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Estimation of Isolated Triterpenoid - Ursolic Acid in *Verbena officinalis* L. Aerial Parts Using TLC Densitometry

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Abstract: *Verbena officinalis* L. (Vervain; family-Verbenaceae),a traditionally used and medicinally promising plant, has been reported to contain triterpenoids as major class of phytoconstituents. But no work has ever been carried out on *V. officinalis* to isolate major chemical constituent(s), and to standardize plant material on the basis of isolated constituent(s) using TLC densitometry.The chloroform extract of *V. officinalis*aerial parts was prepared by extracting properly identified plant in a Soxhlet apparatus.Preliminary phytochemical screening of chloroform extract showed presence of triterpenoids. Column chromatography of chloroform extract using solvent systems viz., hexane:chloroform, chloroform and chloroform:methanol in different concentrationsyielded five fractions (F_1-F_5). Preliminary phytochemical screening of various fractions of chloroform extract revealed presence of triterpenoids in F_2 and F_3 fractions. Therefore, these fractions were further subjected to column chromatography. White colored crystals were obtained in SF_1 sub-fraction separated from $F_2$and was designated as VOC-1. Structure of VOC-1 was elucidated by IR and NMRspectral studies and was characterized as ursolic acid. Further, ursolic acid was quantified in *V. officinalis*aerial parts by validated TLC densitometric method. The content of ursolic acid was found to be 0.1580% in *V. officinalis* aerial parts.The linearity range, correlation coefficient, intra-

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day precision, inter-day precision, LOD, LOQ and accuracy were found to be 300-1800 ng, 0.997, 1.2% CV, 1.6% CV, 40 ng/spot, 130 ng/spot and 98.27% respectively.

Keyword: HPTLC, Ursolic acid, Verbenaceae, *Verbena officinalis*.

1. INTRODUCTION

Before any herbal medicine is screened for pharmacological testing, phytochemical investigations are essential because sometimes a critical constituent may be missing from the herb due to reasons like storage, geographical distribution or processing (Singh, 2002). The chemical constituents in component herbal products may vary depending on stage of collection, part of plant collected, harvest season, plant origin, drying process and other factors (Watchel-Galor and Benzie, 2011). Thus, it seems necessary to standardize herbal products on the basis of their main chemical constituents in order to ensure the reliability and repeatability of pharmacological and clinical research, and also to understand their bioactivities and possible side effects. Chemical assay gives the quantitative evaluation of active constituents by using different techniques like HPTLC and HPLC (Kumar *et al.*, 2008). Quality control of raw materials play vital role in establishing quality and stability of herbal preparations (Calixto, 2000). Thus, proper standardization and quality control of raw material and herbal preparation should be carried out. In case where active principles are unknown, marker substances should be established for analytical purposes.

Verbena officinalis L. (Vervain; Verbenaceae) has been traditionally used in the treatment of various ailments especially in nervous disorders such as anxiety, insomnia, migraine and depression (Burkhart, 2007; Haughton, 2012). But no systematic phytochemical and pharmacological work has ever been carried out on *V. officinalis* aerial parts with a view to isolate main constituent(s) from the plant. Thus, it was planned to isolate major phytoconstituent(s)from chloroform extract of *V. officinalis*using column chromatography, to characterize the isolated major constituent(s) using spectral techniques and tostandardize the plant on the basis of the isolated major chemical constituent(s) using validated TLC densitometric method.

2. MATERIALS AND METHODS

2.1 Plant material

The whole dried plant of *V. officinalis* (10 kg)was procured from Himalaya Herbs Store, Madhav Nagar, Saharanpur, (Uttar Pradesh), India in the month of

September, 2012. The identity of plant was confirmed through Dr H.B. Singh, Scientist F, Head of Raw Material Herbarium and Museum (RMHD), National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (Ref No. NISCAIR/RHMD/Consult/-2012-13/2069/77, dated 22/08/2012).

