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Research Article

Spectroscopic Elucidation and Anticancer Potential of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl Chromenone Isolated from *Haplanthodes tentaculatus* (L.) R. B. Majumdar

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ABSTRACT

In this research, we report on the isolation and structural elucidation of a new flavonoid glycoside called 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone. This flavonoid glycoside was obtained from *Haplanthodes tentaculatus*, a plant that is native to the Western Ghats of India. The molecule was purified via the use of chromatographic procedures, and its structure was validated by the use of spectroscopic investigations, which included HRMS, ^{1}H-NMR, ^{13}C-NMR, FTIR, and UV-visible technologies. With the use of HRMS analysis, the chemical formula $C_{23}H_{24}O_{10}$ was verified. The isolated molecule demonstrated anticancer activity *in-vitro* against the MDA-MB-231 breast cancer cell line, with an IC_{50} value of 58.99 ± 0.495 µg/mL. This indicates that the drug is very effective in curing cancer. The natural origin of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone and the possibility that it has a reduced toxicity level imply that it has the potential to be a lead molecule for the creation of anticancer drugs despite the fact that it is less effective than the conventional medicine cisplatin. The findings of this research add to the increasing body of evidence that bioactive chemicals derived from natural sources have the potential to be used in therapeutic medicine.

Introduction

The area of natural product chemistry arises from the intersection of environmental studies and organic chemistry, where they interact in a fascinating manner. The primary emphasis of this discipline is the study of plant chemistry, with the objective of understanding and using the bioactive substances present in the natural environment. Ancient systems such as Ayurveda recognized the medicinal properties of plants much before the development of modern scientific methods, thereby establishing the origins of employing plants for medical reasons. Today, the task at hand is to objectively verify these old assertions by utilizing rigorous approaches such as compound isolation. An intriguing botanical specimen found in this particular area is *Haplanthodes tentaculatus* (L.) R.B. Majumdar, also known as *H. tentaculata*, and has many local aliases.[1,2] This species is prevalent in India, particularly in the picturesque sceneries of the Western Ghats, which span from Madhya Pradesh to Kerala and Tamil Nadu. [3] The Western Ghats of Maharashtra were selected arbitrarily for the research project. Nevertheless, the region is ideal for the study due to its remarkable biodiversity and abundance of medicinal plants that thrive throughout the year. [1]

H. tentaculatus is regarded as an exclusive species to a certain area by some taxonomists, and historical

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documents and research are suggesting its possible use in traditional medicine. [4] The importance of plants such as *H. tentaculatus* in medicine rests in their capacity to generate bioactive compounds that have potential therapeutic uses. These chemicals have shown substantial promise in the therapeutic management of several disorders, such as cancer, diabetes, and inflammation, and also possess antibacterial properties.[5]

Cancer is a prominent global issue, leading to a substantial number of fatalities in 2008, especially in poorer nations. The increase in cancer incidences is often linked to lifestyle factors such as smoking and poor dietary habits. Cancer formation is marked by the accelerated and unregulated proliferation of cells, which possess the capacity to avoid apoptosis and metastasis to distant sites within the body.[6]

H. tentaculatus, belonging to the Acanthaceae family and originating from the Western Ghats, has a significant impact on India's unique floral legacy.[7] The phenolic and flavonoid content of this plant has the potential to give anticancer effects by affecting gene expression associated with cell transformation, metastasis, and angiogenesis. However, the specific mechanisms by which plant extracts exert their anticancer properties are not fully understood and are still being actively studied.[8] To investigate these characteristics, it is essential to start by isolating the chemical constituents. After being removed, these components might undergo further separation and characterization. Preparative high-performance liquid chromatography, often known as prep-HPLC,[9,10] is a contemporary chromatographic technique that plays a vital role in this approach.[11] These techniques enable the isolation of pure molecules that are crucial for thorough assessment and the potential for creating novel medications.[6,7]

