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Research Article

# Analytical Method Development, Characterization, Evaluation of *In-vitro* Antioxidant and Anticancer Activity of Flavone Chrysin in HeLa Cells

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ABSTRACT

Chrysin is a very effective naturally occurring polyphenol belonging to the subclass of flavones showing abundant biological activities, including potential antioxidant and anticancer activity in numerous cancerous cells. In the present study, the properties of Chrysin were evaluated by performing the analytical method development using UV spectrophotometry and comparing its validation parameters in methanol ($\lambda_{max}$-219 nm), distilled water ($\lambda_{max}$-267 nm), 1.2pH buffer ($\lambda_{max}$-269.6 nm) and 6.8pH buffer ($\lambda_{max}$-268.2 nm). It was analyzed by high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC) and fourier transform infrared spectroscopy (FT-IR) studies. The characterization of Chrysin was performed using differential scanning calorimetry (DSC), scanning electron microscopy (SEM), X ray diffraction (XRD) studies and its log P value was evaluated. The *in vitro* antioxidant activity of Chrysin was evaluated using Ferrous ion chelation assay. The anticancer effect of Chrysin was studied on henrietta lacks immortal cell line (HeLa) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium assay. The analytical method development and validation parameters confirmed the linearity, method reliability, accuracy, precision, and stability of Chrysin with RSD values < 2%. The HPLC analysis showed a sharp symmetric peak for Chrysin at a retention time of 3.036 min in the mobile phase, acetonitrile: methanol (65:35 v/v). The HPTLC analysis indicated the $R_f$ value of 0.50 and 0.53 at 254 and 366 nm respectively for 5 µL sample in the mobile phase toluene: n-hexane: isopropyl alcohol (7:2:1 v/v). The FTIR and DSC studies confirmed the functionality and thermal behavior of Chrysin. The log P value of 3.236 indicated the high lipophilicity of Chrysin. SEM and XRD study suggested its crystalline behavior. The *in-vitro* Ferrous metal ion chelation was found in the range from 96.23 to 88.23% for concentrations ranging from 20–200 µg/mL. This indicated the increase in antioxidant activity of Chrysin with increase in its concentration. The MTT assay depicted the increasing concentration of Chrysin on HeLa cells and confirmed its anticancer effect by inhibiting the cell growth at $IC_{50}$ value of 15 µM.

## Introduction

Polyphenols are a large group of chemicals obtained from various plant sources and possess a wide variety of biological activities. Flavonoids are a subclass of polyphenols characterized by one or more aromatic rings having one or more phenolic hydroxyl groups and connected by a carbon bridge. Furthermore, it is divided into a subgroup called flavones, based on the position of the aromatic rings, functional groups present, and the presence of a double bond in the carbon ring. One such attention-worth and potent flavone is Chrysin (5,7-Dihydroxyflavone), possessing effective antioxidant and anticancer activities.[1] Chrysin acts as a naturally occurring dietary phytoconstituent and it is found in Passiflora flowers (*Passiflora caerulea* and *Passiflora incarnata*), propolis, honey, *Oroxylum indicum*, chamomile and in the mushroom, *Pleurotus ostreatus.*[2]

Chrysin acts as a free radical scavenger, an oxidative stress inhibitor, and transition metal ion chelator, indicating its potential antioxidant activity.[3] As an anticancer agent, it inhibits apoptosis and acts as an anti-proliferative agent in various cancers like malignant glioma, breast

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carcinoma, esophageal squamous carcinoma, leukemia, cervical cancer, colon cancer, etc.[4-11]

Analytical method development is the basic necessity and an integral part of the analysis of any compound to check its properties, reliability, and suitability so that it can meet the guidelines of the regulatory authorities. During the manufacturing process and drug development, the main purpose of analytical methods is to provide information about potency, impurity, and key drug characteristics. Drug approval by regulatory authorities requires the applicant to prove control of the entire drug development process using validated analytical methods.[12]

Chrysin can chelate $Fe^{2+}$ ions, which indicates its potential as an antioxidant agent. Chrysin being a phenolic compound binds with a certain quantity of $Fe^{2+}$ ions and the remaining $Fe^{2+}$ ions react with ferrozine, a ferroin compound that forms a magenta-colored complex with the ferrous ion. In the presence of Chrysin, there is disruption of metallic complex formation owing to its antioxidant activity.[13] Such activity of Chrysin in isolated form has been evaluated in the present study.

The literature survey depicted that Chrysin has been reported to cause apoptosis and inhibit cell proliferation in HeLa cells, an immortal artificially cultivated cell line originating from cervical cancer cells.[14-16] An MTT tetrazolium reduction assay was performed to test the cell viability of Chrysin. Viable cells with active metabolism can convert MTT into a purple-colored formazan product with $\lambda_{max}$ of 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus colour formation is a useful and convenient marker of the viable cells only.[17]

The present study includes the systematic and complete analytical method development of Chrysin, evaluating and comparing its validation parameters using UV spectrophotometry by calibration of Chrysin in different solvents (methanol, water, 1.2 pH acidic buffer, and 6.8pH phosphate buffer). These solvents were selected for studying the properties of Chrysin and its analysis for *in vitro* and *in vivo* release kinetic study and its partition in the water and oil phase. Such comparison of calibration and validation parameters for Chrysin in different solvents has not yet been reported. Further, Chrysin was analyzed by chromatographic techniques of HPLC and HPTLC by modifying the reported method to evaluate its system suitability. The HPTLC analysis in the following mobile phase at the wavelengths of 254 and 366 nm has not yet been reported. Its properties were then studied by FT-IR, DSC, SEM, X-ray diffraction, and its partition coefficient was evaluated.

Furthermore, in the present study, the *in-vitro* antioxidant activity of Chrysin was evaluated by Ferrous ion chelation assay. The anticancer property was evaluated by MTT cell viability assay on HeLa cell lines which has not been reported yet using Minimum essential medium Eagle (MEM). Hence, the compiled data of Chrysin and its properties is depicted in this study at a single glance.

## Materials and Methods

### Materials

Chrysin was purchased from the company Sigma-Aldrich, USA. The solvents used for HPLC analysis i.e., acetonitrile, methanol, and water, were of HPLC grade. Ferrozine was purchased from SDFCL. HeLa cell line was obtained from National Centre for Cell Science (NCCS), Pune. MEM medium for MTT assay was purchased from Himedia company. All the reagents and solvents used were of the analytical grade.

