RESEARCH PAPER

Design and Spectroscopic Characterization of a Novel N-Benzylacetamide Hesperetin Derivative Derived from *Citrus sinensis* Peel

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Abstract

Aim:

This study aimed to synthesize and characterize a novel N-benzylacetamide derivative of hesperetin obtained from *Citrus sinensis* (orange peel) and to evaluate its structural modification, including intermediate formation (MIHT). **Materials and Methods:** Hesperidin was extracted from dried *Citrus sinensis* peel through successive solvent extraction using petroleum ether followed by methanol under reflux conditions. The crude extract was purified by recrystallization and subsequently subjected to acid hydrolysis to obtain hesperetin. Preliminary identification and separation, including formation of MIHT (mixture of hesperetin intermediates), were performed using thin-layer chromatography (TLC) with an optimized solvent system of toluene:ethyl acetate:acetic acid (3:2:0.4). Further purification and isolation of the target compounds were achieved using silica gel column chromatography. The synthesized hesperetin derivative was prepared via multi-step reactions involving ethyl 2-bromoacetate, benzylamine, and coupling agents (EDC and HOBt). Structural characterization of MIHT and the final synthesized compounds was carried out using UV–Visible spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy, and mass spectrometry. **Results:** The extraction process yielded crystalline hesperidin, which upon hydrolysis produced hesperetin with characteristic physicochemical properties. TLC analysis demonstrated effective separation of MIHT and standard hesperetin with distinct Rf values, confirming the suitability of the selected solvent system. Column chromatography resulted in multiple fractions, among which fraction F7 was identified as the principal compound derived from MIHT based on spectral evaluation. FT-IR analysis revealed characteristic functional groups including hydroxyl (–OH), carbonyl (C=O), and aliphatic (C–H) moieties. NMR and mass spectral data further confirmed the structural integrity and molecular composition of MIHT-derived products and their successful transformation into a novel hesperetin derivative. The analytical and spectral findings confirmed the purity and structural identity of both intermediate and final synthesized compounds. **Conclusion:** A novel N-benzylacetamide derivative of hesperetin was successfully synthesized through MIHT as a key intermediate stage. Spectroscopic characterization confirmed structural modification without alteration of the core flavanone framework, validating the effectiveness of the synthetic pathway.

Keywords: Synthesis, Hesperetin , Orange peel , UV-visible spectroscopy, FT-IR, ^{1}H NMR

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1. Introduction

Citrus (family Rutaceae) fruits, oranges are rich sources of hesperidin (3′,5,7-Trihydroxy 4′-methoxyflavanone 7-rutinoside, hesperetin 7-rutinoside**(Ávila et al., 2021).** It is structurally a flavanone glycoside made up of the disaccharide rutinose and the aglycone hesperetin. Hesperidin's pharmacokinetics, bioavailability, and absorption have all been covered in several research. (**Minakshi et al.,2025)**According to these studies, hesperidin taken orally by humans or animals is absorbed as aglycone (hesperetin) **(Olas , 2021),** following the removal of rutinose or via hesperetin 7-glucoside from hesperidin, which is then converted to glucuronidated and sulfated metabolites that can be found in blood or urine by bacterial enzymes in the intestine **(Haidari eta l., 2009).** It is commonly recognized that hesperidin and hesperetin are strong natural antioxidants that effectively lower oxidative stress. **(Cho, 2006).** Numerous pharmacological characteristics, including as anti-inflammatory, antibacterial, anticarcinogenic, antithrombotic, and antiviral activity, have been reported for hesperidin and hesperetin **(Hirata et al., 2005).**Therefore, in this work, we report the extraction of Hesperitin ad synthesis of hesperetin derivatives and evaluate the antimicrobial effects, anti-inflammatory effect of compounds. We look forward to finding compounds that can simultaneously enhance a variety of biological activities in these hesperetin derivatives and to promoting further application development.

2. Materials and methods

2.1 Chemical and instruments used

All chemicals and reagents were of analytical grade, used without purification. Key substances included ethyl 2-bromoacetate, benzylamine, K_2CO_3, Na_2CO_3, KI, HCl, and coupling agents EDC and HOBt, sourced from standard suppliers. Analytical-grade solvents such as ethanol, methanol, chloroform, ethyl acetate, and petroleum ether were utilized. Anhydrous Na_2SO_4 served as a drying agent, while silica gel (60–120 mesh) and pre-coated TLC plates were employed for chromatographic analysis.

