

# STRUCTURAL ANALYSIS OF COUMARIN GLYCOSIDE EXTRACTED FROM *PHASEOLUS VULGARIS* LINN. LEAVES

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## Abstract

This study focused on the extraction, purification, and analysis of a coumarin glycoside isomer from a water-based extract of *Phaseolus vulgaris* L. leaves, which belong to the Fabaceae family. The extraction process employed Sephadex LH-20 resin and reverse-phase high-performance liquid chromatography (RP-HPLC) to separate the compound from the aqueous extract. Preparative HPLC was used to purify the isolated compounds. Various spectroscopic methods, including LC-MS/MS, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, DEPT, and D<sub>2</sub>O, have been utilized to determine the structure of the isolated compound. The results revealed that The extracted compound was a mixture of coumarin glycosides, specifically Skimmin and Mahaleboside. This study enhances our knowledge of the structural variety and potential biological uses of coumarin glycosides.

**Keywords:** Aqueous extract; Coumarin glycoside; Isomers; Mahaleboside; *Phaseolus vulgaris* Linn.; Skimmin

## Introduction

Coumarins are naturally occurring compounds. This is formally known as 2H-Chromen-2-one. It is a heterocyclic compound that belongs to the benzopyrone class. It has a sweet odor and bitter taste. Owing to its olfactory properties, it has extensive applications in flavor and fragrance industries. The latest findings in phytochemistry and pharmacology reveal that coumarin and its derivatives possess diverse biological functions, encompassing antioxidant, anticancer, anti-inflammatory, and antidiabetic capabilities

(Sharma, 2017). Despite its beneficial properties, coumarin can induce hepatotoxicity at high doses. Consequently, the applications of coumarins in the food industry are limited. The biosynthesis of coumarin in plants contributes to the development of self-defense mechanisms that protect plants from herbivores and pathogens, thereby facilitating the maintenance of ecological balance.

The aim of this study was to isolate, purify, and characterize bioactive compounds from aqueous extracts of the leaves of *Phaseolus vulgaris* Linn.

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Bioactive compounds were first isolated from the aqueous extracts of the leaves of the plant. The identification and examination of coumarins is of utmost importance because of their diverse applications and significant biological activities. By thoroughly comprehending its characteristics and interactions, researchers can exploit the potential of coumarin for therapeutic purposes, improve its industrial use, and obtain deeper insights into its natural functions. This extensive knowledge will ultimately contribute to advancements in medicine, agriculture, and chemical research.

## Materials and Methods

### Authentication of Plant

Plant samples were collected from Dapoli, Maharashtra, India. An herbarium was prepared and deposited in the herbarium section of the Xavier's College, Mumbai. The institution's verification report confirmed that the submitted herbarium specimen was *Phaseolus vulgaris* Linn., which belongs to the Fabaceae family. This specimen matches exactly with the Blatter herbarium sample number 2275 of the D.P. Panthaki.

### Extraction

Aqueous extracts of *Phaseolus vulgaris* Linn. leaves were used to isolate the bioactive compounds. The aqueous extract was prepared employing the sequential method described in our previous study (Shaikh and Badole, 2024).

### Isolation of Bioactive Compound from Aqueous Extract

Isolation of bioactive compounds from the aqueous extract was performed using LH-20 gel column chromatography (Mottaghipisheh and Iriti, 2020). This technique is based on the separation of compounds according to their molecular size. The LH-20 resin was regenerated by washing it with water and subsequent overnight soaking. Precisely weighed crude aqueous extract (13.5 g) was dissolved in distilled water and subjected to sonication for 10 min. The resulting filtrate was loaded onto the column apex. It is imperative that the columns do not desiccate; therefore, they require careful handling. Distilled water served as the mobile phase, and fractions were collected in 50 ml test tubes. Fractions 38-52 were analyzed by HPLC and were determined to be pure. These fractions were lyophilized, and the compound eluted at a retention time of 7.2 min exhibited

approximately 50% purity under HPLC conditions. The compound was subsequently purified to greater than 90% purity by preparative HPLC.