2.2 Solvents

Methanol (S.D. Fine Chemicals, Mumbai, India), chloroform (Loba Chemie Pvt. Ltd., Mumbai), ethyl acetate (E Merck, Delhi, India),dimethyl sulfoxide (E-Merck Ltd., Mumbai), hexane (Loba Chemie Pvt. Ltd., Mumbai), toluene (Loba Chemie Pvt. Ltd., Mumbai) and petroleum ether (60-80ºC) (RFCL Ltd., New Delhi, India), all of LR grade, were employed in present investigation.

2.3 Chemicals and instruments

Rotary vacuum evaporator (BUCHI, Switzerland) was used for recovery of solvent under reduced pressure. Silica gel (#100-200), sulphuric acid, calcium chloride fused (E-Merck Ltd., Mumbai), acetic anhydride (Sigma-Aldrich Chemicals Pvt. Ltd., Mumbai) and potassium bromide (S.D. Fine Chemicals, Mumbai) were used in present research work. The silica gel G used for thin layer chromatography was obtained from E. Merck (India) Ltd. The final TLC fingerprint profile was developed using precoated silica gel G plates of 0.2 mm thickness, aluminum base of E. Merck (India) Ltd. IR spectroscopy (PerkinElmer Spectrometer, USA),^{1}H-NMR and ^{13}C-NMR spectroscopy (Bruker Spectrometer, Singapore) of isolated compound was performedat Central Sophisticated Instrumentation Laboratory, Panjab University, Chandigarh.Quantitative analysis by TLC-densitometry was carried out using Camag Linomat 5, Camag TLC scanner 4, Wincats software and Camag UV chamber (254/366 nm) fitted with camera available in Sophisticated Instrument Laboratory (DBT supported), Punjabi University, Patiala, India. Hot air oven (Universal Instrument, India) was used for heating TLC plates sprayed with reagent.

2.4 Preparation of various extracts of *V. officinalis* aerial parts

Dried coarsely powdered *V. officinalis* aerial parts (2 kg) were successively Soxhlet extracted with solvents in order of increasing polarity *viz.*, petroleum ether (60-80ºC) and chloroform. Solvents from extracts were recovered using rotary vacuum evaporator, and dried extracts were preserved in a vacuumdesiccator containing fused calcium chloride.Petroleum ether and chloroform extracts of *V. officinalis* aerial parts were screened for the presence

of different classes of phytoconstituents using specific standard reagents (Farnsworth, 1966).

2.5 Isolation of constituents using column chromatography

The slurry of 280 g silica gel (#100-200) was prepared in hexane and packed in column (length 100 cm, internal diameter 3.5 cm). The column was initially run with hexane for 24 h to get a compact silica gel bed. The chloroform extract (14 g) of *V. officinalis* aerial parts was then loaded onto the column by wet packing method and eluted using hexane-chloroform, chloroform and chloroform-methanol in different concentrations as mobile phases. A total of 135 fractions, 250 ml each, were collected. These were pooled, based on similar thin layer chromatograms, to get 5 fractions (F_1-F_5).

The fraction F_2 (1.8 g) was loaded onto a column (length 50 cm, internal diameter 3 cm) packed with 100 g of silica gel (#100-200) as described above and eluted using solvents viz., hexane, hexane-chloroform, chloroform, chloroform-methanol in different concentrations as mobile phases. A total of 85 fractions, 250 ml each, were collected. These were pooled, based on similar thin layer chromatogram, to get 4 sub fractions (SF_1-SF_4). The fraction F_3 (3.5 g) was also loaded onto a column (length 50 cm, internal diameter 3 cm) packed with slurry 100 g ofsilica gel (#100-200) and eluted using solvents viz., hexane, hexane-chloroform, chloroform, chloroform-methanol in different concentrations as mobile phase. A total of 105 fractions, 250 ml each, were collected. These were pooled, based on similar thin layer chromatograms, to get 4 sub fractions (SUF_1-SUF_4). Thin layer chromatograms were obtained on pre-coated silica gel plates using solvent system chloroform:methanol (9:1) and visualized in UV chamber (254/366 nm).The structure of isolated compound was elucidated by IR and NMR spectral data. The structure was further confirmed by comparingits spectral data with the available literature.