2.2 Extraction process for Hesperidin:

The Orange peel was grinded into powder after air drying and extracted successively amount 12 cycle (total mass of powder is 960 g), in a reflux condenser 80 grams of this powder was placed. 600 ml of petroleum ether was added and refluxed for 1.5 h. after filtration of hot mixture through a Buchner funnel, the powder was allowed to dry at room temperature. The powder was placed putted back to the flask and 600 ml of methanol was added. The contents were heated under reflux for 2h again and then hot

mixture was filtered. The filtrate was concentrated with distillation column, leaving a syrup residue crystallized from dilute acetic acid (6%), and yielding orange needles, melting point was 268°C.

2.3 Purification process for Hesperidin:

Before adding the acetic acid solution, which was made by heating to roughly 60 °C and adding a little amount of acetic acid, the aforementioned orange needles were added to dimethylformamide (7 mL g-1 of syrup). After passing through a Buchner funnel, the mixture was diluted with an equivalent amount of water and left to crystallize for four hours. The compound's crystals were removed by filtering. When the compound was added to chloroform. The white crystalline hesperidin was then filtered through a Buchner funnel. As, Pure Hesperidin has melting point 240-253 °C which is obtained similar to our inferences in our prepared mixture of compound.

2.4 Conversion of obtained compound Hesperidin into Hesperetin:

For eight hours, the aforementioned mixture of substance (9 g), methanol (250 mL), and concentrated sulfuric acid (9 mL) was heated at reflux. After cooling and concentrating the resulting homogenous solution, 500 milliliters of ethyl acetate were added to dilute it. Magnesium sulfate is used to dry each 100 ml of the organic solution after it has been cleaned four times with water.

2.4.1 Hesperetin was purified by the following procedure:

Dissolve the crude product in minimum of acetone, and the resulting solution was added to a vigorously stirred mixture of water (200 mL)and acetic acid (3mL). In an ice bath, precipitated mixture was washed and cooled with water. Pure yellow powder obtained has melting point of 220-221 °C. It's may be mixture of hesperetin. After that we proceed for TLC.

2.5 Evaluation of Hesperetin

2.5.1 Preliminary Thin layer chromatography of MIHT:

Thin-layer chromatography is a "solid-liquid adsorption" chromatography. The polarity of the material, solid phase, and solvent all affect this upward traveling rate. **(Ozlem *et al.*, 2016 and Bele *et al.*, 2011).**

$$\mathbf{R_f\ Value} = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

In the preliminary TLC analysis of the mixture of hesperetin (MIHT), the solvent system in which distinct spots were observed was Toluene: Ethyl acetate: Acetic acid (3:2:0.4). This mobile phase was further evaluated using a standard hesperetin marker purchased from Sigma.

2.3.1 Isolation of hesperetin from MIHT by column chromatography

1. **Coloum size -** Glass column, 250 x 30 mm
2. **Stationary Phase -** Silica gel (60 to 120 mesh size)
3. **Elution mode -** Isocratic elution
4. **Mobile Phase -** Toluene: Ethyl: acetate: Acetic acid (3:2:0.4)

5. **Compound –** MIHT (Mixture of compound hesperetin)
6. **Visualized by -** Short UV (254nm), long UV (365nm) and Visible light

Identification of fractions: Visualized by Short UV (254nm), long UV (365nm) and visible light.

2.3.2 Column chromatography

MIHT compound were subjected to silica gel column chromatography for the isolation of the target compound from MIHT. The fractions were collected sequentially, concentrated, and analyzed by thin-layer chromatography (TLC) to identify the presence of different compounds **(Srivastava *et al.,* 2021 and Mukherjee *et al.,* 2024).**

2.4 Preparation of Hesperetin derivative

The reaction of above obtained hesperetin with ethyl bromoacetate in ethanol provided intermediate 1 in 75% yields. Then, treatment of intermediate 1 with excess sodium carbonate in ethanol and H2O, followed by acidification with aqueous HCl, resulted in intermediate 2 in 62% yield. Finally, the reaction of intermediate 2 with benzylamine produced 48% yields Hesperetin derivative - N-Benzyl-2-[5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yloxy]-acetamide **(Luo et al., 2016; Li et al., 2017).**

2.4.1 Synthesis of Intermediate 1

Hesperetin (3.02 g, 10 mmol), ethyl 2-bromoacetate (1.66 g, 15 mmol), anhydrous K_2CO_3 (2.76 g, 20 mmol) and catalytic amount KI were added in anhydrous ethanol (150 mL), the mixture was refluxed for 1 h. The solution was evaporated, and the residue was poured into water and extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, and then concentrated to give white solid 2, which was used without further purification.

