ISOLATION AND HPTLC ESTIMATION OF KAEMPFEROL FROM *OXYSTELMA ESCULENTUM*

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ABSTRACT

Objectives: *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 diuretic, laxative and anti-ulcer. The present work deals with the isolation and structure elucidation of Kaempferol from *O. esculentum* and HPTLC method development for its estimation. **Methods**: The petroleum ether extract of the entire plant of *O. esculentum* was subjected to further fractionation followed by pilot TLC experiments and column chromatography. **Results**: This yielded a pure, yellow, crystalline solid which resolved at R_f 0.70 upon TLC of methanol fraction of the petroleum ether extract using the mobile phase toluene: methanol (3.5:6.5). This compound was subjected to UV, IR and ^{1}H-NMR spectral analysis and its structure elucidation revealed it to be Kaempferol. A novel HPTLC method for the estimation of Kaempferol from *O. esculentum* was developed, which was found to be 0.879 ± 0.05%w/w. Conclusion: 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, HPTLC, Kaempferol, *Oxystelma esculentum*

1. 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] It has been studied for its pharmacognostic characteristics.[3] However, negligible phytochemical investigations of this plant have been carried out so far. This work deals with the isolation of Kaempferol from *O. esculentum* and 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.

2. MATERIALS AND METHODS

2.1 Collection and Authentication

Oxystelma 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, RK 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.

2.2 Extraction and Fractionation

The extraction process followed the evaluation of pharmacological potential of various extracts of *O. esculentum,* which revealed that the petroleum ether extract has the best pharmacological activity.[4, 5] Thus, extraction of 1kg 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 dried petroleum ether extract was further fractionated first between n-hexane and water, then carbon tetrachloride and water, then toluene and water, then diethyl ether and water, then dichloromethane and water, then n-butanol and water, then chloroform and water, then ethyl acetate and water and finally between methanol and water.

2.3 Chromatography

Each of the above organic solvent fractions of the petroleum ether extract were subjected to a series of pilot 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, 1g dried methanol fraction of the petroleum ether extract was subjected to column chromatography and loaded on a glass column (60 x 3cm) packed with silica gel G (40g, 60-120#, Spectrochem Pvt. Ltd., Mumbai, India) as the stationary phase. Gradient elution was performed using toluene: methanol (10:0 to 0:10) as the mobile phase. Total 200 fractions were collected in test tubes. Upon evaporation of the mobile phase from the test tubes, pure, yellow crystals of a compound were obtained in test tubes of toluene: methanol (3.5:6.5) fraction. A single spot resolved at R_f 0.70 using the mobile phase toluene: methanol (3.5:6.5).

2.4 Spectral Analysis and Structure Elucidation

This compound was subjected to spectral analysis: UV (Labtronic, RKCP), IR (KBr; CSMCRI, Bhavnagar) and ^{1}H-NMR ($CDCl_3$; CSMCRI, Bhavnagar). The UV λ_{max} was observed directly from the instrument. Upon the analysis of melting point and spectral data, the compound was suspected to be a flavonol. This was confirmed when the compound gave Shinoda test positive. The structure of the compound was elucidated on the basis of the spectra.

2.5 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 100µl HPTLC syringe, Camag twin trough chambers (20 x 10cm), Camag TLC Scanner 3, Camag CATS 4

Integration software and Camag Reprostar-3.

Stationary phase used was precoated silica gel 60 F_{254} plate (E. Merck, Germany, methanol washed, thickness 0.2mm, 20 x 20cm) and the mobile phase used was toluene: methanol (3.5:6.5).

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

The spotting volume for calibration curve was 3-15µl and for methanol fraction of Pet. ether extract was 40µl. The amount sprayed for standard curve was 120-600ng. The mobile phase used was toluene: methanol (3.5:6.5).

Densitometric scanning was carried out in Absorbance/Reflectance mode at 254nm using Mercury Lamp and Slit dimension 4 x 3mm.