2.6 TLC densitometric studies

2.6.1 Test solutions

The coarsely powered plant material, 5 g of aerial parts, was extracted by refluxing on water bath thrice with chloroform (3 ×50 ml) for 2 h each time. The extract was filtered, concentrated under reduced pressure and the volume was adjusted to 50 ml with chloroform. Similarly, the methanol extract of plant and its test dilutions were prepared.

2.6.2 Standard solution of marker

A stock solution of 0.3 mg/ml concentration of the marker was prepared in chloroform for TLC studies.

2.6.3 TLC fingerprint profile

A fixed volume, 10 µl, of the extract and marker was applied on pre-coated TLC plate using Camag Linomat 5. The plate was developed using solvent system toulene:ethyl acetate (7:3) in a chamber, saturated for 10 min, to a distance of 8 cm. The plate was dried, and visualized after spraying with Liebermann Burchard reagent followed by heating at 105ºC for 2 min in hot air oven.

2.6.4 Analytical studies

2.6.4.1 Preparation of standard plot

A standard solution of VOC-1 was prepared by dissolving accurately weighed 3 mg of VOC-1 in 10 ml of chloroform. The stock solution was diluted with chloroform to get six dilutions of different concentrations (300 ng, 600 ng, 900 ng, 1200 ng, 1500 ng, 1800 ng). A volume of 10 µl from each dilution was applied in triplicate on pre-coated TLC plate. The plate was developed in solvent system toulene:ethyl acetate (7:3) in a chamber saturated for 10 min, to a distance of 8 cm. The developed plate was dried in a current of hot airand then scanned after derivatization in TLC scanner at 366 nm. The AUC of the peak corresponding to VOC-1 was noted in each track.

2.6.4.2 Preparation of test sample

Test samples of *V. officinalis* aerial parts (Chloroform and methanol extracts) were prepared as described above. These test solutions (10 µl) were applied separately in triplicate on pre-coated TLC plate (20 × 10 cm). The plate was developed and scanned following the same procedure as used for the preparation of standard plot. The average AUC of the peak corresponding to VOC-1 was noted at 366 nm in the test sample, and its concentration was calculated from the standard plot.

2.6.5 Method validation studies

2.6.5.1 Instrument precision

Instrumental precision was checked by repeated scanning (n=7) of the same spot of VOC-1 (600 ng/spot) and expressed as percent coefficient of variance (% CV).

2.6.5.2 Repeatability

The repeatability of the method was affirmed by analyzing 600 ng/spot of VOC-1 individually on a TLC plate (n=5) and expressed as % CV.

2.6.5.3 Intra-day and Inter-day variation

Variability of the method was studied by analyzing aliquots of standard solution containing 600 ng/spot of VOC-1 on same day (Intra-day precision) and on different days (Inter-day precision).

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

LOD and LOQ were determined by applying different concentrations of standard solutions of VOC-1 along with chloroform as blank and determined on the basis of signal-to-noise ratio. LOD was determined at S/N of 3:1 and LOQ at S/N of 10:1.

2.6.5.5 Recovery studies

The accuracy of the method was assessed by performing recovery studies at three different levels (50, 100 and 150% addition of VOC-1). The percentage recovery and average percent were calculated.

2.6.5.6 Specificity

This was ascertained by analyzing the standard compound and sample. The band for VOC-1 from sample solutions was confirmed by comparing the R_f and spectra of the bands to those of the standard. The peak purity of VOC-1 was analyzed by comparing the spectra at three different levels, i.e., start, middle and end positions of the bands.

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

Aerial parts of *V. officinalis* were defatted by extracting with petroleum ether (60-80ºC) in Soxhlet apparatus. The marc of plant was then extracted with chloroform in a Soxhlet apparatus. The chloroform extract of plant was subjected to standard phytochemical screening procedures in order to ascertain various classes of phytoconstituents present therein. The results of phytochemical screening showed presence of triterpenoids.As triterpenoids are the major class of phytoconstituents present in chloroform extract, thus, it was planned to isolatetriterpenoids from chloroform extractusing column chromatography. The percentage yields (w/w) of petroleum ether (60-80ºC) and chloroform were found to be 1.15 and 1.59% respectively.

