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HPLC Method Development and Characterization of Bio-Active Molecule Isolated from *Andrographis paniculata*

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Abstract: The main objective of the investigation was to optimize a suitable extraction method and to isolate and characterize the compound obtained from the leaves of *Andrographis paniculata* (Burm. F.) Nees (Acanthaceae). Preliminary phytochemical screening of the various extracts of the leaves revealed the presence of flavones and terpenoids in them. The active compound was isolated and identified by its melting point (227°C) and TLC analysis. R_f values of test sample was found to be 0.64 and that of standard was 0.65 in solvent system chloroform : methanol (7:1). The maximum absorbance (λ_{max}) of the active compound was found to be 224 nm. From the FTIR graph the compound was found to possess an asymmetric –CH stretch of $–CH_3$ at 2977.89, an asymmetric –CH at 2848.78, an asymmetric $=CH_2$ stretch at 2927, a free OH group attached to the lactone ring demonstrating a peak at 3398.34, a C=C bond of the diterpene ring demonstrating a peak at 1456.16, a O-C=O group in the lactone ring at 1356.12, when compared with C-O of the diterpine ring (1306.00). By HPLC analysis a sharp peak was obtained with the retention time of 2.3 minutes. Significant (92.8%) accuracy was found, when the results were compared with the reference standard. The Relative Standard Deviation (R.S.D.) value of the active compound was found to be 0.1202% with 5 replications. The calibration curve resulted in a linear plot with regression coefficient of 0.999. These findings gave an idea about the structure of the isolated compound, which will be subjected to further analysis for its biological activities.
Key words: *Andrographis paniculata*, Isolation, Method development.

Introduction

Andrographis paniculata (Burm. F.) Nees (family, Acanthaceae) grows widely in many Asian countries, such as China, India, Thailand and Sri Lanka and has a long history of therapeutic usage in Indian and Oriental medicine.[1,2] The herb is official in Indian Pharmacopoeia[3] as a predominant constituent of at least 26 Ayurvedic formulations used to treat liver disorders. It is one of the herbs, which can be used to treat neoplasm as mentioned in ancient Ayurvedic literature.[4] *Andrographis paniculata* is reported as a cold property herb in Traditional Chinese medicine (TCM) and is used to get rid of body heat and to expel toxins. The plant is particularly known for its extremely bitter properties (often called King of Bitters) and is used traditionally as a remedy against common cold, dysentery, fever, tonsillitis, diarrhoea, liver diseases, inflammation, herpes and so on.[5,6] The traditional uses and pharmacological aspects of *A.*

paniculata have been well-documented in an extensive review recently.[7] A number of active principles are reported from the plant, which mainly include diterpenoid lactones, flavonoids and polyphenols.[8,9] However, the prime constituent andrographolide has been mainly attributed for its therapeutic properties. The diterpenoid lactone andrographolide, the principle compound found in *A. paniculata*, is mainly concentrated in leaves and can be isolated from the crude plant extracts as crystalline solid.[10,11] The structure of the compound has been elucidated by X-Ray crystallographic analysis and the molecular stereochemistry; bond distances and bond angles were determined.[12]

Experimental

Isolation of andrograhpolide from *Andrographis paniculata*: Leaves of *Andrographis paniculata* Ness (Acanthaceae) was collected from Kolkata in July 2008. The dried leaves (300 g) were macerated in methanol and kept at room temperature (25±2°C) for 3 days. After filtration, the solution was evaporated under reduced pressure to give the methanol extract (20.5 g). The extract was partitioned between ethyl acetate and water to give a water soluble portion and ethyl acetate soluble portion. The latter portion was extracted with n-butanol to give the n–butanol soluble portion. Filtration of the precipitate formed in the n-butanol soluble portion gave crude andrographolide (1.96 g). The part of crude andrographolide (780 mg) was chromatographed on silica gel column (50 g) using chloroform : methanol (20:10) as the solvent system to yield pure andrographolide.

Physico-chemical properties of the drug:
Melting point of the drug: The melting point of the drug, determined by melting point apparatus, was found to be between 227-228°C, which matches with the value (231°C) of pure andrographolide.[13]

Determination of moisture content by Karl-Fischer method: The moisture content of the isolated drug was found to be 0.112% by Karl–Fischer method.