The study report investigates the anticancer properties of *H. tentaculatus* by conducting the MTT test on MDA-MB-231 cells, which are a kind of human breast cancer cell line. The MTT test is a widely used technique for assessing the vitality and proliferation of cells. The process entails quantifying mitochondrial activity in the presence of several chemicals that might potentially be harmful to cells.[12] This work offers natural-products chemists valuable recommendations for selecting optimal extraction and analysis methods for bioactive compounds derived from plants. Furthermore, it emphasizes the significance of *H. tentaculatus* as a potential reservoir of anticancer drugs.[5] In the current pharmaceutical landscape, there is an urgent need for new, effective anticancer agents with improved safety profiles and reduced resistance compared to traditional chemotherapy drugs. Natural products, particularly plant-derived compounds, offer a promising source of such novel therapeutics.[13] The isolation of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone from *H. tentaculatus*, with its demonstrated anticancer activity against the MDA-MB-231 breast cancer cell line, highlights its potential as a lead compound for drug development. This flavonoid glycoside may provide an alternative to conventional treatments, offering advantages such as lower toxicity and the ability to target cancer cells through distinct mechanisms. In the present scenario, where cancer remains a leading cause of mortality, our findings contribute to the discovery of plant-based compounds that could fill the gap in the development of safer and more effective anticancer therapies.

Materials And Methods

Plant Material Collection

The study was centered on *H. tentaculatus*, which was collected from Uttan, Bhayandar in Maharashtra, India. In August 2022, the plant's aboveground components were collected. Once gathered, these components underwent a meticulous purification procedure to remove any dirt and waste. Subsequently, the specimens were subjected to a drying process in a sheltered area to ensure the preservation of their chemical composition. Ultimately, the substances were meticulously pulverised into a fine powder in order to enhance their exposed area, hence permitting a very efficient extraction procedure.

Plant Material Authentication

The *H. tentaculatus* that was collected underwent authentication at the Blatter Herbarium Department of St. Xavier's Autonomous College. This was done to confirm its validity and ensure consistency in the plant material used for the study. Plant authentication no. 21599.

Process of Extracting

Reflux extraction was used to extract compounds from a 4 kg batch of dehydrated plant material. This was accomplished using a configuration including a three-neck round-bottom flask, heating mantle, condenser, and electric motor stirrer. The solvent used was methanol, with a volume of 5 L required per kg of plant material. The temperature of the heating mantle was precisely controlled within the range of 35 to 45°C to optimize the effectiveness of the extraction process and avoid any solvent loss caused by condensation. The methanol was retrieved after the extraction procedure and reused to maintain environmental sustainability. The extraction method yielded a total of 800 g of crude extract, resulting in a 16% yield.

Extraction of Compounds via Liquid-Liquid Separation

The unprocessed extract underwent a liquid-liquid extraction (LLE) process to separate and isolate certain components.

Commencement of Suspension: In order to ensure a

uniform dispersion, 30 g of the crude extract was combined with 750 mL of water and exposed to sonication for a duration of 10 minutes.

Procedure for Extraction

Petroleum ether extraction included adding 300 mL of petroleum ether to the aqueous solution, thoroughly mixing them, and then separating them using a funnel. The layer of pet ether was collected and then evaporated at room temperature.

The aqueous layer underwent a dichloromethane (DCM) extraction by introducing 400 mL of DCM, vigorously agitating the solution, and then separating and evaporating the organic layer.

The process of ethyl acetate extraction included the addition of 300 mL of ethyl acetate to the residual aqueous layer, followed by vigorous mixing of the solution and subsequent evaporation of the resultant organic layer.

Lyophilization: The fractions that were separated were transferred to round-bottom flasks and treated to vacuum drying for 16 to 18 hours in order to get crystalline compounds.

Chromatographic separation is the method of separating various components of a mixture by using their distinct interactions with a stationary phase and a mobile phase. In order to further the purification process, the compounds were subjected to preparative liquid chromatography using a Waters Prep LC system fitted with a 2487 dual wavelength UV detector. The chromatographic separation included.

Two C18 columns used in the study: a Knauer C18 column (250 × 62 mm, 10 µ) for larger separations and a Supelco C18 column (250 × 20 mm, 5 µ) for finer separations.

The mobile phase was originally composed of 0.05% trifluoroacetic acid (TFA), with a gradual rise in the concentration of acetonitrile up to 15% during a duration of 60 minutes.

A total of 112 fractions were collected, and every fifth tube was examined for purity using HPLC to ensure consistency among the fractions.

Methods of Analysis

NMR spectrometry

NMR analysis was performed using an Avance III HD 400 MHz NMR Spectrometer to investigate ^{1}H, ^{13}C, and DEPT spectra in DMSO-D6.

The study was conducted utilizing a Bruker Impact II UHR-TOF system equipped with ESI ionization for mass spectrometry. The mass range was defined as 20 to 1200 m/z, and the resolution was specified as 50,000 FSR.