3.2 Column chromatography of chloroform extract of *V. officinalis* aerial parts

The column chromatography of chloroform extract of *V. officinalis* aerial parts using hexane-chloroform, chloroform, chloroform-methanol as mobile phases

in different concentrations yielded five fractions (F_1-F_5).Results of column chromatography of chloroform extract are shown in table 1. Preliminary phytochemical screening of all fractions showed presence of triterpenoids in fractions F_2 and F_3. Further, column chromatography of F_2 and F_3 fractions each yielded 4 sub-fractions ($SF_{1-}SF_4$) and (SUF_1-SUF_4), respectively,as

Table 1: Column chromatography of chloroform extract of *V. officinalis* aerial parts.

Fraction	Eluent	Yield (g)
F_1	Hexane : Chloroform (1 : 1) Hexane : Chloroform (2 : 3)	2 g
F_2	Hexane : Chloroform (3 : 7) Hexane : Chloroform (1 : 4) Hexane : Chloroform (1 : 9) Chloroform	1.8 g
F_3	Chloroform : Methanol (49 : 1) Chloroform : Methanol (19 : 1)	3.5 g
F_4	Chloroform : Methanol (93 : 7)	1 g
F_5	Chloroform : Methanol (23 : 2) Chloroform : Methanol (9 : 1) Chloroform : Methanol (17 : 3)	1.8 g

Table 2: Column chromatography of F_2 fraction separated from chloroform extract of *V. officinalis* aerial parts.

Sub-fraction	Eluent	Yield (g)
SF_1	Hexane : Chloroform (4 : 1) Hexane : Chloroform (7 : 3)	0.57 g
SF_2	Hexane : Chloroform (3 : 2)	0.02 g
SF_3	Hexane : Chloroform (1 : 1) Hexane : Chloroform (2 : 3) Hexane : Chloroform (3 : 7) Hexane : Chloroform (1 : 4) Hexane : Chloroform (1 : 9) Chloroform Chloroform : Methanol (49 : 1)	0.14 g
SF_4	Chloroform : Methanol (19 : 1) Chloroform : Methanol (23 : 2)	0.28 g

Table 3: Column chromatography of F_3 fraction separated from chloroform extract of *V. officinalis* aerial parts.

Sub-fraction	Eluent	Yield (g)
SUF_1	Hexane : Chloroform (4 : 1) Hexane : Chloroform (7 : 3) Hexane : Chloroform (3 : 2) Hexane : Chloroform (1 : 1) Hexane : Chloroform (2 : 3)	0.015 g
SUF_2	Hexane : Chloroform (3 : 7) Hexane : Chloroform (1 : 4) Hexane : Chloroform (1 : 9) Chloroform	0.58 g
SUF_3	Chloroform : Methanol (49 : 1)	0.36 g
SUF_4	Chloroform : Methanol (19 : 1) Chloroform : Methanol (23 : 2) Chloroform : Methanol (17 : 3) Chloroform : Methanol (3 : 1)	0.87 g

shown in table 2 and 3. A compound in the form of white colored crystals was separated out from SF_1 (0.57 g). The compound was designated as VOC-1. An another compound (pale yellowish crystals) was separated out from $SUF_{1,}$ but the yield of the compound (VOC-2) was too less (15 mg) to subject for structure elucidation studies.

3.3 Characterization of VOC-1

The structure of VOC-1 was elucidated by analyzing IR and NMR spectral data. Specific peaks for particular functional groups present in the compound obtained in IR spectra are presented as below:

IR cm^{-1} (KBr disk):Absorption bands assigned to C-O stretching (1030.45), olefinic system (1453.23), carbonyl system (1685), methyl group (2920.20) and hydroxyl group (3364).