### Methods

#### *I. Qualitative Identification Test for Flavonoids*

Accurately weighed, 10 mg of Chrysin was added to 10 mL of ethanol and distilled water, respectively and ultrasonicated for proper mixing to obtain ethanolic and aqueous samples. To 2 mL each of ethanolic and aqueous sample solutions, 1 mL of 2N sodium hydroxide was added. The presence of yellow color was indicative of the presence of flavonoids.[18]

#### *II. Determination of Melting Point using Digital Melting Point Apparatus*

Accurately weighed 5 mg of Chrysin was added to a small glass capillary tube, closed at one end and introduced in the digital melting point apparatus. It was heated slowly, and the temperature at which the first drop of liquid was observed was recorded as the beginning of the melting point range. The temperature at which all the drug samples melted was recorded as the end of the melting point range.[19]

#### *III. Analytical Method Development*

1. UV Spectrophotometry-

*a. Calibration of Chrysin and Determination of Absorption Maxima ($\lambda_{max}$)-*

Calibration of Chrysin was performed in four different solvents - methanol, distilled water, 1.2 pH buffer and 6.8 pH buffer. A common standard stock solution was prepared by dissolving accurately weighed 25 mg of pure Chrysin in 25 mL methanol to obtain a solution of 1000 ppm. The stock solution, 10 ppm solution was prepared and scanned using a UV spectrophotometer in the range 200–400 nm to obtain a spectrum and $\lambda_{max}$ value from the stock solution. Same procedure was followed for distilled water, 1.2pH acidic buffer and 6.8pH phosphate buffer.[20]

- *Preparation of 1.2 pH Buffer:* For this, 250 mL of 0.2 M potassium chloride was placed in a 1000 mL volumetric flask and 425 mL of 0.2 M hydrochloric acid was added to it and the remaining volume was made up using distilled water. The pH of the solution was checked using a pH meter and adjusted using 1M HCl/NaOH.[21]
- *Preparation of 6.8 pH Buffer*: For this, 250.0 mL of 0.2M potassium dihydrogen phosphate was placed in a 1000 mL volumetric flask and 112 mL of 0.2M sodium hydroxide was added to it and the remaining volume

was made up using distilled water. The pH of the solution was checked using a pH meter and adjusted using 1M HCl/NaOH.[21]

- *Preparation of Working Solutions*: From the standard stock solution (1000 ppm), solutions of different concentration were prepared and scanned at the $\lambda_{max}$ to obtain the calibration curve and regression equation by plotting concentration vs absorbance for each solvent. Concentrations for methanol- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 ppm; for ultrapure water- 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 ppm; for 1.2 pH buffer- 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 ppm and for 6.8pH buffer- 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 ppm.

*b. Determination of Validation Parameters-*

To determine the accuracy, precision, method suitability and stability of Chrysin, the following validation parameters were evaluated and compared for different solvents-methanol, distilled water, 1.2 pH buffer, and 6.8 pH buffer-

- *Linearity Study:* From the standard stock solution of 1000 ppm, a solution of 100 ppm was prepared. Different concentrations were prepared from this solution and scanned in UV spectrophotometer at $\lambda_{max}$ and their absorbance was noted to obtain the linearity range.[22]
- *Intraday Precision Study:* From the standard stock solution of 1000 ppm, a solution of 10 ppm was prepared. Triplicate absorbance was taken at $\lambda_{max}$ thrice a day at equal interval of time (3 hour) and the mean, standard deviation and %RSD was calculated.[22]
- *Interday Precision Study:* From the standard stock solution of 1000 ppm, a solution of 10 ppm was prepared. Triplicate absorbance was taken at $\lambda_{max}$ for three consecutive days at the same time (12:00 pm) and mean, standard deviation and %RSD were calculated.[22]
- *Robustness:* According to the Q2(R1) guideline i.e. Validation of analytical procedures of the ICH guidelines, the analysis of reliability of the method is done by making deliberate changes in certain method parameters. In the present study, temperature was selected as the deliberate method parameter which was varied. It carried out the analysis under two different temperature conditions, i.e. at room temperature and 8°C (cool condition). From the standard stock solution of 1000 ppm, a solution of 10 ppm was prepared. The respective absorbance was noted at $\lambda_{max}$ and the result was indicated as %RSD.[23]
- *Ruggedness:* The ruggedness of the method was determined by carrying out the analysis by three different analysts. From the standard stock solution of 1000 ppm, a solution of 10 ppm was prepared. The respective absorbance was noted at $\lambda_{max}$ and the result was indicated as %RSD.[23]
- *Limit of Detection and Limit of Quantitation:* From the standard stock solution (1000 ppm), solution of 10 ppm was prepared. Triplicate absorbance was taken and the mean was calculated. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using standard deviation (S) and slope (M) by the formula.[22,23]

$$LOD = [3.3\times S]/M;\ LOQ = [10\times S]/M$$

- *%Recovery Accuracy Study:* Accuracy is defined as the closeness of agreement between the actual (true) value and analytical value and obtained by applying test method for several times. The recovery experiments were carried out in triplicate by spiking previously analysed drug samples with three different concentrations. From the standard stock solution of 1000 ppm, a solution of 100 ppm was prepared. From this solution, three 10 ppm solutions were prepared and triplicate absorbance was taken for each solution. Then subsequently these solutions were spiked externally to obtain solutions of 18, 20 and 22 ppm for methanol, water and 1.2pH buffer and 20, 22 and 24 ppm for 6.8pH buffer and their triplicate absorbance was noted. Then the difference and % recovery was calculated at $\lambda_{max.}$[22]

2. Development of Chrysin using High performance liquid chromatography (HPLC)-

- *Selection of mobile phase* – This study comprised of acetonitrile and methanol in the ratio of 65:35 v/v as the mobile phase. It was filtered through a 0.45µm pore size filter and degassed using ultrasonication before use.[24]
- *Preparation of standard solution of Chrysin* – Chrysin was freely soluble in methanol (HPLC grade). Accurately weighed 1 mg of Chrysin was dissolved in methanol until a clear solution was obtained and the volume was made to 10 mL with methanol to obtain a standard solution of 100 ppm.
- *Instrumentation and chromatographic method* – HPLC with PDA detector of the make Shimadzu, Japan (Model LCMS/MS 8040) was used to study the analysis of Chrysin. It comprised of a binary system with the PDA detector in the range 190-800 nm. The qualitative chromatographic analysis carried out was isocratic in nature and the column used was C-18 (250 mm). From the previously prepared standard solution of Chrysin, 20 µL was injected in the system at a flow rate of 1 mL/min to obtain the chromatogram.[25]

3. HPTLC analysis of Chrysin-

A modified method was developed and used for the HPTLC analysis of Chrysin.[26] For quantification of Chrysin, HPTLC system fitted with Nanomat/ capillary dispenser system was used. The stationary phase was precoated HPTLC plates silica gel 60 F 254 (E. MERCK KGaA) with dimensions 5.0 x 10.0 cm. The standard solution of pure Chrysin was prepared by dissolving 10 mg Chrysin in 10 mL methanol to obtain a 1000 µg/mL solution. From this solution, a working solution of 10 µg/mL was prepared. Two samples of 5 µL and 10 µL were prepared for injection on the plates.

