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Research Article

HPTLC Comparision Studies with Marker Compound and Isolation of Rutin Anti Inflammatory Agent from Ethanolic Leaf Extract of *Rivea Hypocrateriformis (Desr.)* Choisy

Shanthini Nachiar G, Thirumal M*

ABSTRACT

Introduction: To extract the leafy parts with ethanol and perform the HPTLC studies with marker compounds Quercetin, Rutin, and gallic acid then a compound was isolated and susceptible to spectroscopic analysis **Background:** *Rivea hypocrateriformis* (Desr.) Choisy holds Convolvulaceae family the leafy parts underwent a Soxhlet extraction with ethanol which was then subjected to a phytochemical investigation that shows the presence of alkaloids, phenols, flavonoids, terpenoids, tannins, glycosides saponins, sterols, and carbohydrates. **Material and methods:** Firstly, the ethanolic leaf extract is treated with petroleum ether to remove excess lipids, sterols, and impurities. TLC was performed by toluene: ethyl acetate: formic acid and the same solvent system was used for column chromatography. The bands getting separated into the column were collected separately in a test tube. Once the solvent toluene ends, the next solvent system with 18 ml of toluene and 2 ml of ethyl acetate, is introduced into the column, and continues the separation procedure. Collect separated bands and perform TLC of each separate band by using the same solvent system in a concentration of 5:4:1. A single component on a TLC plate with a solvent system of 12 ml toluene and 8 ml ethyl acetate was obtained whose characterization was done by IR, NMR, and mass. **Results:** The RF value of Rutin is 0.17 which correlates with the Retention factor of ethanolic leaf extracts of RH by HPTLC comparative studies and a compound was isolated by column chromatography. **Conclusion:** These studies have concluded that a compound is separated by means of HPTLC and spectroscopic interpretation and identified as Rutin.

Key words: HPTLC studies, Marker compound, Column chromatography, *Rivea hypocrateriformis* ethanolic leaf extract, TLC plates.

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INTRODUCTION

Rivea hypocrateriformis is a Traditionally useful medicinal plant available in the southern region, with its therapeutic values. These leaves consist of markable phytoconstituents such as active compounds like flavonoid, terpenoids, phenol, and glycosides which have been used to treat a wide range of infectious diseases, cancer, and neuropathy in the olden days the root parts have been used to treat snake bites and poisonous insect bites it is the creeper variety of plant with the velvety texture of leaves. The tribal people, karakas used to eat these leaves freshly due to their extensive nutraceutical value by means of LC-MS and GC-MS lot of constituents had been screened out, among these bergeninie, isobergenine, phenolic compounds, have been reported in the stem part. So far, a wide variety of compounds are identified from *Rivea hypocrateriformis. Rivea hypocrateriformis* was grown throughout India, it requires cold environmental conditions, and the leaves bloom in the evening with a floral nostalgic scent smell. One of the widely used active compounds present within the *Rivea hypocrateriformis* leaves are flavonoids, and phenols both play a vital role in curing many disease ailments. Flavonoids are commonly known as phenyl benzopyrones and phenyl chromones it consists of low molecular weight substances which are widely distributed among most Plants. *Rivea hypocrateriformis* leaf extract consists of flavonoids, flavanol, bioflavonoids, terpenoids, and phenolic compounds (proansamitocin, symlandine, sphingosine, cochlearine,6 C-Glucopyranosylpilloin, Artelastochromene,) and also linked with kaempferol attached to 3-rhamnosides, 3-rutinosides, or p-coumaric esters. These leaves were called Phang, they had been used to make traditional dishes in Pakistan and Afghanistan, they are used to treat nervous, and sexual disorders and also to cure infertility. Flavonoids play a vital role in curing all ailments in the human body. They are widely distributed among plant species. Phytonutrients like Rutin act as an antioxidant in curing hemorrhoids, autism, aging skin, and airway infections even beneficial in treating inflammation and swelling in joints. Flavonoids are derived from the Latin word *flavus*, which means yellow in color they are the class of polyphenolic secondary metabolites widely distributed in angiosperm and gymnosperm varieties of plants and also commonly found in the diets of humans.[1-10]

MATERIAL AND METHODS

Shimadzu UV Visible spectrophotometer, Model 1800, FTIR, Toluene, ethyl acetate, Formic acid, and 10% aluminum chloride,1M potassium acetate and Standard Rutin.

Plant material

The plant *Rivea hypocrateriformis* Choisy (Desr) was collected from the bharathiyar university campus at Coimbatore during the month of

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July and was authenticated by Taxonomist. The leaves are washed thoroughly to remove the debris and impurities and then dried in shade and powdered in coarse form using (sieve no10/40). By means of the cold maceration process, the extracts are prepared with ethyl acetate, petroleum ether, hexane, chloroform, ethanol, and water and subjected to the following phytochemical screening tests, which show a presence of sterols, carbohydrates, coumarin glycosides, flavonoids, and phenolic compounds.

The estimation of total flavonoid content present can be determined by linear regression analysis in table 1 and figure 1 was expressed in mg of rutin equivalents to gms of extract. HPTLC studies were performed with marker compounds like rutin, quercetin, and gallic acid with a wavelength of 200 to 600nm and the start RF value is 0.06 to 0.97 and the results are tabulated. The marker compound Rutin at 333 nm and 362 nm shows the RF value as 0.17 which was compared with the ethanolic leaf extract of *Rivea hypocrateriformis* that gives a 0.16 RF value correlates with the marker compound represents in figure 4 and figure 5.