Determination of λ_{max}: The drug was scanned in Shimadzu, UV-1700 Pharmaspec UV-vis-Spectro-photometer (with methanol as blank). The λ_{max} was found to be 224 nm which matches with standard value of andrographolide.

Determination of the active compound by TLC:
TLC is carried out by using solvent system (chloroform : methanol = 7:1). The TLC runs were made in the laboratory conditions of 25±5°C and 50% relative humidity. The R_f value of the compound was 0.65, the R_f value of the standard being 0.66.

FTIR study of andrographolide:
IR transmission spectra were obtained using a FTIR spectrophotometer (FTIR-84005, Shimadzu, Japan). 5% sample w/w was mixed with dry KBr and KBr disc was prepared. Each KBr disc was scanned at 4 mm/s at a resolution of 2 cm at wave number region of 400-4500 cm^{-1}. The characteristic peaks were recorded using the FTIR spectrophotometer.

HPLC method development:
Materials and methods: All solvents were of HPLC grade and all reagents were of analytical reagent grade. Standard andrographolide was procured from Emami Limiled, Kolkata. Water used for chromotographic separation was purified using millipore millipurified system.

HPLC instrument: Shimadzu Model-SPD-20A Col No.228-4500-38, Serial No. L-20-1344036675AE 220-230 (240V-50-60 Hz A HPLC instrument was used for the chromatographic separation using C 18 column (250 nm x 4.6 nm). Isocratic elution was carried out with methanol at a flow rate 1 ml/min. The detection was performed with a D2 lamp at 230 nm wavelength. Class VP software was used for integration and calibration. Evaluation was via peak areas with linear regression.

HPLC study of standard and test andrographolide:
Reference solution: 30 mg andrographolide reference standard (RS) was dissolved in 50 ml of ethanol (stock solution-1). From stock solution-1, 5 ml solution was taken and diluted up to 25 ml to get concentration of 0.12 mg/ml. 20 µl of the standard solution was injected in the HPLC instrument and HPLC chromatogram was obtained.

Test andrographolide: The stock solution of test andrographolide was prepared by dissolving 30 mg andrographolide in 50 ml of ethanol. The sample was sonicated for 5 minutes to ensure complete dissolution and allowed to equilibrate at room temperature. From this, 5 ml solution was taken and was diluted up to 25 ml with methanol. 20 µl of the above sample was injected in 5 replicates. Relative Standard Deviations (RSD values) were calculated.
Similarly 0.168 mg/ml, 0.216 mg/ml, 0.264 mg/ml solutions were prepared for making a calibration curve of andrographolide.

Table 1: Determination of moisture content of andrographolide by Karl –Fischer method

Formulation Code	Wt. of drug taken	% Moisture content
Sample andrographolide	2gm	0.112

Table 2: Calculation of RSD value of andrographolide with 5 replications

Experiment No.	Concentration (mg/ml)	Peak Area (mV.s)
1	0.120	2605.0276
2	0.120	2611.127
3	0.120	2613.331
4	0.120	2608.391
5	0.120	2610.491
Average of the 5 peak areas = 2609.6696 mV.s		

