Isolation and Characterization of an isolated flavonoid from *Averrhoa bilimbi*

Abstract

Averrhoa bilimbi is a multipurpose, long-lived tropical plant commonly known as Bilimbi. The plant has an enormous fiscal value since most of the parts like leaves, bark, flowers, fruits, seeds, roots or the whole plant are used as alternative medicine to treat a variety of diseases. In the present work attempt was made to isolate a flavonoid compound from *Averrhoa bilimbi*. From the methanolic extract of the fruits of *Averrhoa Bilimbi*, a pentahydroxyl flavanonol has been isolated as a major compound for the first time in this plant. The isolate was purified, analyzed and characterized by using UV, FTIR, Mass, NMR, HPTLC and HPLC. The Rf value for HPTLC was found to be 0.24, λ max of UV spectra was obtained at 277 nm and retention time in HPLC was 2.55. The structure of this isolated compound has been characterized as dihydromyricetin i.e (2*R*,3*R*)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one with molecular formula $C_{15}H_{12}O_8$ and molecular mass 320.0529. The structure is established on the basis of 1D and 2D Nuclear Magnetic Resonance (NMR) as well as High Resolution Mass Spectral (HRMS) data.

Key words: *Averrhoa bilimbi*, HPTLC, HPLC, UV, and FTIR

1. Introduction

Averrhoa bilimbi belonging to the family Oxalidaceae. It is a small tree up to 15 meters high. Fruits are fairly cylindrical with five broad rounded longitudinal lobes, and produced in clusters. During maturity stage occurs the maximum increase in fruits weight and dimensions, and their external green colour changes into light yellow. The fruits of *A.bilimbi* possess antibacterial, antiscorbutic, astringent and postpartum protective properties. The decoction of the leaves is being used as medicine for treating fever, inflammation of the rectum, diabetes, mumps and pimples. The paste of leaves is being used for the treatment of itches, boils, rheumatism, cough and syphilis. The juice of preserved fruits is being used for the treatment of scurvy, stomach ache, bilious colic, whooping cough, and hypertension. Moreover, the syrup of flowers is being given to treat children's cough. The plant is known for its antidiabetic, antihyperlipidemic and

antibacterial activity [1,2]. The fruit extracts contain Saponins, Flavonoids, and Triterpenoid. The chemical constituents of A. bilimbi includes Amino acids, citric acid, cyanidin–3–O–h–D–glucoside, phenolics, potassium ion, sugars and Vitamin A [3]. This work has been carried out in the view to isolate flavonoids from the methanolic extract of the fruits of *Averrhoa Bilimbi.*

2. Materials and Methods

2.1.Procurement of Plant Material

The whole plant was collected from Palakkad, Kerala in South India. The specimen was identified by Prof. V. Dhanapal, Professor, Department of Pharmacognosy, Sanjo College of Pharrmaceutical Studies, Palakkad, Kerala. A voucher specimen was prepared in our laboratory and maintained with voucher no. PPG/DPC/01, for further reference. Immediately after collection, the fruit was washed thoroughly with water and then sliced, shade-dried at room temperature. The sliced fruit was then pulverized to form a coarse powder and used for extraction.

2.2.Preparation of fruit extract

250 gm sample is taken in a thimble and kept it in a soxhlet apparatus. It is consecutively extracted with 250 ml of each petroleum ether, hexane and methanol till the extraction is complete. The methanolic extract was used for the screening and isolation of compound [4].

2.3.Preliminary phytochemical screening

Qualitative phytochemical tests (colour reaction) were performed in methanolic extract to determine the presence of major classes of phytochemicals such as alkaloids, flavonoids, tannins, amino acids, phenolic compounds and triterpenes which were then further confirmed by thin layer chromatography.