Procedure for total flavonoid content estimation

An aliquot quantity of quercetin was dissolved in ethanol to get a stock solution of 1mg/mL. Further dilutions were made to get concentrations ranging from 20-100µg/mL. 1ml of the above standard solutions was taken in different volumetric flasks, 0.1mL of aluminum chloride solution, 0.1mL of potassium acetate solution, and 2.8 mL of ethanol were added and the final volume was then made up to 5mL with distilled water. After 20 min the absorbance was measured at 415nm. A sample without aluminum chloride was used as a blank. A calibration curve was constructed from the absorbance obtained by plotting the concentration versus absorbance of quercetin (Figure 1). 1mL of methanolic extract at concentrations 40µg/mL and 80µg/mL were taken and the reaction was carried out as above the absorbance was measured at 415nm after 20min and the readings were tabulated in Table 1. The number of flavonoids present can be determined by linear regression analysis. The total flavonoid content was expressed as mg quercetin equivalents /g of extract.

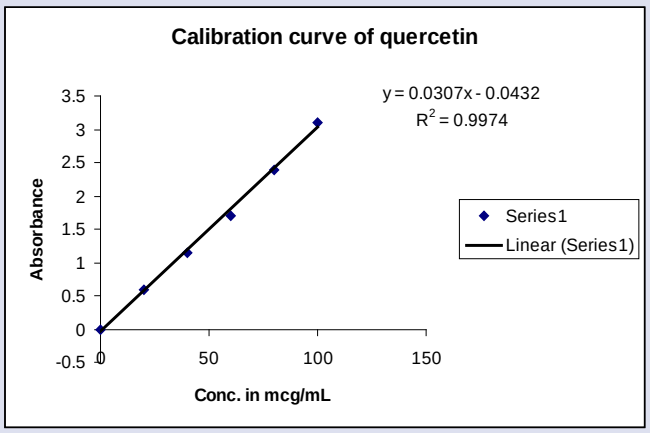


Figure 1: Calibration curve of quercetin

Isolation of flavonoid by column chromatography

The column was prepared by mixing a sufficient amount of silica gel with Toluene, the slurry mixture was transferred into the glass column, and waited until the adsorbent settles down. Cotton was plugged into the top of the column to remain undisturbed while the sample and mobile phase instigate into the column. 20 gm of ethanolic extract of *Rivea hypocrateriformis* was mixed with silica and transferred into the column and should remain undisturbed for half an hour then transfer 20 ml of toluene was into the column and allow to drain a solvent through the column at a speed of 20 drops per minute. Observe bands separately and separated bands were collected separately into a test tube. once 20 ml solvent toluene come to an end add the next solvent system containing 18 ml toluene and 2 ml of ethyl acetate and pour into the column and continue the separation procedure. Collect separated bands and TLC was performed for each separated band by using the same solvent system toluene: ethyl acetate: formic acid in a concentration of 5:4:1 and confirm for a single component on the TLC plate. In this solvent system with 12 ml toluene and 8 ml ethyl acetate we get a separated spot[11-19] whose characterization was done by IR, NMR, and mass to confirm the spot represents at Figure 8,9,10.

PURIFICATION AND IDENTIFICATION OF MIXTURE OF COMPOUNDS BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY

In this process, a plate of 20cm X 20cm is taken, cleaned, and dried. In another beaker take a sufficient qty of silica gel for TLC to it add a

Table 1: Total flavonoid content of quercetin Eq/gm of extract.

STANDARD		ABSORBANCE AT 415 nm	TEST		ABSORBANCE			TOTAL FLAVONOID CONTENT
Sample	Conc.in mg/ml		Sample	Conc in mg/ml	I	II	III	mg of quercetin Eq/gm of extract
Quercetin	2	0.589	EERH	100	0.217	0.220	0.206	84.07 ± 1.21
	4	1.151						
	6	1.710						
	8	2.390						
	10	3.112						

Mean of three readings ± SEM
EERH- Ethanolic extract of *Rivea hypocrateriformis*

Table 2: Bands at different ratios of solvent system.

Sr. No	Toluene in ml	Ethyl acetate in ml	Bands appear	Rf
1	20	00	1	
2	18	02	2	
3	16	04	0	
4	14	06	0	
5	12	08	1	0.92
6	10	10	2	
7	08	12	0	