### RP-HPLC

The analysis was conducted using high-performance liquid chromatography (HPLC) with a Waters 2695 separation module and photodiode array (PDA) detector (2996). A Hemochrom C18 column (150×4.6 mm with a 5-micron particle size) served as the stationary phase. The mobile phase consisted of a gradient program using solvents A (acetonitrile, ACN) and B (0.05% trifluoroacetic acid, TFA). The gradient program lasted 24 min, with a 6 min delay time, and followed the following parameters: 0-20 min, 0-100% ACN; 20-24 minutes, 100% ACN; 24-25 min, 0% ACN; and 25-30 min, 0% ACN. The maximum absorption wavelength ( $\lambda_{\text{max}}$ ) was set to 210 nm and the flow rate was maintained at 1 mL/min (Nag *et al.*, 2020).

### Prep HPLC Method

The compound, weighing 238 mg, extracted from the Sephadex LH-20 column, was further purified using preparative HPLC. The substance was solubilized in a 1:1 mixture of HPLC water and acetonitrile, which served as the eluent. The HPLC system, comprising an autosampler, pump, and photodiode array detector, was linked to an analytical workstation. A Supelco C18 reverse-phase column (250×20 mm, 5-micron) was used for the separation. The flow rate was set at 18.0 mL/min, with detection at 210 nm. The autosampler tray was maintained at 35°C, and the column was maintained at room temperature. A step gradient of ACN and 0.05% TFA was used as the mobile phase. The photodiode array detector identified flavonoids at 210 nm and distinct peaks were observed. A fraction collector was used to collect and isolate the fractions. Subsequently, a lyophilizer was used to remove the solvent. Before lyophilization, a single peak of the compound with 96% purity was obtained, but after lyophilization, three peaks were observed in the chromatogram. Therefore, it can be concluded that an isomeric mixture was present in the given compound.

### Spectroscopic Condition

NMR experiments were conducted using an Avance III HD 400 MHz NMR Spectrometer equipped with Top Spin software version 3.5pl6 and a Probe PA BBO 500S1BBF-H-D-05 Z (Bruker Biospin Ltd., Switzerland) at ambient temperature. <sup>1</sup>H NMR, <sup>13</sup>C NMR, and DEPT spectra were acquired in DMSO-D<sub>6</sub> (99.8 atom % D, Eurisotop).

## LC-MS

The Acquity Triple Quadrupole (TQD) LC-MS/MS system with Mass Lynx V4.2 software (Waters Corp. UK) was used.

## LC-MS Analysis Parameters

An Inertsil ODS-3 column (150×4.6 mm, 3 µm) was utilized. The column effluent was directed to the MS inlet via an electrospray ionization source operating in the positive mode with a cone voltage of 30 V. The capillary voltage was set to 3 kV. The source and desolvation temperatures were maintained at 150 and 500°C, respectively. Nitrogen served as both the cone and desolvation gas, flowing at 50 L h<sup>-1</sup> and 500 L h<sup>-1</sup>, respectively. Collision-induced dissociation (CID) experiments employed argon as the collision gas at 0.19 mL min<sup>-1</sup>. The collision energy ranged from 10 to 12 V with a 0.2 s scan time. The mass spectrometer was operated at unit mass resolution. Mobile phases consisted of 0.05% TFA (AR Grade, Merck) for 'A' and Acetonitrile (HPLC grade, Finar) for 'B'. Separation was achieved using the following gradient program: time (min)/mobile phase 'B' (%): 0.01/0, 1/0, 20/100, 24/100, 25/0, and 30/0, with a 1 mL min<sup>-1</sup> flow rate. The column temperature was maintained at 30°C. A Valco valve split the eluent 1:4, with a small portion sent to MS, and the majority monitored at 210 nm. The injection volume was 10 µL. Acetonitrile was used as

the diluent. Analytical Solution Preparation The test solution was formulated at a concentration of 1,000 µg mL<sup>-1</sup>.

## Result and Discussion

### Characterization of Compound

Compounds isolated from the aqueous extract were characterized using spectroscopic methods. The data obtained from this analysis, in conjunction with data from literature, were compared to elucidate the structure of the compound.