Evaluation of Anticancer Activity

The individual compounds were evaluated for their biological activity by conducting the MTT test on MDA-MB-231 breast cancer cells.

Cell culture

Cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and antibiotics in a 5% CO_2 atmosphere at a temperature of 37°C.

Treatment

The cells were subjected to various concentrations of the *H. tentaculatus* extract (range from 5–100 µg/mL) for a period of 48 hours.

Viability assessment

After treatment, cell viability was assessed using the MTT method. This included replacing the treatment medium with an MTT reagent and measuring the absorbance after the formation of formazan crystals.

IC_{50} calculation

The IC_{50} value, which indicates the concentration needed to inhibit 50% of cell viability, was calculated by linear regression analysis of the dose-response curve.

Result And Discussion

Isolation and Characterization of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl Chromenone

The isolation of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone was achieved from the plant *H. tentaculatus* using a series of chromatographic techniques, yielding 29.3 mg of the compound with 96.39% purity. The compound was obtained as a pale-yellow crystalline solid, and its structure was elucidated through comprehensive spectroscopic methods, including HRMS, ^{1}H-NMR, ^{13}C-NMR, and HPLC analysis. The NMR chemical shifts of the isolated compound are summarized in Table 1. Fig. 1 depicts the molecular structure of a newly discovered flavanone glycoside with anticancer properties. It shows the specific arrangement of hydroxy, methoxy, and

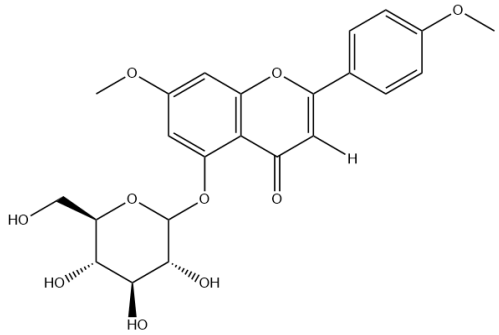


Fig. 1: Structure of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone; Chemical structure of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone, with the IUPAC name 7-methoxy-2-(4-methoxyphenyl)-5-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-4H-chromen-4-one, isolated from H. tentaculatus. The structure features a flavone core with methoxy and glycosyl substitutions, crucial for its biological activity

Table 1: NMR spectroscopy data for 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone: proton and carbon chemical shifts and environments

S. No.	*^{13}C Chemical shift (ppm)*	*^{1}H Chemical shift (ppm)*	*Proton environment*
1	129.6	7.10	Aromatic H
2	128.2	6.92	Aromatic H
3	121.4	6.84	Aromatic H
4	138.1	-	Aromatic C
5	129.6	7.10	Aromatic H
6	128.2	6.92	Aromatic H
7	55.8	3.86	OCH_3
8	-	-	-
9	162.2	-	C=O (Flavonoid ring)
10	98.3	6.36	H on Flavonoid ring
11	164.5	-	C-OH
12	96.6	6.22	H on Flavonoid ring
13	164.5	-	C-OH
14	181.3	-	C=O (Flavonoid ring)
15	55.8	3.86	OCH_3
16	102.4	-	C in glycosidic linkage
17	76.7	-	CH in glucose
18	76.7	-	CH in glucose
19	76.7	-	CH in glucose
20	72.1	-	CH in glucose
21	68.4	-	CH_2 in glucose
22	68.4	-	CH_2 in glucose
23	12.1	2.45	CH_3 (Acetyl group)
24	102.4	4.38	Anomeric H (Glucose)
25	76.7	3.83	CH in glucose
26	76.7	3.66	CH in glucose
27	72.1	3.55	CH in glucose
28	68.4	3.75	CH_2 in glucose
29	68.4	3.75	CH_2 in glucose
30	12.1	2.45	CH_3 (Acetyl group)
31	55.8	3.86	OCH_3
32	72.1	3.66	CH in glucose
33	68.4	3.75	CH_2 in glucose
34	12.1	2.45	CH_3 (Acetyl group)

glycosidic functional groups that are responsible for its biological action.