2.4.2 Synthesis of Intermediate 2

Compound 2 (2.82 g, 7.1 mmol), 5% Na_2CO_3 (10 mL), and ethanol (40 mL) were stirred at 50 °C for 8 h. Then the reaction mixture was poured into 10% HCl (200 mL), and filtered. The filter cake was recrystallized from EtOH to give yellow solid.

2.4.3 Procedures for the Preparation of hesperetin derivative

Compound 3 (0.37 g, 1 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC, 0.29 g, 1.5 mmol), 1-hydroxybenzotrizole (HOBT, 0.20 g, 1.5 mmol) and $CHCl_3$ (20 mL) were stirred at room temperature for 1 h, and then benzylamine (3 mmol) were added. The solution was stirred at room temperature overnight. Then the solvent was poured into water and extracted with ethyl acetate (20 mL × 3). The solution was dried over anhydrous Na_2SO_4 and concentrated. The residues were purified by flash chromatography with chloroform/petroleum ether (2:1, *v*/*v*) elution. White solid (48% yield) was obtained finally.

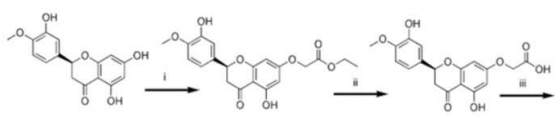


Figure 1: Synthesis of hesperetin derevative

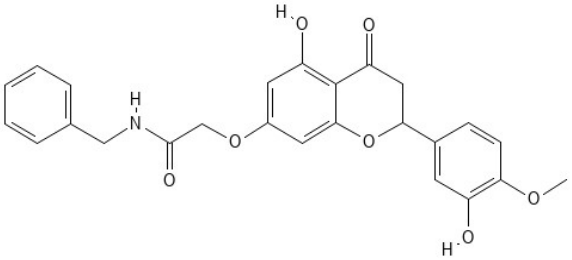


Figure 2: Structure of obtained hesperetin derivative (- N-Benzyl-2-[5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yloxy]-acetamide).

2.5 Characterization hesperetin derivative and hesperitin

2.5.1 UV-visible Spectroscopy:-

The synthesized hesperetin derivative were scanned over a wavelength range of 200 to 800 nm using a UV-Visible spectrophotometer (Shimadzu UV-1700), and the characteristic peaks were detected and recorded**(Kapoor et al., 2022).**

2.5.2 Characterization of synthesized hesperetin derivative by FT-IR spectroscopy:-

FT-IR spectroscopy was performed using a Perkin Spectrum 95763 spectrophotometer to identify functional groups in the synthesized hesperetin derivative **(Sant et al., 2025).**

2.5.3 Characterization of hesperetin derivative by NMR spectroscopy:-

The synthesized compound was characterized using nuclear magnetic resonance (NMR) spectroscopy. ^{1}H NMR spectra was recorded on a JNM EC-500 NMR spectrometer operating at [400/500/600] MHz for ^{1}H, respectively **(Mistry et al., 2017).**

2.5.4 Characterization of synthesized hesperetin derivative by Mass spectroscopy:-

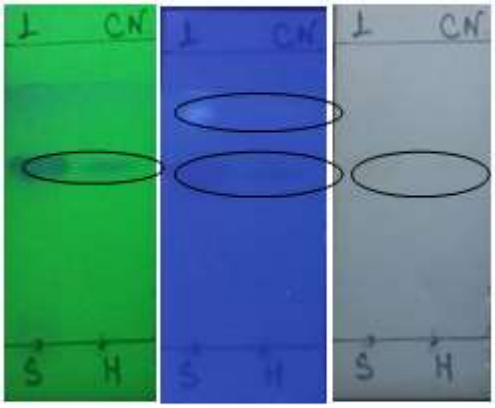
Mass spectrometry converts molecules into ions, which are then separated and detected based on their mass-to-charge (m/z) ratios. It was used to determine the molecular weights of the synthesized hesperetin derivative. The measurements were recorded using a micrOTOF-Q mass spectrometer **(Kapoor et al., 2022).**