### LCMS/MS

Mass spectrometry facilitates fragmentation of compounds and yields valuable information about their molecular structures. This process aids in elucidating the structures of unknown compounds, particularly when utilized in conjunction with other analytical techniques such as NMR (Prichystal *et al.*, 2016). Figure 1 LCMS/MS analysis revealed that positive full scan ESI mode yields a molecular weight of 324.2 for the molecular formula C<sub>15</sub>H<sub>16</sub>O<sub>8</sub>. A review of the literature indicates that the molecular weight of the isolated compound corresponds precisely to that of a coumarin glycoside known as Mahaleboside. LCMS/MS studies demonstrated a fragment of mass 146, which was potentially attributable to the loss of a sugar

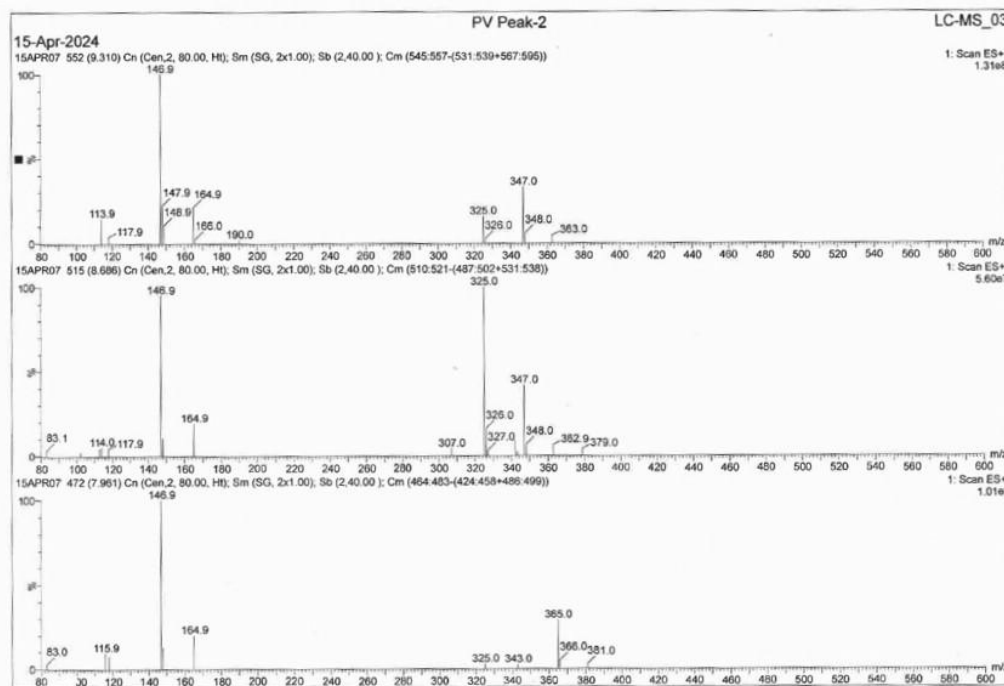


Figure 1. LCMS/MS of an isolated compound

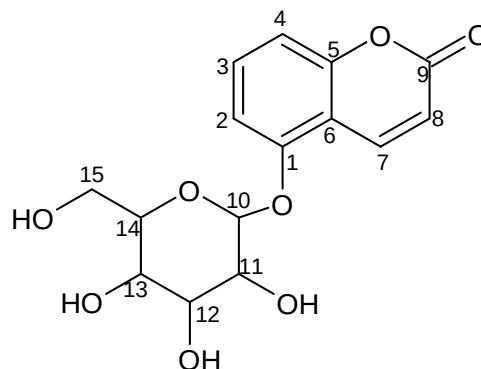
moiety to form coumarin, further corroborating the predicted structure (Singh and Vellapandian, 2023). Figure 2 depicts the structure of Mahaleboside.

#### **<sup>1</sup>H-NMR Data: (DMSO-d<sub>6</sub>)**

<sup>1</sup>H NMR spectroscopy was used to predict the environment of the hydrogen atoms in a compound. The data obtained from the Figure 3. <sup>1</sup>H-NMR spectra are listed below in Table 1. The NMR signals obtained from the experiment were compared with those reported in the literature. Aromatic protons (2,3,4,7, and 8) appear in the range of 6.75 to 7.69  $\delta$  ppm. Protons from the sugar moiety (10,11,12,13,14,15) exhibit chemical shift values from 4.04 to 4.50  $\delta$  ppm in multiplets. The hydroxyl group on the sugar moiety appeared in the range of 1-5 ppm. The hydroxyl groups present on (11, 12, 13, and 15) appear in the range of 5-5.97  $\delta$  ppm.