A 5 µL and 10 µL samples were applied as spots at the application position at a distance of 20 and 30 mm, respectively, from the bottom with 2 tracks and 10 mm distance between the tracks using a 100 µL Hamilton syringe. The plates were developed in a twin trough chamber (20 x 10 cm) by first saturating the chamber with the mobile phase for 10 min. The mobile phase used was toluene: n-hexane: isopropyl alcohol in the ratio of 7:2:1. The volume of mobile phase used was 10 mL with solvent front position of 80.1 mm. After that, the plates were dried in an oven at 60°C for 5 minutes. The detection was performed using CAMAG TLC Scanner with slit dimension of 4.00 x 0.30 mm with a light scanning speed of 20 mm/s with a data resolution of 100 µm/step. The samples were scanned at 254 and 366 nm using a D2 lamp with voltage of 273V and data filtering of Savitsky-Golay 7. The software used for computing the data was WinCATS Planar Chromatography manager.

4. Fourier Transform Infrared (FT-IR) Spectroscopy of Chrysin

The FTIR spectrum of Chrysin was obtained using Shimadzu – IRAFFINITY – 1 Fourier transform infrared spectrophotometer. Chrysin was accurately weighed and triturated well with potassium bromide in the ratio 1:100 using a small mortar and pestle. The mixture was then introduced in the sample holder of the FT-IR instrument and scanned to obtain the spectrum in the range of 4500–500 $cm^{-1}$.[27]

### *IV. Characterization of Chrysin-*

*1. Determination of Solubility of Chrysin by Partition Co-efficient Method*

A modified method was used to determine the partition coefficient of Chrysin[28] Accurately weighed 10 mg of Chrysin was taken and dispersed in 10mL of distilled water. It was then stirred on a magnetic stirrer for 30 minutes. This dispersion was added to 10 mL n-octanol in a 50 mL volumetric flask and it was subjected to mixing for 24 hours on a 5 stationed rotary shaker (REMI). After 24 hours, the dispersion was transferred in a 60 mL separating funnel and the layers of both the phases were allowed to separate for 1 hour. When two distinct layers were formed, they were separated and filtered. The solution in both phases were diluted and assayed using UV spectrophotometer by recording absorbance in triplicate. Concentration in both the phases was calculated using the regression equation and partition coefficient was calculated by using the formula.[29,30]

$$\text{Partition coefficient (log P)} = \log C_o/C_w$$

Where, $C_o$ – Concentration in oil phase and $C_w$ – Concentration in water phase

*2. Differential Scanning Calorimetry of Chrysin-*

The DSC thermogram of Chrysin was recorded using the Netzsch DSC 204 F1 differential scanning calorimeter with a temperature range of -150 to 600°C (STIC, Cochin). The thermal behavior was studied by heating 4.555 mg of Chrysin in a concavus aluminium crucible with a pierced lid under nitrogen atmosphere (40.0 mL/min). The analysis was carried out over the temperature range 0–320°C at a rate of 20°C/10.0(K/min)/400°C.[29,31]

*3. Scanning Electron Microscopy of Chrysin-*

The surface morphology of Chrysin was studied by SEM imaging using a Jeol 6390LA/ OXFORD XMX N scanning electron microscope (STIC, Cochin) having a tungsten filament with an accelerating voltage of 0.5 to 30 mV and magnification of x 300000.[32]

*4 X-Ray Diffraction of Chrysin-*

The crystallinity was evaluated using X-Ray powder diffraction by obtaining the diffraction patterns of Chrysin using Bruker D8 Advance (STIC, Cochin). The X-ray generator was allowed to operate at 40 mA tube current and 40 kV tube voltages. The scanning angle was adjusted from 3 to 60° in the step scan mode with a step time of 59.7s. The diffraction pattern was studied based on 2-Theta angle values.[28]

### *V. Evaluation of In-vitro Antioxidant Activity of Chrysin by Ferrous Ion Chelation Assay-*

A modified method was used for the evaluation of *in vitro* ferrous ion chelation activity of Chrysin[13,33]

- *Preparation of 5 mM Ferrozine Solution*: Accurately weighed 23.26 mg of ferrozine was dissolved in 25 mL of distilled water to obtain a solution of 5 mM concentration and kept in the dark for 1-hour.
- *Preparation of 2 mM Ferric Chloride Solution*: Accurately weighed 8.11 mg of ferric chloride was dissolved in 25 mL of distilled water to obtain a solution of 2 mM concentration and kept in the dark for 1 hour.
- *Preparation of Different Concentrations of Chrysin*: Accurately weighed 10 mg of Chrysin was dissolved in 10 mL methanol to obtain a stock solution of 1000 µg/mL. From this solution different concentrations were prepared (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL).
- *Assay Procedure*: From each concentration of Chrysin 0.25 mL was withdrawn. To it 1.75 mL methanol, 0.25 mL ferric chloride solution (2 mM) and 0.25 mL ferrozine solution (5 mM) were added and shaken to mix well. After this the solutions were kept in dark for 10 minutes and absorbance of each Chrysin concentration was recorded at 562 nm. For control, 2 mL of distilled water was used as blank and a solution of 1 mL each of ferric chloride and ferrozine were taken as sample and the absorbance was noted at 562 nm. The %ferrous ion chelation was calculated as follows –

$$\text{Ferrous ion chelation (\%)} = [(A_c\text{-}A_t)/A_c] \times 100$$

where, $A_c$–Absorbance of control and $A_t$–Absorbance of test sample

### *VI. Evaluation of Anticancer Activity of Chrysin by MTT Tetrazolium Cell Viability Assay on HeLa Cell Line*

The anticancer activity of Chrysin was evaluated using MTT tetrazolium cell viability assay on HeLa cell line by modifying the method.[14,17] MEM (Minimum essential medium Eagle) was used as the cell culture medium. The stock solution was prepared by dissolving 1 mg of Chrysin in 1 mL DMSO. From this solution 20 µL was taken and diluted 250 times using MEM medium. Final concentration of Chrysin used was 80µM. The sample solutions of Chrysin were co-cultured with HeLa cells. HeLa cells harvested in the log phase of growth were inoculated in 96-well plate and the final volume of each well was 200 µL. After 24h of incubation at 37°C, the cell cultures were treated with 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µM concentrations of Chrysin. To each, 10 µL of MTT solution was added. A concentration of 22 µM was used as the negative control. The MTT absorbance value for each solution was detected at 590 nm and the results were expressed as optical density and $IC_{50}$ value.