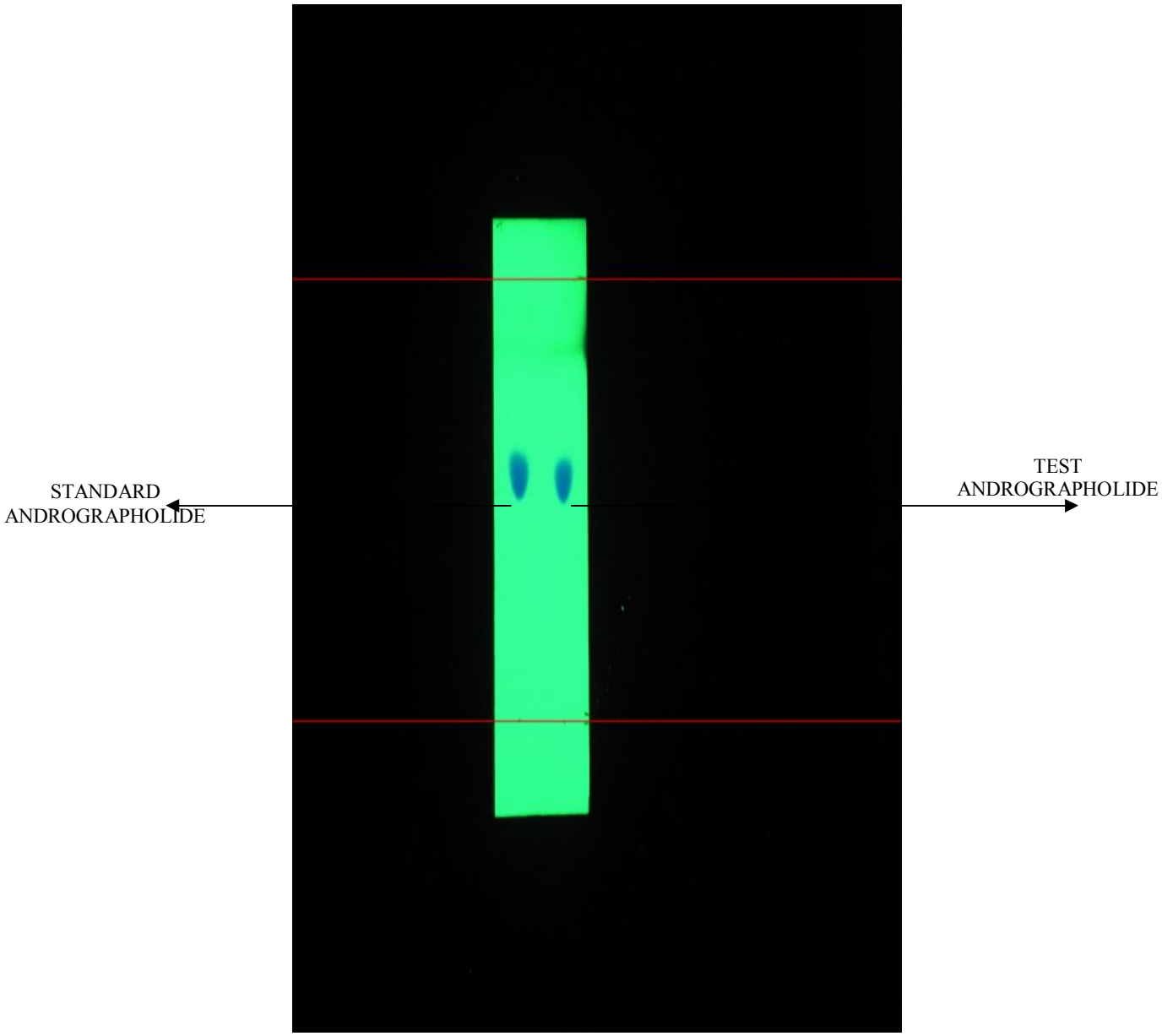


Fig. 1: TLC chromatogram of standard and test andrographolide

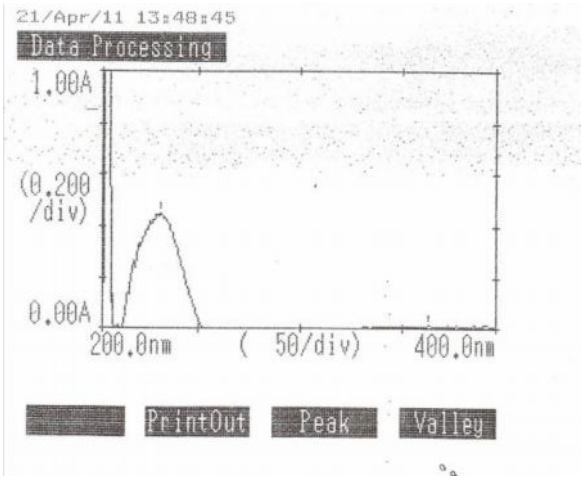


Fig. 2: Determination of λ_{max} for andrographolide

Table 3: Linear regression data of andrographolide by HPLC method

Conc. of the drug (µg/ml)	Area of the peak (mV.s)
0	0
0.120	2401.174
0.168	3173.254
0.216	4181.174
0.264	4988.955

