

Anticancer Activity and Cell Mechanism of a Novel Flavonoid Derivative from *Chrysanthemum morifolium* (Ramat). Hems! Leaves Against the MCF-7 Breast Cancer Line in vitro

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Background: Breast cancer has the highest number of cases and deaths in women. Cell cycle dysregulation and decreased cell apoptosis are among the causes of cancer cell growth. An ideal anticancer drug has selective toxicity, meaning it can kill cancer cells without damaging normal tissue cells. Several cultivars of *Chrysanthemum morifolium* leaves (CML) contain the main active compounds, such as flavonoids, as active ingredients of drugs that have potential as anticancer agents.

Purpose: The main objective of this study was to investigate the potential anticancer effects of a compound extracted from CML.

Methods: Chromatographic isolation of compounds using different separation techniques, including extraction, separation, and purification; characterization using ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and mass spectroscopy; and evaluation of anticancer activity in vitro (WST-8 assay), cell mechanism: cell cycle inhibition, and apoptosis activity (flow cytometry), and analysis of the *ESR1* gene expression (RT-qPCR).

Results: Bioactive components found in the ethyl acetate extract of CML were acaetin (1), 3,5,5'-Trihydroxy-4'-methoxy flavone (2), and kaempferide-7-O-glycoside (3). The lowest percentage of cell viability was shown in isolate (2) with an IC₅₀ value of 49.37 and 225.045 ppm in MCF-7 and HEK-293 cells. Isolate 2 at a concentration of 1 × IC₅₀ caused a significant increase in the G2/M phase (50.73 ± 4.13%), indicating G2/M arrest. Meanwhile, in the apoptosis assay, the same treatment increased early apoptosis of up to 38.87 ± 11.43%. Isolate 2 CML showed increased *ESR1* gene expression at a dose of 1 × IC₅₀ of 1.34 ± 0.13 and at a dose of ½ × IC₅₀ of 1.01 ± 0.14.

Conclusion: Isolate 2 CML showed selective potential as an anticancer agent through G2/M cell cycle inhibition, early apoptosis, and suppression of *ESR1* gene expression.

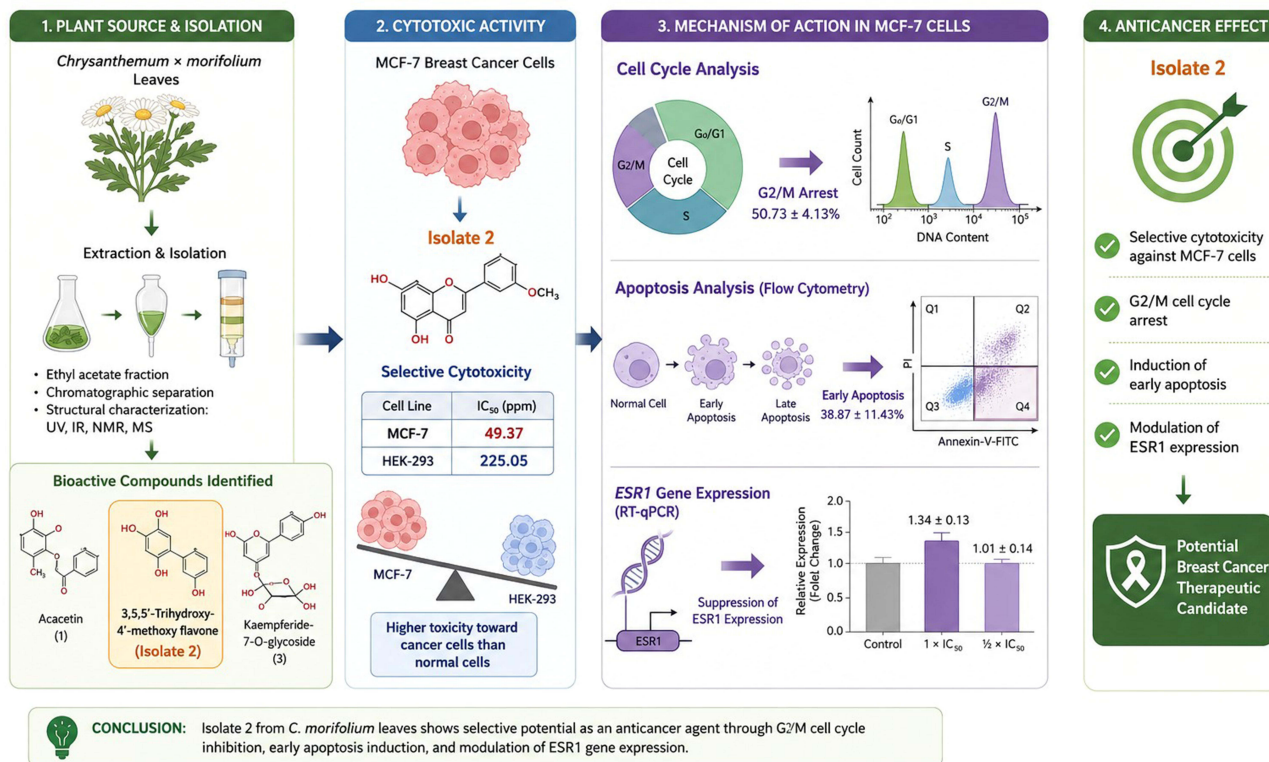
Keywords: *Chrysanthemum morifolium*, cytotoxicity, MCF-7 cells, in vitro, cell mechanism

Introduction

Cancer is a non-communicable disease that poses a global health burden. According to the World Health Organization (WHO), cancer is one of the main causes of mortality globally. According to data from the World Health Organization's Global Burden of Cancer (GLOBOCAN), there were 20 million cancer cases and 9.7 million cancer-related deaths in 2022. Cancer deaths are expected to continue to increase to 35 million cases by 2030. After heart disease and stroke, cancer is the third most common cause of death in Indonesia. In 2022, the Global Cancer Observatory (GLOBOCAN) reported 242,988 cancer-related deaths and 408,661 new cases. The highest number of cases was breast cancer in women and lung cancer in men, with the highest number of deaths caused by breast, cervical, and lung cancer.¹ Hormonal factors, genetics, oxidative stress, advanced age, and unhealthy lifestyles influence the high incidence of breast cancer.²

Graphical Abstract

Novel Flavonoid Derivative from *Chrysanthemum × morifolium* Leaves Induces G2/M Arrest and Early Apoptosis in MCF-7 Breast Cancer Cells



Breast cancer is thought to be caused by excessive exposure to endogenous estrogen during a woman's life. Estrogen is a steroid hormone that contributes to female sexual characteristics, affects various specialized organs and tissues, and regulates cell proliferation and differentiation in both women and men. Estrogen stimulates the establishment of an extensive ductal system, the development of breast stromal tissue, and the accumulation of breast fat.³ Estrogen receptors (ER) and androgen receptors are often co-expressed in breast cancer.^{4,5}

Research to identify potential new anticancer drug candidates is essential to address these issues. Developing drugs from natural sources is expected to offer a solution in selecting therapies for breast cancer patients with a high selectivity index. One plant with potential as an anticancer agent for breast cancer cells is *Chrysanthemum morifolium*. *Chrysanthemum morifolium* has been reported to have produced 60 isolates of flavonoid compounds, most of which are glycoside derivatives of luteolin, apigenin, acacetin, diosmetin, or eriodictyol at the C7 position. Detection of active compounds, especially with high flavonoid levels in *Chrysanthemum*, provides its pharmacological effects.⁶ Flavonoids and phenolic acids are the main components of secondary metabolites that are essential for providing therapeutic activity in *Chrysanthemum* species.^{7,8} Several leaf cultivars of *Chrysanthemum morifolium* contain main active compounds such as flavonoids, galuteolin, quercitrin, chlorogenic acid, and 3,5-O-caffeoylquinic acid as active ingredients with potential as anticancer agents.⁹

The anticancer activity of *Chrysanthemum morifolium* and *Chrysanthemum indicum* extracts has been reported in several in vitro and in vivo studies. Flavonoids such as luteolin, apigenin, and acacetin showed strong cytotoxicity, with IC₅₀ values ranging from 1.08–25 µg/mL against breast (MCF-7), lung (A549), and liver (HepG2) cancer cells. These compounds modulate key molecular pathways, including NF-κB, PI3K/AKT/mTOR, STAT3, and MAPK/ERK1/2, leading to apoptosis through mitochondrial dysfunction, activation of caspase-3 and -9, as well as altered BAX/BCL-2 ratios.¹⁰

In the study of the metabolite profiling test on the ethyl acetate fraction of *Chrysanthemum morifolium* leaves using UHPLC-HRMS¹¹ was reported that *Chrysanthemum morifolium* has the most dominant compounds, namely flavonoid compounds that can act as breast cancer anticancer, such as glycitein,^{12–16} diosmetin,^{17–21} kaempferol,^{22–28} esculetin,^{29–33} scopoletin,^{34,35} dihydroartemisinin,^{36–40} and chrysin.^{41–47}

Flavonoids have significant impacts on chemotherapy and cancer prevention. Numerous genes and enzymes, including those involved in antiproliferation, cell cycle arrest, apoptosis, angiogenesis, and multidrug resistance, have been demonstrated to interact with flavonoids.⁴⁸ Flavonoid compounds play a role in inhibiting cell proliferation and regulating tumor suppressor genes that play an important role in the cell cycle and p53 in cancer cells and can increase cell apoptosis in breast cancer.⁴⁹ One type of phytoestrogen that functions as a selective estrogen modulator (SERM) is flavonoids. Because flavonoids require a phenolic ring to connect to estrogen receptors, these SERMs can interact with receptors to induce modest oestrogenic or antiestrogenic effects.^{50–52} After passing through the cell membrane, these SERMs attach to cytosolic estrogen receptors to form a complex. The estrogen response element (ERE), which is close to the gene whose transcription is being regulated, is then bound by this complex. The complex then binds to the corepressor protein NCoR, thereby suppressing transcription of genes that regulate cell proliferation.^{53,54}

Until now, there has been no research that examines the isolation and elucidation of the structure and anticancer bioactivity test of *Chrysanthemum morifolium* leaves against MCF-7 cells. Therefore, this study was conducted to examine the analysis, compound isolation, cytotoxic activity, and cell mechanisms of *Chrysanthemum morifolium* leaf isolates in inhibiting MCF-7 cell proliferation through cell cycle modulation, apoptosis induction, and changes in *ESR1* gene expression for breast cancer therapy.

The novelty of this study lies in: (1) the isolation and structural elucidation of flavonoid derivatives from *Chrysanthemum morifolium* leaves, (2) evaluation of their cytotoxicity and selectivity against MCF-7 and HEK-293 cells, and (3) investigation of their effects on gene *ESR1* expression, apoptosis, and cell-cycle progression.

Materials and Methods

Materials

Chrysanthemum × morifolium (Ramat). Hemsl. Leaves were collected from a chrysanthemum plantation in the Bandung area of Semarang, Central Java, Indonesia. The plants were identified and stored in the Biosystematics and Molecular Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University (No.190/LBM/IT/V/2023(No.IV.K.78a)) and identified by Mr. Joko Kusmoro. The distilled organic solvents used for extraction, separation, and purification included ethanol, n-hexane, ethyl acetate, acetone, methanol, chloroform, and distilled water, while the chemicals for spectroscopic analysis were pro-analyzed (p.a.) grade. Chromatographic separation was performed on silica gel 60 (230–400 mesh) (Merck), silica gel 60 (30–70 mesh) (Merck), silica gel 60 PF254 (Merck), silica gel 60 F254 thin film plates (Merck) were used for thin-layer chromatography (TLC) and detection was achieved by spraying with 5% H₂SO₄ in ethanol (v/v), followed by heating. Breast cancer cell line MCF-7 (ECACC) and normal cell line HEK-293 (ECACC), cisplatin, and Dulbecco's Modified Eagle Medium (DMEM) growth medium. In addition to the above materials, 0.25% Trypsin-EDTA (Gibco), DMSO (Sigma), PBS (Sigma), high glucose (Sigma), fetal bovine serum (Sigma), penicillin–streptomycin (Sigma), WST-8 [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma), Annexin V (BioLegend), Propidium Iodine (PI) (BioLegend), Total RNA kit (Geneaid), Rever Tra-Ace (Toyobo), wash buffer, β-mercaptoethanol, DNase, water-free RNase, EGTA, β-actin primer, REa primer, and GoTaq[®] Green Master Mix (Promega) were also used.