HRMS Analysis

The high-resolution mass spectrometry (HRMS) analysis of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone confirmed the molecular formula $C_{23}H_{24}O_{10}$ through the observed molecular ion peak at m/z 461.1444 $[M+H]^+$ (Fig. 2). Key fragment ions at m/z 299.0914 (loss of glycosyl moiety) and m/z 314.0712 (loss of a methoxy group) provided further validation of the proposed structure. These fragmentation patterns are consistent with the expected breakdown of glycosylated flavonoids and suggest that the glycosylation occurs at the C-5 position. The identification of these fragments reinforces the presence of both the flavonoid core and the glycosylated side chain, which is critical for its biological activity. These observations are consistent with the proposed structure of the flavone glycoside, reinforcing the presence of both the flavonoid core and the glycosylated side chain.[14]

^{1}H-NMR Analysis

The ^{1}H-NMR spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone provided critical information on the proton environments, confirming the flavonoid core structure. The aromatic proton signals at δ 7.86 and δ 7.62, observed as doublets with coupling constants J = 8.7Hz and J = 8.6Hz, respectively, are indicative of a typical para-substituted phenyl ring. The singlet at δ 6.84 corresponds to an aromatic proton on the chromenone core. The singlet at δ 3.70 evidences the methoxy group. The multiplet in the δ 3.0 to 4.0 ppm region, particularly the distinct anomeric proton doublet at δ 5.11 (J = 7.9 Hz), confirms the presence of a glycosyl unit attached to the flavone, supporting glycosylation at C-5. The distinct chemical shifts align with known flavonoid glycosides, providing strong evidence for the proposed structure (Fig. 3).

To further confirm the presence of exchangeable protons, such as hydroxyl groups, a D_2O exchange experiment was conducted. After the addition of D_2O, the broad signals around δ 9.0 to 7.0 ppm, which correspond to hydroxyl protons, diminished significantly or disappeared. This confirmed the presence of hydroxyl groups within both the flavone core and the glycosyl moiety, further supporting the proposed structure.[15]

^{13}C-NMR Analysis

The ^{13}C-NMR spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone provided a detailed mapping of the carbon environments. Key signals in the δ 150 to 100 ppm range represent the aromatic carbons of the flavone core, with the quaternary and methoxy-substituted carbons at δ 55.5 ppm (Fig. 4). The DEPT analysis further distinguished between the CH, CH_2, and CH_3 groups, with the aromatic CH groups appearing as positive peaks in the DEPT-135 spectrum, while the CH_2 groups of the

Table 2: IC_{50} values of isolated compound and cisplatin against MDA-MB-231 breast cancer cell line

S. No.	*Sample name*	*IC_{50} (µg/ml)*
		MDA-MB-231
1	HT3C	58.99±0.495
2	Cisplatin	23.38±0.621

The IC_{50} values are shown as mean ± standard deviation, with '±' indicating the variability across replicates, reflecting the precision of the measurements.

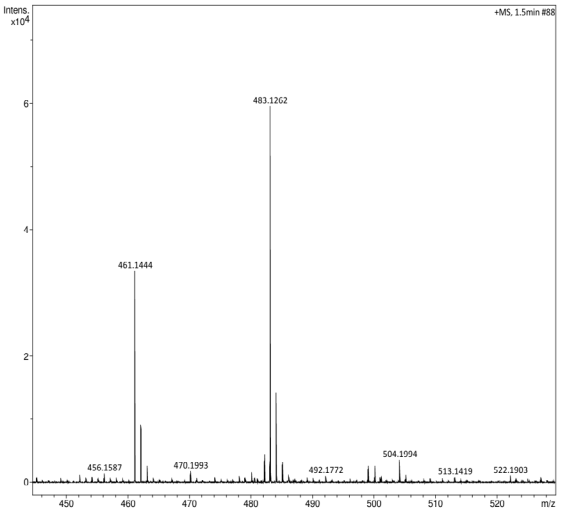


Fig. 2: HRMS spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone - HRMS spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone, showing the molecular ion peak at m/z 461.1444 $[M+H]^+$, corresponding to the molecular formula $C_{23}H_{24}O_{10}$ Key fragment ions at m/z 299.0914 and m/z 314.0712 indicate the loss of the glycosyl moiety and a methoxy group, respectively, supporting the proposed structure.

glycosyl moiety showed negative peaks. The DEPT-90 spectrum confirmed the presence of CH_3 groups, including the methoxy substitution. These data corroborate the placement of glycosyl and methoxy groups, reinforcing the structural elucidation.