## Results and Discussion

### I. Qualitative identification test for flavonoids

Both the ethanolic and aqueous samples of Chrysin showed yellow color confirming the presence of flavonoid as shown in Fig. 1.

### II. Determination of melting point using Digital melting point apparatus

The melting point range of Chrysin using digital melting point apparatus (Equiptronics) was found to be 285–288°C which was near the reported melting point range of Chrysin (284–286°C).

### III. Analytical method development

*1] UV spectrophotometry-*

a) *Calibration of Chrysin and determination of absorption maxima ($\lambda_{max}$)-*

The absorption maxima of Chrysin in methanol, distilled water, 1.2pH acidic buffer and 6.8pH phosphate buffer was found to be 212, 267, 269.6, and 268.2 nm, respectively.

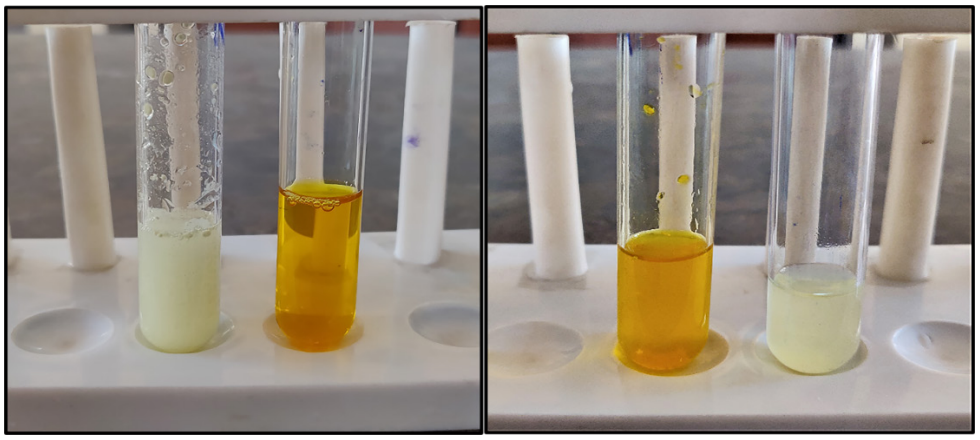

**Fig. 1:** Test for flavonoids – Yellow colour formation in aqueous (left) and ethanolic (right) samples of chrysin

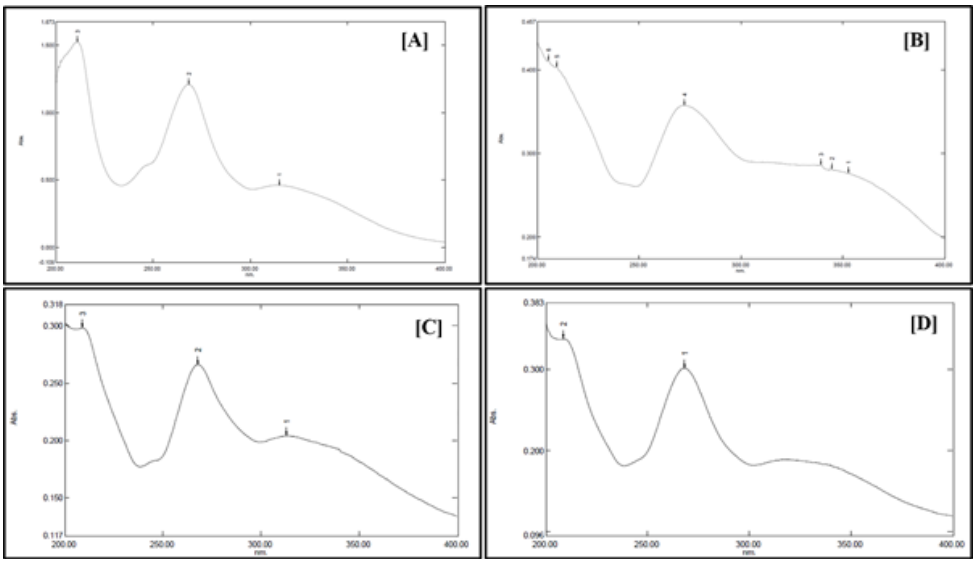


**Fig. 2:** Absorption maxima peaks of Chrysin in [A] Methanol, [B] Distilled water, [C] 1.2pH buffer and [D] 6.8pH buffer

Although Chrysin was insoluble in distilled water, method validation was performed to analyze its partition in water as seen in Fig. 2 (C). This was done using methanol as a cosolvent and hence a blunt and diffused solvent peak is visible in the calibration curve of water. The maximum peak and calibration curves and the regression equations and correlation coefficient values is shown in Figs. 2 and 3. The calibration and regression data of Chrysin in all four solvents is shown in Tables 1 and 2. The result showed a correlation coefficient value near to 1 in all solvents indicating that it followed Lambert Beer's law. The highest correlation coefficient was found in the case of distilled water.

*b) Determination of Validation parameters-*

The selected concentrations for all the validation parameters in all four solvents lied within the linearity range and the observed RSD was found to be < 2 % as per ICH guidelines. The graphical comparison is shown in Fig. 4.