Instruments

The chemical structures of isolated compounds were determined using infrared and ultraviolet (UV) spectra, which were recorded using a Perkin-Elmer Spectrum 100 FTIR spectrometer and a Perkin-Elmer Lambda 35 UV/VIS Double Beam spectrometer. Mass Spectrometry uses Waters LCT Premier XE-MS. NMR spectra were obtained using an Agilent DD2 console system spectrometer at 500 MHz for ¹H and 125 MHz for ¹³C. Chemical shifts were measured in δ (ppm), with a δH signal of 7.26 and a δC signal of 77.16 for the reference solvents acetone and DMSO, respectively. Column

chromatography (CTC) was performed using silica gel 60 (Merck, 70–230 and 230–400 mesh) and octadecylsilane (Fuji Silysia Chemical Ltd, Chromatorex® C18 DM1020 M, 100–200 mesh). Thin-layer chromatography (TLC) was performed on pre-coated silica gel 60 F254 plates (Merck) and RP-18 F254 plates (Merck). TLC spots were observed under UV light (254 and 365 nm), and heated after soaking with 10% H₂SO₄ in ethanol. For the cytotoxicity assay, 96-well microplates, micropipettes, microtubes, incubators, and Biochrom microplate readers were used. FACScan *flow cytometer*, PCR *multibox* (Proflex, Applied Biosystems), *thermal cycler* (ProFlex™ 3x32-well PCR System, Applied Biosystems).

Isolation of Components *Chrysanthemum morifolium* (Leaves (CML))

Chrysanthemum × morifolium (Ramat). Hemsil leaves (8.9 kg) that had been air-dried were ground up and extracted using 70% ethanol (EtOH) at room temperature (3 × 12 L each, for 24 hours). After the solvent was removed under reduced pressure, the EtOH extract (3 kg) was partitioned between n-hexane and ethyl acetate (EtOAc). The organic layer was evaporated to yield crude n-hexane (187.5 g) and EtOAc extracts (750 g), respectively. Ethyl acetate fraction (2 g) was extracted by silica gel column chromatography and eluted with a gradient of n-hexane–chloroform–ethyl acetate (3:3:4) to obtain an eluate collected in 40 vials. The composition of the column chromatography results was monitored by TLC with n-hexane:chloroform:ethyl acetate (3:3:4) mixture. Spot visualization was monitored under UV light at 254 and 365 nm, both without spraying and with spraying using 10% H₂SO₄ ethanol and heating, to detect the flavonoid compound group. The results of the column chromatography TLC test are shown in Figure 1. From the TLC results, the column chromatography results that had the same spot pattern were combined to obtain 3 isolates, namely isolate 1 CML (12 mg), isolate 2 CML (10 mg), and isolate 3 CML (20 mg). The purity test for the isolates was carried out by two-way TLC using mobile Phase I n-hexane:chloroform:ethyl acetate (3:3:4) and mobile Phase II n-hexane:ethyl acetate (5:5) with a pre-coated plate stationary phase and a suitable reagent spot visualizer. From the elution results, there was one spot in each mobile phase, which was used to indicate that the isolate was pure. To ensure that an isolate is truly pure (consisting of only one compound), several supporting methods are required, such as chromatography (two-dimensional /two-way TLC), HPLC, and thermal analysis with melting point determination. In this study, only two-way TLC was used to determine compound purity. Melting point determination and HPLC was not performed due to the limited number of isolates obtained.

Cytotoxicity Assay and Selectivity Index of Compounds 1, 2, and 3 *Chrysanthemum morifolium* Leaves (CML)

The cytotoxicity of compounds 1–3 CML was evaluated using the Water-Soluble Tetrazolium salt-8 (WST-8) technique against two human cancer cell lines, MCF-7 and HEK-293. These cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 IU/mL penicillin, 10 µg/mL streptomycin) in 5% CO₂. Following a 24-hour incubation period, both were seeded into 96-well plates at an initial density of roughly 3 × 10⁴ cells cm⁻³. Meanwhile, the compounds were dissolved in 2% dimethyl sulfoxide (DMSO) solutions at the required concentrations of 120, 60, 30, 15, and 12.5 ppm. Cisplatin was utilized as a positive control, and the cells were subjected to the test chemicals in triplicate for 48 hours. Following a 24-hour incubation period, 10 µL of WST-8 (Sigma) Cell Viability Reagent was added, and the mixture was then incubated for an additional 1–2 hours until the colour changed. The IC₅₀ value was ascertained by comparing the graph of the percentage of living cells with the control, which was given only phosphate-buffered saline (PBS) and DMSO, at different doses of the tested substance (ppm). The observed absorbance was analysed using a microplate reader at 570 nm.

$$Selectivity\ Index = \frac{IC_{50}\ HEK - 293\ Cells}{IC_{50}\ MCF - 7\ Cells}$$

Cell Cycle Assay

The culture medium from each sample was transferred into a 15 mL centrifuge tube. The cells were subsequently washed twice with cold PBS (1 mL each wash) and then detached using trypsin. After detachment, the cells were collected into

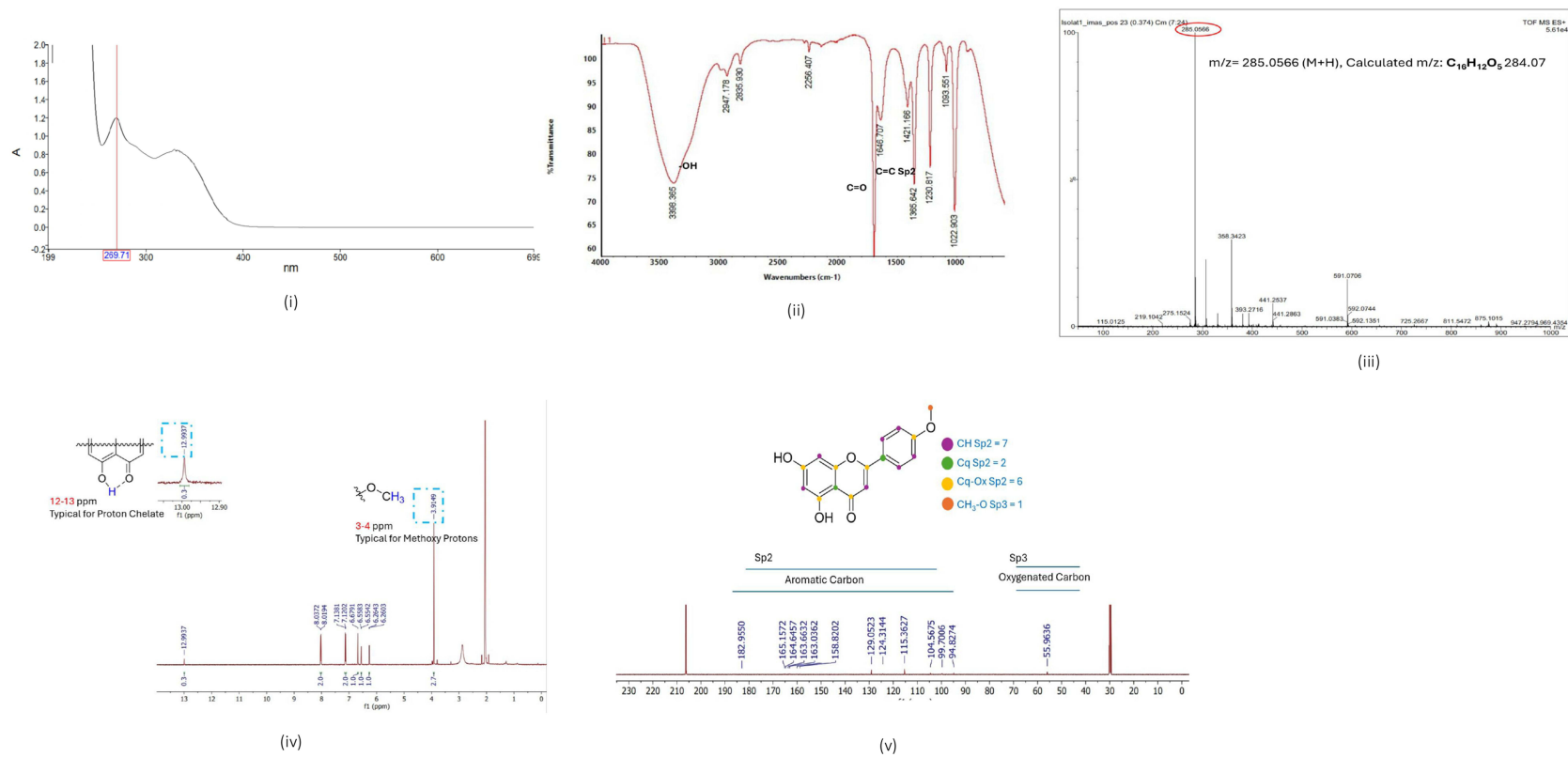


Figure I Elucidation of the structure of isolates 1: (i) UV-Vis spectrum, (ii) IR spectrum, (iii) MS spectrum, (iv) ¹H-NMR spectrum analysis, (v) ¹³C-NMR spectrum analysis.

a tube and centrifuged. The resulting pellet was washed more with cold PBS and centrifuged again. The supernatant was discarded, and the cell pellet was vortexed. The cells were then resuspended in 300 μ L of PBS. Subsequently, 1.2 mL of cold absolute ethanol was added, followed by vortexing, and the samples were stored at -20°C for at least 1 to 24 hours. After incubation, the samples were centrifuged, the ethanol was removed, and the remaining cell pellet was retained. Finally, PBS was added, and the cells were resuspended and incubated for 15 minutes. The samples were centrifuged, the supernatant was discarded, 100 μ L of RNaSe A was added, suspended, and incubated at 37°C . The tubes were lined with aluminum foil, and 400 μ L of PI reagent (50 $\mu\text{g}/\text{mL}$) was added and vortexed. Incubated at $2-8^{\circ}\text{C}$ for 30 minutes. The samples were read on a flow cytometry device within 1 hour. The assay procedure was according to the Cell Cycle kit (Elabscience).

Apoptosis Assay

The cell medium from each test was collected in a 15 mL centrifuge tube, washed twice with cold PBS (1 mL volume), and then released with trypsin. The cells were collected and placed in a tube and then centrifuged. The cells were washed again with cold PBS once and centrifuged. The PBS was discarded, then the cell pellet was vortexed. 1 mL of binding buffer was added; the cells were resuspended with 500 μ L of $1\times$ binding buffer. The samples were placed in 1.5 mL tubes lined with aluminum foil. To each tube, 5 μ L of Annexin V FITC Conjugate and 5 μ L of propidium iodide were added, then vortexed. Next, they were incubated at room temperature for 20 minutes and protected from sunlight. Samples were measured using a flow cytometer every 1 hour. The test procedure was according to the Annexin V-FITC PI Apoptosis Kit (Elabscience).

ESR1 Gene Expression

The preparation was carried out following the working instructions of the SensiFAST™ SYBR® No-ROX Kit. *ESR1* expression was examined by taking cDNA, adding up to 20 μ L of PCR Master Mix (2x SensiFAST SYBR® No-ROX Mix 10 μ L final concentration $1\times$; 10 nM forward primer 0.8 μ L final concentration 400 nM; 10 nM reverse primer 0.8 μ L final concentration 400 nM; template up to 8.4 μ L; H_2O (NFW) as needed). *ESR1* and ACTB Gene Sequence Primers are shown in Table 1. The mixed samples were inserted into the real-time PCR device and operated according to the protocol. One cycle of polymerase activation was carried out for 2 minutes at 95°C . Forty cycles of amplification were performed, with each cycle consisting of 5 seconds at 95°C (denaturation), 10 seconds at the annealing temperature according to the optimization results (60°C for β -actin, 48°C – 54°C), 5–20 seconds at 72°C (extension), and a melting curve stage with automatic temperature and time. ACTB is a housekeeping gene used as an internal control to standardize the relative expression levels for all biomarkers.

Gene Expression Analysis and Data Processing

All CT data were analyzed to determine target gene expression using the Livak method (delta-delta CT) after normalization using a housekeeping gene (beta-actin). Several controls were included in this assay, including a negative reverse transcription, no reverse transcription, and a no-template control. These controls were used to detect the presence of gDNA and possible reagent contamination. The $\Delta\Delta\text{CT}$ method was used to normalize particular gene expression in relation to an endogenous control. This process involves comparing the quantification cycle (ΔCT) values of the target gene with those of an endogenous control, a reference gene that exhibits stable expression levels. Beta-actin gene

Table 1 *ESR1* and ACTB Gene Sequence Primers

Gen		Primary Sequences
<i>ESR1</i>	F	5'-TGATGAAAGGTGGGATACGA-3'
	R	5'-AAGGTTGGCAGCTCTCATGT-3'
ACTB	F	5'-TGGAATCCTGTGGCATCCATGAAAC-3'
	R	5'-TAAACGCAGCTCAGTAACAGTCCG-3'

expression served as the selected endogenous control. The $\Delta\Delta CT$ method provides a precise method for quantifying and assessing changes in gene expression levels under different experimental conditions.

The normalized ΔCT values of the treated samples were compared with those of the omitted reference control (ΔCT (ref)) to obtain $\Delta\Delta CT$ values, which were then used to calculate the relative fold change compared to the control. Quantification of estrogen receptor alpha (ER α) by RT-qPCR in MCF-7 cells treated for 24 hours with or without 2 μM E2. Data presented are the mean $\pm$ standard deviation (SEM) obtained from a minimum of three different experiments, with each experiment involving three replicates.