The number and position of protons and carbons in VOC-1 were deduced based on specific peaks observed in ^{1}H NMR and ^{13}C NMR spectra, respectively, as given below:

^{1}H NMR (400MHz, DMSO): δ_H = 0.6265 (3H, s, CH_3, H-27), 0.6993 (3H, s, CH_3, H-25), 0.7555 (3H, d, *J*=6.36 Hz, $CH_{3,}$ H-30), 0.8169 (3H, s, CH_3, H-26), 0.8461 (3H, d, *J*=6.84 Hz, CH_3, H-29), 0.8632 (3H, s, CH_3, H-23),

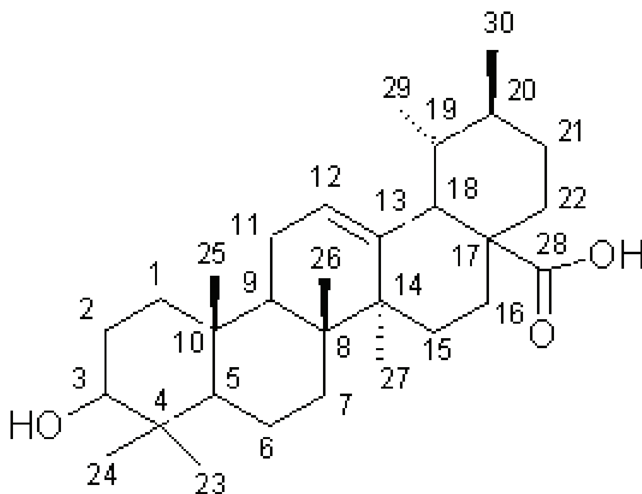

Figure 1: Structure of VOC-1.

0.9873 (3H, s, CH_3, H-24), 1.1772-1.8663 (22H, m, H-1, 2, 5, 6, 7, 9, 11, 15, 16, 19, 20, 21, 22), 2.0550 (1H, d, *J*=10.96 Hz, H-18), 2.9590 (1H, m, H-3), 5.0769 (IH, m, olefinic proton, H-12).

^{13}C NMR(400MHz, DMSO): δ_C =38.22 (C-1), 26.92 (C-2), 76.89 (C-3), 38.32 (C-4), 54.76 (C-5), 17.93 (C-6), 32.65 (C-7), 39.06 (C-8), 46.18 (C-9), 36.47 (C-10), 22.79 (C-11), 124.58 (C-12), 138.05 (C-13), 41.58 (C-14), 28.14 (C-15), 23.76 (C-16), 46.79 (C-17), 52.31 (C-18), 38.47 (C-19), 38.83 (C-20), 30.22 (C-21), 36.28 (C-22), 27.49 (C-23),15.16 (C-24), 15.89 (C-25), 16.82 (C-26), 23.19 (C-27), 178.34 (C-28), 16.89 (C-29), 21.00 (C-30).

VOC-1 was characterized as ursolic acid based on IR and NMR spectral data, and the structure was further confirmed by comparing with spectral data of ursolic acid given in literature (Figure 1).

Ursolic acid, a natural pentacyclic triterpenoid carboxylic acid, is major component of some traditional medicinal herbs and is well known to possess a wide range of pharmacological activities such as anti-inflammatory (Ikeda *et al*., 2008), antioxidant (Liobikas *et al*., 2011), antitumor (Novotny *et al*., 2001; Muthu *et al*., 2013), apoptotic activity (Kwon *et al*., 2010), antidepressant (Machado *et al*., 2012), antidiabetic (Alqahtani *et al*., 2013), hepatoprotective (Rajendrasozhan *et al*., 2006), antibacterial (Fontanay *et al*., 2008), antiviral activities (Kong *et al*., 2013). It is suggested that ursolic acid is one of the bioactive constituents of *V. officinalis*, which may be responsible for most of pharmacological activities of the plant. Therefore, it is selected as a marker to standardize *V. officinalis.*

3.4 Standardizationof *V. officinalis* using TLCdensitometric method

High Performance Thin Layer Chromatography is a sophisticated instrumental technique with merits of easy method development and validation, automation, scanning, full optimization, selective detection principle, minimum sample preparation, etc., enable it to be a powerful analytical tool for quantitatively determination of particular compound(s) in complex mixtures of inorganic, organic and biomolecules (Srivastava, 2011).