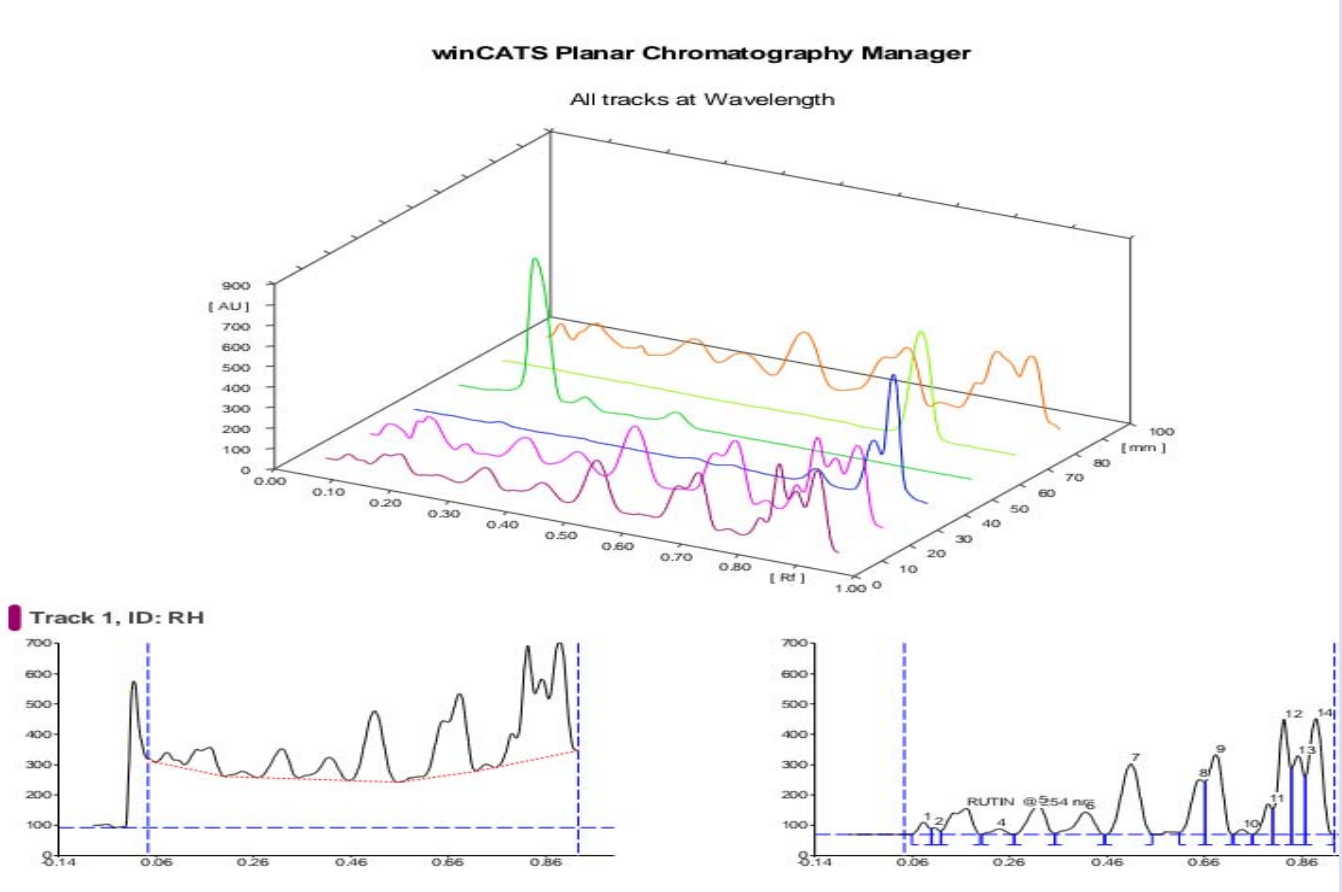


Figure 2: Tracks of the peak at wavelength 200 to 400 nm

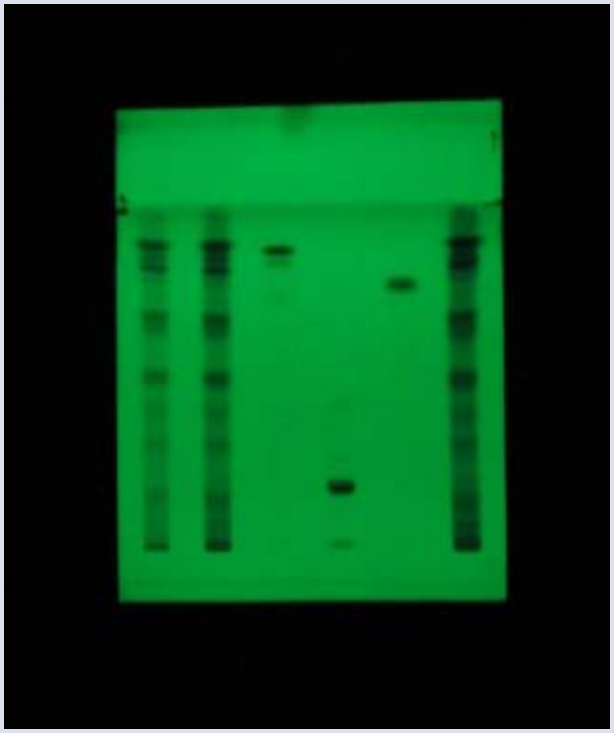


Figure 3: Sequence of the sample

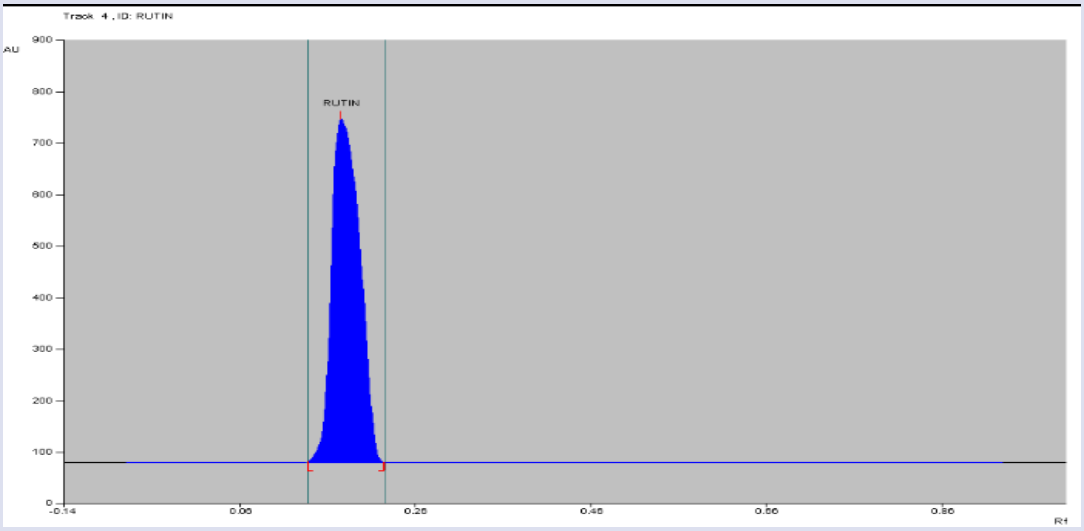


Figure 4: Marker compound Rutin at 362nm with RF value 0.17

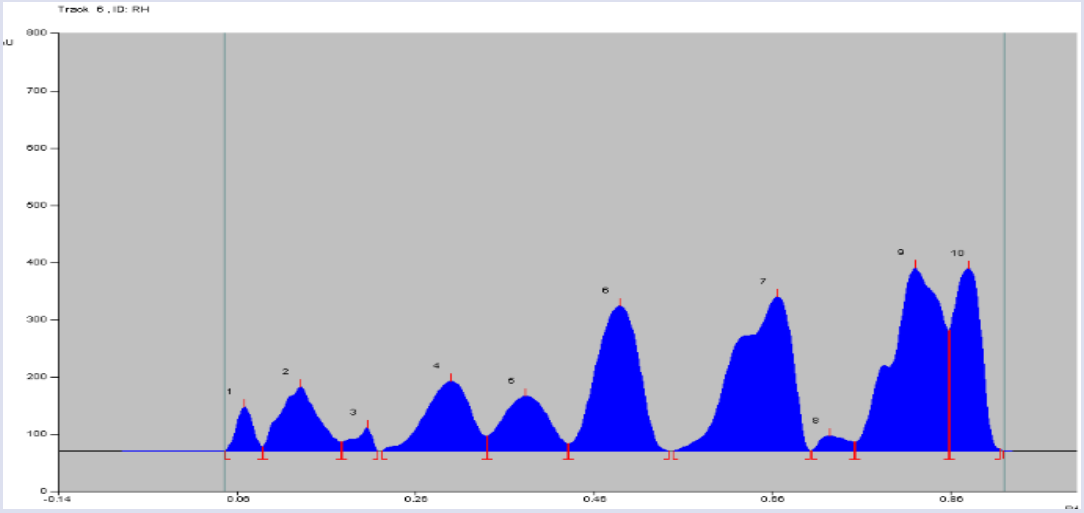
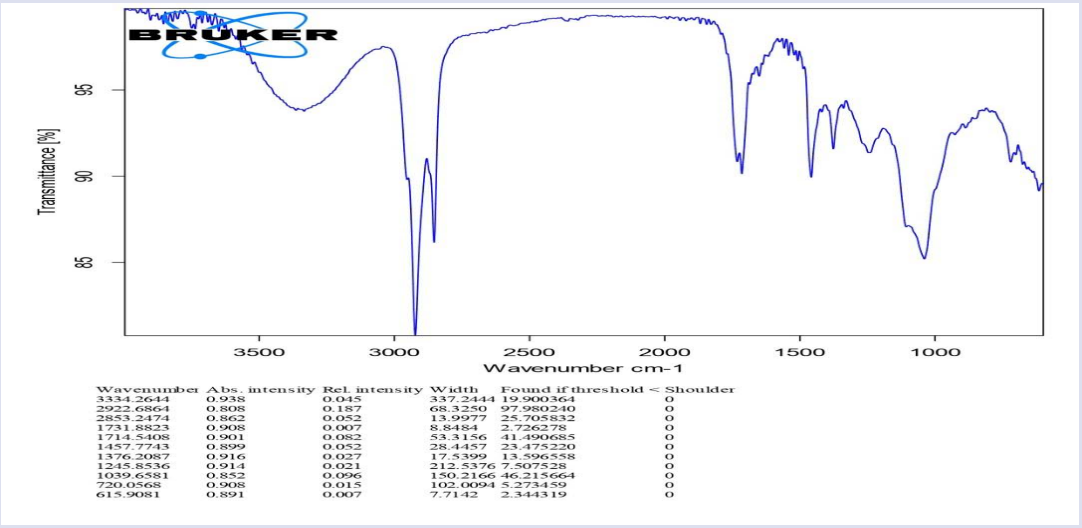


Figure 5: Peaks of RH plant extracts at different concentrations

Figure 6: Preparative TLC and the spot observed at UV chamber at longer wavelength

Figure 7: CO-TLC with rutin standard



Wavenumber	Abs. intensity	Rel. intensity	Width	Found if threshold <	Shoulder
3334.2644	0.938	0.045	337.2444	19.900364	0
2922.6864	0.808	0.187	68.3250	97.980240	0
2853.2474	0.862	0.052	13.9977	25.705832	0
1731.8823	0.908	0.007	8.8484	2.726278	0
1714.5408	0.901	0.082	53.3156	41.490685	0
1457.7743	0.899	0.052	28.4457	23.475220	0
1376.2087	0.916	0.027	17.5399	13.596558	0
1245.8536	0.914	0.021	212.5376	7.507528	0
1039.6581	0.852	0.096	150.2166	46.215664	0
720.0568	0.908	0.015	102.0094	5.273459	0
615.9081	0.891	0.007	7.7142	2.344319	0