Y = 18936 x+40.09

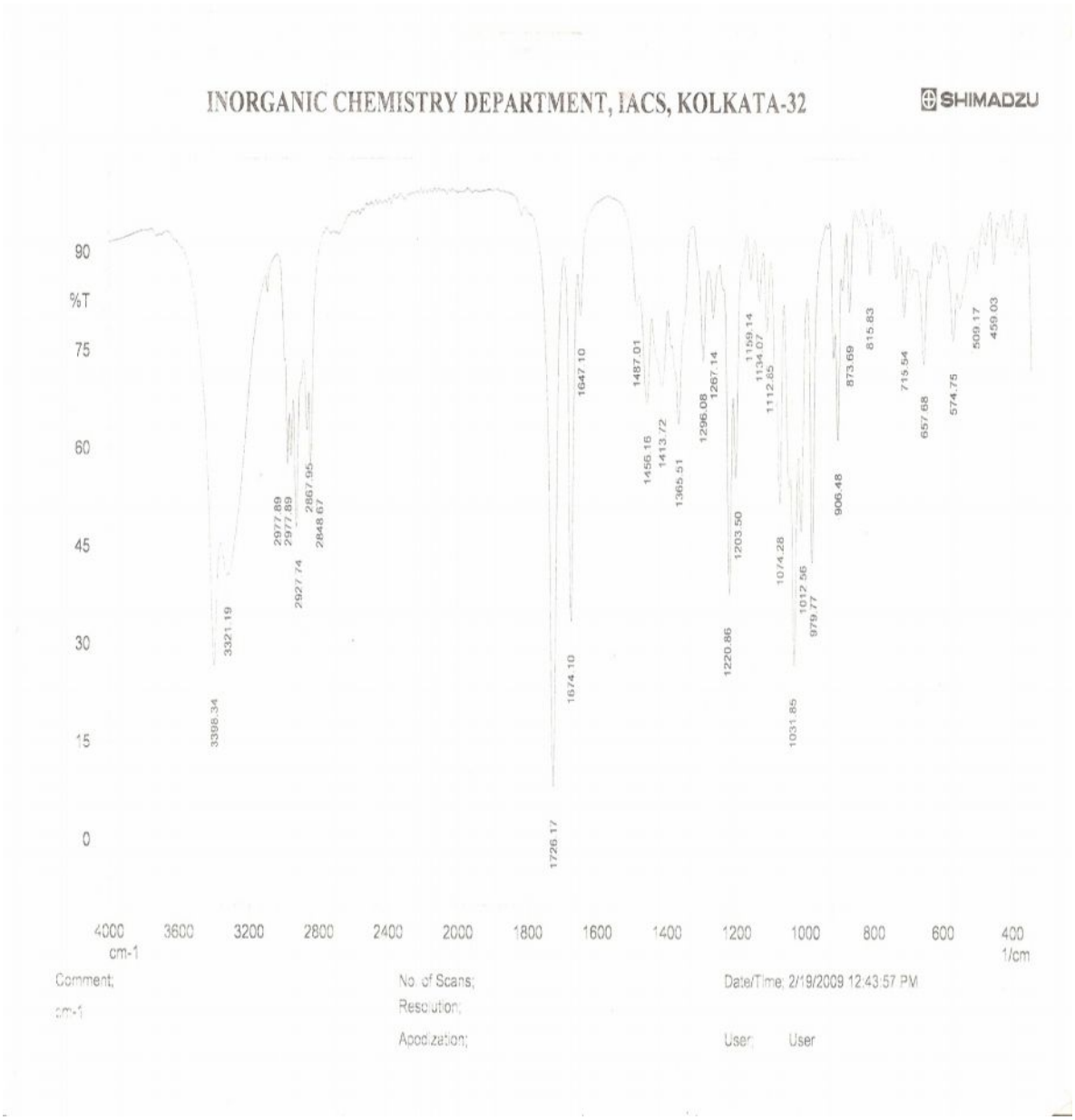


Fig. 3: FTIR spectrum of isolated andrographolide