The content of ursolic acid was determined in *V. officinalis* aerial parts using developed TLC densitometric method. Eight solvent systems were tried to develop TLC method for resolving components of chloroform and methanol extracts but the best resolution of components was obtained in solvent system toulene:ethyl acetate (7:3). List of solvent systems employed to resolve components of chloroform and methanol extracts is shown in table 4. Figure 2 shows comparative TLC fingerprint profile of ursolic acid, chloroform and methanol extracts of *V. officinalis* after derivatization with Liebermann Burchard reagent followed by heating. A standard plot was prepared between different concentrations of marker and their peak areas after scanning at 366 nm (Figure 3). The estimation of ursolic acid was done from the regression equation of standard plot. The content of ursolic acid in *V. officinalis* aerial parts using chloroform and methanol extract was found to be 0.158 and 0.0005% respectively (Table 5).From these observations, it

Table 4: Mobile phases employed for TLC of chloroform and methanol extracts of *V. officinalis.*

Plant part	Solvent system	Composition
Aerial parts	Toluene : Ethyl acetate : Glacial acetic acid	7 : 2 : 1 8 : 1 : 1
	Hexane : Chloroform	5 : 5 7 : 3 3 : 7
	Hexane : Ethyl acetate	5 : 5
	Hexane : Chloroform : Methanol	4.5 : 4.5 : 1
	*Toluene : Ethyl acetate	7 : 3

*Best resolution

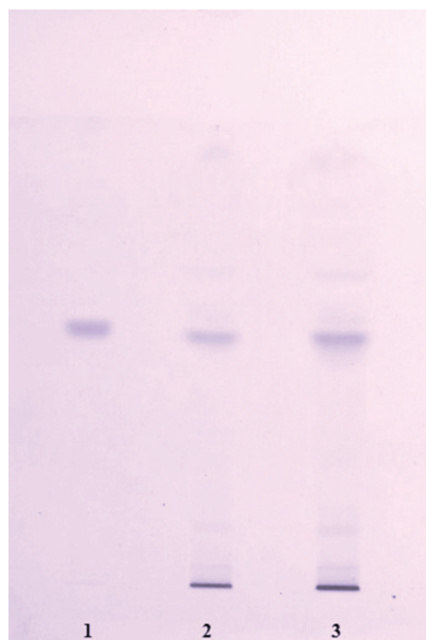

Figure 2: Comparative TLC fingerprint profile of ursolic acid, chloroform and methanolextractsof *V. officinalis* after derivatization with Liebermann Burchard reagent followed by heating. 1: Ursolic acid; 2: Chloroform extract; 3: Methanol extract.

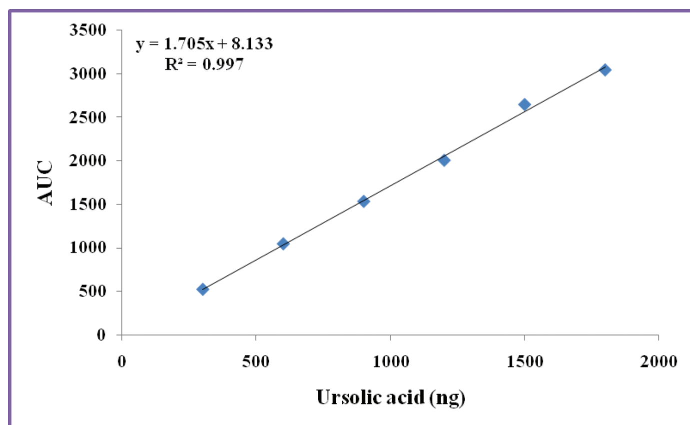

Figure 3: Standard plot of ursolic acid in TLC densitometric analysis.

Table 5: Percentage yield of ursolic acid in *V. officinalis* aerial parts.

Plant	Plant part	Extract	Percentage yield (w/w) (Mean^n ± S.D.)
V. officinalis	Aerial parts	Chloroform	0.1580 ± 0.0031
		Methanol	0.0005 ± 0.0000

n=3

is clearly evident that chloroform is solvent of choice for extracting ursolic acid from plant material as the content of ursolic acid in chloroform extract was found to be 316 times higher than the methanol extract of *V. officinalis* aerial parts.