For calibration curve, accurately weighed 4mg of the isolated standard compound was dissolved in methanol and the volume was adjusted up to 10ml with methanol in a volumetric flask (0.4mg/ml). From this 1ml was diluted up to 10 ml with methanol in a volumetric flask to give a final concentration of the standard solution (40µg/ml). Graded concentration of standard solution (40µg/ml) in 3, 6, 9, 12 and 15µl volume were applied on a pre-coated TLC silica gel 60 F_{254} plate (E. Merck, Germany) using Camag Linomat IV automatic spotter. The concentration of the compound was 120, 240, 360, 480 and 600ng/spot. The plate was developed in a mobile phase, toluene: methanol (3.5:6.5). Data of peak area of each the compound spot was recorded. The calibration curve was obtained by plotting area V/s concentration of each peak corresponding to the respective spot.

50mg methanol fraction of petroleum ether extract was dissolved in methanol and volume was adjusted to 5ml using methanol in a volumetric flask to get 10mg/ml concentration. 200µl of this test sample of methanol fraction of petroleum ether extract was spotted along with standard solution of the compound (3-15µl) on pre-coated silica gel 60 F_{254} plate. The plate was developed in mobile phase and scanned at 254nm. Peak area was noted and concentration was determined by comparing the area of standard solution from calibration curve.

2.6 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 40µg/ml standard solution and 30µl 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 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. Different amount of minimum concentrations of standard solution were used and Limit of Quantification was measured at a signal to noise ratio of 10:1.

3. RESULTS

On TLC, the compound resolved at R_f 0.70 using the mobile phase toluene: methanol (3.5:6.5) as a blue spot upon spraying anisaldehyde sulfuric acid and heating for 10min. It gave a yellowish-blue fluorescence in long-wave UV light.

Upon the analysis of melting point and spectral data, the compound was suspected to be a flavonol. This was confirmed when the compound gave Shinoda test positive. With the help of the data (Table 1) of melting point, UV λ_{max}, IR (Fig. 1) and NMR (Fig. 2) and chemical tests, the structure of marker the compound was elucidated and it was found to be Kaempferol (Fig. 3).

The HPTLC densitometric chromatogram (Fig. 5, 6) obtained by scanning at 254nm shows super-imposable peaks of the standard concentrations of Kaempferol at R_f 0.70. A calibration curve (Fig. 4) was obtained by plotting the peak area against the concentration of Kaempferol. Correlation coefficient was found to be 0.9997 (Table 2). Kaempferol was estimated to be 0.879 ± 0.05%w/w (Table 3). The inter-day and intra-day coefficient of variation for Kaempferol varied from 1.34 to 2.91% and 1.45 to 3.05% respectively (Table 4). Relative Standard Deviation for repeatability of measurement of peak area based on 7 times measurement of the same spot was found to be 0.255 (Table 5). Relative Standard Deviation for repeatability of sample application of peak area on 7 times measurement of the same spot was found to be 0.282 (Table 6). The % recovery of Kaempferol was found to be 99.25 to 99.56%, which was satisfactory (Table 7). It was observed that the other constituents present did not interfere with the peak of Kaempferol. Therefore, the method was specific. The minimum detectable limit (Limit of Detection) was found to be 150ng/spot whereas the minimum quantifiable limit (Limit of Quantification) was found to be 200ng/spot.

4. DISCUSSION

In its IR spectrum, a very intense broad band at 3317cm^{-1} and a moderately intense band at 1177cm^{-1} indicated O-H vibrations of the hydroxyl group. The out of plane C-H vibrations of the unsaturated part was observed at 819cm^{-1}. Aromatic C-H stretching is proven by absorption at 2951cm^{-1}. IR band at 1660cm^{-1} indicates a C=O linkage of a phenolic ring, whereas absorption at 1008cm^{-1} indicates a C-O linkage in a heterocyclic aromatic ring. δ 6.89, 4.86 and 3.31 indicate the presence of a flavonoid ring structure. 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 the flavonol Kaempferol.