Figure 8: Infrared spectroscopy analysis

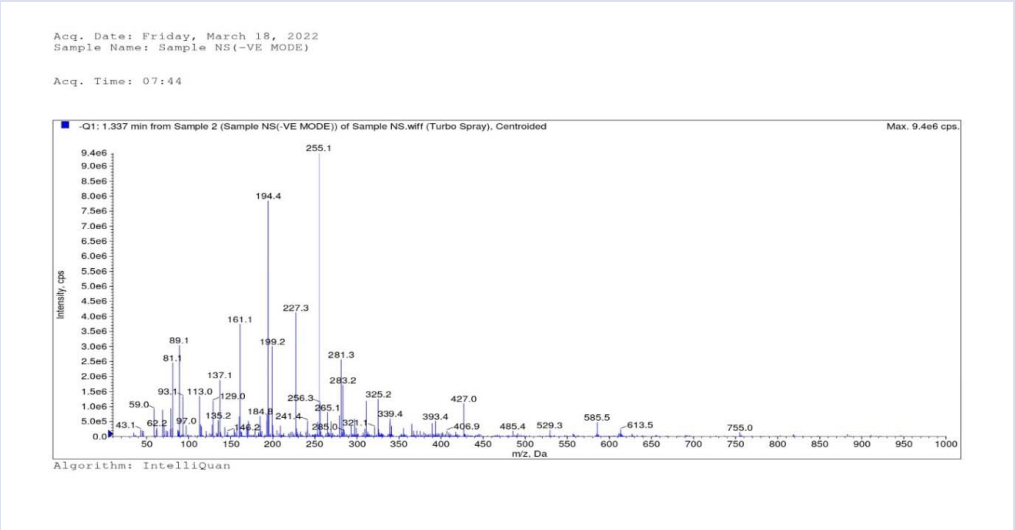
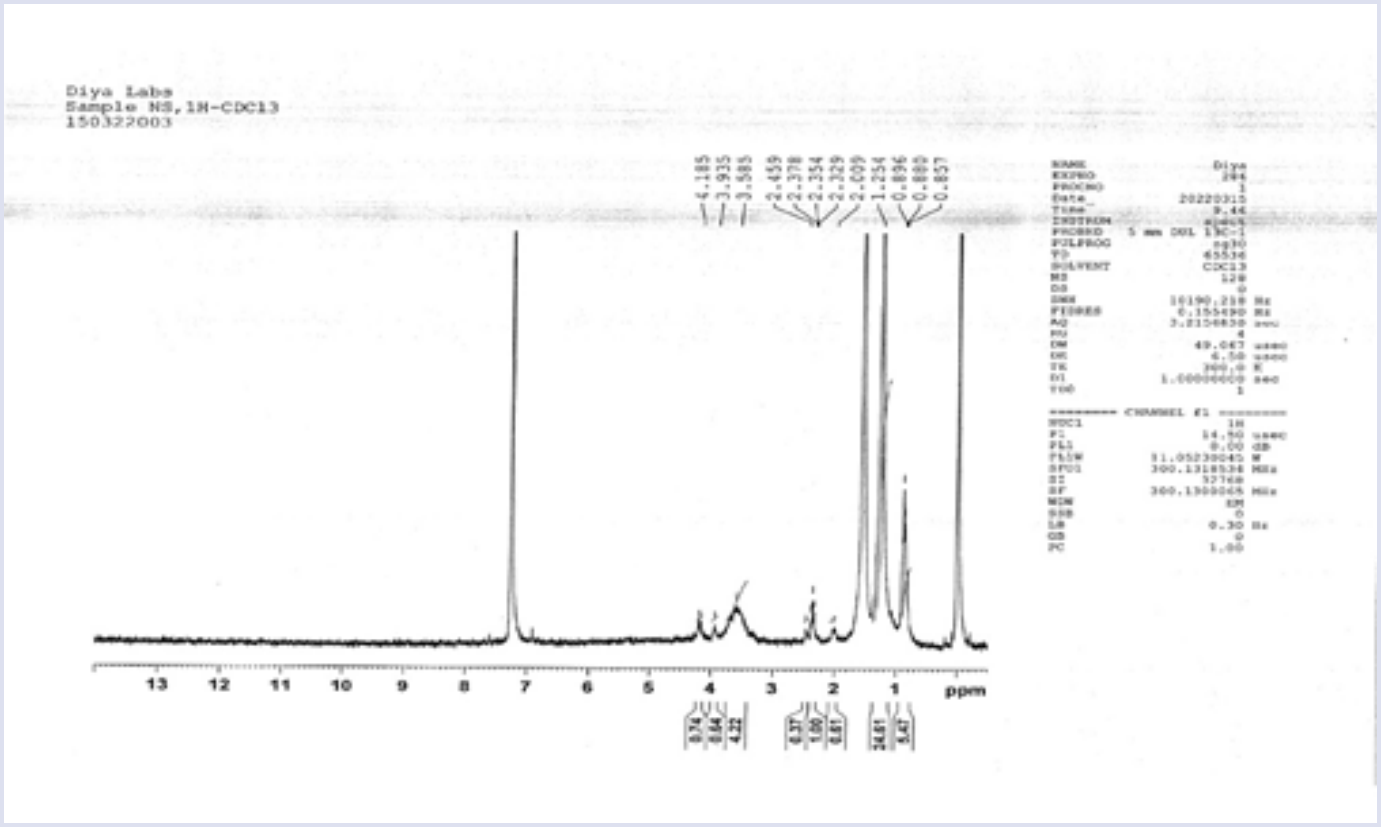