2.4.Detection of phytoconstituents by TLC

The spotted TLC plates were developed using different mobile phases to detect the various classes of phytochemicals. The proportion of the chemicals in the mobile phases is as follows. Linear ascending development was carried out in 10 x 10 cm twin trough glass chamber equilibrated with mobile phase. The optimized chamber saturation time for

mobile phase was 20 minutes at 25±2 with a relative humidity of 60±5%. Ten millilitres of the mobile phase (5 ml in trough containing the plate and 5 ml in other trough) was used for the development and allowed to migrate a distance of 85 mm from the point of sample application. After development, TLC plate was dried and the chromatogram was viewed at 254 nm and 366 nm to visualize and detect various phytochemical constituents [5].

Table 1. Protocol for detection of various compounds by TLC

S. No.	Compounds	Mobile Phase	Visualizing Agent
1.	Alkaloids	Toluene: Methanol: Diethyl amine (8:1:1)	Dragendorff reagent
2.	Flavanoids	Toluene: Ethyl acetate: Formic acid (7:3:0.1)	NP/PEG Reagent
3.	Tannins	Ethyl acetate: Acetic acid: Ether: Hexane (4:2:2:2)	Fast Blue Salt B
4.	Triterpenes	Toluene: Chloroform: Ethanol (4:4:1)	Anisaldehyde sulphuric acid
5.	Amino acids	1-Butanol: Acetic acid: Water: Formic acid (28:9:8:2)	Ninhydrin
6.	Essential oil	Toluene: Ethyl acetate (8.5:1.5)	Anisaldehyde sulphuric acid

2.5.Screening of phytochemical groups using HPTLC

HPTLC is a flexible, reliable, and cost-efficient separation technique ideally suited for the analysis of botanicals and herbal drugs. A Camag HPTLC instrument consisting of Linomat V automatic spotter equipped with a 100 μL syringe connected to a nitrogen cylinder, Scanner-III, twin-trough developing chambers, and viewing cabinet with dual wavelength UV lamps (Camag, Muttenz, Switzerland) were used. HPTLC plates used were of aluminium backed silica gel 60 F_{254} with 0.2mm thickness. Before analysis, HPTLC plates were cleaned by predevelopment with methanol and activated at 110 for 5min for solvent removal. Specific mobile phases were used for each phytochemical [6-8].

2.5.1 Sample application

Sample was spotted on pre-coated TLC plate in the form of narrow bands (8 mm) with 10 mm from the bottom and at least 15 mm from left and right edges of the plate using Linomat V spotter. Samples were applied under continuous dry stream of nitrogen gas at constant application amount 10 µl. HPTLC was performed using mobile phase Toluene:

Ethyl acetate: Formic acid (7:3:0.1) on precoated silica gel 60 F254 plates as stationary phase.

2.6 HPLC

Thermo HPLC system consisted of Quaternary gradient pumps (LC – 10ATvp); Photodiode Array (PDA) detector (SPD – M10Avp) with built-in system controller was used. The analysis was performed on a 250 x 4.6 mm, 5 µm particle size CNW, Athena C18-WP column. The data acquisition was done on ChromQuest 5 software. The isolated compound from HPTLC was analyzed by using Methanol: Water: Acetonitrile (40:40:20) as mobile phase and UV detector set at 254 nm. The injection volume was 20 µl, flow rate was 1 ml/min and run time was 10 minutes. The retention time of the fruit extract was compared with that of the isolated compound acting as the reference standard.

2.7.Isolation of flavonoid compound from methanolic extract

A specific phytochemical compound with Rf 0.24 was identified in the screening of flavonoid compounds and that compound was selected for further study. Extract was subjected to repetitive HPTLC using aluminium backed silica gel 60 F_{254} as stationary phase (20 × 10 cm plates) and Toluene: Ethyl acetate: Formic acid (7:3:0.1 v/v/v) as mobile phase. A band under 254 nm at Rf value 0.24 was identified and were scraped. The compound was separated from silica gel by treating with methanol, filtered through Whatman filter paper, and filtrates were combined, concentrated, and dried. Isolated compound was subjected to HPTLC, HPLC, UV spectroscopy, IR spectroscopy, and LC MS.