5. CONCLUSION

The HPTLC method developed for estimation of Kaempferol 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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Table 1. Data of Melting Point, UV λ_{max}, IR & NMR of the compound.

Parameter	Experimental data
Melting Point	274-277˚C
UV λ_{max}	266nm and 366nm in methanol
IR peaks (cm^{-1})	3317 (O-H vibrations of the hydroxyl group), 3198, 2951 (Aromatic C-H stretching), 2613, 1660 (C=O linkage of a phenolic ring), 1611, 1504, 1381, 1307, 1249, 1229, 1177 (O-H vibrations of the hydroxyl group), 1089, 1008 (C-O linkage in a heterocyclic aromatic ring), 819(C-H vibrations of the unsaturated part), 723, 673.
NMR peaks	δ 6.39 (9, 1H, d), δ 8.09 (15, 1H, ddd), δ 8.08 (19, 1H, ddd)

Table 2. Calibration data of Kaempferol (Concentration Vs Mean Peak Area)

Sr. No.	Concentration of Kaempferol (ng/spot)	Mean peak area ± SD (n=5)	% CV
1	120	878.34 ± 14.67	1.77
2	240	1112.91 ± 18.11	1.38
3	360	1436.22 ± 21.55	1.61
4	480	1698.45 ± 16.92	2.45
5	600	2018.41 ± 23.73	2.36

CV = Coefficient of Variance; SD = Standard Deviation
Correlation coefficient: 0.9997, Slope: 13.18,y- Intercept: -4.3

Table 3. Estimation of Kaempferol

Mean peak area (n=5)	Avg. amount of Kaempferol (µg/spot).	Avg. % w/w of Kaempferol ± SD	% CV
1415.67	20	0.879 ± 0.05	1.3

CV = Coefficient of Variance; SD = Standard Deviation

Table 4. Data for Inter-day and Intra-day precision for Kaempferol

Concentration (µg/spot)	Inter-day Precision (n = 5)		Intra-day Precision (n = 3)	
	Peak area (Mean ± SD)	% CV	*Peak area* (Mean ± SD)	% CV
10	871.29 ± 12.65	1.34	923.67 ± 14.69	1.87
20	1277.34 ± 15.33	1.39	1289.31 ± 17.26	1.45
30	1498.21 ± 13.41	2.91	1462.76 ± 21.34	3.05
40	1733.90 ± 16.87	2.28	1805.29 ± 19.68	2.39
50	2145.69 ± 19.41	2.56	2217.36 ± 18.40	2.81

Table 5. Data of repeatability of measurement for Kaempferol

No. of measurement	Area
1	1782.4
2	1734.2
3	1729.1
4	1767.2
5	1778.3
6	1731.5
7	1791.9
Mean	1759.3
% CV	**0.255**

CV = Coefficient of Variance; RSD = Relative Standard Deviation

Table 6. Data of repeatability of application for Kaempferol

No of measurement	Area
1	1746.2
2	1758.6
3	1778.4
4	1735.9
5	1745.7
6	1754.3
7	1782.1
Mean	1757.3
% CV	**0.282**

CV = Coefficient of Variance; RSD = Relative Standard Deviation

Table 7. Data of accuracy for Kaempferol

Conc. of Kaempferol (ng/spot)		**Amount of Kaempferol found Mean ± SD (n = 3)**	**% Recovery (n = 3)**
Taken	Added		
200	0	197.45 ± 1.45	99.34
200	50	247.77 ± 1.67	99.25
200	100	296.51 ± 1.81	99.56
200	150	346.87 ± 1.74	99.43
200	200	396.20 ± 1.79	99.50