3. Results

3.1 Extraction of Hesperidin

Figure 2: Extracted hesperidin compound

3.2 Preliminary Thin layer chromatography of mixture of hesperetin (MIHT)



Short-UV (365 nm) **Long-UV** **Visible (254 nm) Light**

Figure : 3 TLC estimation by UV lamp for MIHT (Mixture of hesperetin) with Std. Hesperetin (Std. = Standard, H=MIHT)

TLC of MIHT was performed using different solvent systems, which were selected based on a literature survey. Among the tested systems, Toluene: Ethyl acetate: Acetic acid (3:2:0.4) produced clearly visible bands of MIHT when compared with standard hesperetin. The Rf values of MIHT and hesperetin were found to be 0.92 and 0.73, respectively. Based on these findings, Toluene: Ethyl acetate: Acetic acid (3:2:0.4) was considered a suitable mobile phase for MIHT and was selected for further use in column chromatography.

3.3 Column chromatography of MIHT

Table 1: Fractions collected from Column Chromatography of MIHT

Sr. No.	Eluent composition	Fraction collected	Remarks
1	Toluene: Ethyl Acetate: Acetic acid (3:2:0.4)	F1, F2	Transparent coloured mixture of compound
2		F3	Light yellow coloured mixture of compound
3		F4	Pale yellow coloured mixture of compound
4		F5	Light yellow coloured mixture of compound
5		F6	Yellow coloured mixture of compound
6		F7	Transparent colored mixture of compound
7		F8	Light yellow coloured mixture of compound
8		F9	Transparent coloured mixture of compound
9		F10	Transparent coloured mixture of compound

3.4 Spectral analysis of isolated fraction F7 (hesperetin).

3.4.1 UV spectroscopy o isolated fraction F7 (hesperetin)

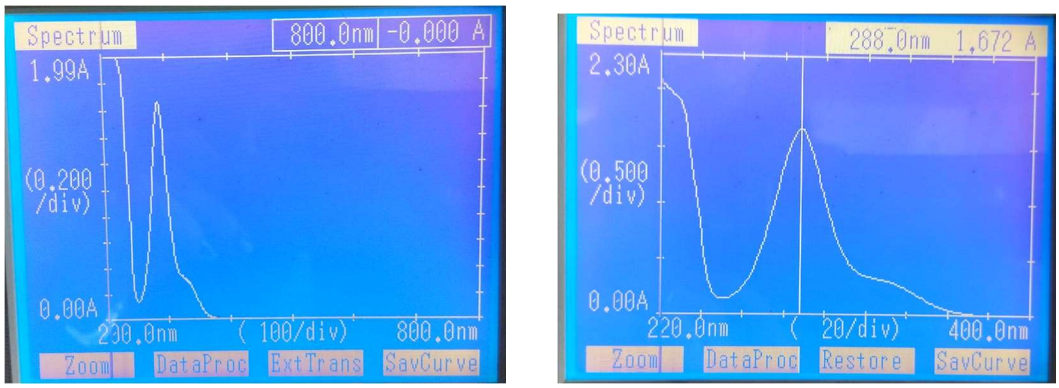


Figure 4: UV Graph of isolated fraction F7 (hesperetin)

3.4.2 IR spectra of isolated fraction F7 (hesperetin)

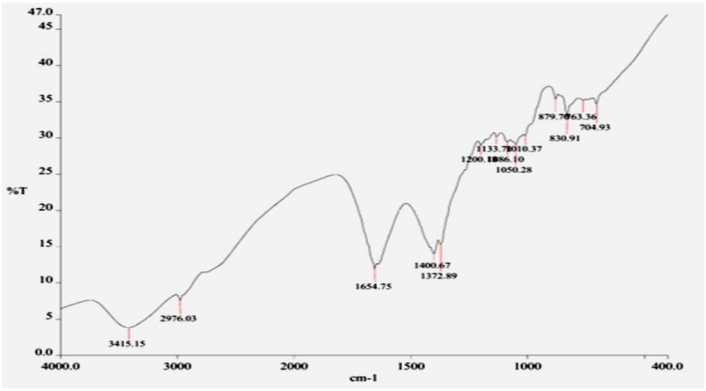


Figure 5: IR spectra of isolated fraction F7 (hesperetin)