Results

Structural Characterization of Compounds 1, 2, and 3 from Chrysanthemum morifolium Leaves (CML)

Based on the interpretation of spectroscopic data, including UV-Vis, IR, MS, 1H -NMR, ^{13}C -NMR, HMBC, and HSQC, as shown in Figure 1 for the structural characterization of compound 1, Figure 2 for the structural characterization of compound 2, and Figure 3 for the structural characterization of compound 3. According to High Resolution Electrospray Ionization Mass Spectrometry (HR-ESI-MS) data of m/z $[M+H]^+$ 285.0566 (calculated for 284.07), Compound 1 CML was isolated as an amorphous powder with the chemical formula $C_{16}H_{12}O_5$, which represents 10 double bond equivalents (DBE). Spectral data of compound 1 CML are as follows: IR: 3398.365 cm^{-1} contained OH functional groups, 1646.707 cm^{-1} , and 1421.166 cm^{-1} indicated the presence of C=O (carbonyl) and C=C Sp 2 aromatic functional groups, respectively. 1H -NMR: The chemical shift of isolated compound 1 δH 12.9937 ppm indicates the presence of typical chelate protons, and δH 3.9149 ppm indicates the presence of methoxy protons. Typical aromatic Sp 2 protons are found in the chemical shift region δH 6.2603 ppm and δH 6.2643 ppm (H-6 and H-8) with a bond constant value of $j = 1.60$, where H-6 and H-8 indicate meta positions, each in a splitting doublet pattern (d) which shows the characteristics of ring A in the flavonoid framework. Chemical shift area δH 7.1202 ppm and 7.1381 ppm (H-3' and H-5') with a bond constant value $j=7.14$, where H-3' and H-5' indicate the ortho position; δH 8.0194 ppm and 8.0372 ppm (H-2' and H-6') with a bond constant value $j=7.14$, where H-3' and H-5' indicate the ortho position. Each in a doublet splitting pattern (d), which shows the characteristics of ring B in the flavonoid framework. Then the chemical shift δH 6.6791 ppm (H-3) in ring C of the flavonoid framework. ^{13}C -NMR: Based on the spectrum, it shows a chemical shift for the carbon atoms that comprise the flavonol compound framework, which has 16 carbons. In the chemical shift areas, respectively δC 164.65 ppm (C-2); δC 104.57 ppm (C-3); δC 182.96 ppm (C-4); δC 163.04 ppm (C-5); δC 165.16 ppm (C-7); δC 104.57 ppm (C-10); δC 124.31 ppm (C-1'); δC 158.82 ppm (C-4') there are two methine carbons (CH) in a singlet (s) splitting pattern. In the chemical shift areas, δC 99.70 ppm (C-6); δC 94.83 ppm (C-8); δC 129.05 ppm (C-2'); δC 115.36 ppm (C-3'); δC 115.36 ppm (C-5'), and δC 129.05 ppm (C-6') each have two methine carbons (CH) in a doublet splitting pattern (d). Quaternary carbon appears in the chemical shift region of δC 55.96 ppm (C-4' OCH $_3$). Based on the description above, it can be assumed that the structural framework of the compound obtained is a flavonol compound.

The chemical formula of Compound 2 CML, which was produced as an amorphous powder, was determined to be $C_{16}H_{12}O_6$ with 12 degrees of DBEs based on the molecular ion peak at m/z $[M+H_2]$ 301.0459 (calculated for 300.07). The following are compound 2 CML's spectral data: IR: has the aromatic functional groups C=O (carbonyl) and C=C Sp 2 at wave numbers 1636.869 cm^{-1} and 2126.776 cm^{-1} , respectively, and the OH functional group at wave number 3320.191 cm^{-1} .

1H -NMR: The chemical shift of isolated compound 2 CML δH 13.0036 ppm indicates the presence of typical chelate protons, and δH 3.9496 ppm indicates the presence of methoxy protons. Typical aromatic protons Sp 2 are found in the chemical shift region δH 6.6520 (H-6) with a bond constant value of $j = 1.19$, chemical shift δH 6.6484 (H-7) with a bond constant value of $j = 1.19$ and chemical shift δH 6.5642 (H-8) with a bond constant value of $j = 1.19$ where H-6 and H-8 indicate meta positions, each in a splitting doublet pattern (d) which shows the characteristics of ring A in the flavonoid framework. The chemical shift area δH 7.5835–7.5618 (H-2') with the bond constant value $j_1=8.5$ and $j_2=2.3$; and the chemical shift δH 7.1438–7.1267 (H-6') with the bond constant value $j=2.3$. The bond constant value $j=8.5$, where H-2' and H-3' indicate the ortho position; with the

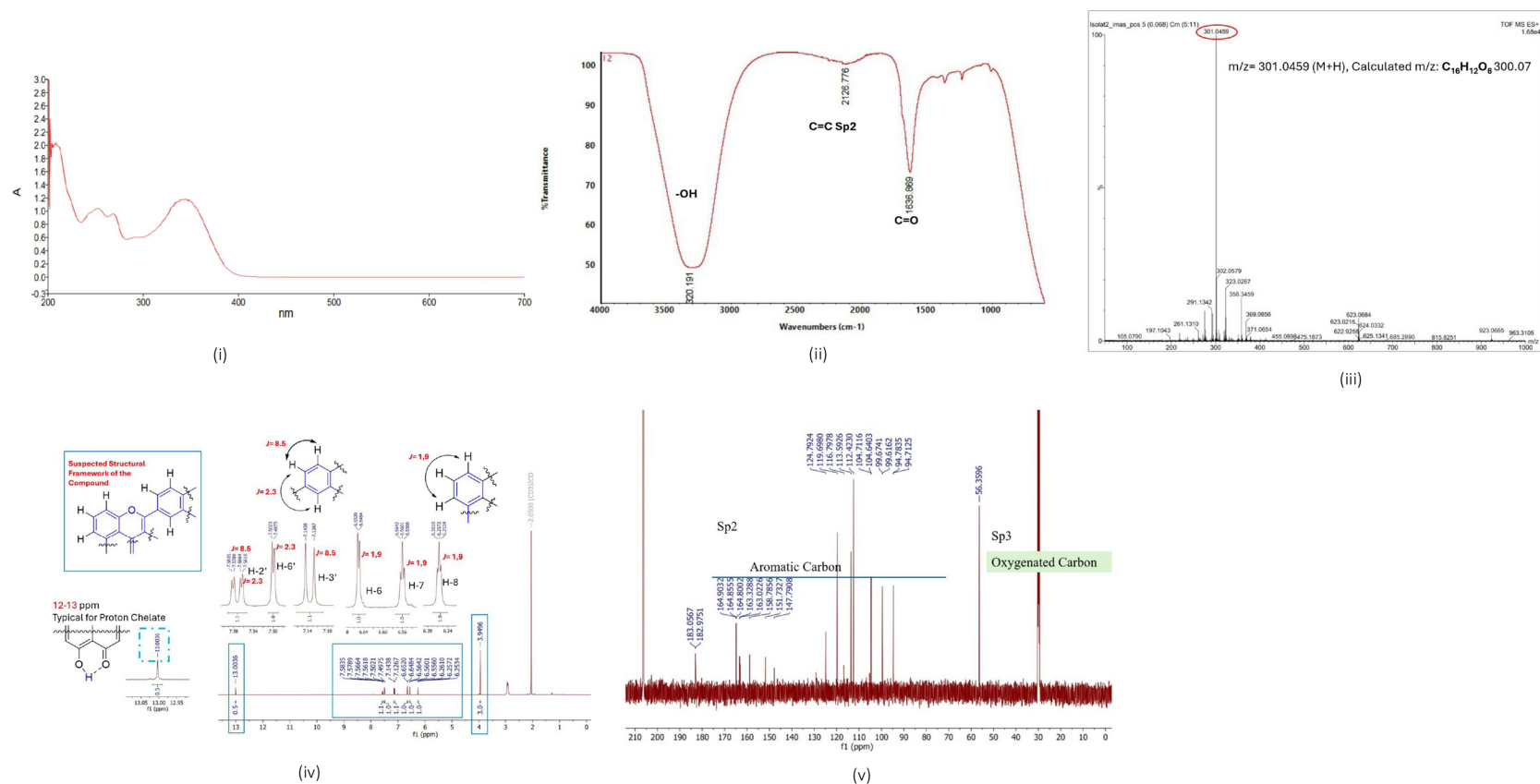


Figure 2 Elucidation of the structure of isolates **2**: (i) UV-Vis spectrum, (ii) IR spectrum, (iii) MS spectrum, (iv) ¹H-NMR spectrum analysis, (v) ¹³C-NMR spectrum analysis.

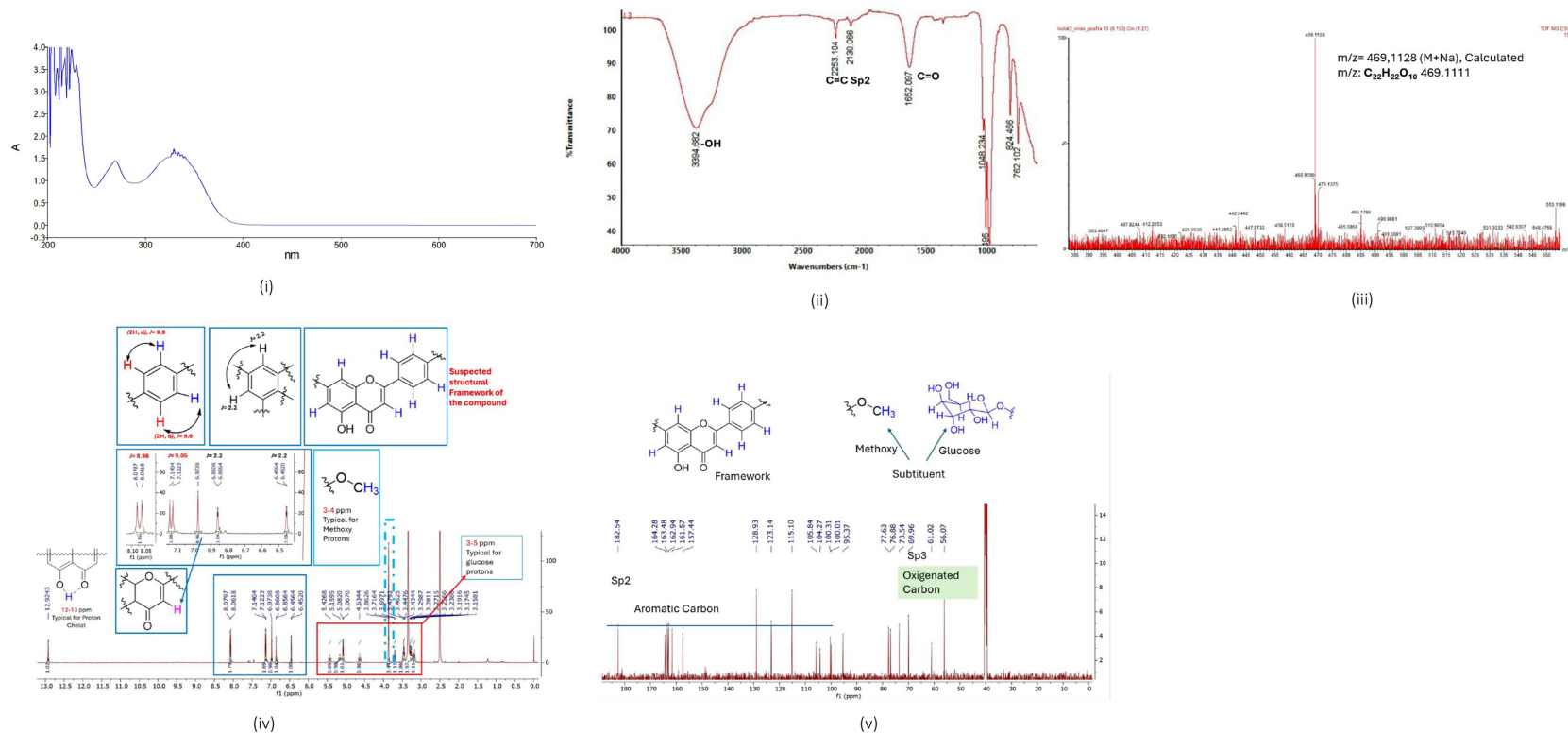
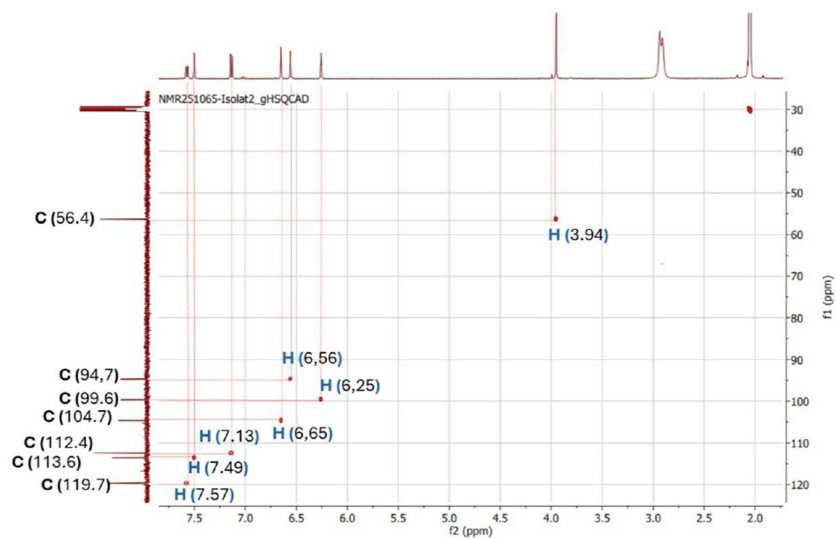