SD = Standard Deviation

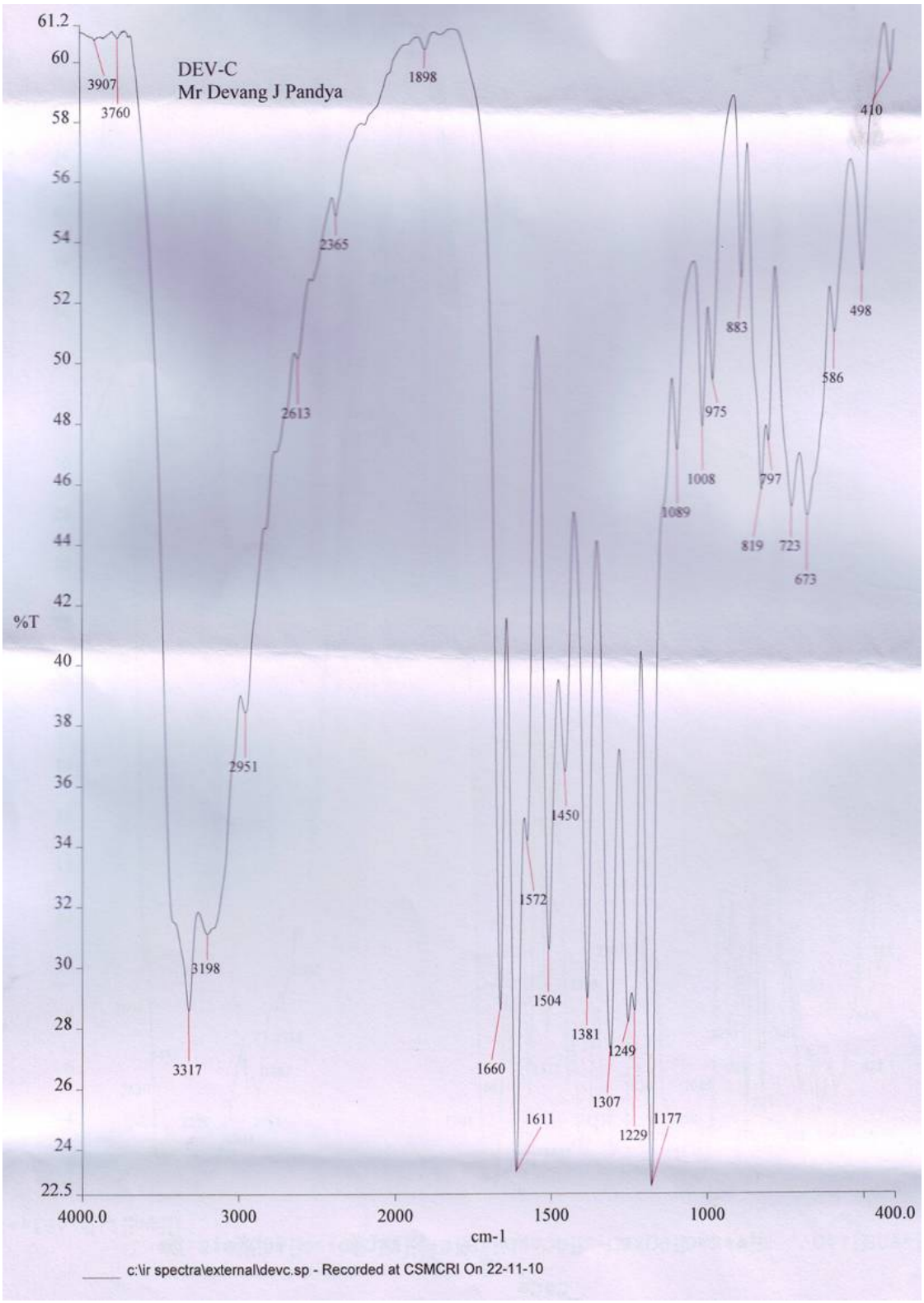

Figure 1. IR spectra

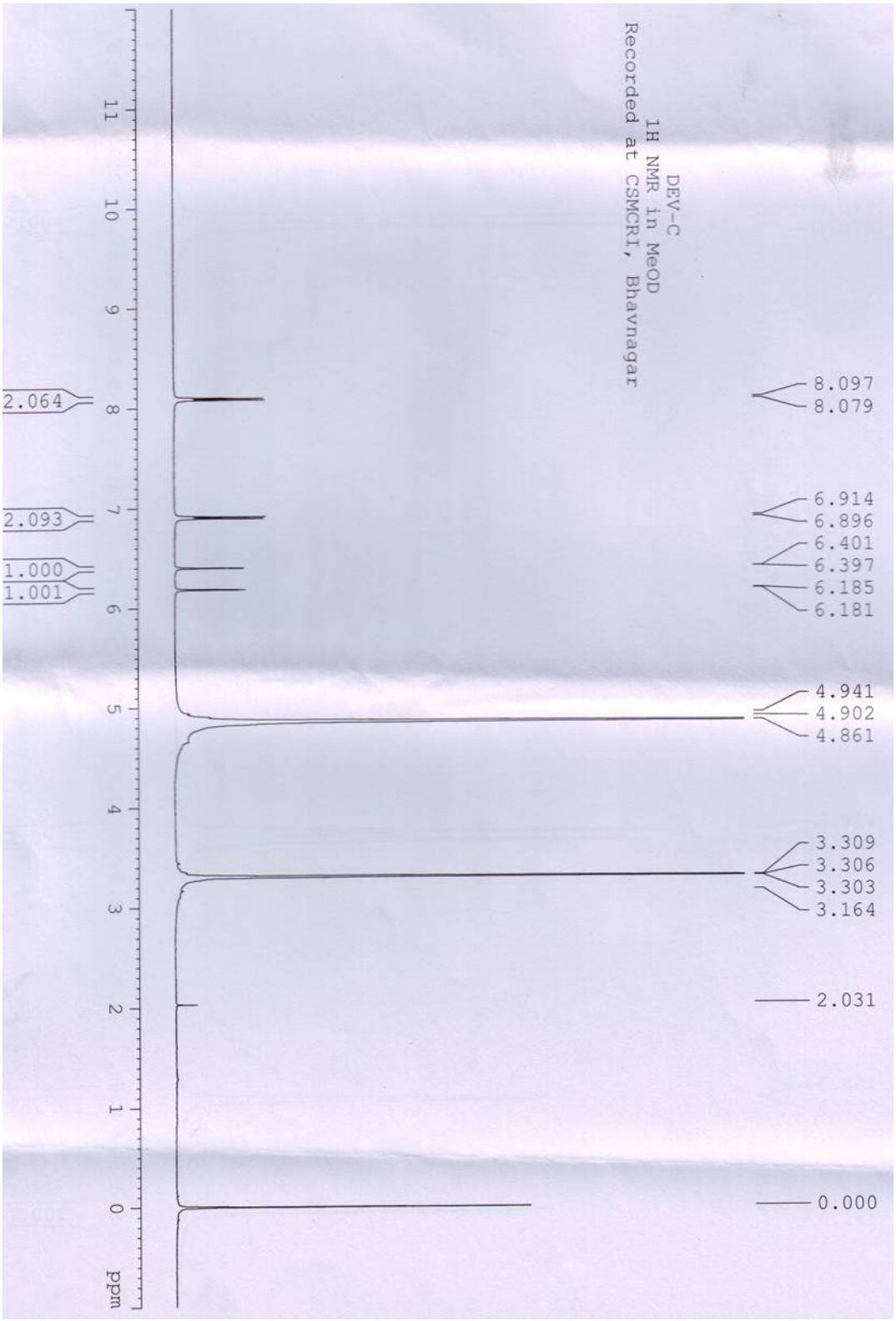