Figure 9: Mass spectroscopy


Diya Labs
Sample NS,1H-CDCl3
150322003
4.185
3.935
3.585
2.459
2.378
2.354
2.329
2.009
1.254
0.896
0.880
0.857
13 12 11 10 9 8 7 6 5 4 3 2 1 ppm
0.74
0.64
4.22
0.37
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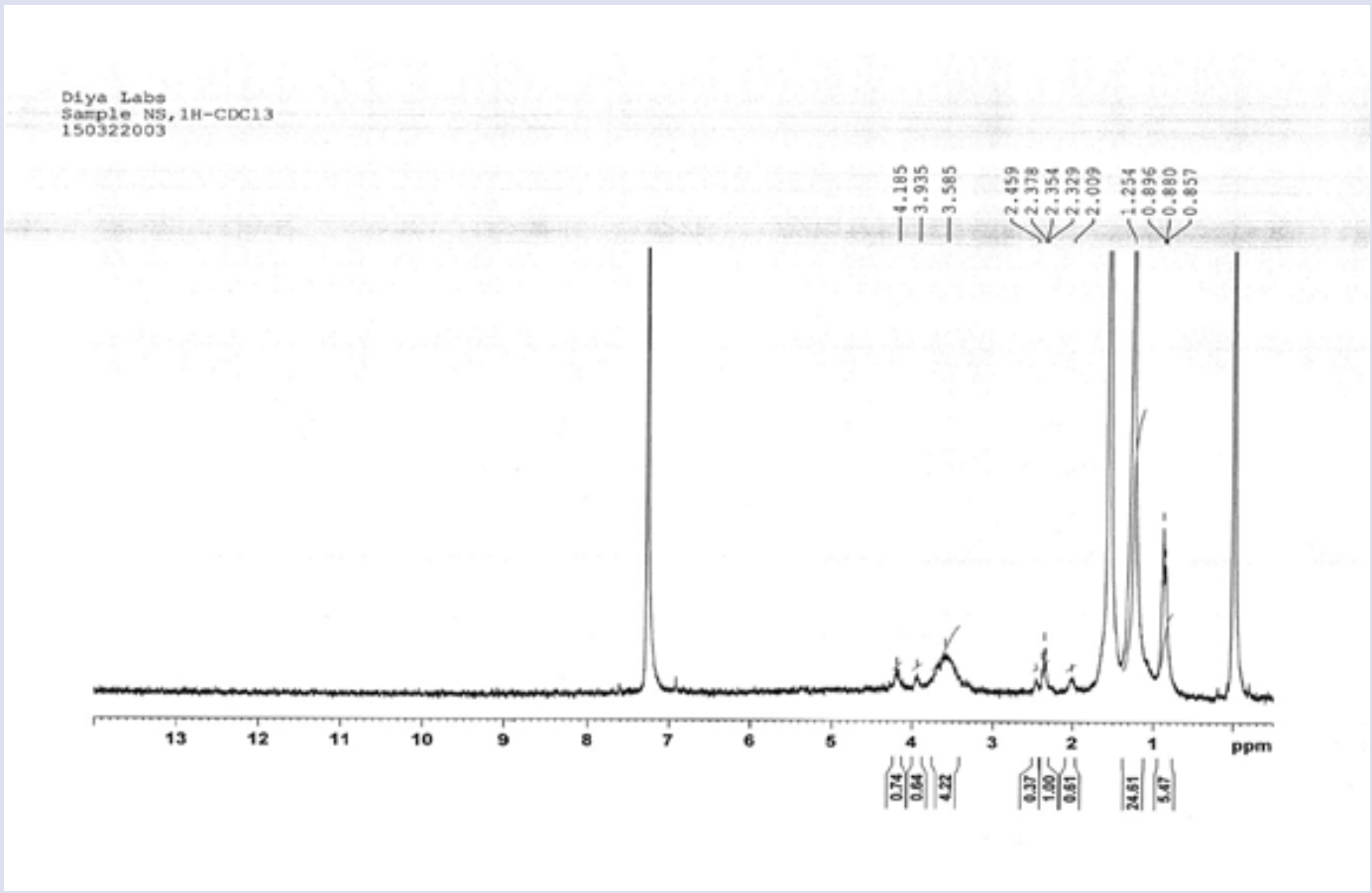
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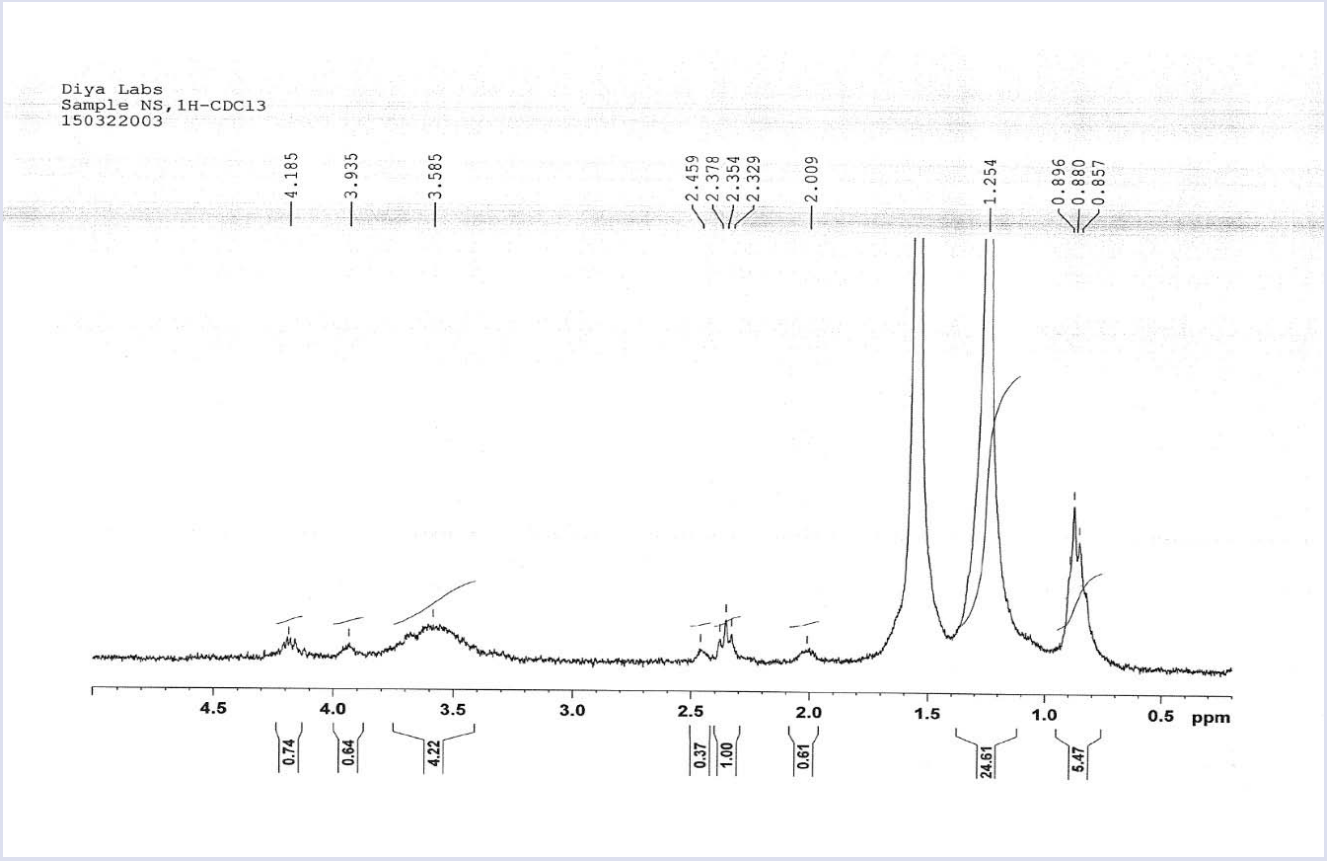