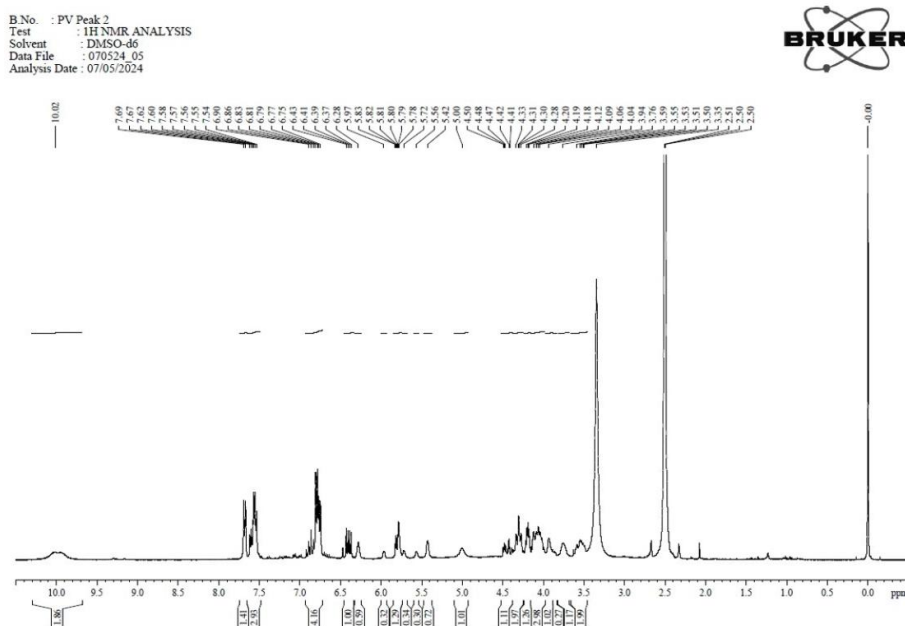
#### **D<sub>2</sub>O NMR Data**

Figure 4 indicates the D<sub>2</sub>O NMR spectra of an isolated compound. D<sub>2</sub>O NMR spectroscopy was used to determine the number of hydroxyl