3.4.3 ^{1}H NMR spectra of Hesperetin–

(A)$_{1}$H NMR spectra of Hesperetin –

Figure 2: lH-NMR spectra of the isolated fraction F7 (hesperetin)

3.4.4 Mass – Spectroscopy-

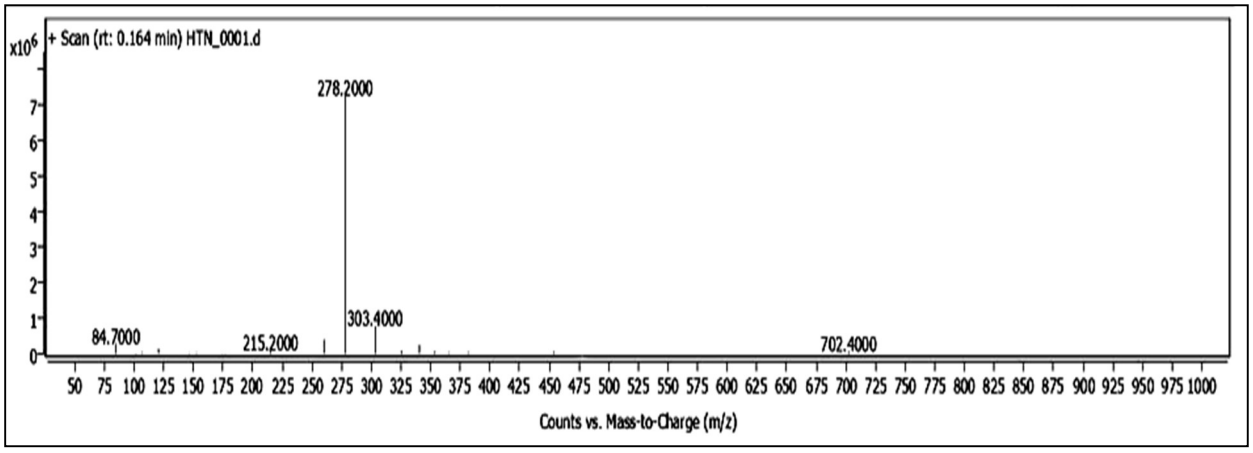
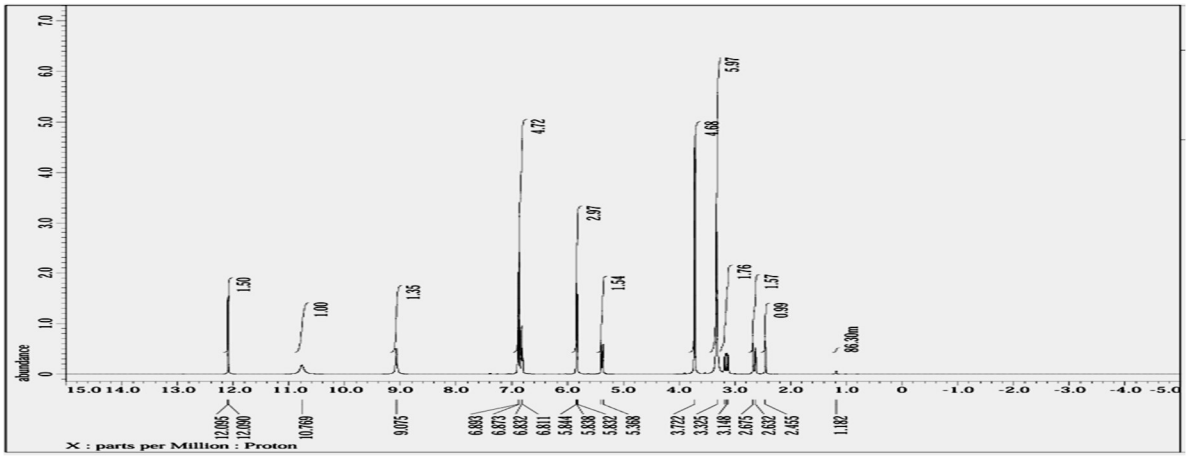
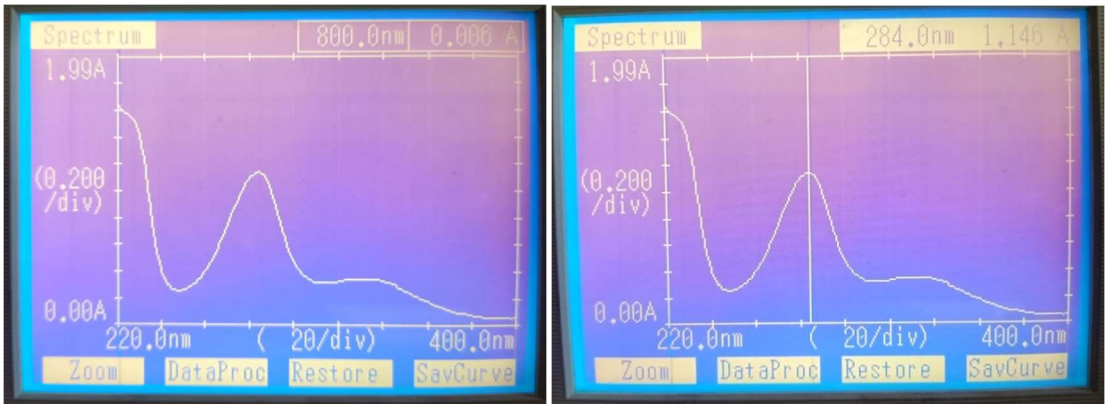


