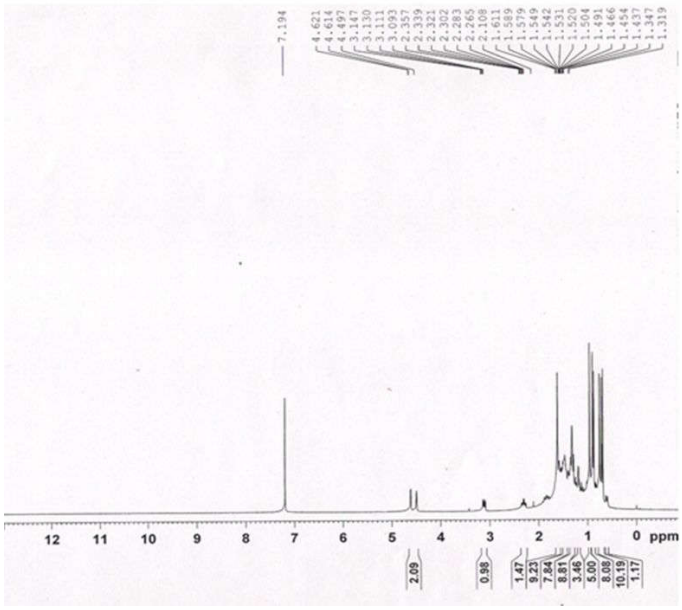

Fig 3 ^{1}H NMR spectra of the isolated compound

In the ^{13}C NMR spectrum of lupeol (fig. 4) showed δ_C: δ 37.17 (C-1), δ 20.93 (C-2), δ 79.02 (C-3), δ 38.05 (C-4), δ 55.2 (C-5), δ 18.31 (C-6), δ 27.99 (C-7), δ 38.87 (C-8), δ 50.43 (C-9), δ 34.29 (C-10), δ 19.31 (C-11), δ 20.93 (C-12), δ 35.56 (C-13), δ 40.01 (C-14), δ 25.1 (C-15), δ 29.83 (C-16), δ 40.86 (C-17), δ 48.28 (C-18), δ 48 (C-19), δ 151.13 (C-20), δ 27.96(C-21), δ 38.87 (C-22), δ 25.1 (C-23), δ 15.38 (C-24), δ 15.38 (C-25), δ 15.38 (C-26), δ 14.5 (C-27), δ 17.96 (C-28), δ 109.42 (C-29) and δ 18.31 (C-30).

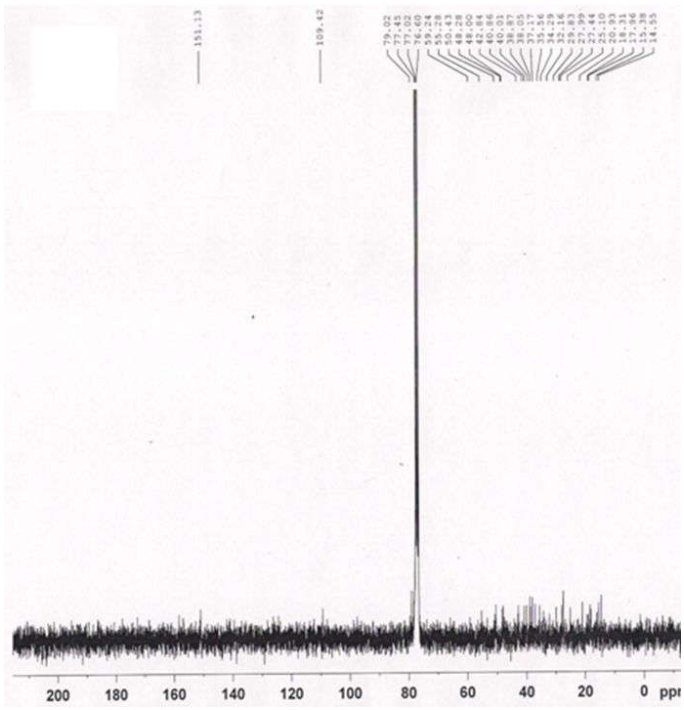

Fig 4 ^{13}C NMR spectra of the isolated compound

Mass spectrum of isolated compound lupeol showed parent molecular ion [M^+] peak at mlz 426 which corresponds to the molecular formula $C_{30}H_{50}O$ (fig 5). The GCMS spectra of these isolated compounds revealed the characteristic fragments m/z with % abundance 261.1(14), 213.3(15), 161.2(16), 55(100), 408.1(9), 382.2(9), 369.4(14), 281.2(8), 207.1(37), 191(50), 173.2(37), 161.2(41), 147.1(48), 134.4(64), 121.3(71), 95.2(78), 81.1(36), 69.1(68), 41.1(64). The molecular weight and fragmentation pattern indicate that the compounds presenting lupeol respectively.