- *Linearity study:* The linearity parameter and the corresponding regression data, indicated good linear

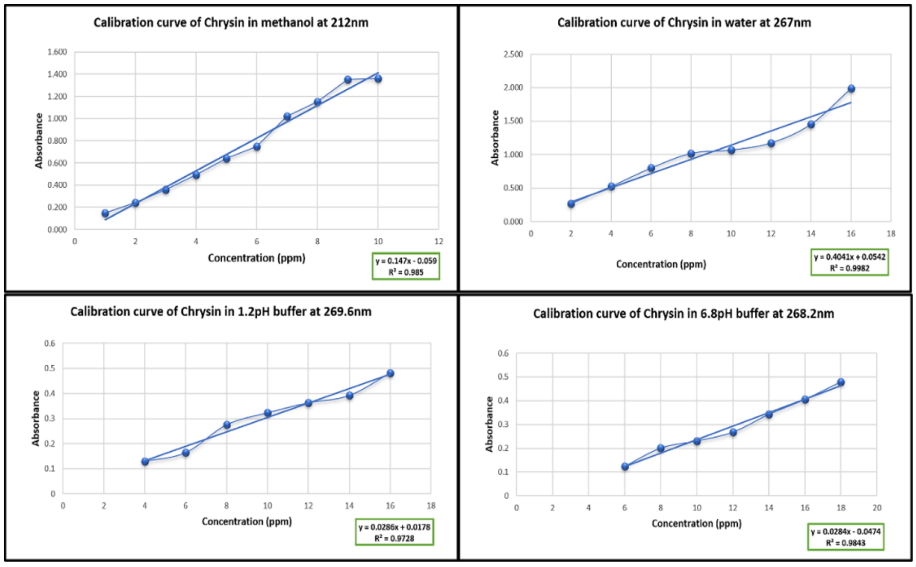


**Fig. 3:** Calibration curves of Chrysin in different solvents

**Table 1:** Calibration of Chrysin in different solvents

| *Methanol* | | *Distilled water* | | *1.2 pH buffer* | | *6.8 pH buffer* | |
|---|---|---|---|---|---|---|---|
| *Concentration (ppm)* | *Absorbance* | *Concentration (ppm)* | *Absorbance* | *Concentration (ppm)* | *Absorbance* | *Concentration (ppm)* | *Absorbance* |
| 1 | 0.150 ± 0.001 | 2 | 0.271 ± 0.001 | 4 | 0.129 ± 0.003 | 6 | 0.124 ± 0.001 |
| 2 | 0.242 ± 0.001 | 4 | 0.524 ± 0.001 | 6 | 0.164 ± 0.003 | 8 | 0.201 ± 0.001 |
| 3 | 0.359 ± 0.002 | 6 | 0.799 ± 0.001 | 8 | 0.275 ± 0.002 | 10 | 0.231 ± 0.001 |
| 4 | 0.491 ± 0.012 | 8 | 1.017 ± 0.001 | 10 | 0.323 ± 0.002 | 12 | 0.268 ± 0.001 |
| 5 | 0.640 ± 0.001 | 10 | 1.068 ± 0.001 | 12 | 0.363 ± 0.002 | 14 | 0.343 ± 0.001 |
| 6 | 0.747 ± 0.001 | 12 | 1.174 ± 0.001 | 14 | 0.392 ± 0.006 | 16 | 0.406 ± 0.002 |
| 7 | 1.019 ± 0.003 | 14 | 1.453 ± 0.001 | 16 | 0.482 ± 0.005 | 18 | 0.480 ± 0.001 |
| 8 | 1.151 ± 0.001 | 16 | 1.986 ± 0.002 | - | - | - | - |
| 9 | 1.353 ± 0.002 | - | - | - | - | - | - |
| 10 | 1.359 ± 0.002 | - | - | - | - | - | - |

Data is represented as mean value ± SD (n=3)

**Table 2:** Regression data of Chrysin in different solvents

| *Sr. no* | *Solvent* | *$\lambda_{max}$* | *Regression equation* | *Slope* | *$R^2$ value* |
|---|---|---|---|---|---|
| 1 | Methanol | 212 nm | y = 0.147x – 0.059 | 0.147 | 0.985 |
| 2 | Distilled water | 267 nm | y = 0.4041x + 0.0542 | 0.4041 | 0.9982 |
| 3 | 1.2 pH acidic buffer | 269.6 nm | y = 0.0286x + 0.0178 | 0.0286 | 0.9728 |
| 4 | 6.8 pH phosphate buffer | 268.2 nm | y = 0.0284x – 0.0474 | 0.0284 | 0.9843 |

relationship over the working concentration range of Chrysin in all the four solvents as shown in Table 3.

- *Intraday precision study:* The intraday precision study of Chrysin in four solvents confirmed the adequate sample stability and method reliability throughout 24 hours as shown in Table 4.
- *Interday precision study:* The interday precision study of Chrysin in four solvents confirmed the adequate sample stability and method reliability over a period of 3 days as shown in Table 5.
- *Robustness:* The comparative robustness data of Chrysin in four solvents at two different temperatures is shown in Table 6. It confirmed the method's reliability at room temperature and cold conditions.

**Table 3:** Linearity data of Chrysin

| *Sr. no* | *Solvent* | *Beer's law linearity range* |
|---|---|---|
| 1 | Methanol | 1 – 10 ppm |
| 2 | Distilled water | 2 – 20 ppm |
| 3 | 1.2 pH acidic buffer | 4 – 22 ppm |
| 4 | 6.8 pH phosphate buffer | 6 – 24 ppm |

- *Ruggedness:* The comparative ruggedness data of Chrysin in four solvents performed by three different analysts is shown in Table 7. It confirmed the method reliability for three different analysts.
- *Limit of detection (LOD) and Limit of quantitation (LOQ):* The detection and quantitation limits of Chrysin in four solvents are depicted in Table 8 at the concentration

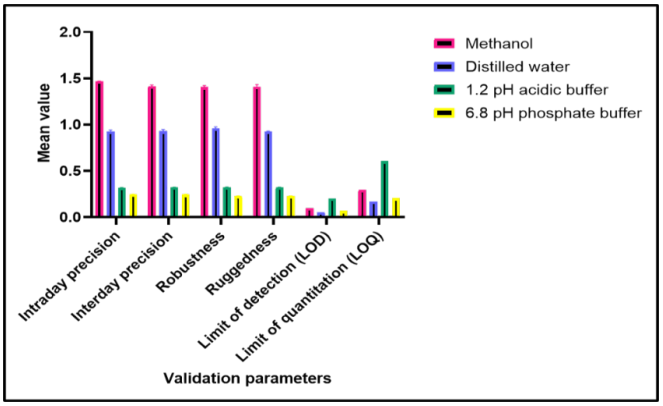


**Fig. 4:** Compiled validation data of Chrysin in different solvents

**Table 4:** Intraday precision data of Chrysin

| | *Mean absorbance in solvents (10ppm concentration of chrysin)* | | | |
|---|---|---|---|---|
| | *Methanol* | *Distilled water* | *1.2pH buffer* | *6.8pH buffer* |
| A1 (10:00 am) | 1.4665 | 0.942 | 0.32 | 0.248 |
| A2 (01:00 pm) | 1.4666 | 0.925 | 0.318 | 0.245 |
| A3 (04:00 pm) | 1.4703 | 0.913 | 0.312 | 0.24 |
| Mean | 1.4678 | 0.927 | 0.317 | 0.244 |
| SD (σ) | 0.0022 | 0.0146 | 0.0042 | 0.004 |
| %RSD | 0.1475 | 1.5725 | 1.315 | 1.65 |