Figure 7: Mass spectrum of isolated fraction F7 (hesperetin)



3.5 Spectral analysis of hesperetin derivative

3.5.1 UV spectra of hesperetin derivative-



Graph 8: UV Graph of hesperetin derivative

3.5.2 IR spectra of hesperetin derivative

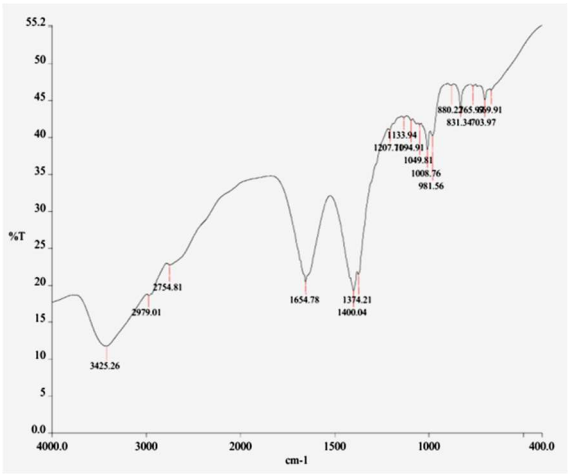


Figure 9: IR spectra of hesperetin derivative

3.5.3 NMR – Spectroscopy hesperetin derivative–

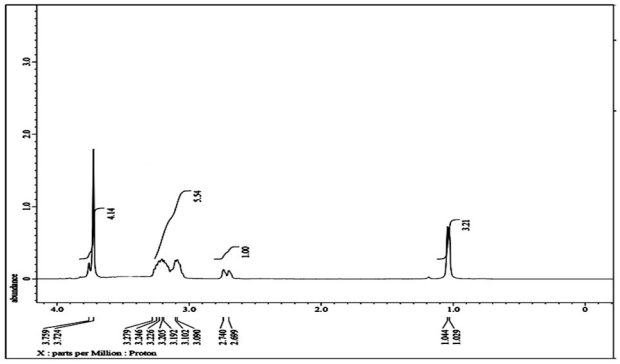


Figure 10: ^{1}H-NMR spectra of the hesperetin derivative.

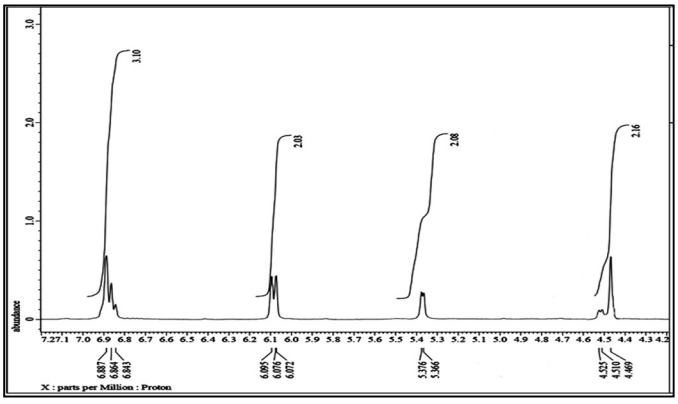


Figure 11: ^{1}H-NMR spectra-2 of the hesperetin derivative.