**Figure 2. Structure of Mahaleboside**

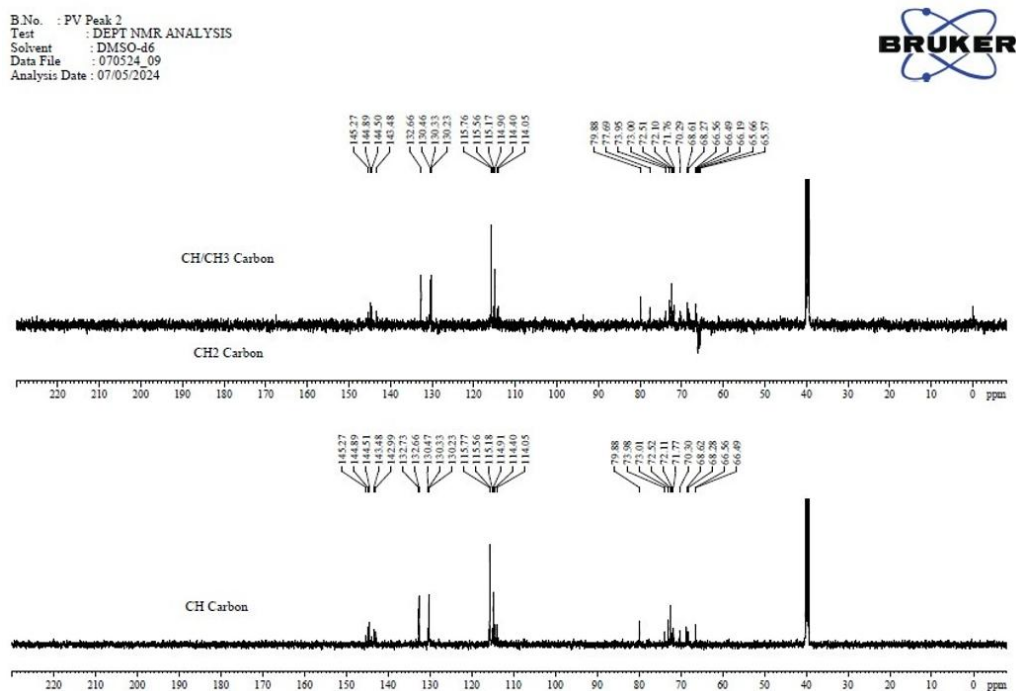
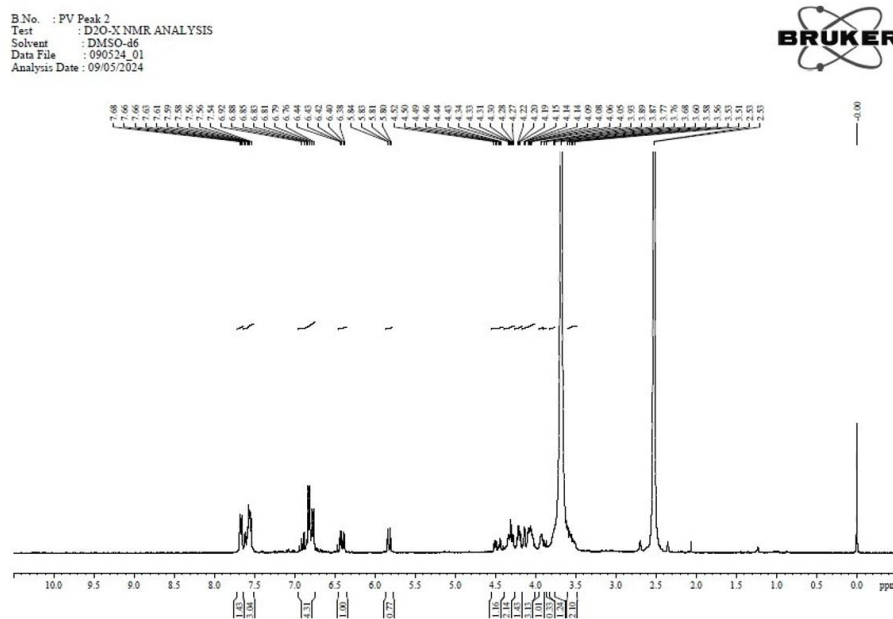
groups. Deuterium exchange with the hydrogen of the hydroxyl groups enables the estimation of the number of -OH groups in the given structure. In D<sub>2</sub>O-X NMR, signals at  $\delta$  5.97, 5.56, 5.42 and 5.00 ppm disappeared, indicating that these are exchangeable protons from the -OH groups. This observation confirmed that the structure contained four hydroxyl groups.



**Figure 3. Represents the <sup>1</sup>H-NMR spectra of isolated compound**

**Table 1. <sup>1</sup>H-NMR data interpretation of isolated compound from the aqueous extract of *Phaseolus vulgaris* Linn**

| Atom Number (Position) | Chemical shifts ( $\delta$ ppm) | No. of protons |
|------------------------|---------------------------------|----------------|
| H(2,3,4)               | 7.54 – 7.69(d,t,d)              | 3              |
| H(7,8)                 | 6.75 – 6.81 (dd)                | 2              |
| H (10,11,12,13,14,15)  | 4.04 – 4.50 (m)                 | 7              |
| OH (11,12,13,15)       | 5.97, 5.56, 5.42, 5.00(s)       | 4              |



CH<sub>3</sub> signals as positive and CH<sub>2</sub> signals as negative (Hawaiz, 2014). Quaternary carbon atoms cannot be detected using DEPT. However, the presence of a corresponding carbon peak in C13 and its absence in DEPT confirmed the existence of a quaternary carbon atom. Signals oriented upward (positive

signal) indicate the presence of -CH, whereas signals oriented downward indicate the presence of -CH<sub>2</sub>. Table 2. shows interpretation of DEPT NMR-135 spectra of an isolated compound.

### C13 NMR Shift

Figure 6 shows an C13 NMR spectra of an isolated compound. Carbon-13 nuclear magnetic resonance (<sup>13</sup>C NMR) spectroscopy is employed to determine the number of carbon atoms in a molecular structure. According to published data, the following spectral patterns are observed:

The coumarin ring's aromatic carbons typically produce signals between 110 and 160 ppm. The lactone carbonyl carbon of the coumarin structure is expected to resonate around 160-180 ppm due to conjugation effects. For the glycosidic portion, the anomeric carbon usually appears between 90 and 105 ppm, while the remaining sugar carbons generally show signals in the 60-80 ppm range (Lou *et al.*, 2020).