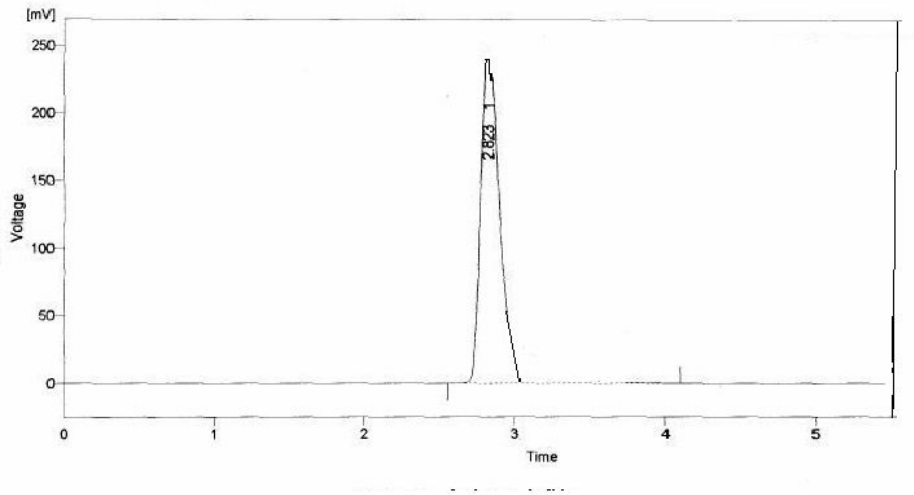
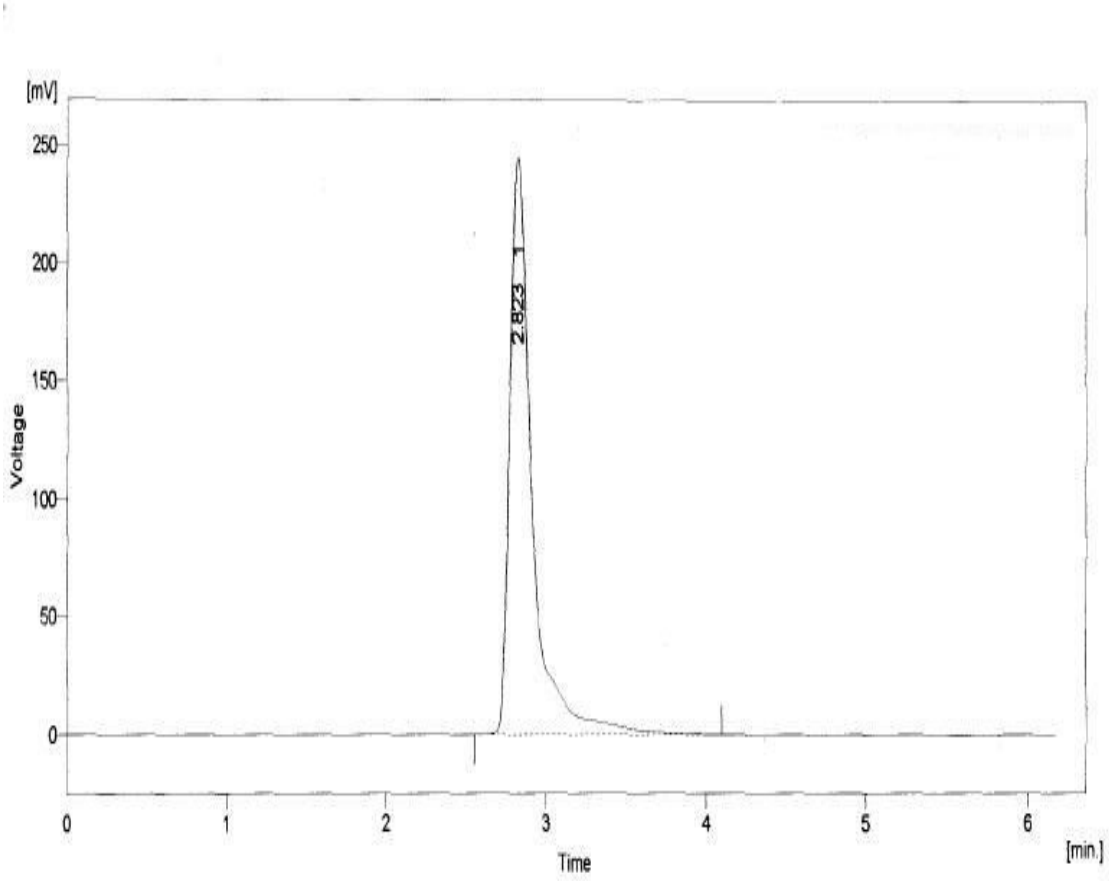