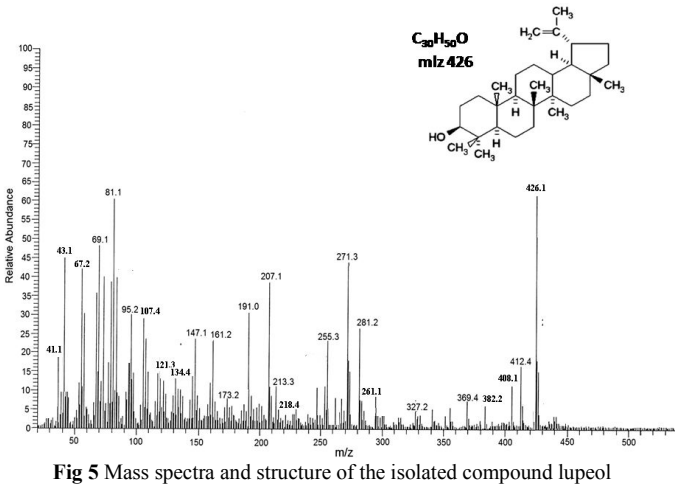

Fig 5 Mass spectra and structure of the isolated compound lupeol

Purification of isolated compound by HPTLC and HPLC

HPTLC fingerprint patterns have been therefore evolved to check the purity of isolated compound from methanolic extract of sample. The Rf value of standard lupeol 0.55 was matched with the Rf value of isolated compound was about 0.55 was shown in peak (fig 6).

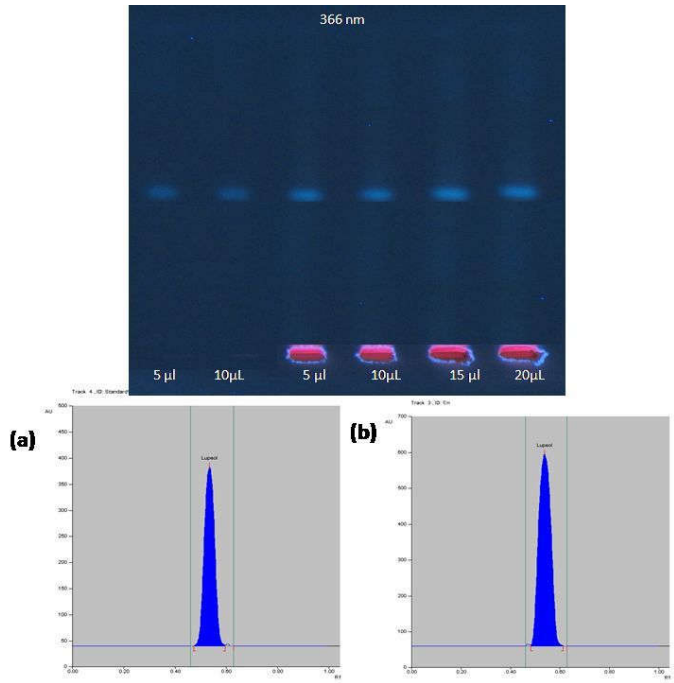

Fig 6 HPTLC chromatogram of purity of the isolated compound (a) standard lupeol (b) isolated lupeol in methanolic extracts of leaves of *Andrographis echioides*.

The Retension time of lupeol isolated from the methanolic extract of sample was about 3.750 was shown by HPLC peak (fig 7).