2.8.Characterization of isolated compound[9]

2.8.1 UV Spectroscopy

The absorbance of the isolated compound was read using one cm cell in a UV – Vis - NIR spectrophotometer (Varian, Cray 5000, and Netherlands). The instrument have a spectral range of 175 nm to 3300 nm, wavelength accuracy of ± 0.1 nm (UV –Vis), ± 0.4 nm (NIR), Wavelength reproducibility of 0.025nm and a limiting resolution of 0.05nm(UV-Vis), 0.2nm(NIR).The maximum range of absorbance of isolated compound in the methanolic solution was noted by comparing it against HPLC grade methanol as a blank.

2.8.2. Fourier Transform Infra Red spectrometer (FTIR)

FTIR analysis was performed using Thermo Nicolet, Avatar 370 spectrophotometer system, which was used to detect the characteristic peaks and their functional groups. The Spectral range was between 4000-400 cm $^{-1}$ and resolution was 4 cm $^{-1}$ with KBr beam splitter, DTGS Detector and HATR Assembly for convenience of measurement. The finger print region extended between 400 – 1600 cm $^{-1}$. The spectrum of the isolated compound was elucidated against a blank of HPLC grade methanol.

2.8.3. LC-MS

The LC-MS system (Varian, USA-410 Prostar Binary LC with 500 MS IT) consisted of two pumps (LC-10ATvp), PDA detectors (SPD-M10Avp) and auto sampler (SIL-HTA) with built-in system controller. The analytical column was a C18, 250x4.6 mm ID, 5 µ particle size (Lichrospher 100 RP-18e, Merck, Germany) protected by a compatible guard column. For the characterization of isolated compound the HPLC method was same as that used in HPLC with CNW, Athena C18-WP column.

2.8.4 NMR/MS Studies

The 1D and 2D NMR spectral data were acquired using standard pulse sequences on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instrument instruments. The NMR spectra were performed in C2D6SO (d6-DMSO). The chemical shifts were given in δ (ppm), and coupling constants were reported in Hz. MS and MS/MS data were generated with a Thermo LTQ-FTMS mass spectrometer (100,000 resolutions) equipped with a Nano spray ionization source. The samples were diluted with methanol and introduced via infusion using the onboard syringe pump.

3. Results and Discussion

3.1.Preliminary Phytochemical Screening

Methanolic extract was subjected to preliminary qualitative tests for the detection of major phytochemical groups using standard protocols. The analysis revealed the presence of alkaloids, Flavanoids, Saponins, Triterpenoids, Tannins and Phenolic compounds. The results are presented in Table 2.

Table 2. Preliminary Phytochemical Studies of *Averrhoa bilimbi*

S. No.	Constituents	Test	Aqueous Extract	Methanolic Extract
1.	Alkaloids	Dragendroff's test	+	+

		Mayer's test	+	+
		Wagner's test	-	+
2.	Flavanoids	With 1% KOH	-	+
		With H_2SO_4	+	+
		Legal's test	-	-
3.	Amino acids	Ninhydrin test	+	+
4.	Triterpenoids	Salkowski's test	+	+
		Libermann's Burchard test	-	+
5.	Tannins and Phenolic compounds	FeCl3 test	-	+

3.2.Screening of phytochemical groups using HPTLC

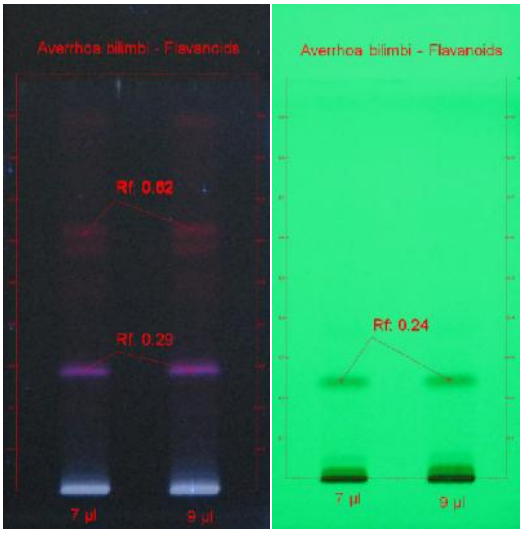
The results obtained from HPTLC analysis of the methanolic extract of *Averrhoa bilimbi* with respect to Alkaloids, Flavonoids, Tannins, Triterpenes, Amino acids and Essential oil are given below (Table 3).