(n=3)

**Table 5:** Interday precision data of Chrysin

| | *Mean absorbance in solvents (10 ppm concentration of chrysin)* | | | |
|---|---|---|---|---|
| | *Methanol* | *Distilled water* | *1.2 pH buffer* | *6.8 pH buffer* |
| A1 (Day 1) | 1.399 | 0.942 | 0.324 | 0.248 |
| A2 (Day 2) | 1.4151 | 0.936 | 0.321 | 0.242 |
| A3 (Day 3) | 1.428 | 0.908 | 0.322 | 0.239 |
| Mean | 1.414 | 0.929 | 0.322 | 0.243 |
| SD (σ) | 0.0145 | 0.0181 | 0.0015 | 0.005 |
| %RSD | 1.028 | 1.954 | 0.474 | 1.89 |

(n=3)

**Table 6:** Robustness data of Chrysin

| | *Mean absorbance in solvents (10ppm concentration of chrysin)* | | | |
|---|---|---|---|---|
| | *Methanol* | *Distilled water* | *1.2 pH buffer* | *6.8 pH buffer* |
| A1 (R.T) | 1.3967 | 0.969 | 0.323 | 0.231 |
| A2 (8°C) | 1.4247 | 0.943 | 0.315 | 0.228 |
| Mean | 1.4107 | 0.956 | 0.319 | 0.230 |
| SD (σ) | 0.0198 | 0.0184 | 0.0057 | 0.0021 |
| %RSD | 1.4035 | 1.923 | 1.773 | 0.92 |

(n=3)

**Table 7:** Ruggedness data of Chrysin

| | *Mean absorbance in solvents (10ppm concentration of chrysin)* | | | |
|---|---|---|---|---|
| | *Methanol* | *Distilled water* | *1.2 pH buffer* | *6.8 pH buffer* |
| Analyst 1 | 1.3849 | 0.929 | 0.323 | 0.231 |
| Analyst 2 | 1.4179 | 0.920 | 0.322 | 0.228 |
| Analyst 3 | 1.4287 | 0.924 | 0.322 | 0.226 |
| Mean | 1.4105 | 0.924 | 0.322 | 0.228 |
| SD (σ) | 0.0228 | 0.0045 | 0.0006 | 0.00252 |
| %RSD | 1.6178 | 0.4878 | 0.179 | 1.102 |

(n=3)

**Table 8:** LOD and LOQ data of Chrysin

| | *Mean absorbance in solvents (10ppm concentration of chrysin)* | | | |
|---|---|---|---|---|
| | *Methanol* | *Distilled water* | *1.2pH buffer* | *6.8pH buffer* |
| A1 | 1.357 | 1.069 | 0.324 | 0.231 |
| A2 | 1.36 | 1.068 | 0.321 | 0.231 |
| A3 | 1.36 | 1.067 | 0.324 | 0.232 |
| Mean | 1.359 | 1.068 | 0.323 | 0.231 |
| SD (σ) | 0.0017 | 0.001 | 0.00173 | 0.00058 |
| %RSD | 0.1275 | 0.094 | 0.536 | 0.2496 |
| LOD | 0.0969 | 0.056 | 0.200 | 0.0671 |
| LOQ | 0.2936 | 0.169 | 0.606 | 0.2033 |

(n=3)

**Table 9:** Accuracy data of Chrysin by UV spectroscopy

| *Concentration* | *Mean absorbance* | *Concentration of spiked sample (µg/mL) ± SD* | *Recovery (%)* |
|---|---|---|---|
| For Methanol – 10 ppm | 1.468 ± 0.002 | | |
| 18 ppm | 2.549 ± 0.004 | 7.74 ± 0.030 | 96.75 ± 0.331 |
| 20 ppm | 2.850 ± 0.067 | 9.30 ± 0.462 | 93.02 ± 0.463 |
| 22 ppm | 3.081 ± 0.064 | 11.13 ± 0.430 | 92.72 ± 0.360 |
| For Distilled water – 10 ppm | 1.068 ± 0.001 | | |
| 18 ppm | 1.782 ± 0.001 | 1.63 ± 0.006 | 20.41 ± 0.030 |
| 20 ppm | 2.069 ± 0.003 | 2.34 ± 0.012 | 23.43 ± 0.079 |
| 22 ppm | 2.205 ± 0.004 | 2.68 ± 0.010 | 22.33 ± 0.087 |
| For 1.2 pH buffer – 10 ppm | 0.323 ± 0.002 | | |
| 18 ppm | 0.490 ± 0.005 | 5.21 ± 0.142 | 65.06 ± 1.768 |
| 20 ppm | 0.525 ± 0.001 | 6.45 ± 0.081 | 64.52 ± 0.803 |
| 22 ppm | 0.605 ± 0.004 | 9.25 ± 0.162 | 77.08 ± 1.351 |
| For 6.8 pH buffer – 10 ppm | 0.231 ± 0.001 | | |
| 20 ppm | 0.482 ± 0.002 | 10.48 ± 0.023 | 104.84 ± 0.202 |
| 22 ppm | 0.534 ± 0.001 | 12.34 ± 0.006 | 102.82 ± 0.006 |
| 24 ppm | 0.558 ± 0.004 | 13.16 ± 0.056 | 94.00 ± 0.387 |

Data is represented as mean value ± SD (n=3)

of 10 ppm. The LOD and LOQ values signified the lowest concentrations at which Chrysin can be reliably detected in a sample in all solvents.