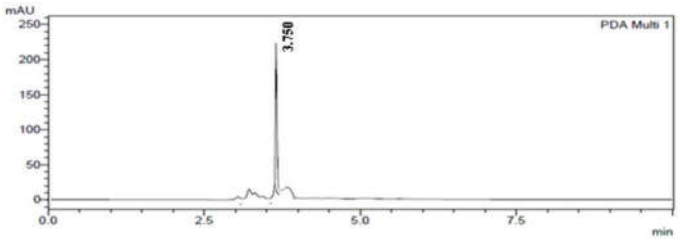

Fig 7 HPLC spectra of purity of the isolated compound

DISCUSSION

Pentacyclic triterpenes are based on a 30-carbon skeleton comprising five, six-membered rings (ursanes and lanostanes) or four, six-membered rings and one, five-membered ring (lupanes and hopanes). Pentacyclic triterpenes are produced by arrangement of squalene epoxide molecules (Sholichin *et al*., 1980). Lupeol, a pentacyclic triterpene was isolated and characterized for the first time, from the methanolic leaves extract of *Andrographis echioides* by chromatographic techniques. The spectral and physical data generated were found to be in accordance with the earlier literature data of lupeol (Saleem, 2009). Triterpenoid, lupeol (3ˇ-hydroxylup-20(29)-ene), is a bioactive compound present in different medicinal plants (Agarwal and Rangar, 2003). Lupeol has been reported to have potent pharmacological properties antiangiogenic, antioxidative and anti-inflammatory in nature (Sudhahar *et al*., 2008). It inhibits early responses of tumor growth induced by benzoyl peroxide (Saleem, 2008). It also plays very important role in normalization of lipid profile (Sudhahar *et al*., 2007), wound healing activity, protective effect in hypercholesterolemia associated with renal damage (Sudhahar *et al*., 2008) and suppression of immune factors (Bani *et al*., 2006; Vasconcelos *et a*l., 2008).

In IR spectrum, a very intensely broad and at 3432 and moderately band at 1106 and 632 were observed for the O-H bond vibrations of hydroxyl group. The out of plane C-H vibrations of the unsaturated part was observed at 887. The corresponding C=C vibrations was shown around 1661 as weakly intense band. The stretching and ending vibrations of methyl part were noticed by the intense band 2943 and medium intensity band at 1416. The vibration of the methylenic part was shown by the band at 2356 and the medium band at 1446. The moderately intense band at 632 was attributed to the rocking movement of methylenic part. The corresponding C-C vibration was shown as weak intense band at 1035. In ^{1}H NMR spectrum of lupeol, H-3 proton appeared as a triplet of a double doublet (tdd) at 3.14 (J=4.5 and 1.1 MHZ) and H-29 olefinic proton showed a multiplet at 4.62 and 4.61, respectively. Seven methyl protons also appeared at 1.46, 1.45, 1.44, 1.43, 1.34 and 1.31 (3H each, s, CH_3). Mass spectrum of isolated compound lupeol showed parent molecular ion [M^+] peak at mlz 426 which corresponds to the molecular formula $C_{30}H_{50}O$. These assignments are in good agreement for the structure of lupeol (Vasconcelos *et a*l., 2008; Imam *et al*., 2007; Fernández *et al*., 2001).

Previous study has reported that quantitative analysis of andrographolide in *Andrographis paniculata* herb samples by high-performance thin-layer chromatographic methods (Meenu and Sharma, 1994). The whole plant of *Andrographis echioides* contains more number of phyto-constituents (alkaloids, flavonoids, glycosides, phenols, phytosterols, proteins, saponins, tannins and triterpenoids, volatile-oils, aminoacid, cardiac glycosides, gums and phytosteroids) that are extracted using various solvents depending upon the polarity of these compounds (Padma *et al*., 2015; Ramasubramania, 2014). Lupeol, a triterpene compound has been isolated from *Crataeva nurvala* by HPTLC and also showed antioxaluric and anticalciuric effects in rats against hydroxyproline-induced hyperoxaluria (Suthar *et al*., 2001).The earlier investigators isolated lupeol from the methanol extract of stem bark of *Grewia titiaefolia* and evaluated the cytotoxic properties on *in vitro* cell lines (Badami *et al*., 2004). Recently, the isolation of andrographolide, 14-deoxyandrographolide, 14-deoxy-12-hydroxyandrographolide, β-sitosterol, stigmasterol and chlorophyll a, β-sitosterol, stigmasterol, 5,2'-dihydroxy-7,8-dimethoxyflavone, long chain transcinnamateesters and β-sitosteryl fatty acid esters, β-sitosterol, monogalactosyl diacylglycerols, lupeol, and triacylglycerols from the pods; and 14-deoxyandrographolide of *A. paniculata* (Maria Carmen *et al*., 2016).

The presented study clearly gave evidence of the bioactive quantitative of lupeol in methanolic extracts of leaves of *Andrographis echioides* for the first time. The developed HPTLC method for the quantification of above lupeol compounds is simple, precise, specific, sensitive, and accurate. Further, this method can be effectively used for routine quality control of herbal materials as well as formulations containing any or both of these compounds.

CONCLUSION

From the above physical, chemical and spectral evidences, the compound isolated from methanolic leaves extract of *Andrographis echioides* is confirmed as lupeol. This is the first ever report of these terpenoid compounds from this plant.

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Author Contribution

All authors 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.

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