Table 2 IC₅₀ Value and Selectivity Index of Isolates 1, 2, 3 CML

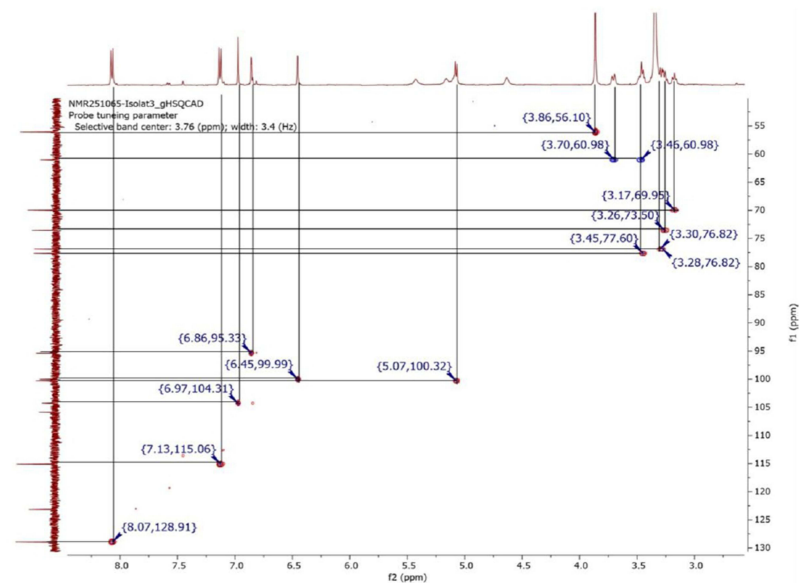
Sample	IC ₅₀ value (ppm) 24 Hours		Selectivity Index
	MCF-7 Cells	HEK-293 Cells	
Isolate 1 CML	189.289	439.600	2.322
Isolate 2 CML	49.37	225.045	4.558
Isolate 3 CML	268.031	444.019	1.657
Cisplatin	6.530	9.295	1.423

bond constant value $j=2.3$, where H-2' and H-6' indicate the meta position. Each in the doublet splitting pattern (d) shows the characteristics of the B ring in the flavonoid skeleton. ¹³C-NMR: Based on the spectrum, it shows a chemical shift for the carbon atoms that make up the flavonol compound framework, which selects a total number of carbons. In the chemical shift areas of δC 116.8 ppm (C-2); δC 163.0 ppm (C-3); δC 182.9 ppm (C-4); δC 163.4 ppm (C-5); δC 158.7 ppm (C-9); δC 164.8 ppm (C-10); δC 123.9 ppm (C-1'); δC 151.7 ppm (C-4'); δC 147.9 ppm (C-5') there are two methine carbons (CH) in a splitting singlet (s) pattern, respectively. In the chemical shift areas of δC 99.70 ppm (C-6); δC 94.83 ppm (C-8); δC 129.05 ppm (C-2'); δC 115.36 ppm (C-3'); δC 115.36 ppm (C-5') and δC 129.05 ppm (C-6'), each contains two methine carbons (CH) in a doublet splitting pattern (d). Quaternary carbon appears in the chemical shift region of δC 55.96 ppm (C-4' OCH₃). Based on the description above, it can be assumed that the structural framework of the compound obtained is a flavonol compound, as shown in Table 2. The results of ultraviolet spectrophotometric analysis of isolate 2 CML showed maximum absorption in band II with a λ_{max} wavelength of 201 nm and band I at a λ_{max} wavelength of 300–400 nm.

Compound 3 CML is a yellow amorphous powder having the formula C₂₂H₂₂O₁₀ with 16 degrees DBEs, as determined by the molecular ion peak at m/z [M+Na] 469.1128 (calculated for 469.1111). Spectral data of compound 3 are as follows: IR: contains an OH functional group at wave number 3394.682 cm⁻¹, C=O (carbonyl) and aromatic C=C Sp² functional groups at wave numbers 1652.097 cm⁻¹ and 2253.104 cm⁻¹, respectively. The presence of OH, C=O (carbonyl); and aromatic C=C Sp² functional groups is a characteristic of flavonoid compounds. ¹H-NMR: The chemical shift of the isolated compound 2 CML δH 12.9243 ppm indicates the presence of typical chelate protons, δH 3–4 ppm indicates the presence of methoxy protons, and δH 3–5 ppm indicates typical sugar protons. The typical aromatic Sp² protons are found in the chemical shift region δH 6.8 (H-6) and chemical shift δH 6.4 (H-8) with a bond constant value of $j=2.2$ where H-6 and H-8 indicate the meta position, each in a splitting doublet pattern (d) which shows the characteristics of ring A and chemical shift δH 8.05 (H-6) with a bond constant value of $j=8.98$ and chemical shift δH 7.1 (H-8) with a bond constant value of $j=9.05$ where H-6 and H-8 indicate the ortho position, each in a splitting doublet pattern (d) which shows the characteristics of ring B in the flavonoid framework. The chemical shift region δH 6.9738 (H-3) shows the characteristics of ring C in the flavonoid framework. ¹³C-NMR: Based on the spectrum, it shows a chemical shift for the carbon atoms that make up the flavonol compound framework, which indicates the presence of these carbons. In the chemical shift regions of δC 164.3 ppm (C-2); δC 104.3 ppm (C-3); δC 182.9 ppm (C-4); δC 161.5 ppm (C-5); δC 163.4 ppm (C-7); δC 157.4 ppm (C-9); δC 105.8 ppm (C-10); δC 123.4 ppm (C-1'); δC 162.9 ppm (C-4') there are two methine carbons (CH) in a splitting singlet (s) pattern, respectively. In the chemical shift regions of δC 99.9 ppm (C-6); δC 95.3 ppm (C-8), δC 128.9 ppm (C-2'), δC 115.1 ppm (C-3'), δC 115.1 ppm (C-5'), and δC 126.9 ppm (C-6'), each have two methine carbons (CH) in a doublet splitting pattern (d). Quaternary carbon appears in the chemical shift region of δC 56.1 ppm (C-4' OCH₃). In the chemical shift region of δC 100.3 ppm, there are two methine carbons (CH) in a doublet splitting pattern (d). In the chemical shift regions of δC 77.6 ppm; δC 60.9 ppm; δC 69.9 ppm; δC 73.5 ppm; δC 76.8 ppm; δC 77.6 ppm, each contains two methine carbons (CH) in a singlet (s) splitting pattern. Based on the description above, it can be assumed that the structural framework of the compound obtained is a flavonol glycoside compound.



(i)



(ii)

Figure 4 2D HSQC NMR spectrum of isolate **2**: (i) 2D HSQC NMR spectrum of isolate **3** (ii).

^1H - ^{13}C -HSQC: The relationship between carbon and protons in isolates **2** and **3** CML indicates a relationship between the signals of the carbon nucleus and protons, as shown in Figure 4. The same spot in the spectrum indicates that both are directly connected by a bond. Two C-H bonds in Ring A are formed from chemical shifts of δH 6.56 ppm (H-8), which correlates with δC 94.7 ppm (C-8); δH 6.25 ppm (H-6), which correlates with δC 94.7 ppm (C-6); δH 6.65 ppm (H-7), which correlates with δC 104.7 ppm (C-7). Four C-H bonds in Ring B are formed from chemical shifts of δH 3.94 ppm (H-4') which correlates with δC 56.4 ppm (4'-OCH₃); δH 7.13 ppm (H-6') which correlates with δC 112.4 ppm (C-6'); δH 7.49 ppm (H-3') which correlates with δC 113.6 ppm (C-3') and δH 7.57 ppm (H-2') which correlates with δC 119.7 ppm (C-2'). The correlation of carbon with proton ^1H - ^{13}C -HSQC for isolate **3** can be seen. Two C-H bonds in Ring A are formed from chemical shifts of δH 6.86 ppm (H-8), which correlates with δC 95.3 ppm (C-8) and δH 6.45 ppm (H-6), which correlates with δC 99.9 ppm (C-6). Four C-H bonds in Ring B are formed from chemical shifts of δH 7.13 ppm (H-3' and H-5'), which correlate with δC 115.1 ppm (C-3' and C-5') forming two C-H bonds (overlapping) and δH 8.07 ppm (H-2' and H-6') which correlate with δC 128.9 ppm (C-3' and C-5') forming two C-H bonds (overlapping).

HMBC: The results of the HMBC spectrum analysis show a relationship between protons and carbons that are more than one bond apart, as shown in Figure 5. The ordinate axis in the HMBC spectrum is plotted with the ^1H chemical shift of the proton nucleus. The HMBC spectrum of isolate compound **2** CML gives a proton signal at δH 6.25 ppm (H-6) when a vertical line is drawn from the signal, 4 bonds will be obtained, which are the result of long-distance relationships with carbon. Each of these spots belongs to carbon from the position of C-8 (δC 94.7); C-7 (δC 104.7); C-5 (δC 163.4); and C-7 (δC 104.7). If drawn from δH 6.56 ppm (H-8), four spots will be obtained at C-6 (δC 99.6); C-7 (δC 104.7); C-9 (δC 158.7); C-7 (δC 104.7); C-10 (δC 164.8) and C-4 (δC 182.9). If drawn from δH 7.57 ppm (H-2'), then three spots will be obtained at C-1' at C-1' (δC 123.9 ppm); C-3' (δC 113.6 ppm); C-4' (151.7 ppm); C-2 (116.8 ppm). Then for δH

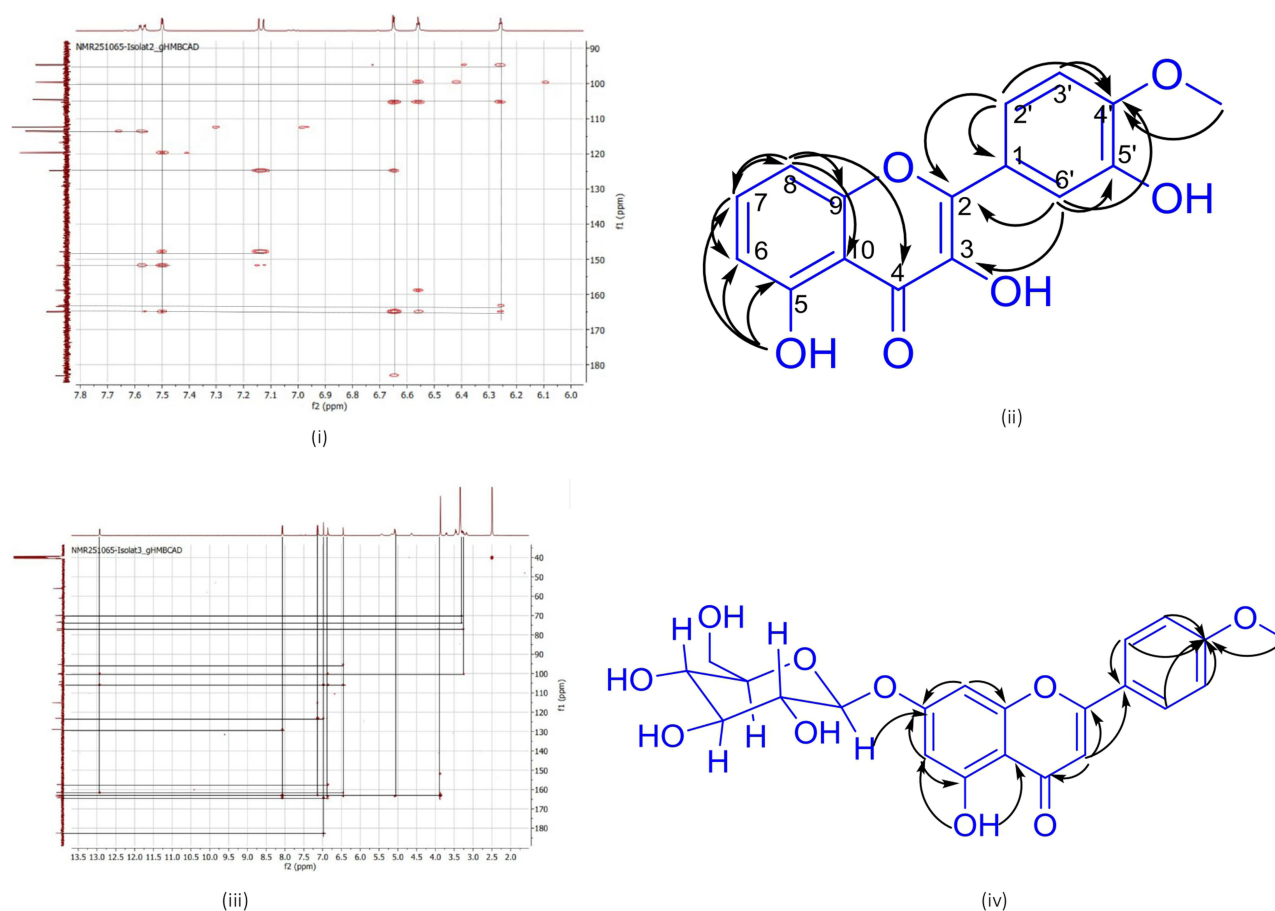


Figure 5 2D HMBC NMR spectrum of isolate **2** (i), HMBC isolate **2** correlation diagram (ii), 2D HMBC NMR spectrum of isolate **3** (iii), HMBC isolate **3** correlation diagram (iv).