The distortionless enhancement by polarization transfer (DEPT) NMR experiment was employed to distinguish between CH, CH_2, and CH_3 groups. In the DEPT-135 spectrum, positive peaks indicated the presence of CH groups, including aromatic carbons and some glycosyl carbons. Negative peaks in the DEPT-135 spectrum corresponded to CH_2 groups, particularly within the glycosyl moiety, while CH_3 groups, such as those in the methoxy group, were observed as positive peaks in the DEPT-90 spectrum.

This comprehensive analysis confirmed the structure of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone, validating the presence of methoxy groups, aromatic CH groups, and the glycosyl moiety, with specific carbon environments and proton exchanges characteristic of the proposed molecular structure.

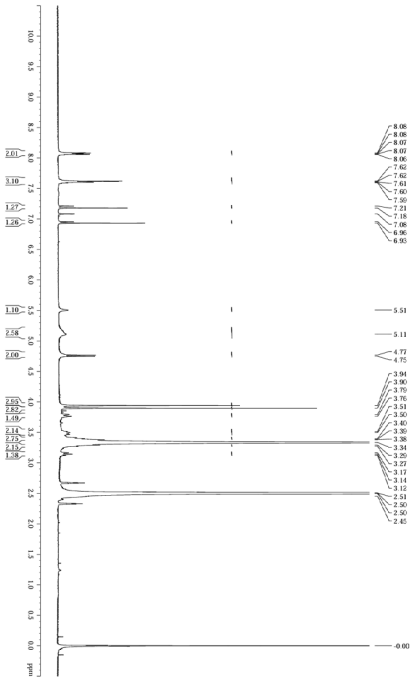


Fig. 3: ^{1}H-NMR Spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone in DMSO-d6 - ^{1}H-NMR spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone, highlighting the aromatic proton signals at δ 7.86 and δ 7.62, a methoxy proton at δ 3.70, and glycosyl protons in the δ 3.0–4.0 ppm range, with the anomeric proton appearing as a doublet at δ 5.11.

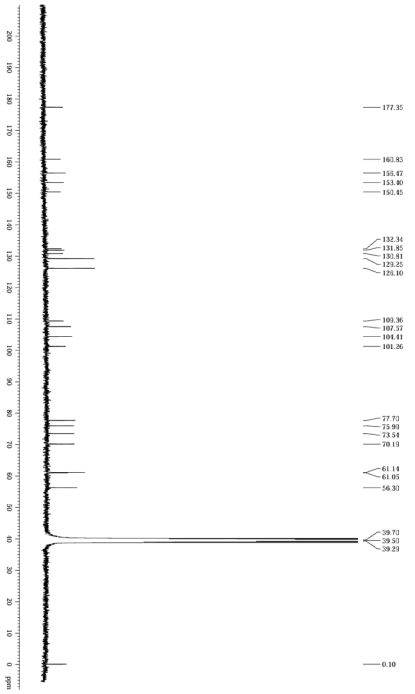
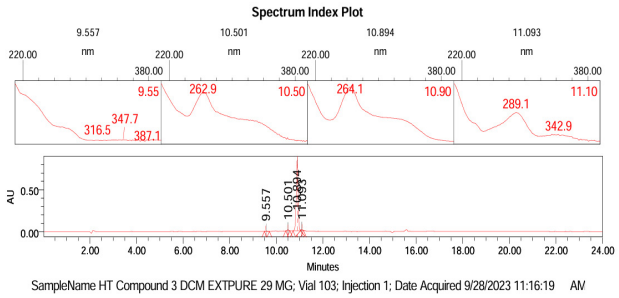


Fig. 4: ^{13}C-NMR Spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone in DMSO-d6 -^{13}C-NMR spectrum showing the carbon environments within 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone, with aromatic carbons in the δ 150–100 ppm range and a methoxy carbon at δ 55.5 ppm. The spectrum confirms the structure of the flavone glycoside.v



	RT	Area	% Area	Height
1	9.557	38200	0.78	7771
2	10.501	64251	1.32	11737
3	10.894	4704978	96.39	842411
4	11.093	73996	1.52	15419

Fig. 5: HPLC Chromatogram of 7-7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone - HPLC chromatogram demonstrating the high purity (96.39%) of the isolated 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone, with a single major peak confirming the efficacy of the purification process and also UV-vis spectra.