<sup>13</sup>C NMR shows two set of signals for each carbon atom indicating that, it may be mixture of two isomers. When literature search was done,

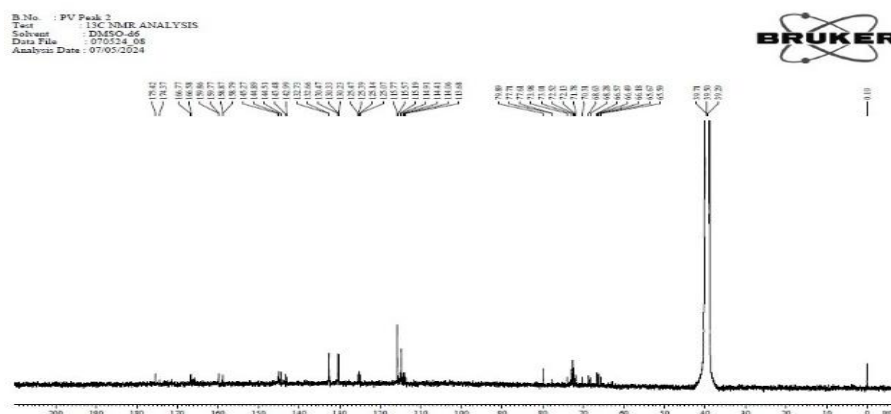


Figure 6. C13 NMR spectra of an isolated compound

Table 2. DEPT NMR-135 spectra interpretation of isolated compound

| Atom Number (Position) | Chemical shifts ( $\delta$ ppm)     | Nature of Carbon |
|------------------------|-------------------------------------|------------------|
| C (1)                  | 166.58-166.77                       | Quaternary       |
| C (2, 4)               | 115.77                              | -CH              |
| C (3)                  | 114.91                              | -CH              |
| C (5)                  | 158.79-159.86                       | Quaternary       |
| C (6)                  | 125.07-125.47                       | Quaternary       |
| C (7)                  | 130.23-130.47                       | -CH              |
| C (8)                  | 132.66-132.73                       | -CH              |
| C (9)                  | 174.37-175.42                       | Quaternary       |
| C (10)                 | 79.88                               | -CH              |
| C (11, 12, 13, 14)     | 73.98, 73.01, 72.52,<br>66.49-66.56 | -CH              |
| C (15)                 | 66.19                               | -CH <sub>2</sub> |

Table 3. C13 NMR interpretation of isolated compound

| Atom Number (Position) | Chemical shifts ( $\delta$ ppm)            | No. of Carbons |
|------------------------|--------------------------------------------|----------------|
| C (1)                  | 166.58-166.77                              | 1              |
| C (2, 4)               | 115.77                                     | 2              |
| C (3)                  | 114.91                                     | 1              |
| C (5)                  | 158.79-159.86                              | 1              |
| C (6)                  | 125.07-125.47                              | 1              |
| C (7)                  | 130.23-130.47                              | 1              |
| C (8)                  | 132.66-132.73                              | 1              |
| C (9)                  | 174.37-175.42                              | 1              |
| C (10)                 | 79.89                                      | 1              |
| C (11, 12, 13, 14, 15) | 73.98, 73.01, 72.52,<br>66.49-66.57, 66.18 | 5              |

it was found that one more isomer of Mahaleboside was reported where sugar is attached to C<sub>2</sub> instead of C<sub>1</sub> and it is called as Skimmin as shown in the Figure 7. Table 3 shows C13 NMR interpretation data.

### Prep HPLC

Prep HPLC was used to purify the compounds to a higher percentage. The fractions obtained from prep HPLC were analyzed for purification using analytical HPLC, and the isolated compound was found to have a purity of more than 95%, as shown in Figure 8. All the pure fractions were lyophilized using a lyophilizer and injected again by analytical HPLC to maintain the purity of the fractions.