Figure 2. NMR spectra

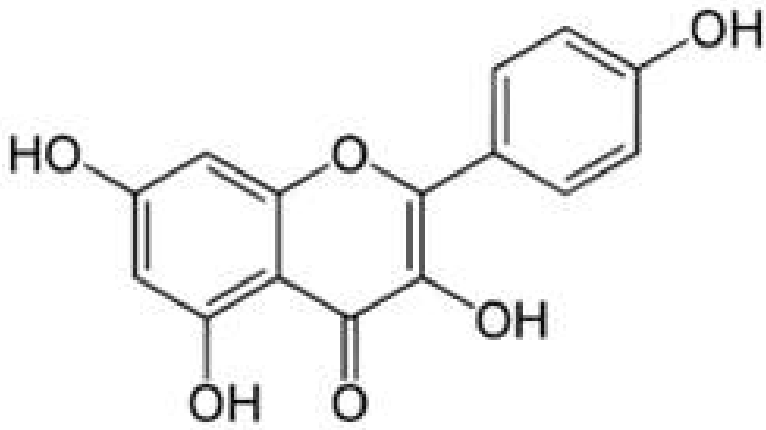

Figure 3. Kaempferol

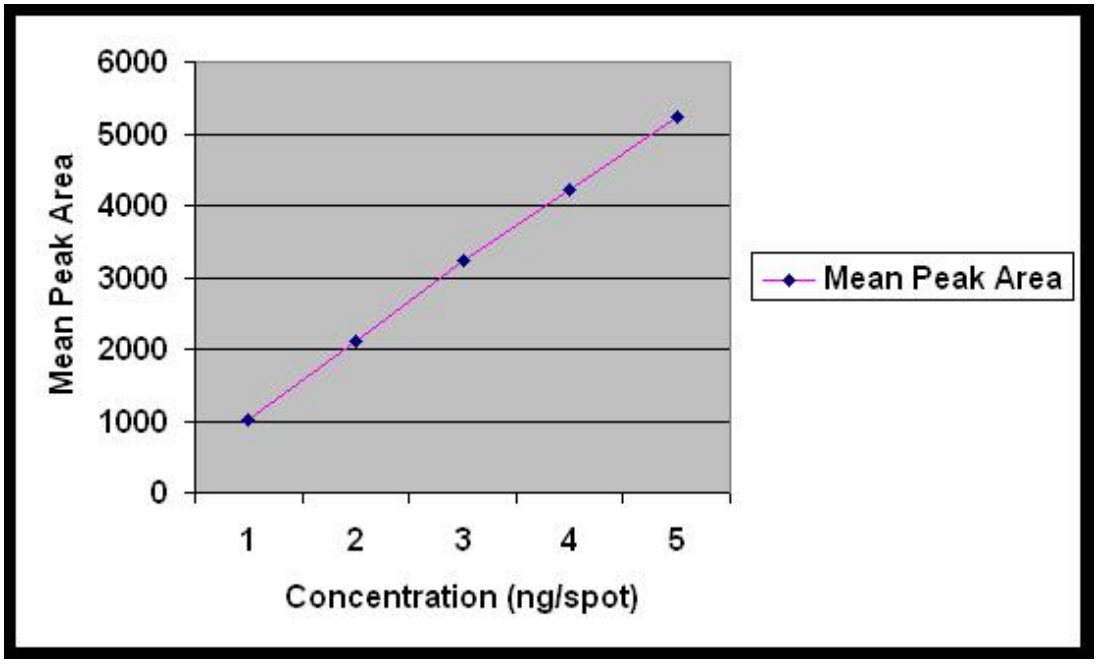

Figure 4. Calibration curve of Kaempferol

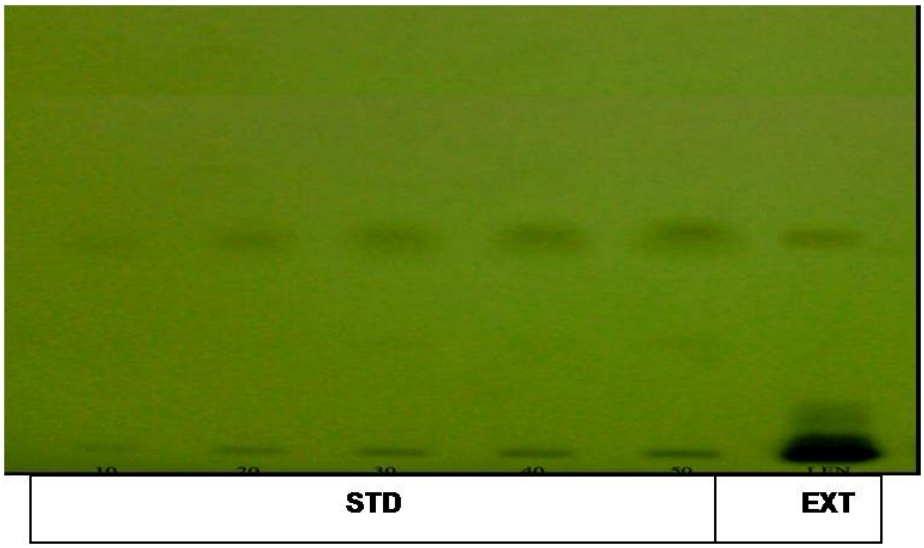