Figure 10: HNMR spectorscpopy

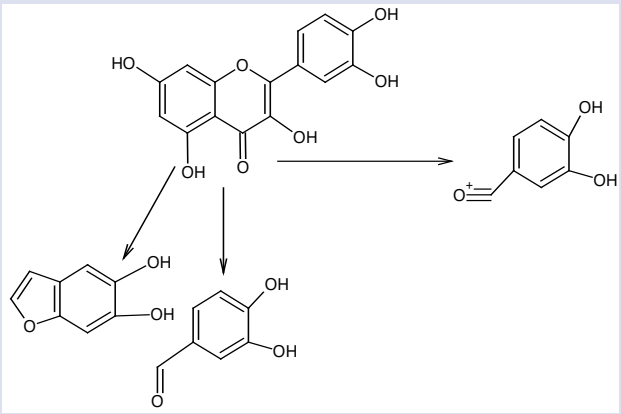

Figure 11: Fragment of a compound Rutin

sufficient qty of methanol and stirred at optimum speed so as to have uniform mixing then spread this slurry on a declined slide so as to spread silica all over the slide then dry slide in the oven at 80^{0}C temp. dissolve the extract in a sufficient quantity of methanol and tale in the prepared capillary and apply a spot on the dried TLC plate at a constant height from the bottom near about 1.5cm. Take a big chamber for TLC in that add solvent system toluene: ethyl acetate and formic acid at a proportion of 50:40:10 place a filter paper at one side in chambers so as to raise the solvent system on paper and maintained uniform saturation by closing the chamber after 20 min. the plate is placed in a chamber and allowed to run 80%of the total length of the plate. After attaining 80% height remove the plate from the chamber and dry it. And observed under UV light at a long wavelength and mark the observed corresponding spot and remove the plate from UV light and then scrapped all the marked spots on a plane paper dissolved the compound in ethanol and filtered through Whatman filter paper and dry the solvent to get an isolated spot.[20-30]

HPTLC equipment

A CAMAG HPTLC system comprising of a Linomat-5applicator and CAMAG TLC Scanner-3 and single pan balance of Shimadzu model was used, for weighing the samples.

Preparation of standards and extracts from the herbal raw materials

One gram of dried leaf powder of *Rivea hypocrateriformis* material was taken and sonicated with 10ml of ethanol. Filtered and the filtrate solution was used for HPTLC analysis. Standard marker compounds were prepared using ethanol to get a concentration 1mg/1ml.

Application of sample

The sample solutions were spotted in the form of bands of width 6mm with a Hamilton 100µl syringe on precoated plate 60 F254 (10cm × 10cm with 0.2mm thickness, E. Merck) using a Camag Linomat V applicator. The slit dimension was kept 6mm × 0.45mm. Eight µl of each sample and five µl of standard solutions were applied on to the plate. The migration distance was 80mm. TLC plates were dried with air dryer. Densitometric scanning was performed using Camag TLC Scanner-3 at 254nm and 366nm operated by a Wincat software.

Development

The chromatogram was developed in CAMAG glass twin-trough chamber (10-10cm) previously saturated with the mobile phase toluene: ethyl acetate: formic acid: ethanol [3:6:1.6:0.4] for 10 min

(temperature 25°C, relative humidity 40%). The development was done for 8cm from bottom.

Detection

The plate was scanned at UV 254 and 366nm using CAMAG TLC Scanner-3 and LINOMAT-V. Rf value of each compound which were separated on plate and data of peak area of each band was recorded.

RESULTS AND DISCUSSION

Estimation of total flavonoid content from ethanolic leaf extract of *Rivea hypocrateriformis*

The total number of flavonoid content was measured as mg of quercetin Eq/gm of the extract. Different concentration of the sample was measured at absorbance 415nm and compared with 100mg/ml of RH extract and tabulated in the Table:1 and expressed in the graph as absorbance vs concentration of mg/ml RH extracts in figure:1

HPTLC studies with marker compound Rutin, Quercetin, and Gallic acid

The plant extract is subjected to HPTLC studies with marker compounds such as rutin, quercetin, and Gallic acid, six tracks were runned at a particular wavelength of which three tracks were RH plant extracts, by using the solvent system toluene: ethyl acetate: formic acid at ratio 3:6:1.6.The wavelength was set up between 200nm to 400nm, RF value designed from 0.09 to 0.83, following peaks are obtained from the sample tracks when compared with the marker compounds mentioned in figure:2,3,4 and 5. The RF value of RH plant extracts matches the RF value of rutin at wavelength 362nm.

Isolation of flavonoid from ethanolic leaf extracts of *Rivea hypocrateriformis* by column chromatography

The following solvent system toluene: ethyl acetate for the separation of a compound band appeared with solvent system Toluene: Ethyl acetate 12:8 at 0.92 as RF value then subjected to preparative TLC by means of spectroscopic interpretation and analysis the fragment found was Rutin which was tabulated in Table 2 and figure 6 represents the preparative TLC and spot identified at UV chamber.

These compounds were further characterized by IR, MASS, and NMR Spectroscopical interpretation.

FT-IR (cm^{-1})

The alcoholic O-H stretch is usually a broad IR band with strong intensity at 3334.26cm^{-1}, aromatic C-H- stretch at 2952.68 cm^{-1}, methyl group stretch at 2853.24 cm^{-1} carbonyl group at 1731.88 cm^{-1}, carbon-carbon bend at 1245.85 cm^{-1}, -C-O-C- stretching frequency at 1039.65 cm^{-1}. following groups are mentioned with their frequency.

3334.26 (-OH str.)

2952.68 (Ar-CH str.)

2853.24 (-CH3 str.)

1731.88 (-C=O str.)

1245.85 (-C=C bend)

1039.65 (-C-O-C str.)