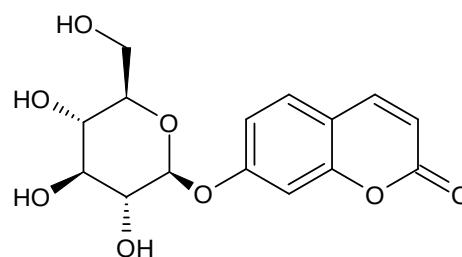


Figure 7. Structure of skimmin

However, after lyophilization, a single peak is converted into three peaks, as shown in Figure 9. This may be due to the lyophilization process, which

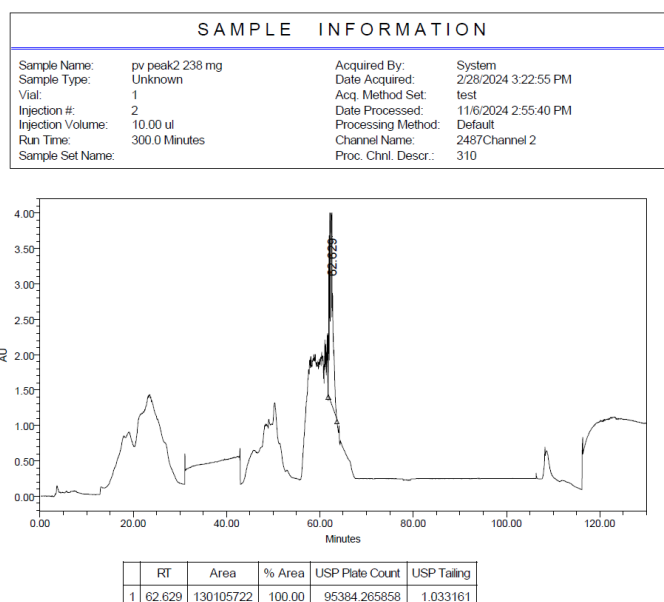


Figure 8. Prep HPLC chromatogram of an isolated compound before lyophilization

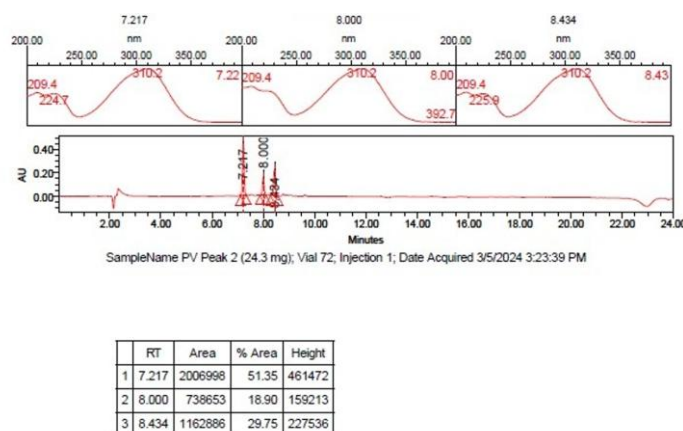


Figure 9. Prep HPLC chromatogram of an isolated compound after lyophilization

involves solvent extraction and freezing and can induce alterations in the structure of a compound. This may result in the formation of various conformers or slight isomeric variation. These structural changes can be detected as distinct peaks when analyzed using HPLC or GC-MS.

Sequential extraction plays a crucial role in the isolation of compounds from aqueous crude extracts. LH-20 Sephadex gel column chromatography works exclusively on the size of the isolated compound. It is mainly used for the separation of compounds present in plants, as they have similar polarity differences. LH-20 works with both organic and aqueous solutions; therefore, it is considered a versatile technique for the isolation of compounds. The fractions obtained from the column were lyophilized using a lyophilizer. The fractions were of 50% purity. The purity of the compound can be increased by Prep HPLC to more than 95%. The isolated pure compound obtained was 28.3 mg. The compound can be characterized by spectroscopic techniques such as NMR, LCMS/MS, and other NMR techniques.

## Conclusions

All the spectral data obtained from the experiment corresponded to those reported in the literature. The <sup>13</sup>C NMR spectrum exhibited two sets of signals for each carbon atom, suggesting that the compound may be a mixture of two isomers. A literature search revealed that an additional isomer of Mahaleboside has been reported, in which the sugar moiety is attached to C2 instead of C1; this isomer is known as Skimmin. Both Mahaleboside and Skimmin are coumarin glycosides. Coumarin glycosides are an important class of phytoconstituents with a wide range of bioactivity. Furthermore, investigation of the bioactivity of the isolated compound is possible; however, separating the isomers is a costly process.

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