- *%Recovery Accuracy Study:* The comparative % recovery data of Chrysin in all four solvents is shown in Table 9. The results signified the high accuracy of the method as the recovery is in the acceptable range for methanol and 6.8pH buffer. As Chrysin is practically insoluble in water, the low recovery values in water and 1.2 pH buffer suggested the instability of Chrysin in them.

### *2. Development of Chrysin using High-Performance Liquid Chromatography (HPLC)-*

The chromatogram of Chrysin using the mobile phase Acetonitrile: Methanol (65:35 v/v) is shown in Fig. 5. The run time followed was of 10 minutes. The chromatogram showed a sharp peak without any tailing and lagging at the selected wavelength of 268 nm. The retention time was 3.036 minutes with a peak area of 6553450, which was a measure of the concentration of Chrysin. Hence, a qualitative analysis was possible at these chromatographic conditions as a symmetric peak was observed for Chrysin with an appreciable resolution.

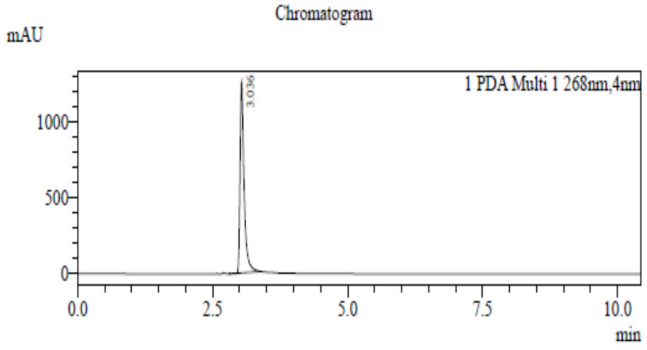


**Fig. 5:** HPLC Chromatogram of Chrysin

### *3. HPTLC analysis of Chrysin-*

The chromatograms were obtained for Chrysin with concentration of 5 and 10 µL at 254 nm and 366 nm, respectively. The HPTLC plates, all-track chromatograms and individual chromatograms are depicted in Figs 6–9. The chromatogram data for highest peaks at 254 and 366 nm at 5 and 10 µL concentrations is shown in Table 10. The $R_f$ values for chrysin at 254 nm for 5 and 10 µL volume of the sample were found to be 0.50 for each. The $R_f$ values for chrysin at 366 nm for 5 and 10 µL volume of sample were found to be 0.53 and 0.54, respectively. Hence, these chromatographic conditions with the selected mobile