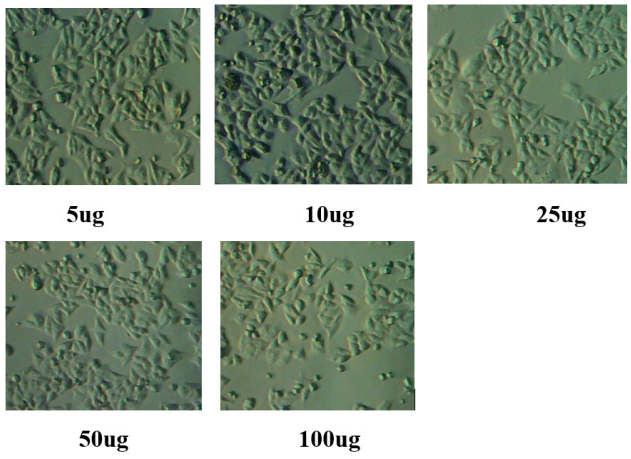


Fig. 6: IC_{50} values of 7-methoxy-5-glycosylflavone against MDA-MB-231 breast cancer cell line -IC_{50} determination of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone against MDA-MB-231 breast cancer cells, showing a dose-dependent reduction in cell viability with an IC_{50} value of 58.99 ± 0.495 µg/mL, indicating its potential as an anticancer agent

FTIR Analysis

The FTIR spectrum provided further confirmation of the functional groups in 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone. The broad absorption band at 3400 cm^{-1} indicates the presence of hydroxyl groups, likely attributed to the glycosyl unit and flavonoid hydroxyl substitutions. The carbonyl (C = O) stretch at 1650 cm^{-1}, characteristic of flavonoid compounds, reinforces the presence of the chromenone core. Additionally, C-O stretching bands between 1250 to 1000 cm^{-1} are consistent with the methoxy and glycosidic linkages, further supporting the glycosylation pattern observed in the NMR data. The spectrum also displayed bands between 1250 to 1000 cm^{-1}, attributed to C-O stretching vibrations, which are consistent with the methoxy and glycosidic functionalities of the compound.[14,16]

UV-vis Analysis

The UV-vis spectrum of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone revealed characteristic absorption peaks that are indicative of the conjugated aromatic system within the flavonoid core (Fig. 5). The major absorption peak at 264.1 nm is attributed to π→π* transitions, which are typical of flavonoid compounds with extensive conjugation. This peak underscores the presence of a highly conjugated system, reinforcing the structural integrity of the flavonoid backbone.[17,18]

Purity Determination by HPLC

The HPLC chromatogram of the isolated 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone demonstrated a high level of purity, with a single major peak accounting for 96.39% of the total area under the curve (Fig. 5). This high purity level validates the effectiveness of the extraction and purification processes used, ensuring the reliability of subsequent biological evaluations.

Evaluation of Anticancer Activity

The anticancer potential of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone was evaluated using the MTT assay on the MDA-MB-231 breast cancer cell line, revealing a dose-dependent reduction in cell viability with an IC_{50} value of 58.99 ± 0.495 µg/mL. While less potent than Cisplatin (IC_{50} = 23.38 ± 0.621 µM), this value still demonstrates significant activity for a natural product (Fig. 6). Flavonoid glycosides are known for their relatively lower toxicity, and the presence of glycosyl and methoxy groups in this compound may enhance water solubility and bioavailability, contributing to its anticancer effects. The compound's structural features, such as its conjugated aromatic system and hydroxyl groups, could be influencing key cancer cell signaling pathways, including apoptosis and cell cycle arrest. This warrants further investigation into its precise mechanism of action and potential synergy with existing chemotherapeutic agents. The results suggest that 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone may act through mechanisms distinct from cisplatin, warranting further investigation into its mode of action. Table 2 displays a comparison between the IC_{50} values of the isolated chemical and cisplatin, a well-established chemotherapeutic therapy, in terms of their efficacy against the MDA-MB-231 breast cancer cell line.

Conclusion

The successful isolation and structural elucidation of 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl

chromenone from *H. tentaculatus* highlight the plant's potential as a source of novel bioactive compounds. The compound demonstrated significant anticancer activity against the MDA-MB-231 breast cancer cell line, though with moderate potency compared to cisplatin. Nonetheless, its natural origin and unique structural properties position 7-methoxy-2-(4-methoxyphenyl)-5-O-glycosyl chromenone as a promising candidate for further development in cancer therapeutics. Future studies should focus on detailed mechanism-of-action studies and the potential for synergistic effects with existing chemotherapeutic agents. This research underscores the importance of exploring endemic plant species for new drug leads, particularly in the ongoing battle against cancer.

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