7.13 ppm (H-6'), two spots will be obtained at C-5' (δ C 147.9 ppm); C-4' (δ C 151.7 ppm); C2 (δ C 116.8 ppm), and C-3 (δ C 163.0 ppm). Isolate compound **3** gives a proton signal at δ H 6.45 ppm (H-6) when a vertical line is drawn from the signal, 4 bonds will be obtained, which are the result of long-distance relationships with carbon. Each of these spots belongs to carbon from the C-8 position (δ C 95.3), C-9 (δ C 157.4), C-5 (δ C 161.5), and C-7 (δ C 163.4). If drawn from δ H 6.86 ppm (H-8), four spots will be obtained at C-6 (δ C 99.9), C-9 (δ C 157.4), and C-7 (δ C 163.4). If drawn from δ H 7.13 ppm (H-3' and H-5'), then three spots will be obtained at C-2' or C-6', giving the same signal at δ C 126.9 ppm; C-3' (δ C 115.1) and C-1' (δ C 123.4). Then for δ H 7.13 ppm (H-3' and H-5'), four spots will be obtained at C-1' (δ C 123.4); at C-3' or C-5' giving the same signal at δ C 115.1 ppm; C-4' (δ C 162.9) and C-1' (δ C 123.4).

Cytotoxic Bioassay and Selectivity Index of Compounds **1**, **2**, and **3** CML

The cytotoxic activities of compounds **1** and **2** CML were evaluated against the MCF-7 breast cancer cell line according to a previously published method.¹¹ The results of the cytotoxic test of the test solution on MCF-7 and HEK-293 cells obtained the % survival rate values shown in Figure 6, and the IC₅₀ values and selectivity index presented in Table 2. The IC₅₀ value (ppm) indicates the concentration of a compound that inhibits cell viability by 50%. The lower the value, the stronger the cytotoxicity. The Selectivity Index is calculated by comparing the IC₅₀ value of HEK-293 with the IC₅₀ value of MCF-7. A Selectivity Index value > 2 indicates good selectivity against cancer cells, an IS value $\approx$ of 1 is categorized as non-selective, and an IS value < 1 is categorized as more toxic to normal cells.

Cell Cycle Assay

From the results of the cell cycle inhibition test using propidium iodide reagent with a flow cytometer, the percentage of inhibition in each phase was obtained, namely the percentage of accumulation in each phase in the cell cycle, as shown in Table 3 and Figure 7.

The results of the cell cycle distribution analysis showed that treatment with cisplatin and isolate **2** CML resulted in significant changes in the distribution of cell cycle phases compared to the control. In control cells, the cell cycle distribution was relatively normal with a predominance in the G1 phase ($49.96 \pm 0.93\%$), followed by the S phase ($22.61 \pm 4.27\%$) and G2/M phase ($25.81 \pm 4.09\%$), indicating stable cell proliferation activity.

Cisplatin treatment at a concentration of $1 \times \text{IC}_{50}$ increased the proportion of cells in the G1 phase to $55.55 \pm 2.52\%$ and decreased the S phase to $19.22 \pm 2.56\%$. At a concentration of $\frac{1}{2} \times \text{IC}_{50}$, the increase in the G1 phase was higher, namely $71.32 \pm 0.52\%$. In contrast, isolate **2** DKP at a concentration of $1 \times \text{IC}_{50}$ showed a significant increase in the G2/M phase to $50.73 \pm 4.13\%$, while at a concentration of $\frac{1}{2} \times \text{IC}_{50}$, there was an increase in the G1 phase by $63.78 \pm 5.66\%$.

The results of the statistical test analysis of the cell cycle distribution showed that treatment using cisplatin and isolate **2** CML in the G0, G1, S, and G2/M cycles resulted in significant changes to the distribution of the cell cycle phases compared to the control, as seen in Figure 8.

The results of this study indicate that cisplatin and isolate **2** CML treatments can inhibit cell proliferation through different cell cycle inhibition mechanisms. Cell cycle distribution is an important indicator in determining the point of action of an anticancer agent in inhibiting cancer cell growth.⁵⁵

Apoptosis Assay

The flow cytometry data, presented as the percentage of live MCF-7 cells, those undergoing apoptosis, and those undergoing necrosis, are shown in Table 4 and Figure 9. In cell cycle analysis, isolate **2** CML at the $1 \times \text{IC}_{50}$ concentration caused a significant increase in the G2/M phase ($50.73 \pm 4.13\%$), indicating G2/M arrest. Meanwhile, in the apoptosis assay, the same treatment increased early apoptosis of up to $38.87 \pm 11.43\%$. This relationship suggests that cell cycle arrest at the G2/M phase contributes directly to the induction of apoptosis.

In contrast, cisplatin showed a dominant effect on the G1 phase with an increase of up to $71.32 \pm 0.52\%$ at a concentration of $\frac{1}{2}$ the IC₅₀. However, the effect on apoptosis was relatively low, with initial apoptosis only around $6.63 \pm 1.77\%$ at the $1 \times \text{IC}_{50}$. This suggests that cisplatin in this condition acts more as a cytostatic agent that inhibits cell proliferation through G1 arrest, without directly inducing significant apoptosis. The results of the statistical test on the apoptosis test results are shown in Figure 10.

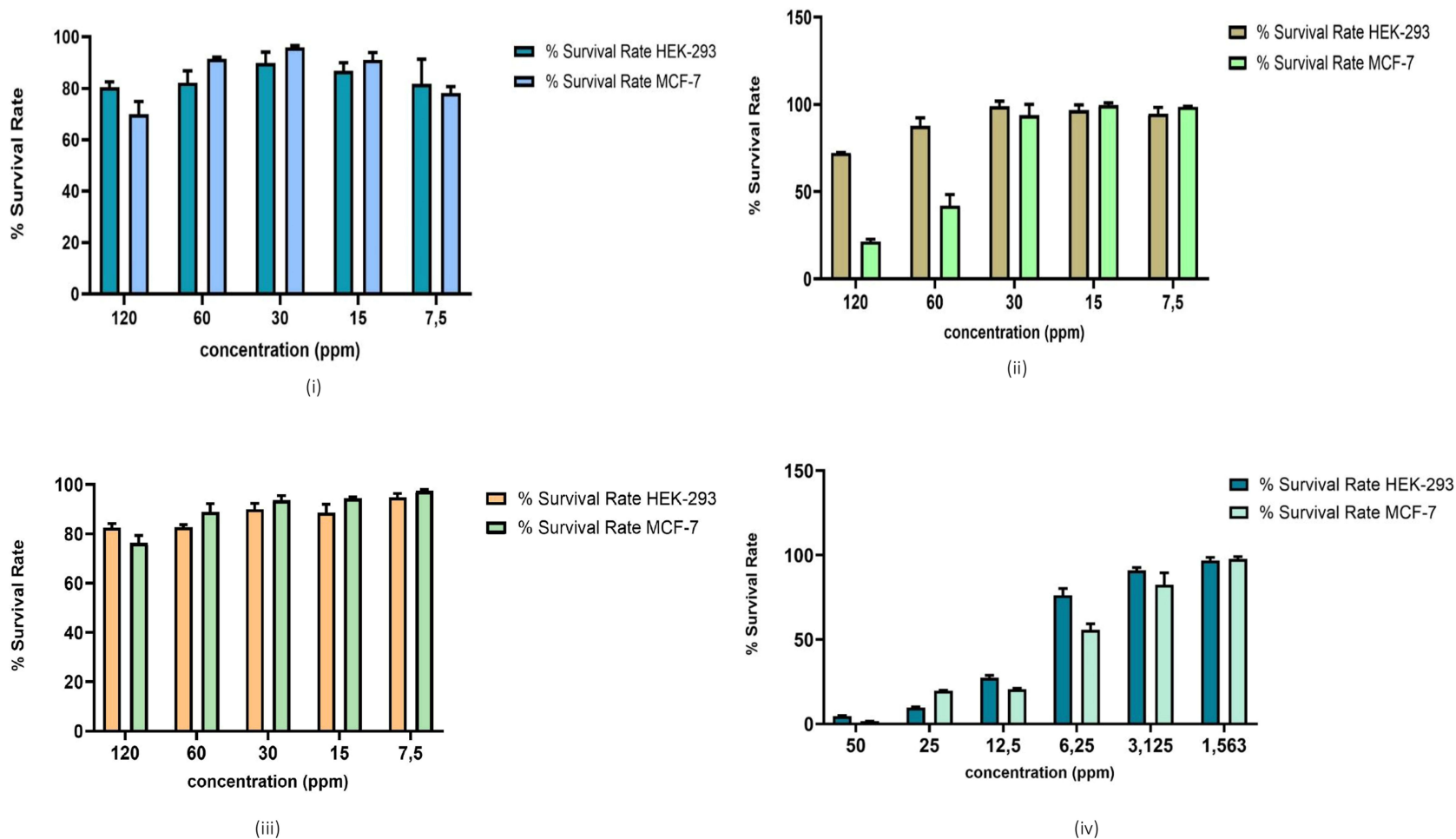


Figure 6 % Survival rate of isolates **1** (i), isolate **2** (ii), isolate **3** (iii), and cisplatin (iv).



Table 3 Percentage of Accumulation of Isolate 2 CML and Cisplatin Against MCF Cell Lines at Each Phase of the Cell Cycle

No	Treatment	Average Cell Cycle Phase (%)			
		G0	G1	S	G2/M
1	Cisplatin (1 × IC ₅₀)	7.72±1.44	55.55±2.52	19.22±2.56	17.51±0.67
2	Cisplatin (1/2 × IC ₅₀)	3.81±0.74	71.32±0.52	14.16±2.11	10.70±2.19
3	Isolate 2 CML (1 × IC ₅₀)	5.64±0.24	30.51±2.42	13.12±2.49	507.3±41.3
4	Isolate 2 CML (1/2 × IC ₅₀)	2.25±0.34	63.78±56.6	8.48±1.45	25.50±56.2
5	Cell Control	1.61±0.06	49.96±0.93	22.61±4.27	25.81±4.09

Notes: Average treatment of 3 replications.

The results of this study indicate that cisplatin and isolate 2 CML treatments can inhibit cell proliferation through different cell cycle inhibition mechanisms. Cell cycle distribution is an important indicator in determining the point of action of an anticancer agent in inhibiting cancer cell growth.⁵⁵

ESR1 Gene Expression

Reverse transcriptase-PCR (RT-PCR) is a method for synthesizing single-stranded DNA from mRNA. The basic principle of RT-PCR is to convert mRNA strands into DNA using the reverse transcriptase enzyme and oligonucleotide primers. The primers used in the PCR process are forward and reverse. The resulting double-stranded DNA is then amplified using standard PCR techniques.⁵⁶ In this test, MCF-7 cells were treated with isolate 2 test compound and cisplatin with concentrations of 1 × IC₅₀ and 1/2 × IC₅₀ in each MCF-7 cell. Measurement of *ESR1* and *ACTB* expression was carried out using RT-qPCR to produce an amplification curve in the form of a melting curve, then the data were analyzed using the $\Delta\Delta C_t$ or 2- $\Delta\Delta C_t$ method to calculate the ratio of expression changes (fold change) between the control and treatment groups. The results of the gene expression test are shown in Table 5.

The results showed that *ESR1* gene expression experienced different changes in each treatment. The control group had a relative expression value of 0.83 ± 1.17. The Isolate 2 CML treatment group at a dose of 1 × IC₅₀ showed an increase in expression to 1.34 ± 0.13, while at a dose of 1/2 × IC₅₀ showed a value close to normal, namely 1.01 ± 0.14. In the cisplatin treatment group at a dose of 1 × IC₅₀, there was a very high increase in expression, namely 4.58 ± 1.87, while at a dose of 1/2 × IC₅₀, there was a significant decrease in expression to 0.19 ± 0.04. These results indicate that the treatment provides a modulatory effect on *ESR1* gene expression that is dose-dependent.