3.5 Method validation studies

The developed TLC densitometric method was validated for linearity, sensitivity, precision, accuracy and specificity for marker. The linearity, range, limit of detection, limit of quantification, inter day precision, intra-day precision and accuracy were observed to be 0.997, 300-1800 ng, 40 ng, 130 ng, 1.6%, 1.2% and 98.27% respectively (Tables 6 and 7). Inter and intra-day precision was well within the acceptable range (% CV), it indicated that proposed method for marker was precise and reproducible. The recovery studies were performed to check accuracy of the method at three levels of spiking, i.e., 50, 100 and 150%

Table 6: Method validation parameters in TLC-densitometric analysis of ursolic acid in *V. officinalis*aerial parts.

Sr. No.	Parameter	Ursolic acid
1	Instrumental precision (% CV, n=7)	0.8
2	Repeatability (% CV, n=5)	1.1
3	Linearity (coefficient of correlation)	0.997
4	Range (ng)	300-1800
5	LOD (ng)	40
6	LOQ(ng)	130
7	Intra-day precision (% CV, n=9)	1.2
8	Inter-day precision (% CV, n-=9)	1.6
9	Accuracy (average % recovery)	98.27
10	Specificity	Specific

Table 7: Recovery studies of ursolic acid.

Compound	Initial amount		Amount detected (mg)	Per cent recovery	Average per cent recovery
	In percent	In recovery			
Ursolic acid	0.158	0.079 (50%)	0.233	98.31	98.27
		0.158 (100%)	0.309	97.78	
		0.237 (150%)	0.390	98.73	

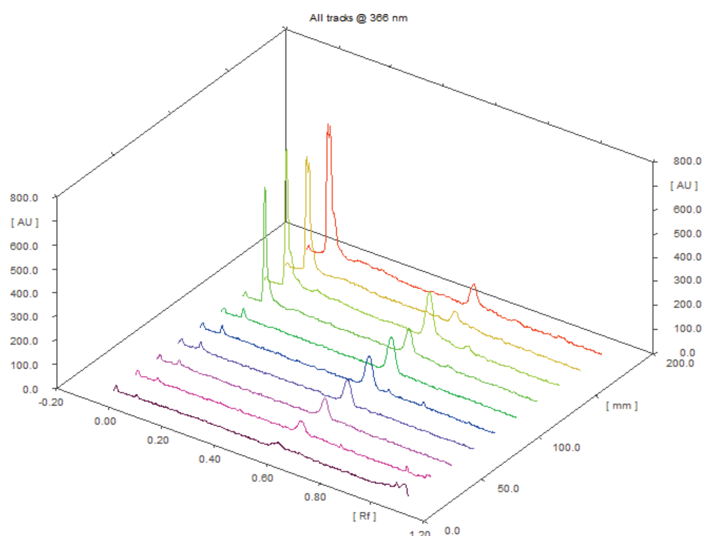

Figure 4: TLC densitometric chromatogram of ursolic acid, chloroform and methanol extracts of *V. officinalis* aerial parts.

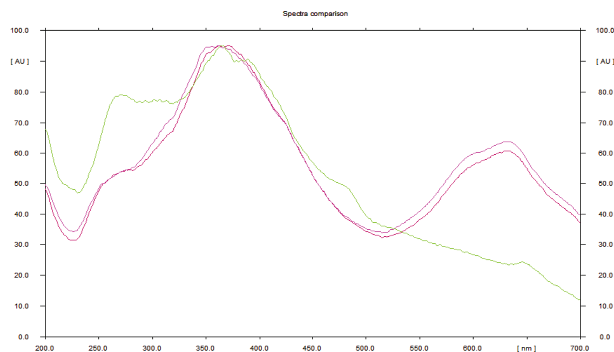

Figure 5: Spectra overlay of ursolic acid with corresponding peak in chloroform and methanol extracts of *V. officinalis* aerial parts.

for the marker. The results indicated that method was accurate as all spiked contents were extracted and quantified with average recovery of 98.27% w/w for VOC-1. It is clearly evident from spectra and TLC chromatogram overlay that there is no interference in quantitative analysis (Figures 4 and 5), thus, confirming specificity of the developed TLC densitometric method.

4. CONCLUSION

It is finally concluded that ursolic acid is one of the major constituent of *V. officinalis*, and may be responsible for most of pharmacological activities of the plant. Therefore, detailed pharmacological research is further needed on ursolic acid to validate traditional claims of *V. officinalis*.

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