Table 3. Rf Values of various phytoconstituents present in *Averrhoa bilimbi*

Sr. No.	Compounds	Rf Values
1.	Alkaloids	0.14, 0.45, 0.62, 0.91
2.	Flavanoids	0.24, 0.29, 0.62
3.	Tannins	0.74, 0.79
4.	Triterpenes	0.31, 0.37, 0.44, 0.47, 0.69, 0.89
5.	Amino acids	0.55, 0.64, 0.79, 0.86
6.	Essential oil	0.10, 0.12, 0.39, 0.53, 0.62, 0.68

3.3.HPTLC of flavonoid compound

Figure 1. HPTLC of the isolated flavanoid compound I

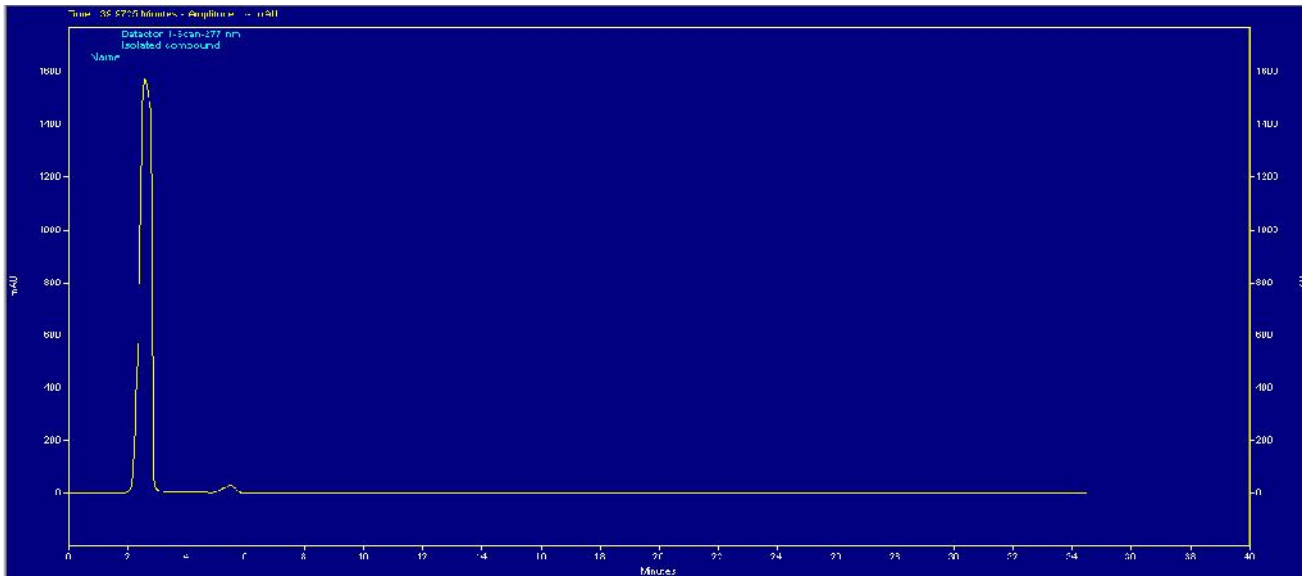


UV 366 nm UV 254 nm

3.4.HPLC of isolated compound