Statistical analysis of the *t*-test comparing the expression of the *ESR1* gene of isolate 2 CML and Cisplatin against the control is presented in Figure 11. The results showed that treatment with isolate 2 CML and cisplatin resulted in differences in gene expression levels as indicated by the normalized expression ratio value. *ESR1* gene expression analysis showed that treatment of isolate 2 CML at concentrations of 1 × IC₅₀ and 1/2 × IC₅₀ did not provide a significant difference compared to the control group (*p* > 0.05). Isolate 2 DKP 1 × IC₅₀ increased *ESR1* expression compared to the control, but the very large variation in the control caused the difference to be insignificant. Isolate 2 DKP 1/2 × IC₅₀ showed *ESR1* expression that was almost the same as the control. Cisplatin treatment 1 × IC₅₀ resulted in the highest increase in *ESR1* gene expression, but this increase was also not statistically significant (*p* > 0.05). Cisplatin 1/2 × IC₅₀ showed a tendency to decrease *ESR1* expression compared to the control.

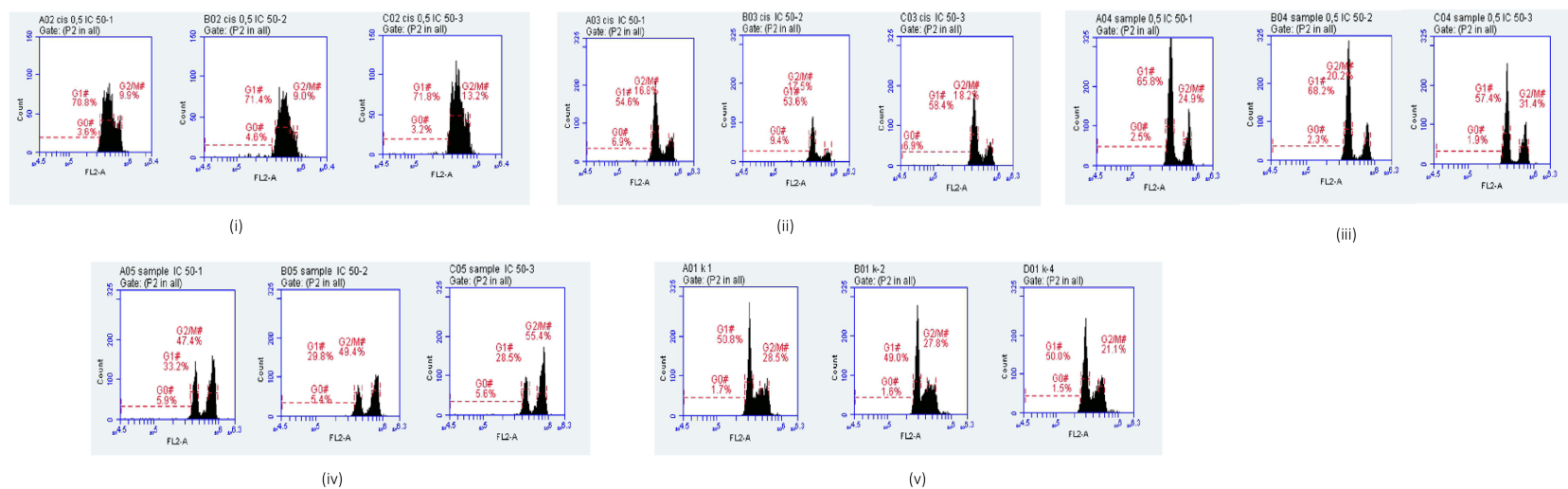


Figure 7 Flow cytometer image results MCF-7 cell cycle: (i) Cisplatin $1/2 \times IC_{50}$, (ii) cisplatin $1 \times IC_{50}$, (iii) isolate 2 CML $1/2 \times IC_{50}$, (iv) isolate 2 CML $1 \times IC_{50}$, (v) Cell control.

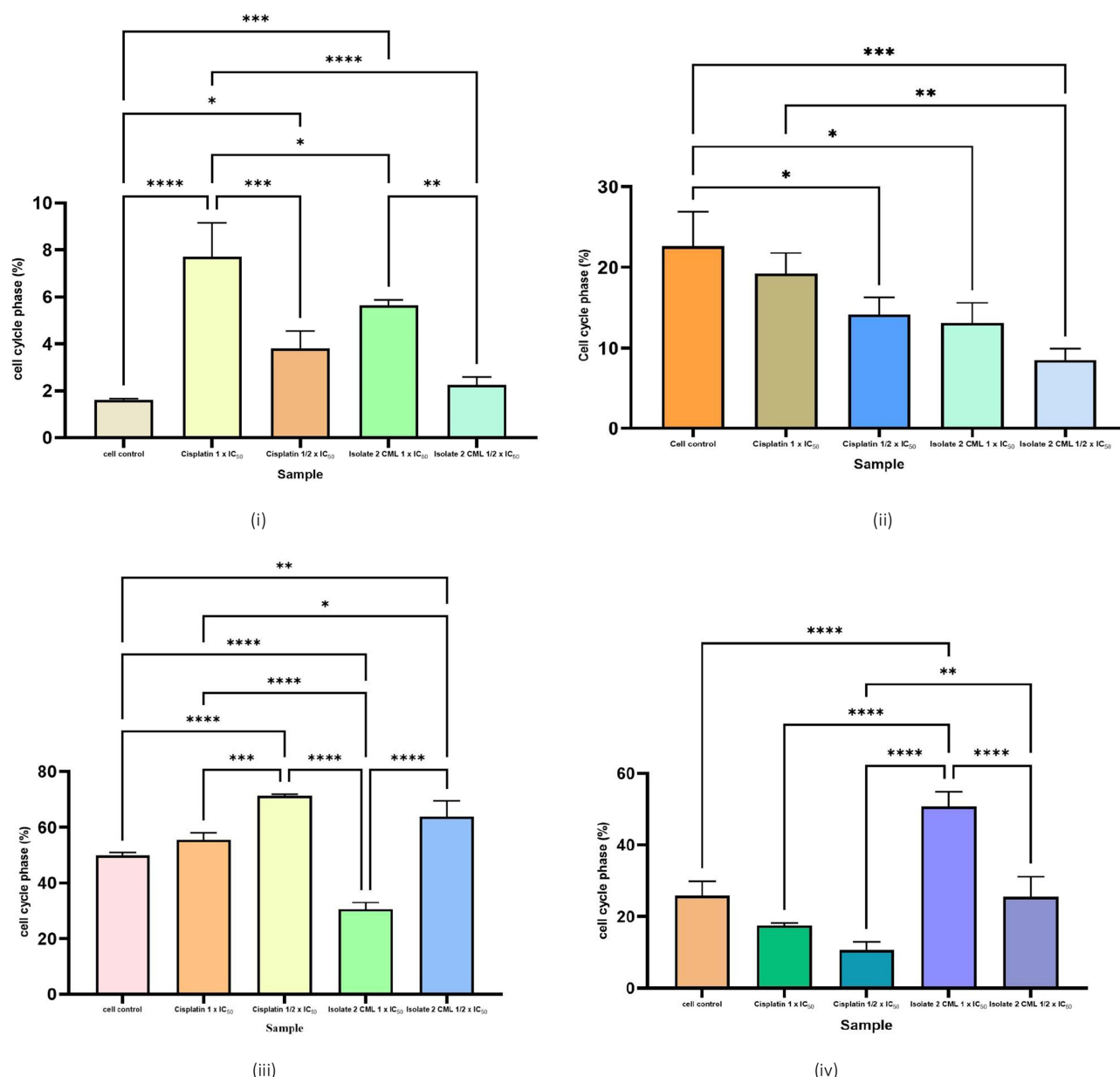


Figure 8 Comparison of cell cycle phases G0 (i), G1 (ii), S (iii), and G2/M (iv) in control, cisplatin (1 × IC₅₀), cisplatin (1/2 × IC₅₀), isolate 2 (1 × IC₅₀), isolate 2 (1/2 × IC₅₀). * (p < 0.05) significant. ** (p < 0.01) highly significant. *** (p < 0.001) very highly significant. **** (p < 0.0001) extremely significant.

Discussion

Based on the UV, IR, 1-dimensional NMR (proton and carbon) spectrum data, mass spectrum, and by comparing the chemical shift data (proton and carbon) with the acacetin compound, the chemical structure of isolate compound **1** can be determined as acacetin compound. Based on the UV, IR, 1-dimensional NMR (proton and carbon), 2-dimensional NMR (HSQC and HMBC), and mass spectrum data on isolate 2, it is predicted to be a 3,5,5'-Trihydroxy-4'-methoxy flavone compound. Meanwhile, isolate 3 is predicted to be a kaempferide-7-O-glycoside compound. The chemical structure of isolate compounds **1**, **2**, and **3** is shown in Figure 12.

From the results of the cytotoxicity test, IC₅₀ value, and selectivity index value, isolate **1** CML has moderate cytotoxic activity with good selectivity against MCF-7. Isolate **2** CML has moderate but selective anticancer activity against MCF-7, and is the best candidate as a selective anticancer compound. Isolate **3** CML has moderate cytotoxic

Table 4 Apoptosis Test Results of Isolate 2 CML and Cisplatin Against MCF Cell Lines

No	Treatment	Test	Gate (%)			
			Life	Early Apoptosis	Late Apoptosis	Necrosis
			Annexin (-) PI (-)	Annexin (+) PI (-)	Annexin (+) PI (+)	Annexin (-) PI (+)
1	Control	Unstain	100.00	0.00	0.00	0.00
		PI	98.73	0.00	1.25	0.00
		Annexin	90.47	9.53	0.00	0.00
		Annexin-PI	74.09	25.00	0.05	0.86
2	1 × IC ₅₀ Isolate 2 (49.37 ppm)	Average	58.27±12.26	38.87±11.43	0.05±0.05	2.81±0.93
3	½ × IC ₅₀ Isolate 2 (24,685 ppm)	Average	61.37±9.51	37.17±9.37	0.02±0.03	1.43±0.14
4	1 × IC ₅₀ Cisplatin (6.530 ppm)	Average	90.82±1.72	6.63±1.77	0.26±0.15	2.29±0.32
5	½ × IC ₅₀ Cisplatin (3.625 ppm)	Average	72.80±13.58	14.93±11.00	0.02±0.03	1.32±0.16

Notes: Average treatment of 3 replications.

activity; selectivity is not optimal, and cisplatin, as a comparison, is very potent but less selective, in line with the toxic effects of cisplatin on normal cells.

The overall results indicate that the cytotoxic activity of isolate compound **2** is higher compared to other flavonoids (isolate compounds **1** and **3**), largely due to the presence of more hydroxyl groups in isolate compound **2**. This claim is fully supported by the literature, proving that flavonoid derivatives with methoxy content and having many free hydroxyl groups are more potent as cytotoxic agents and have a higher ability to inhibit tumor cells than flavonoid derivatives in the form of glycosides.^{57–59} The literature also concludes that methoxy-flavonoids exhibit remarkable chemoprotective properties and are potentially useful as anticancer agents (Mohammed et al, 2021).⁵⁸

Cisplatin is known to work as a chemotherapeutic agent that induces DNA damage through the formation of DNA crosslinks. This damage activates cell cycle checkpoints, particularly in the G1 phase, via the p53 protein pathway, which subsequently increases the expression of p21, a CDK inhibitor.⁶⁰ In this study, the increase in the G1 phase at both 1 × IC₅₀ and ½ × IC₅₀ concentrations indicates that cisplatin causes G1 arrest, preventing cells from progressing to the DNA synthesis phase (S phase). The decrease in the S phase in both treatments further strengthens the inhibition of the G1 to S transition. Furthermore, the stronger effect at ½ × IC₅₀ concentrations indicates that cisplatin at low doses is more cytostatic, meaning it inhibits proliferation without directly causing cell death. This is consistent with reports that cisplatin can induce cell cycle arrest before leading to apoptosis.⁶¹

In contrast to cisplatin, isolate **2** CML at a concentration of 1 × IC₅₀ showed a significant increase in the G2/M phase. This indicates the occurrence of G2/M arrest, which is the arrest of the cell cycle before entering mitosis. The G2/M phase is a critical checkpoint to ensure that DNA has been properly replicated before cell division occurs. Disruption of this phase is often associated with severe DNA damage, impaired mitotic spindle formation, and inhibition of the cyclin B/CDK1 complex. The accumulation of cells in the G2/M phase indicates that isolate **2** CML has potential as an antimitotic agent, acting by inhibiting mitosis. Compounds with this mechanism generally have high cytotoxic potential because cells that fail to pass through this phase will undergo apoptosis.^{62,63} At a concentration of ½ × IC₅₀, isolate **2** CML showed an increase in the G1 phase, indicating that at lower doses, the effect tends to shift to cytostatic. This suggests a dose-dependent relationship with the compound's mechanism of action.