3.5.4 Mass – Spectroscopy hesperetin derivative-

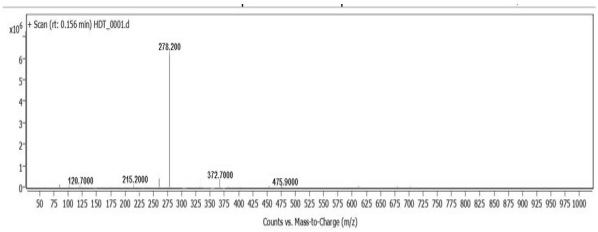


Figure 12: Mass spectrum of hesperetin derivative

4. Discussion

The study effectively extracted, isolated, synthesized, and characterized hesperidin and its derivative, hesperetin, using comprehensive phytochemical and analytical methods. Methanol was identified as an effective polar solvent for extracting hesperidin from orange peel, yielding a crude product melting point of 268°C, consistent with literature values. Further purification through recrystallization with dimethylformamide and acetic acid produced hesperidin with a melting point range of 240–253°C, confirming successful isolation and purity. The conversion of hesperidin to hesperetin through acid hydrolysis was successfully accomplished under reflux conditions. Hesperetin's formation was confirmed by its melting point (220–221°C), aligning with literature. Thin-layer chromatography (TLC) provided preliminary confirmation, with a solvent system of Toluene: Ethyl acetate: Acetic acid (3:2:0.4) showing well-defined spots and Rf values (0.92 for MIHT and 0.73 for standard hesperetin), affirming the presence of target compounds and supporting the mobile phase choice for further separation. Column chromatography effectively fractionated a mixture of hesperetin (MIHT), with fraction F7 identified as the target compound through TLC profiling. The use of silica gel (60–120 mesh) and isocratic elution conditions proved suitable for separating flavonoid derivatives. Spectral characterization confirmed F7 as hesperetin via UV–Visible spectroscopy, which showed characteristic absorption peaks. FT-IR analysis identified functional groups such as hydroxyl, carbonyl, and ether linkages, validating the flavonoid backbone. Additionally, ^{1}H NMR spectroscopy and mass spectrometry supported the molecular structure and confirmed the identity of the isolated compound. The synthesis of a hesperetin derivative was achieved through a multi-step process involving alkylation, hydrolysis, and amide bond formation with EDC and HOBt as coupling agents. The yields for intermediates and the final product were 75%, 62%, and 48%, respectively. The final compound, N-Benzyl-2-[5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yloxy]-acetamide, was obtained as a white solid. Characterization through UV–Visible, FT-IR, NMR, and mass spectroscopy confirmed its structural features, with spectral data aligning with expected attributes, indicating successful synthesis and purification procedures. Overall, the study highlights an efficient approach for the extraction of hesperidin, its conversion to hesperetin, and subsequent derivatization. The combination of chromatographic and spectroscopic techniques ensured accurate identification and characterization of compounds.

5. Conclusion

The study achieved the extraction, isolation, conversion, and derivatization of hesperidin from orange peel, characterizing hesperetin and its derivative. Efficient recovery and satisfactory purity of hesperidin were ensured through appropriate solvent systems, extraction techniques, and purification methods, confirmed by melting point analysis. The conversion of hesperidin into hesperetin through acid hydrolysis was effectively accomplished, and the identity of the obtained compound was validated using chromatographic and spectral techniques. Thin-layer chromatography and column chromatography proved to be reliable methods for separation and isolation, particularly in identifying the active fraction (F7). Furthermore, the successful synthesis of the hesperetin derivative through a multi-step reaction pathway demonstrated the feasibility of chemical modification of flavonoids. The synthesized compound was thoroughly characterized using UV–Visible spectroscopy, FT-IR, NMR, and mass spectrometry, all of which confirmed the presence of expected functional groups and structural integrity. The findings establish a strong basis for further exploration of hesperetin derivatives in pharmaceutical and medicinal chemistry applications, particularly for the development of novel bioactive agents.

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