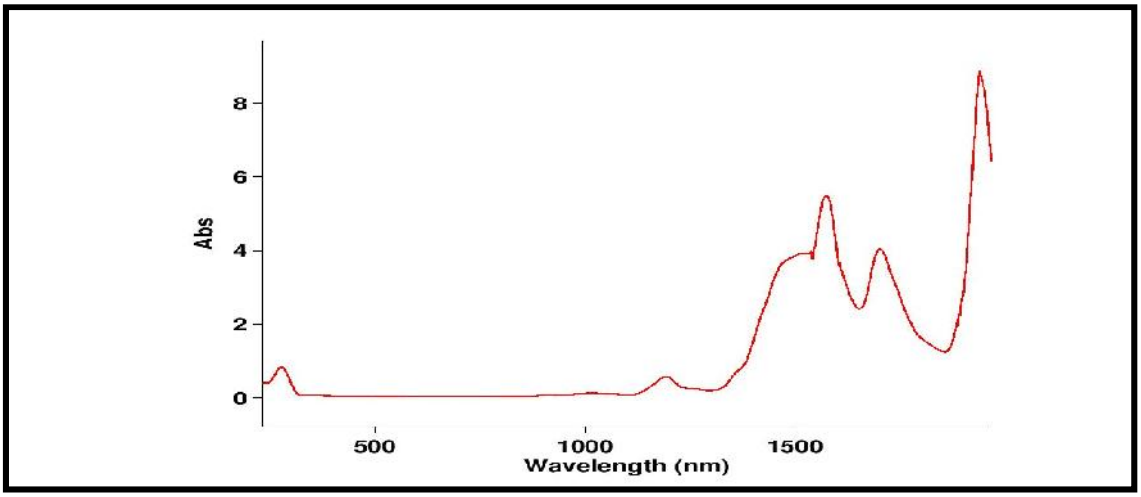
The highest sharp peak with 2.55 retention time is of isolated compound I.

Figure 2 HPLC chromatogram of isolated compound I



3.5.UV spectrum of isolated compound

Figure 3. UV Spectrum of isolated compound I



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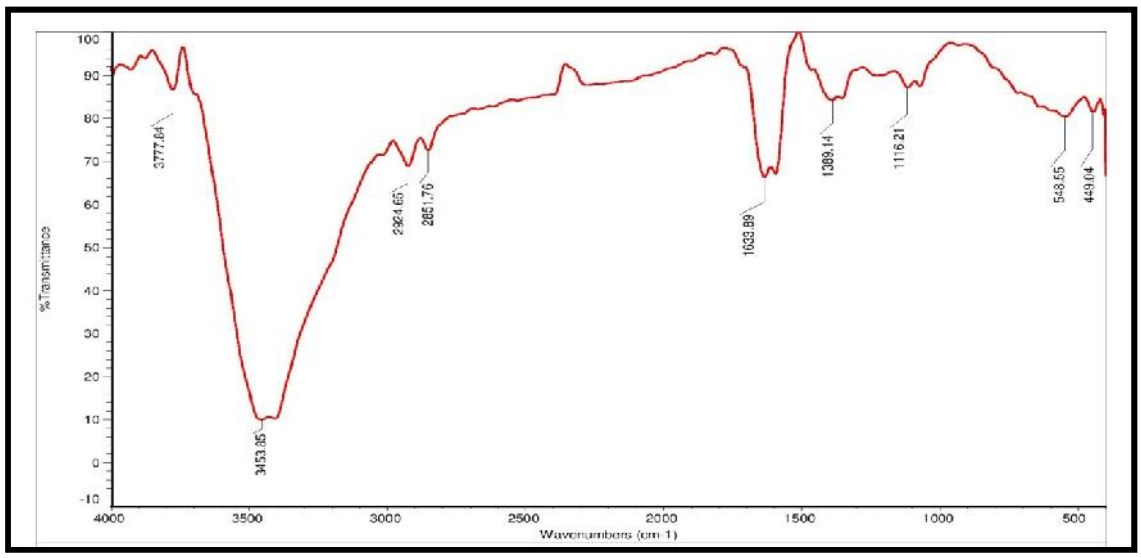
186 **3.6.IR spectrum of isolated compound**

187 Figure 4. IR spectrum of isolated compound I



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199 **3.7.LCMS of isolated compound**