Figure 13 illustrates the differences in cell cycle distribution between the two treatments, indicating different mechanisms of action: cisplatin predominantly causes G1 arrest through DNA damage, while isolate **2** CML

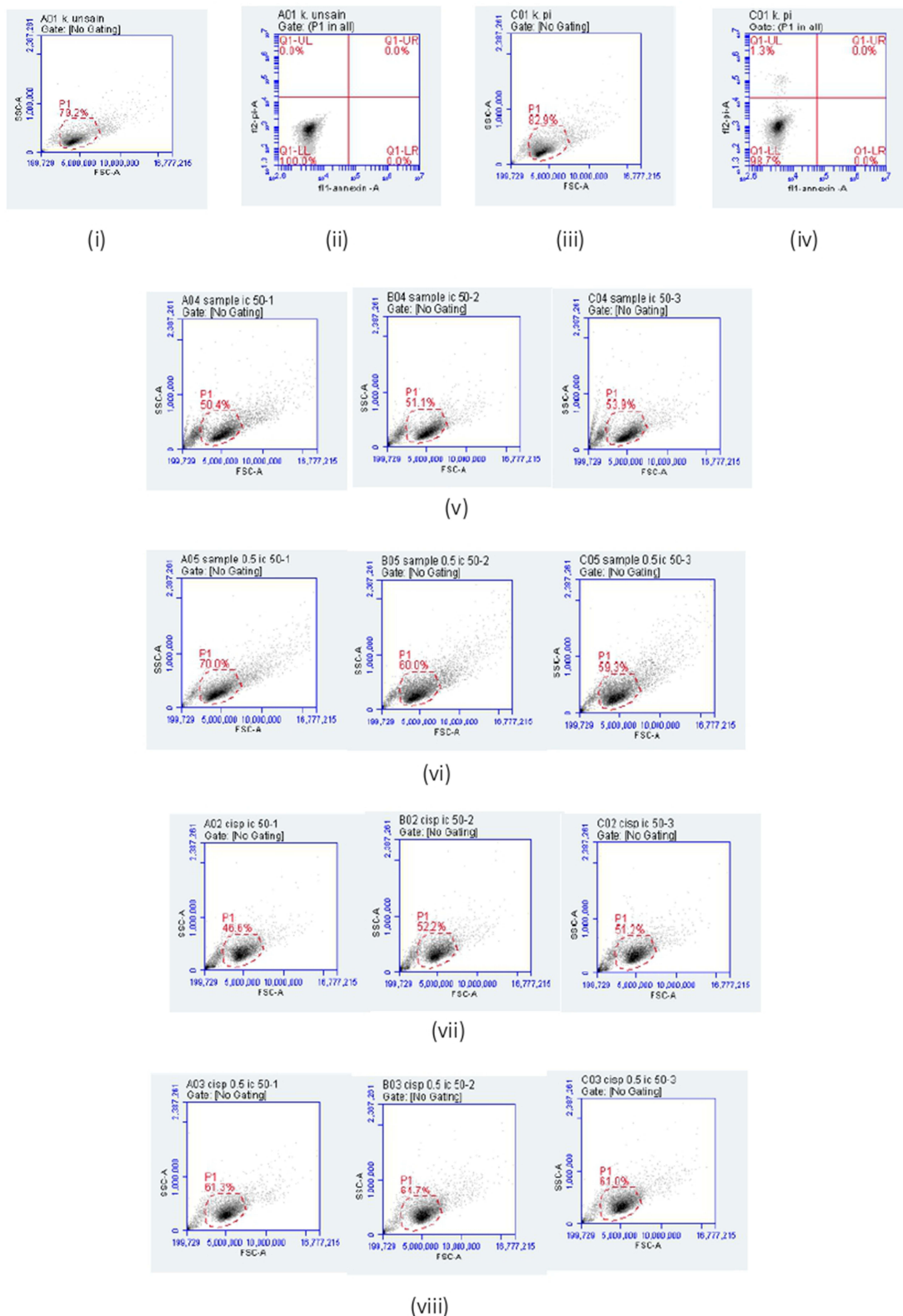


Figure 9 Apoptosis analysis by flow cytometry method of MCF-7 cells. The gated cell population (P1): (i) Unstained control, (ii) PI-stained control, (iii) Annexin V-stained control, (iv) Annexin V/PI dual-stained control, (v) Isolate 2 ($1 \times IC_{50}$)-treated MCF-7 cells, (vi) Isolate 2 ($1/2 \times IC_{50}$)-treated MCF-7 cells, (vii) Cisplatin ($1 \times IC_{50}$)-treated MCF-7 cells, (viii) Cisplatin ($1/2 \times IC_{50}$)-treated MCF-7 cells, (ix) Viable cells distribution unstained control, (x) PI-positive cell distribution, (xi) Annexin V-positive cell distribution, (xii) Annexin V/PI cell distribution. Apoptosis distribution: (xiii) Isolate 2 ($1 \times IC_{50}$) treated MCF-7 cells, (xiv) Isolate 2 ($1/2 \times IC_{50}$)-treated MCF-7 cells, (xv) Cisplatin ($1 \times IC_{50}$)-treated MCF-7 cells, (xvi) Cisplatin ($1/2 \times IC_{50}$)-treated MCF-7 cells.

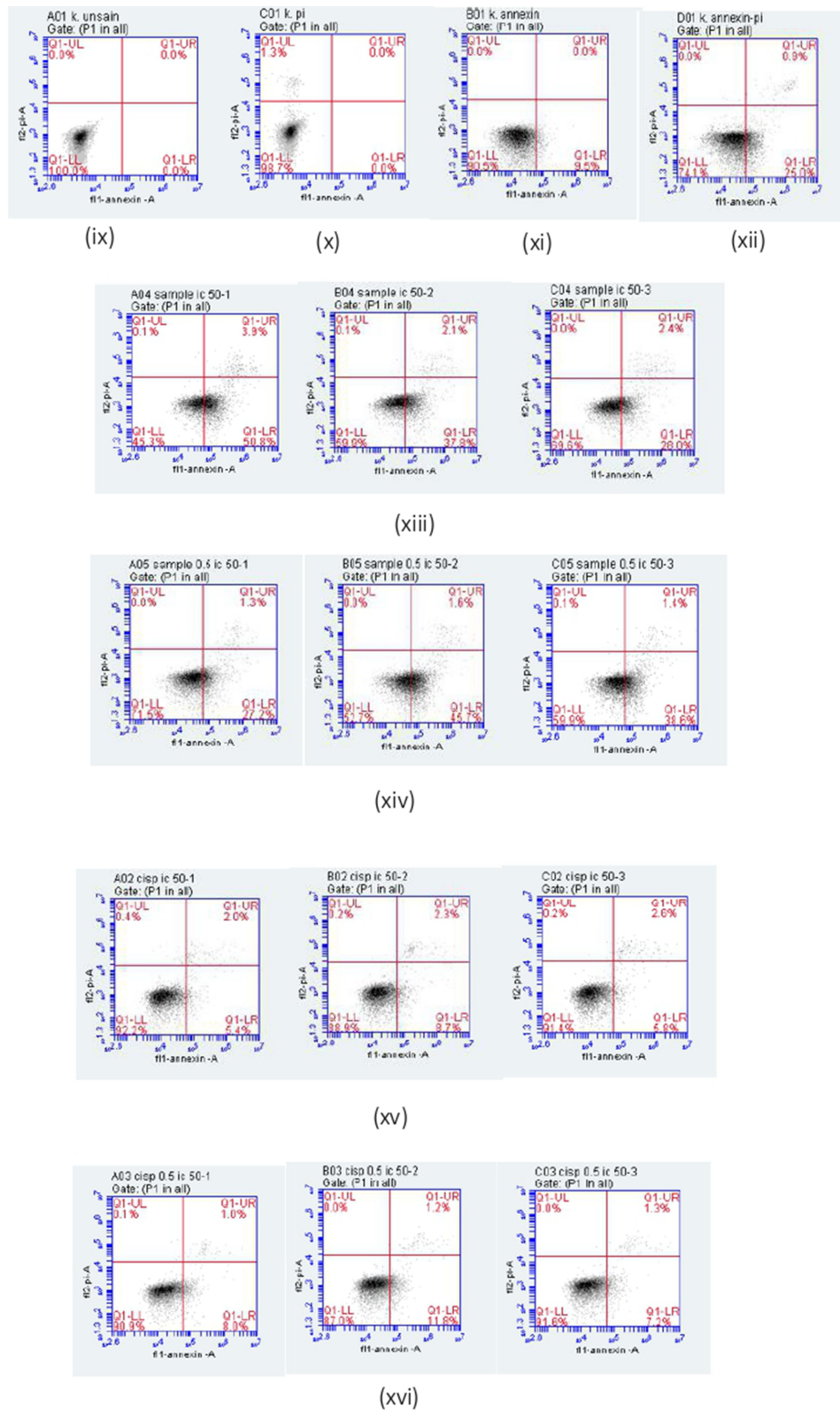


Figure 9 continued.

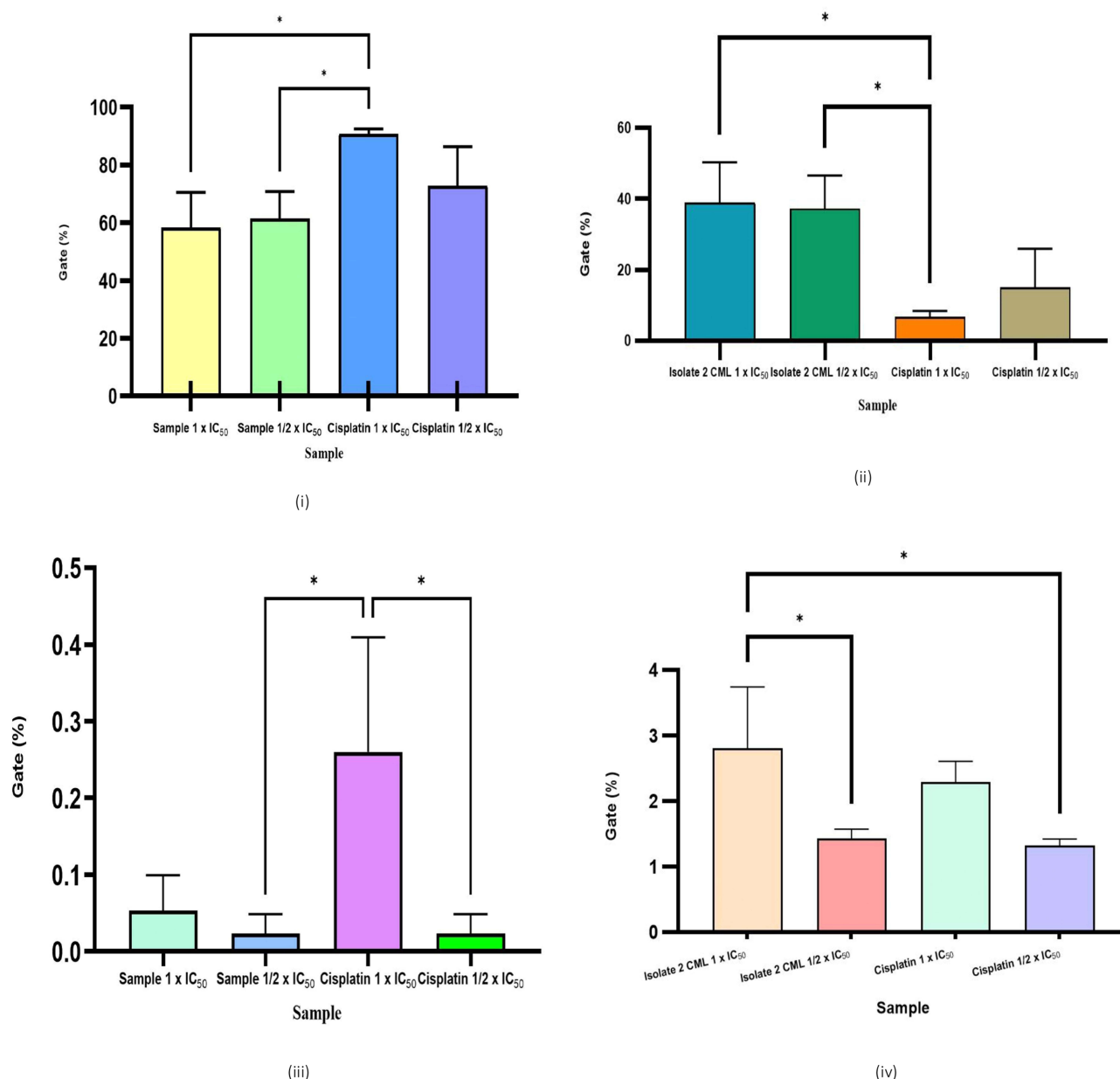


Figure 10 Comparison of the percentage of live cells (i), early apoptosis (ii), late apoptosis (iii), and necrosis (iv) in isolate 2 (1 × IC₅₀), isolate 2 (1/2 × IC₅₀), cisplatin (1 × IC₅₀), and cisplatin (1/2 × IC₅₀). * (p < 0.05) significant.

predominantly causes G2/M arrest through mitosis disruption. This difference indicates that isolate 2 CML has a different target of action than cisplatin. This is crucial in the development of cancer therapies, as the combination of two agents with different mechanisms can increase the effectiveness of therapy and reduce cancer cell resistance.