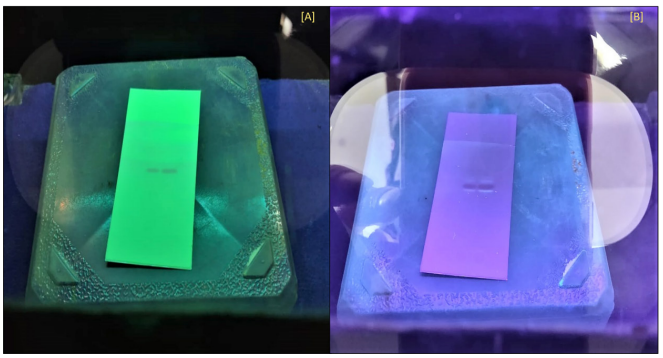


**Fig. 6:** HPTLC plates of Chrysin under UV light at 254 nm [A] and 366 nm [B]

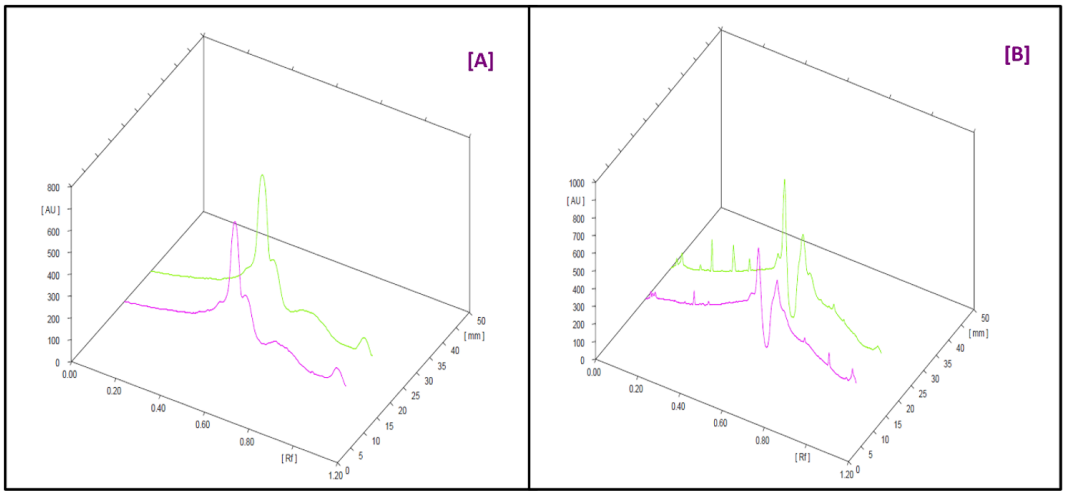


**Fig. 7:** HPTLC all-track chromatograms of Chrysin at 254 nm[A] and 366 nm[B]

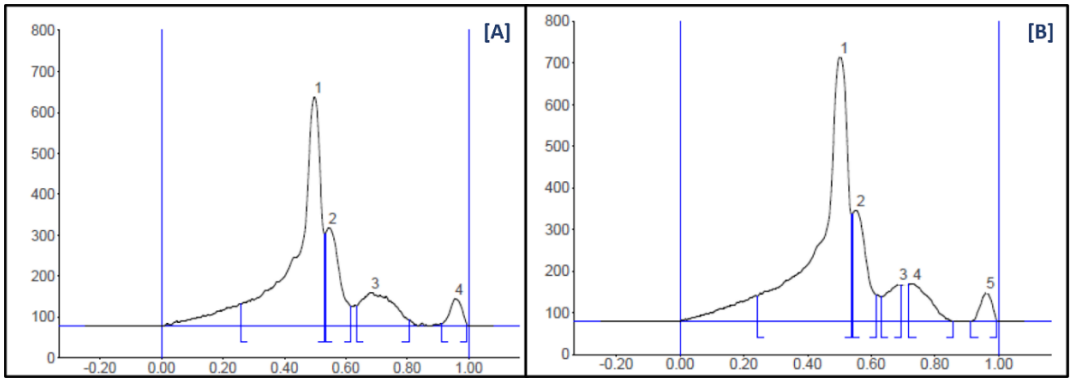


**Fig. 8:** HPTLC chromatograms of Chrysin at 254 nm at 5 µL [A] and 10 µL [B]

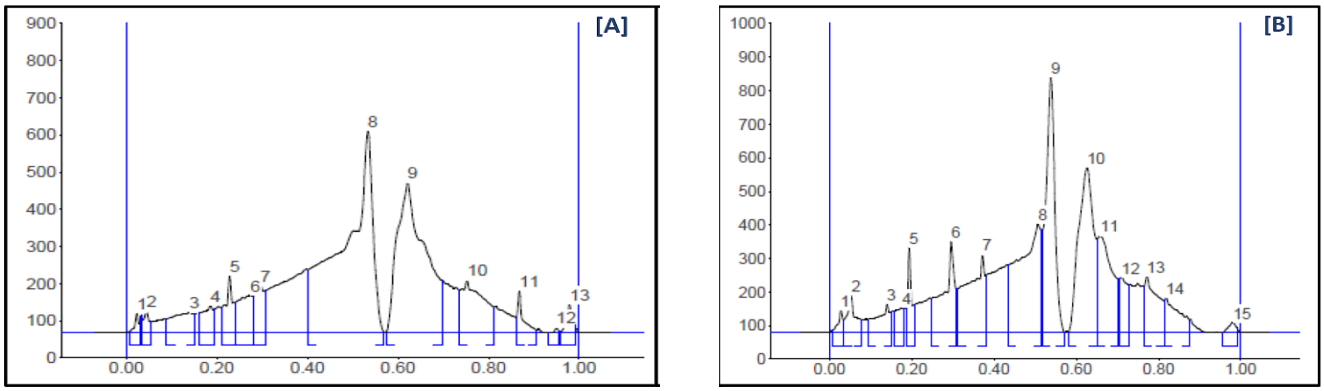


**Fig. 9:** HPTLC chromatograms of Chrysin at 366 nm at 5 µL [A] and 10 µL [B]

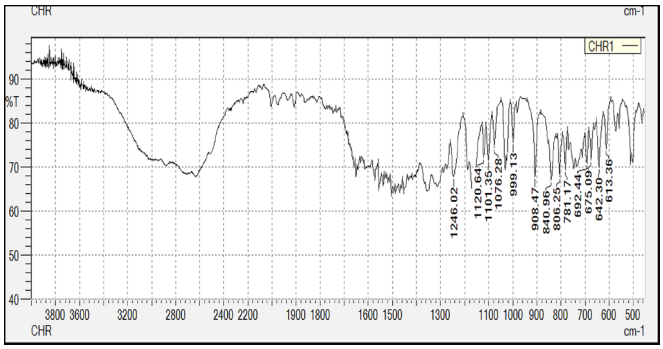

**Fig. 10:** FT-IR spectrum of Chrysin

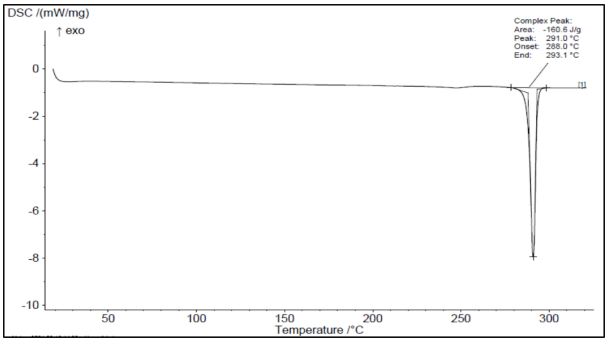


**Fig. 11:** DSC thermogram of Chrysin

**Table 10:** HPTLC chromatogram data of chrysin at 254 nm and 366 nm

| *Peak no.* | *Start Rf* | *Start height* | *Max Rf* | *Max Ht.* | *Max %* | *End Rf* | *End height* | *Area* | *Area %* |
|---|---|---|---|---|---|---|---|---|---|
| *At 254 nm- 5 µL* | | | | | | | | | |
| 1 | 0.26 | 52.8 | 0.50 | 558.0 | 59.08 | 0.53 | 223.7 | 29430.2 | 65.73 |
| At 254 nm- 10 µL | | | | | | | | | |
| 1 | 0.24 | 60.5 | 0.50 | 635.6 | 55.21 | 0.54 | 256.5 | 36751.9 | 68.76 |
| At 366 nm- 5 µL | | | | | | | | | |
| 8 | 0.40 | 167.9 | 0.53 | 540.9 | 28.65 | 0.57 | 0.1 | 27400.0 | 41.63 |
| At 366 nm- 10 µL | | | | | | | | | |
| 9 | 0.52 | 304.8 | 0.54 | 759.8 | 22.24 | 0.57 | 1.3 | 13214.2 | 16.16 |

**Table 11:** FT-IR interpretation of Chrysin

| *Wavenumber ($cm^{-1}$)* | *Groups identified* |
|---|---|
| 2880-3200 | OH group |
| 1650 | C=O stretch |
| 3060-3084 | Aromatic C-H stretch |
| 1101-1246 | Phenyl group |

phase of toluene: n-hexane: isopropyl alcohol (7:2:1 v/v) provided a simple and reliable HPTLC fingerprint for quantification of Chrysin. The same $R_f$ values of Chrysin at both the concentrations for 254 and 366 nm confirmed the analysis to be accurate and authentic.

### *4. Fourier Transform Infrared (FT-IR) Spectroscopy of Chrysin-*

The FT-IR spectrum of Chrysin (Fig. 10) showed fingerprint peaks for -OH group, C=O stretch, aromatic C–H stretch and phenyl groups, characteristic of a flavonoid as interpreted in Table 11.

## IV. Characterization of Chrysin

### *1. Determination of Solubility of Chrysin by Partition Coefficient Method-*

The solubility of Chrysin by partition coefficient method was found to be 0.0048 ± 0.0014 mg/mL and 8.345 ± 0.004 mg/mL in water and oil phases, respectively. The partition coefficient of Chrysin was found to be 3.236. This value indicated that Chrysin was highly lipophilic in nature.

### *2. Differential Scanning Calorimetry of Chrysin-*

The thermal behavior of Chrysin was depicted in its DSC thermogram, which showed a sharp endothermic peak (Fig. 11). The melting point range was found to be 288–293.1°C, which complied with the standard thermogram.