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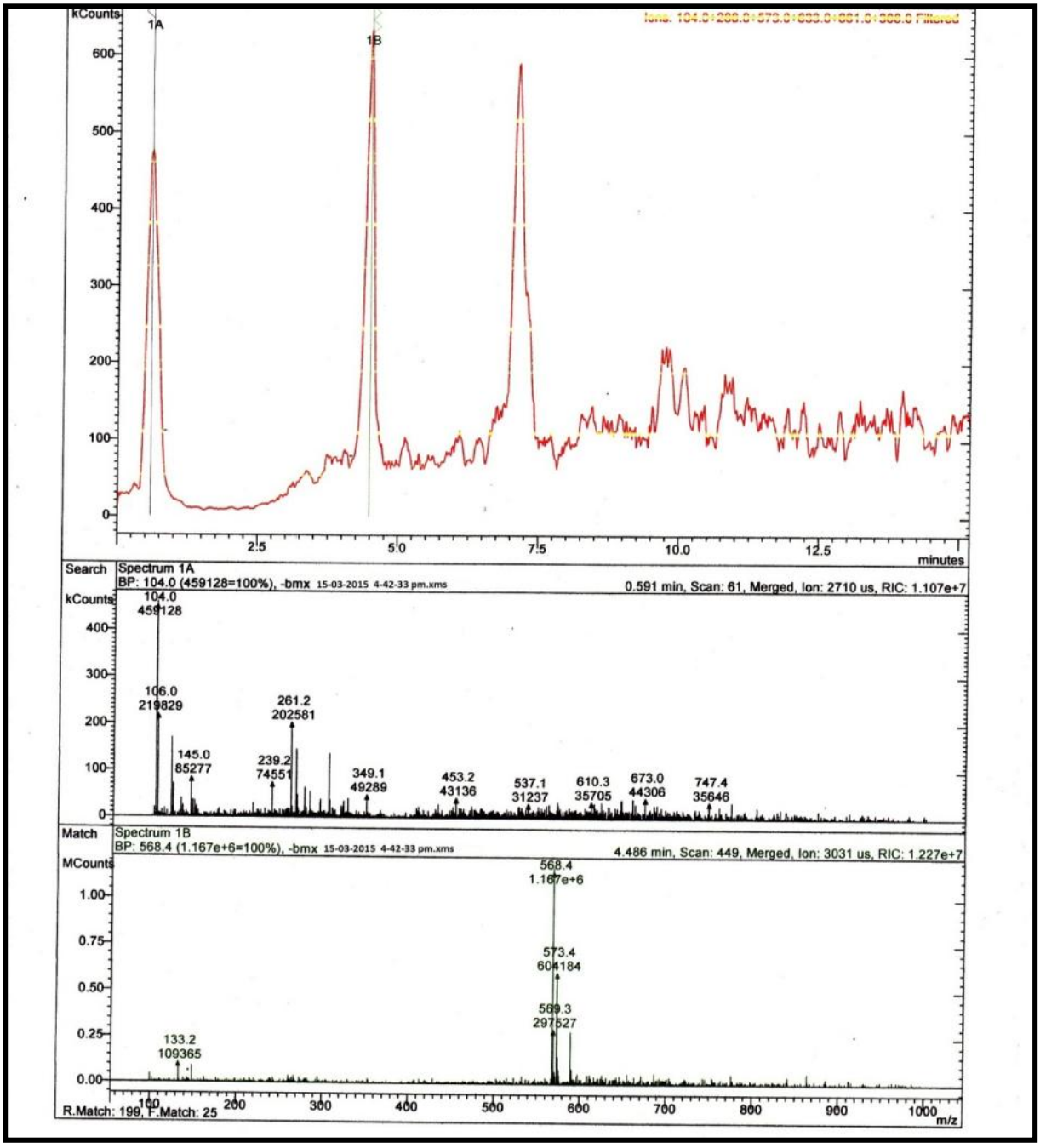
201 Figure 5. LCMS of isolated compound



3.8 NMR Spectra:

Identification and spectroscopic data of dihydromyricetin (5, 7, 3′, 4′, 5′-pentahydroxyl flavanonol, 1) Off-White powder; 1H-NMR (600 MHz, d6-DMSO, δ ppm) and 13C-NMR (150 MHz, d5pyridine/d4-methanol/d6-DMSO, δ ppm) spectroscopic data see Table 3a; HRMS (M+Na) + m/z 343.0426 (calcd. for C15H12O8Na: 343.0424).