Fig. 4: HPLC chromatogram of reference andrographolide



Result Table **Andrographolide**

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.823	2401.174	244.815	100.0	100.0	0.13
	Total	2401.174	244.815	100.0	100.0	

Fig. 5: HPLC chromatograms of test andrographolide

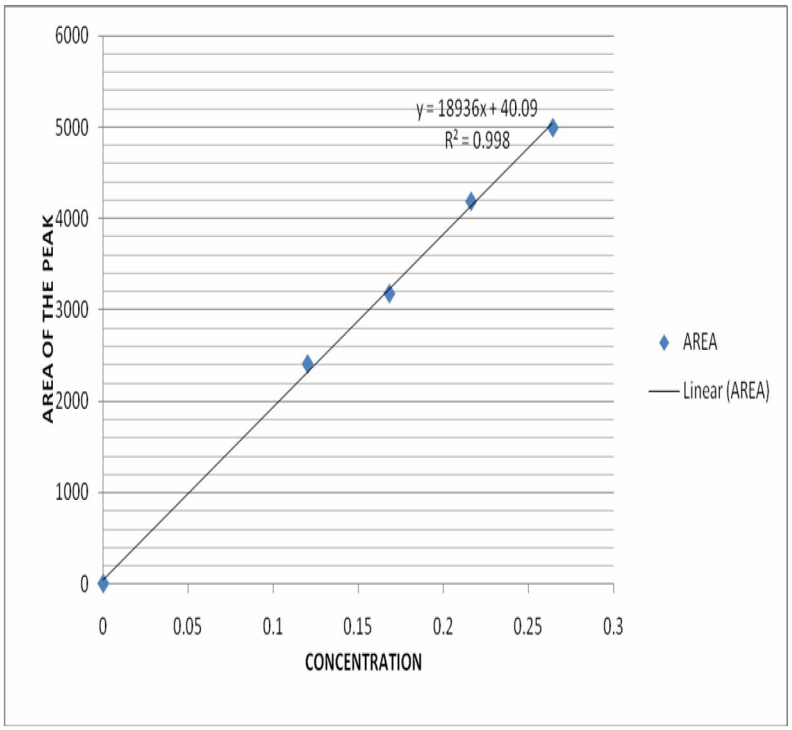


Fig. 6: Standard curve of andrographolide by HPLC

Results and Discussion

From the present study a novel compound andrographolide was isolated and the various parameters determined were melting point (227˚C), R_f value of 6.5 (single compound), as determined by TLC at 25±5°C and 50% relative humidity using solvent system chloroform : methanol (7:1) (Fig. 1) and moisture content 0.112% (Table 1). λmax was found at 224 nm (Fig. 2). FTIR data coincided with that of the standard sample. From the FTIR graph of andrographolide sample the asymmetric –CH stretch of –CH3 was noted at 2977.89. The symmetric –CH was noted at 2848.78, asymmetric =CH2 stretch showed peak at 2927, free OH attached to the lactone ring demonstrated a peak at 3398.34, C=C of the diterpene ring demonstrated a peak at 1456.16. O-C=O in the lactone ring demonstrated a peak at 1356.12, *i.e.*, at a higher frequency compared to C-O of the diterpine ring (peak value 1306.00) (Fig. 3). HPLC study of the isolated compound using methanol as a solvent showed RSD value of 0.102% with 5 replications which was less than 2% (Table 2) that was highly satisfactory. The HPLC peaks of the standard and the isolated compounds were compared and obtained a significant value of 92.8% (Fig. 4 and 5). The peak purity of the sample was found to be 50%. A linear plot was obtained from the calibration curve (Table 3 and Fig. 6).

Conclusion

Thus from the present investigation it can be concluded that the isolated compound andrographolide is a pure compound obtained from leaves of *Andrographis panaculata.* This isolated novel phytoconstituent can be further studied for its various biological activities like anti HIV, anticancer, immunomodulation and so on.

The HPLC method has been found to be simple and fast with less trial and error experimentations. The method shows good accuracy and provides a linear curve. This method has been found to be suitable for quantification of andrographolide.

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