Figure 5. HPTLC chromatogram

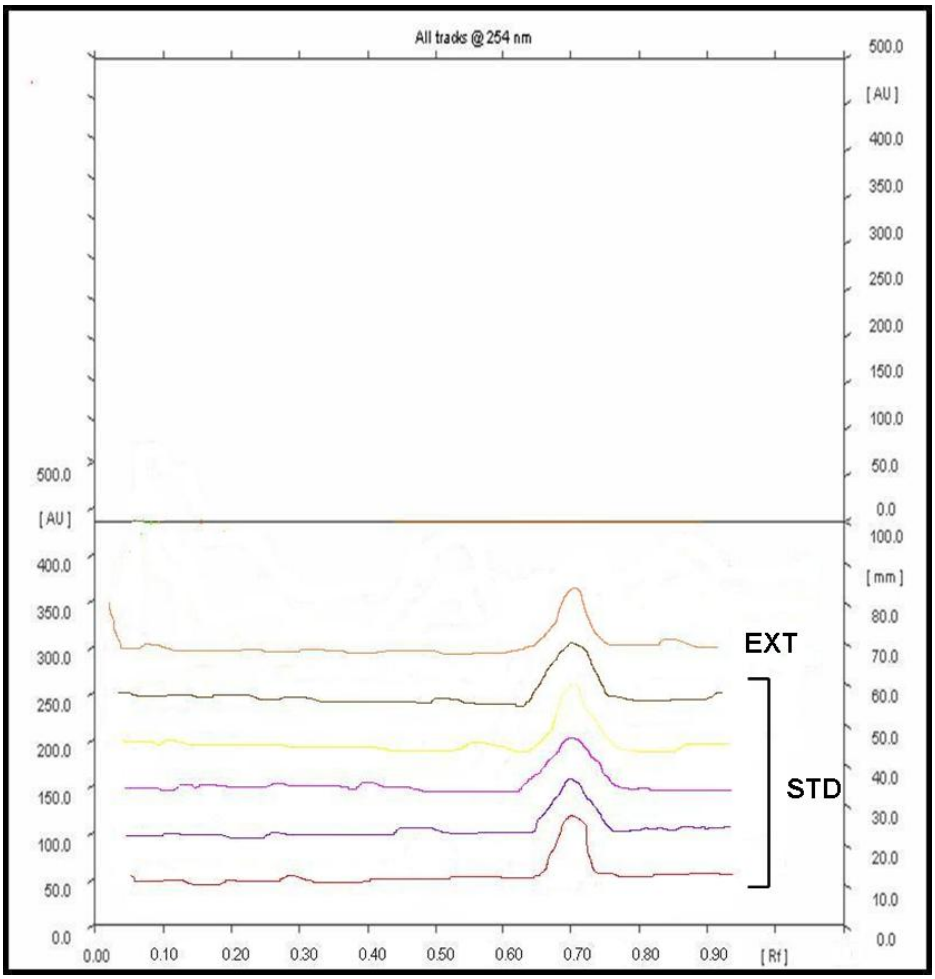

Figure 6. HPTLC densitometric chromatogram