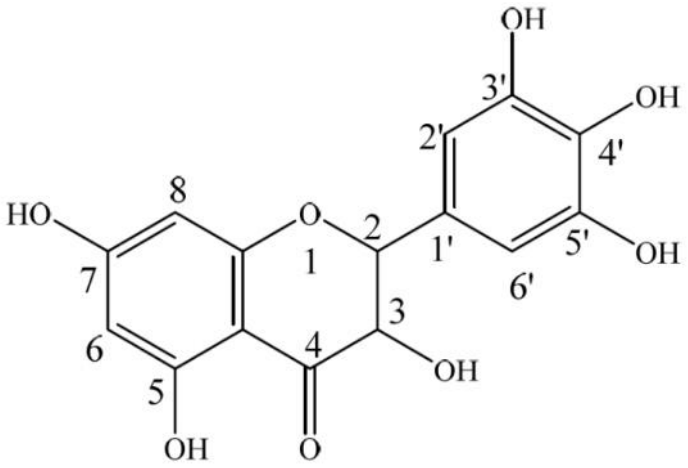


Fig. 6. Structure of dihydromyricetin

Table 3a. ^{1}H and ^{13}C NMR Spectral data (chemical shifts and coupling constants) for dihydromyricetin

Position	**NMR Data in d6-DMSO**	
	δH	δC
2	4.91 (1H,d, 12.6)	83.3
3	4.42 (1H, dd, 12.8, 6.4)	71.7
4		197.7
5		163.4
6	5.86 (1H, d, 2.4)	95.9
7		166.8
8	5.91 (1H, d, 2.1)	95.0
9		162.6
10		100.5
1’		127.2
2’,6’	6.40 (2H, s)	106.9
3’,5’		145.7
4’		133.5
3-OH	5.76 (1H, 6.2)	

Compound 1 was isolated in the form of an off-white powder. The molecular formula of the isolated compound has been deduced as C15H12O8 from the adduct ion corresponding to [M+Na]+ ion observed at m/z 343.0426. This composition was further supported by the 13C NMR spectral data. . The UV spectrum of compound 1 showed λ max at 277nm suggested a flavonoid structure. The 1H NMR spectra data of compound 1 has been acquired in d6-DMSO. The 1H NMR spectra data of compound 1 showed doublet and

doublet of doublets at δ 4.42 and 4.91 in d6-DMSO corresponding to a proton each suggested the 2, 3-dihydroflavonol or 3-hydroxyflavanone skeleton in the structure of compound 1. The presence of 2,3 dihydroflavonol was further supported by the 13C NMR spectral data which showed the presence of oxymethine groups resonating between δ 71.7 and 85.8. In addition, the 1H NMR spectra data of 1 also showed the presence of two meta-coupled aromatic protons as doublets between δ 5.86 and 6.50, and an additional two metacoupled aromatic protons δ 6.40 and 7.24 as singlets corresponds to a pentahydroxyl flavanonol scaffold. on the basis of COSY, HMQC and HMBC correlations the 1H and 13C NMR values for all the protons and carbons for the compound 1 were assigned and are tabulated in Table 1. The HMBC correlations established the position of all the five hydroxyl groups at 5, 7, 3′, 4′, 5′ positions as shown in Figure 6. The structure of compound 1 was determined unambiguously as dihydromyricetin (5, 7, 3′, 4′, 5′-pentahydroxyl flavanonol) on the basis of 1D and 2D NMR spectroscopic data [10].

Conclusion

Based on the UV, FTIR, LCMS and NMR analysis the isolated compound was found to be dihydromyricetin i.e (2*R*,3*R*)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one with molecular formula $C_{15}H_{12}O_8$ and molecular mass 320.0529. Further this compound can be explored to find out the mechanism behind its pharmacological activities like anitdiabetic, and antihyperlipidemic activites.

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