^{1}H-NMR (ppm)

^{1}H-NMR spectrum was obtained on Bruker Advance 400, spectrometer (Bruker, Rheinstetten, Germany) with standard pulse sequences operating at 400 MHz in P1 ^{1}H-NMR. Chemical shifts are shown in δ values (ppm) using a DMSO as a solvent and tetramethyl silane (TMS) as an internal standard. The ^{1}H-NMR spectra show the dominance of signals in the aliphatic region (e.g., methyl proton peak at 0.9 ppm, methylene protons [CH_2] n peaks in the region of 1.2–3.5 ppm). Particular glycosides (e.g., anomeric hydrogen at 4.0 ppm) and some of the aromatic moieties at (7.0-7.2 ppm) are presented in spectra.

MS spectra

(m/z): m/z 613.5 [C27H30O16] + [M + H]+, annotated as rutin; with the loss of –CH2-CH2 m/z 585.5; loss of glycoside will give m/z 427.0; loss of another glycoside will give m/z 281.3; with the loss of 2 –OH m/z 227.3;[30-37] Figure 11.

CONCLUSION

The fragment obtained by interpretation is considered to be as rutin it plays a vital role in curing many diseases occurring in the human body. They are widely distributed among plant species. These had a wide benefit in skin diseases like atopic dermatitis, eczema, and psoriasis and have beneficial anti-inflammatory effects and they protect the cells from oxidative damage that can lead to disease. However, the compound Rutin was isolated and characterized. Rutin has powerful antioxidant properties and many health benefits; rutin cannot be used efficiently because of its high price of the product.[42-44] Nowadays many herbal plants have been formulated and used for their therapeutic value because of various kinds of phytoconstituents which has been a challenging job for new researchers pursuing their studies in traditional plants considering their safety and efficacy.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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ABBREVIATION

HPTLC: High performance liquid Chromatography, UV: UltraVisible Spectroscopy, EERH: Ethanolic extract of *Rivea hypocrateriformis*, IR: Infra-red spectroscopy, MS: Mass spectroscopy, H-NMR: High resonance nuclear magnetic spectroscopy, RF: Retention Factor, Rh: *Rivea hypocrateriformis*

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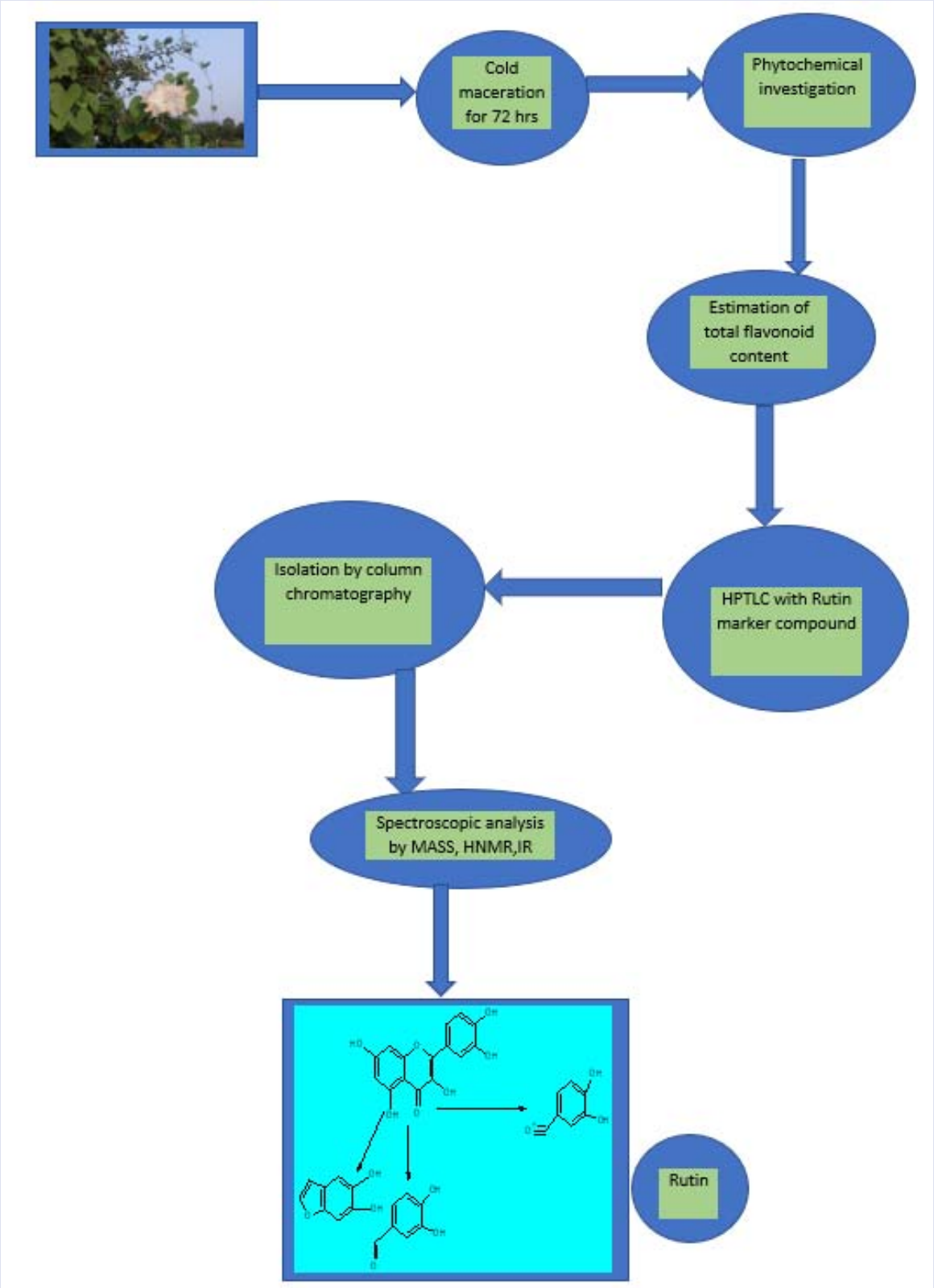
GRAPHICAL ABSTRACT



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