Apoptosis is a desirable mechanism in the development of anticancer agents. Apoptosis can be observed physiologically, including cell shrinkage, plasma membrane damage, and chromatin condensation. Cells that die in this state do not lose their internal contents and do not elicit an inflammatory response. Necrosis, on the other hand, is characterized by an increase in cell volume and a loss of membrane tension. Necrosis is caused by the release of lytic enzymes, followed by an inflammatory response. Necrosis is a pathological process caused by exposure to physical or chemical stress that affects cells.⁶⁴

Treatment with isolate 2 CML increased the percentage of cells in the early apoptotic phase (Annexin V+/PI-). These results indicate that isolate 2 is capable of inducing cell death via the apoptotic pathway. In contrast, the percentage of

Table 5 Results *ESR1* Gene Expression Analysis Test of Isolate 2 CML with Cisplatin

No	Sample	Normalized Expression Ratio $2^{-\Delta\Delta CT}$
1	Control	0.83 ± 1.17
2	1×IC ₅₀ Isolat 2 CML	1.34 ± 0.13
3	1/2×IC ₅₀ Isolat 2 CML	1.01 ± 0.14
4	1×IC ₅₀ Cisplatin	4.58 ± 1.87
5	1/2×IC ₅₀ Cisplatin	0.19 ± 0.04

Notes: $\Delta C_T(\text{test}) = C_T(\text{ESR1}) - \text{average} C_T(\text{ACTB})$; $\Delta\Delta C_T = \Delta C_T(\text{test}) - \Delta C_T(\text{calibrator})$.

necrotic cells (Annexin V–/PI+) was relatively low, indicating that the primary cytotoxic mechanism is apoptosis, not necrosis.

Administration of Isolate 2 CML at a dose of 1×IC₅₀ caused an increase in *ESR1* gene expression compared to the control. This indicates that at high doses, Isolate 2 CML can activate the estrogen receptor pathway. *ESR1* activation plays a role in regulating cell proliferation and survival of breast cancer cells. In contrast, at a dose of 1/2×IC₅₀, *ESR1* gene expression did not experience significant changes. This indicates that the effect of Isolate 2 CML on *ESR1* gene expression is dose-dependent, where lower doses are not strong enough to modulate the hormone pathway. Increased *ESR1* gene expression may reflect a mechanism of cell adaptation to stress due to compound exposure, so that cells try to maintain survival through activation of the proliferation pathway.

In several previous studies, flavone compounds such as apigenin and luteolin demonstrated high binding affinity for *ESR1* ligands in silico.⁶⁵ Another study on Anhydrocaritin (AHI), a prenylated flavonoid, demonstrated that it can

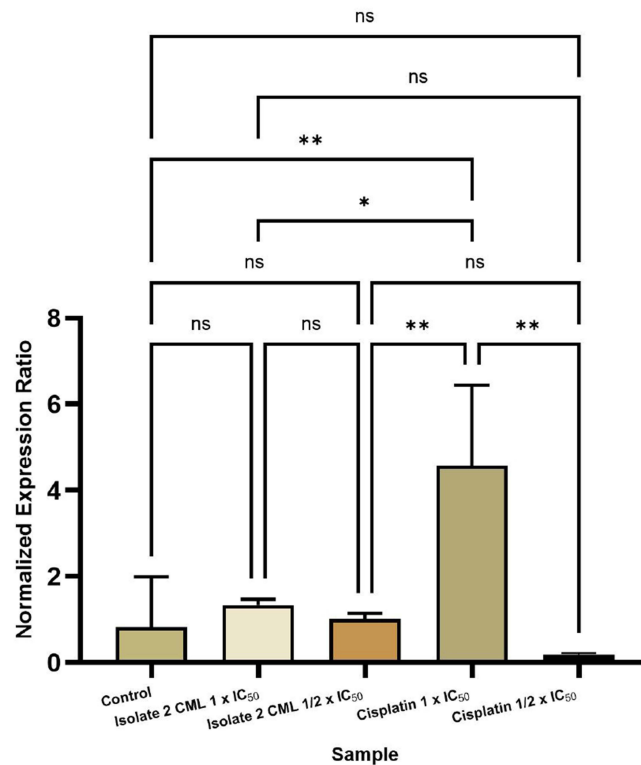


Figure 11 Comparison of *ESR1* gene expression of isolate 2 CML and cisplatin against control. ns ($p > 0.05$) non-significant. * ($p < 0.05$) significant. ** ($p < 0.01$) highly significant.

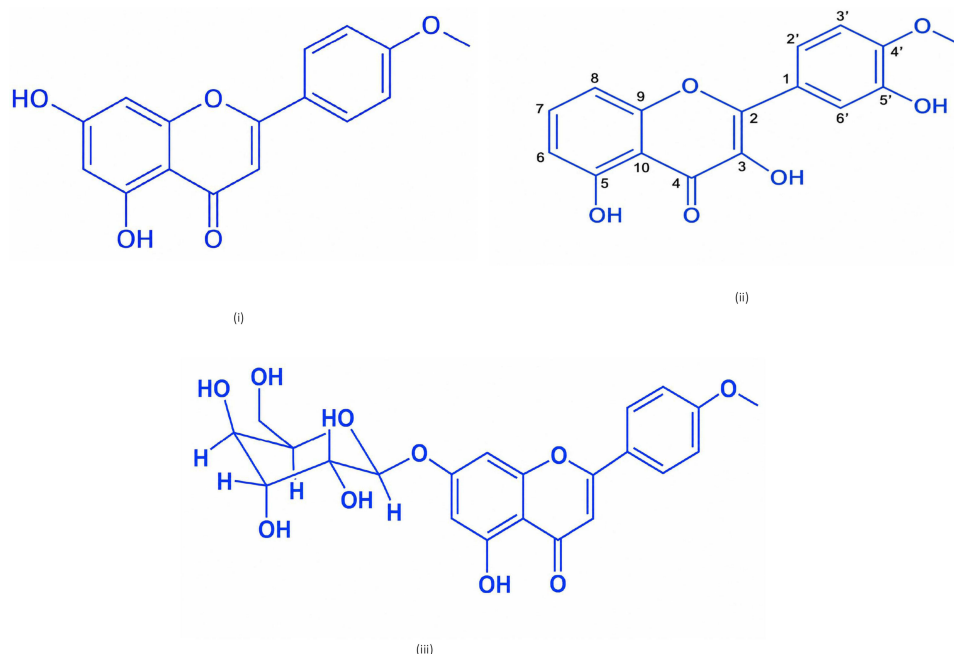


Figure 12 Structure of Isolate 1 (acacetin) (i), isolate 2 (3,5,5-Trihydroksi-4'-metoksi flavon) (ii), isolate 3 (Kaempferide-7-O-glikosida) (iii).

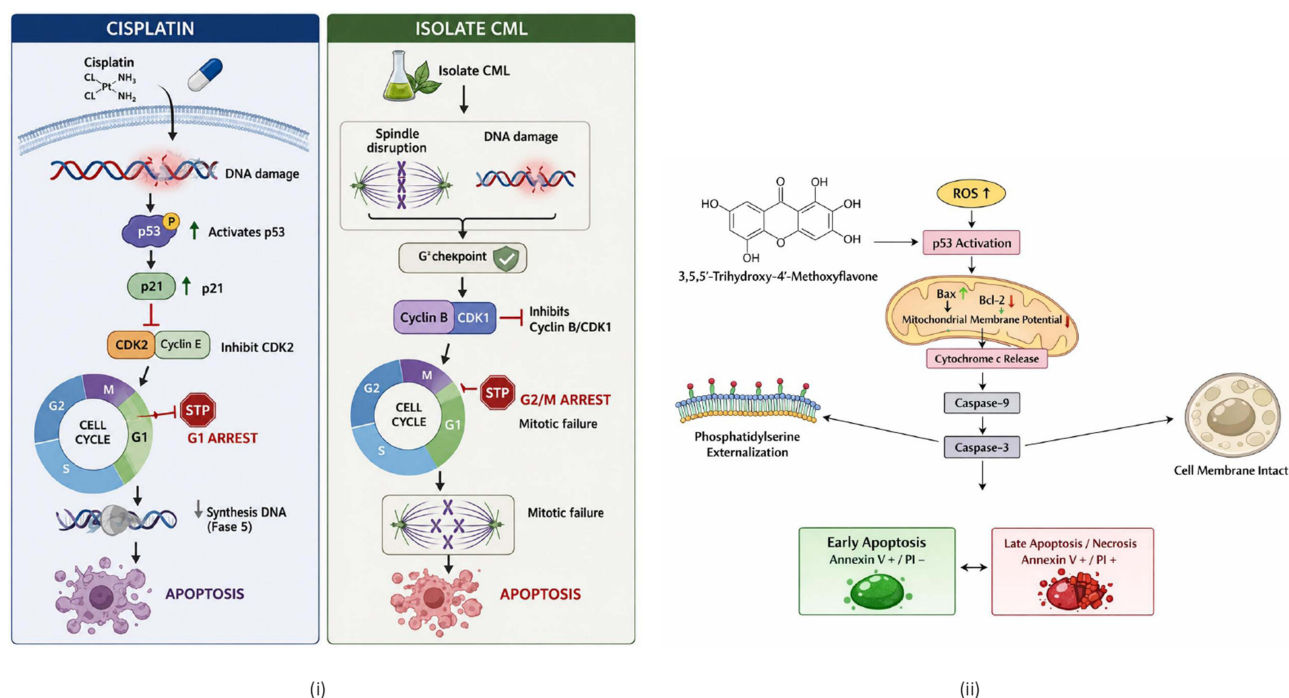


Figure 13 Differences in cell cycle distribution between cisplatin and isolate 2 CML (i); molecular mechanism of early apoptosis of isolate compound 2 CML (ii).

reduce *ESR1* gene expression.⁶⁶ Other studies have also shown that flavonoid phytochemicals act as agonists and antagonists of the human estrogen receptor. Although some of these compounds act by binding directly to the estrogen receptor, certain phytochemicals, such as flavonoid chalcones and flavones, exert antagonistic effects on estrogen signaling independent of direct receptor binding. Chalcones and flavones function as selective, cell-type-specific estrogen receptor modulators. In MCF-7 breast carcinoma cells, chalcones and flavones suppress *ESR1* activity through

stimulation of members of the stress-activated mitogen-activated protein kinase (MAPK) family: c-Jun N-terminal kinase (JNK)1 and JNK2.⁵¹

Cisplatin is a chemotherapy agent that works by inducing DNA damage through the formation of DNA cross-links. This DNA damage inhibits replication and transcription and activates the apoptosis pathway. In this study, cisplatin at a dose of $1 \times IC_{50}$ significantly increased *ESR1* expression. This increase is likely a compensatory response of cells to chemotherapy-induced stress. *ESR1* activation can increase the expression of antiapoptotic genes, thereby helping cells survive. Conversely, at a dose of $1/2 \times IC_{50}$, cisplatin drastically decreased *ESR1* gene expression. This decrease indicates that under certain conditions, cisplatin can suppress the estrogen receptor pathway and increase cell sensitivity to programmed cell death. The difference in response between high and low doses indicates that the effect of cisplatin on *ESR1* gene expression is complex and influenced by the balance between cell adaptation mechanisms and apoptosis induction.

Particularly in ER-positive cells like MCF-7, the *ESR1* gene is essential for controlling the growth and survival of breast cancer cells. *ESR1* activation can prevent apoptosis and boost the production of genes related to the cell cycle. Increased *ESR1* expression in several treatment groups indicates potential activation of the estrogen pathway, which can support cell proliferation. Conversely, decreased *ESR1* gene expression indicates potential induction of apoptosis through suppression of the hormone pathway. The results of this study suggest that modulating *ESR1* expression can influence cancer cell response to therapy, making *ESR1* a potential therapeutic target for ER-positive breast cancer.⁶⁷

Conclusion

Based on UV, IR, 1-dimensional NMR (proton and carbon), 2-dimensional NMR (HSQC and HMBC), and mass spectra data for isolates (1), (2), and (3), it is predicted that the compounds are (1) acacetin, (2) 3,5,5'-Trihydroxy-4'-methoxy flavone, and (3) kaempferide-7-O-glycoside. Isolate 2 CML has an IC_{50} value of 225.045 in HEK-293 cells, thus having a higher selectivity index (4.558) compared to other isolates and cisplatin. Isolate 2 CML can inhibit the cell cycle by causing arrest in the G2/M phase, as indicated by an increase in the percentage of cells in that phase. Isolate 2 CML can significantly induce early-stage apoptosis, as indicated by an increase in Annexin V-positive/PI-negative cells. Administration of Isolate 2 CML at doses of $1 \times IC_{50}$ and $1/2 \times IC_{50}$ caused an increase in *ESR1* gene expression compared to the control, meaning that transcriptional activation occurred in MCF-7 cells.

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Disclosure

The authors report no conflicts of interest in this work.

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